ADVANCES IN THE BIOLOGY, AQUACULTURE, AND CONSERVATION OF THREATENED MARINE SPECIES AND THEIR APPLICATION IN HUMAN HEALTH AND NUTRITION

EDITED BY: Anthony Lee Dellinger, Rachel Tinker-Kulberg, Christopher C. Chabot, Stephen Allen Smith, Siu Gin Cheung, Paul Shin, Kit Yue Kwan and Alan T. Critchley

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Topic Editors:
Anthony Lee Dellinger, Kepley BioSystems, Inc., United States
Rachel Tinker-Kulberg, Kepley BioSystems, Inc., United States
Christopher C. Chabot, Plymouth State University, United States
Stephen Allen Smith, Virginia Tech, United States
Siu Gin Cheung, City University of Hong Kong, SAR China
Paul Shin, City University of Hong Kong, SAR China
Kit Yue Kwan, Beibu Gulf University, China
Alan T. Critchley, Cape Breton University, Canada

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REVIEW
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The Role of Horseshoe Crabs in the Biomedical Industry and Recent Trends Impacting Species Sustainability

*Jordan Krisfalusi-Gannon[1,2], Waleed Ali[1,3], Kristen Dellinger[4], Lee Robertson[1], Terry E. Brady[1], Melinda K. M. Goddard[5], Rachel Tinker-Kulberg[4], Christopher L. Kepley[1,5] and Anthony L. Dellinger[1,4]**

[1] Kepley Biosystems Incorporated, Greensboro, NC, United States, [2] High Point University, High Point, NC, United States, [3] Department of Biology, Columbia University, New York, NY, United States, [4] Joint School of Nanoscience and Nanoengineering, Greensboro, NC, United States, [5] ClienTell® Consulting, LLC, The Valley, Anguilla

Every year the Atlantic horseshoe crab (*Limulus polyphemus*) arrives on shore to spawn, a sight once taken for granted. However, in addition to the gradual climate changes impacting all ecosystems, commercial demand from the widespread application of Atlantic horseshoe crab blood in industrial endotoxin testing and steady use as eel and whelk bait has brought the future of this enduring species into question. In response, regulations have been adopted to enhance the traceability and record keeping of horseshoe crab harvest, which has historically been difficult to track. However, these regulations do not restrict or limit LAL harvest in any significant manner. Still, sometimes-lethal biomedical bleeding process and associated behavioral changes pose a risk to horseshoe crab viability after bleeding and once returned to the waters. As a result, regulators and environmentalists are concerned that current trends and overfishing of this marine arthropod will significantly impact the surrounding ecosystem. This review examines their role and recent trends in the biomedical industry that are impacting these ancient creatures and the derivative effect on shorebirds, while considering emerging alternatives where feasible, as well as ways to ensure sustainable and pragmatic harvesting strategies. Ultimately, healthy populations of horseshoe crabs are vital to restoring and maintaining ecosystems while balancing the need for medical and research applications entirely dependent on these unique creatures.

Keywords: biomedical industry, ecological status, horseshoe crab, *Limulus amebocyte* lysate assay, *Limulus polyphemus*, migrating shorebirds, red knot, ocean ecology

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Edited by:
Elvira S. Poloczanska, Alfred Wegener Institut Helmholtz Zentrum für Polar und Meeresforschung, Germany

Reviewed by:
David Smith, United States Geological Survey, United States
Donald F. Boesch, University of Maryland, United States

***Correspondence:**
Anthony L. Dellinger
adellinger@kepleybiosystems.com

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INTRODUCTION

The American horseshoe crab (*Limulus polyphemus*) is a valuable keystone species distributed across the Atlantic Coast of the United States and the Gulf of Mexico (Botton and Haskin, 1984; Botton and Ropes, 1989; Walls et al., 2002; Botton, 2009; Sekiguchi and Shuster, 2009). Horseshoe crabs play a key role in the eel and whelk fishing industry and an unparalleled, integral part in ensuring environmental safety and that of nearly every drug and medical device in use today (van Holde and Miller, 1995; Loveland et al., 1996).

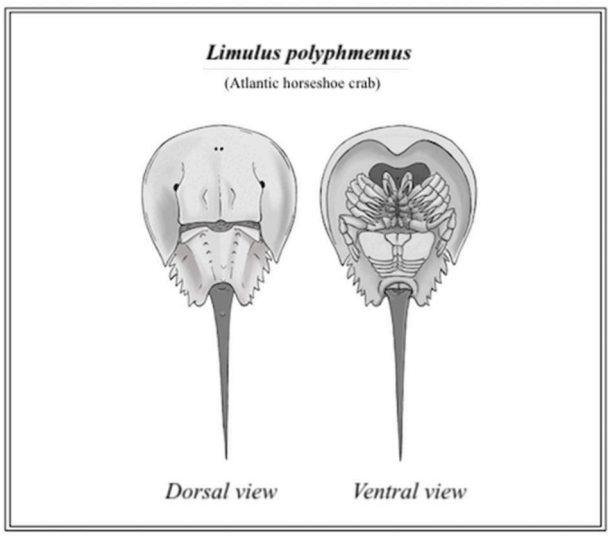

FIGURE 1 | Basic anatomy of the horseshoe crab (*L. polyphemus*) **(Top)**; and photograph of a mature female **(Bottom)**.

This ancient aquatic arthropod, more closely related to scorpions and spiders than to crabs (Størmer, 1952), belongs to its own distinct class, Merostomata (Woodward, 1866), literally meaning "legs attached to mouth." The name "horseshoe crab" is derived from the *Limulus polyphemus*' most recognizable features, its extended prosoma (or *cephalothorax*), a large shell that resembles a horseshoe (**Figure 1**). Commonly referred to as a "living fossil," the horseshoe crab has been able to survive nearly unchanged for an estimated 200 million years (Walls et al., 2002; Kin and Blazejowski, 2014)–prior to recent population dynamics in the face of growing commercial demand. Concerted conservation and research efforts are vital for this species' future wellbeing. Improved or alternative biomedical harvest practices, environmental protection considerations and the suggested partnership of multiple stake-holding organizations are examined further, as discussed herein.

Abbreviations: ^{15}N, Nitrogen-15; ARM, Adaptive Resource Management; ASMFC, Atlantic States Marine Fisheries Council; BET, Bacterial Endotoxin Test; BMP, Best Management Practices; FMP, Fishery Management Plan; LAL, *Limulus* Amebocyte Lysate; LPS, Lipopolysaccharide; MAT, Monocyte Activation Test; PCO_2, Partial Pressure of Carbon Dioxide; PO_2, Partial Pressure of Oxygen; rFC, Recombinant Factor C; TAL, *Tachypleus* Amebocyte Lysate; USP, United States Pharmacopeia; WHO, World Health Organization.

FACTORS AFFECTING HORSESHOE CRAB POPULATIONS

Interest in the horseshoe crab has grown over last half century due to the distinctive nature of its blood and popular publications articulating the species link to migratory shorebirds. The animal's main commercial value is based on a substance found within its light blue blood (van Holde and Miller, 1995). Possessing an open circulatory system with no adaptive immune response, the horseshoe crab has survived through the ages by an "innate immunity" based on granular amebocytes, which comprise 99% of its hemocytes (**Figure 2**) (Shuster, 1978; Iwanaga et al., 1998; Medzhitov and Janeway, 2000). When these granular amebocytes come into contact with an endotoxin or 1,3ß-D-glucan (present in the cell walls of Gram-negative bacteria and fungi, respectively), a cascade of defense molecules is released, triggering coagulation and neutralization of the pathogens. The resulting clot effectively immobilizes the threat and prevents an infection from progressing beyond the wound (Isakova and Armstrong, 2003). While most recent research on horseshoe crabs has been focused on amebocytes and endotoxin detection (http://www.ncbi.nlm.nih.gov/pubmed/), some earlier studies have also yielded insights into human eyesight adaptation, the effect of circadian rhythms on vision, and the process by which sensory information is encoded (Hartline and McDonald, 1947; Barlow et al., 1977).

The unique ability of amebocytes to produce an instantaneous, visible reaction to endotoxins, in particular, has driven commercial demand from pharmaceutical and biomedical companies to confirm drug and medical device safety (Mikkelsen, 1988; Novitsky, 2009) using the *Limulus* amebocyte lysate (LAL) test, which has become the method of choice for endotoxin detection (Novitsky, 2009; Gauvry, 2015). These LAL test applications include quality assurance for: intravenous drugs; biologicals (e.g., clotting factors, insulin, and vaccines); recombinant drugs; and implantable medical devices (e.g., heart valves and orthopedic devices) (Novitsky, 2009). Environmental applications have also increased demand for the LAL test to ensure air quality and detect endotoxin concentrations in fresh water, sea water, and surrounding sediment (Novitsky, 2009).

Such vital benefits are nonetheless dependent on a crude LAL test manufacturing process; whereby the horseshoe crabs are captured, bled, and the collected blood is centrifuged to concentrate the amebocytes. Water is then added to the packed amebocytes, causing them to lyse and release coagulation proteins; thus, the "lysate" nomenclature.

Historically, horseshoe crabs have also been used apart from the extraction of blood for safety testing. They were once harvested for fertilizer and livestock feed; but this widespread practice ended in the 1920s, as the stock of horseshoe crabs began to decline and the public nuisance of the strong odor hastened the adoption of more competitive, alternative fertilizers (Walls et al., 2002). Thereafter, the use of horseshoe crabs as bait in commercial fishing became popular in the 1990s. Horseshoe crabs, particularly egg-bearing female crabs, proved to be an excellent bait for use in eel and whelk pots (Loveland et al., 1996).

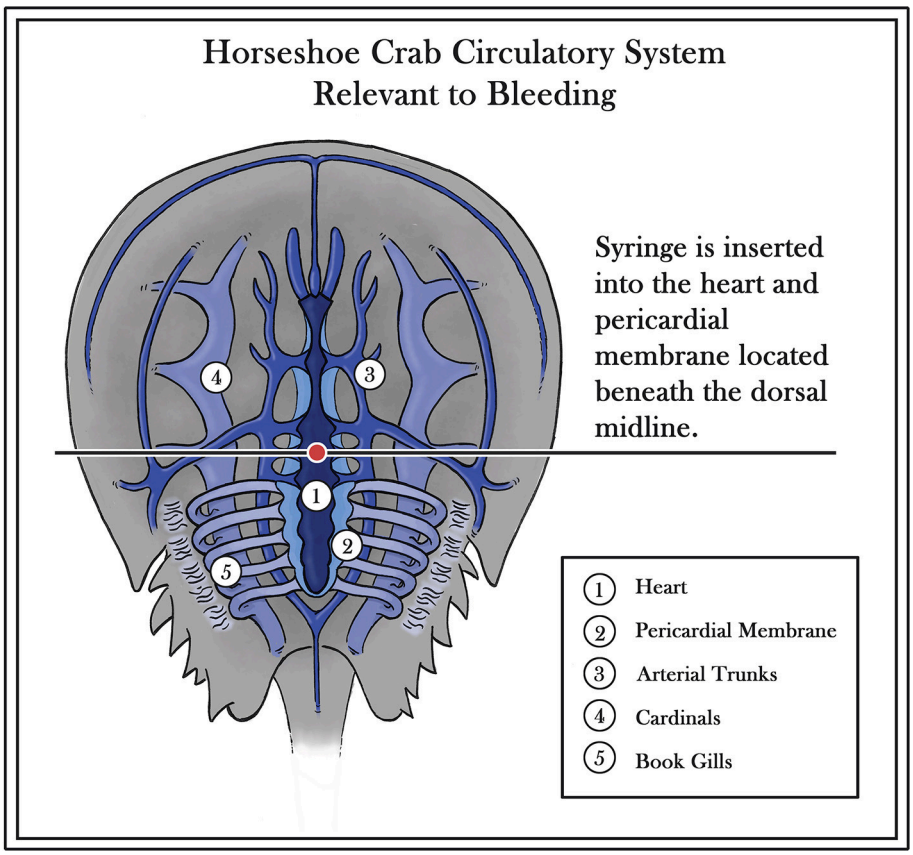

FIGURE 2 | Diagram of horseshoe crab circulatory system (Diagram modified from Patten, 1912; copyright permission, reuse or modifications of **Figure 2** are not required for this image as it is in the Public Domain and holds no copyright).

However, as the increasing biomedical industry requirements of horseshoe crab blood and the species link to migratory shorebirds viability were realized, new oversight agencies were established to mediate the risks from over-harvesting, and restrictions were placed on the number of horseshoe crabs collected for bait in order to regulate populations. These agencies further generated programs for stock management, developed state quota regulations, and established best practices for biomedical harvesting. In 2015, 583,208 horseshoe crabs were harvested as bait for eel and whelk (Atlantic States Marine Fisheries Commission, 2016), a significant reduction from the millions that were once harvested (Atlantic States Marine Fisheries Commission, 2013).

IMPACT OF AMEBOCYTE HARVESTING ON HORSESHOE CRAB BEHAVIOR AND PHYSIOLOGY

The Atlantic States Marine Fisheries Commission (ASMFC) reported that in 2015, 559,903 horseshoe crabs were transported to biomedical facilities for the production of LAL (Atlantic States Marine Fisheries Commission, 2016). The raw materials for the preferred LAL test require careful extraction of blood from horseshoe crabs. Established methods entail introduction of a hypodermic needle placed directly into the exposed pericardial membrane of the horseshoe crab to draw from 50 to 400 mL of blood, depending on the sex and maturity of the horseshoe crab (**Figure 2**). The plasma is centrifuged, and LPS-free reagents, such as Na_2EDTA or 3% NaCl, are added to help prevent clotting after extraction; this can occur as a result of the unintended introduction of endotoxins or other external factors, including undue stress during extraction and exposure to extreme temperatures. Careful handling of the horseshoe crab during bleeding, while maintaining the crab and blood at low and consistent temperatures, can help to prevent such coagulation issues (Armstrong and Conrad, 2008).

The harvest and collection procedure for bleeding horseshoe crabs may appear straightforward, but there are significant risks posed to the crabs at various stages of the process, ranging from transportation and crab storage, to the blood drawing itself. Horseshoe crab mortality rates following such harvesting range from 10 to 30%; however, these figures do not account for any further trauma and/or detrimental behavioral changes once the animals are returned to the ocean, nor the derivative population impact from the disruption of horseshoe crab spawning (Walls and Berkson, 2003; Anderson et al., 2013).

In fact, blood loss may not be the leading cause of death, but rather compounding factors, such as capture, handling and transportation. Biomedical harvest usually entails horseshoe crab collection from the bottom of the shallow seabed with dragging trawls and stacked on the bed of a boat before transferring them into plastic storage containers or bins for extended periods of time. During this process, crabs are crushed under the weight of other stacked crabs resulting in broken telsons and cracked shells, or accidentally impaled by the telsons of neighboring crabs. Each of these parameters must be evaluated when assessing overall mortality, rather than limiting the assessment to estimations. Prolonged survey and evaluation of recently bled horseshoe crabs could also provide more reliable extrapolation of morbidity if extended beyond the established 6-week assessment period.

A study evaluating these factors recorded no such sequelae in horseshoe crabs that were removed from the water only for the bleeding process and immediately returned, without being exposed to the impact of transport and storage (Hurton and Berkson, 2006). These "low stressed" crabs were observed to recover from removal of up to 40% of their estimated blood volume. However, when conventional horseshoe crab capture, transport, storage and handling procedures at biomedical bleeding facilities were simulated, the time spent out of the water and extent of exposure to elevated temperatures appeared to play a role in increased mortality. In controlled laboratory simulations, crabs exposed to capture, transport and holding stressors without bleeding, compared to those exposed to both bleeding and other external influences, yielded 2.6 vs. 8.3% mortality rates; while crabs losing the greatest percentage of blood along with capture, transport and storage exhibited the highest mortality rates overall. When both groups of crabs had 40% of their blood volume extracted, only 6% of non-stressed crabs perished vs. 15.4% of stressed crabs. (Hurton and Berkson, 2006). Thus, improved harvest practices have the potential to reduce mortality rates during biomedical harvest by more than half.

The stress from removing horseshoe crabs from the water during harvest may prove especially lethal. The horseshoe crab breathes through a set of gills and transports oxygen via hemocyanin (Towle and Henry, 2003). The primary function of the gills is to supply oxygen, not to remove CO_2. Because carbon dioxide is water-soluble, it is easily removed when the animal is in an aquatic environment. However, when a crab is removed from the water, it is not able to efficiently remove CO_2, and regulation of PCO_2 results in abnormal hemolymph pH levels (Henry and Wheatly, 1992; Towle and Henry, 2003). While these animals can tolerate low oxygen environments based on various physiologic adaptations, such as a sharp decrease in heart rate and increased affinity of oxygen to hemocyanin (Towle and Henry, 2003); after removal from the water for only 5 min, they can develop severe hypoxia and metabolic acidosis. After 24 h of transportation out of water, horseshoe crabs have been shown to exhibit significantly diminished PO_2 levels and extreme respiratory acidosis (Allender et al., 2010).

A review on the effects of hypoxic conditions on multiple marine organisms demonstrated that survival times are reduced by an average of 74% when an animal experiences hypoxia (Vaquer-Sunyer and Duarte, 2011). Accordingly, hypoxia has been associated with decreased stamina in hermit crabs (Mowles et al., 2009) and with altered fish migration patterns and distance (Ultsch, 1989). Assuming similar side effects are likely in horseshoe crabs, oxygen deprivation and the resultant disturbance in homeostasis has the potential to disrupt normal functions, such as spawning, even after the horseshoe crabs are returned to their natural habitat.

Exposing horseshoe crabs to high temperatures during capture and/or transportation has also been shown to negatively impact both blood quality and overall health (Coates et al., 2012). In a study to determine horseshoe crab response to varying temperatures, crabs held in the highest temperature (23°C) lost the most body weight and were among the only ones to expire. Hemocyanin and amebocyte concentrations were inversely proportional to temperature, with crabs held in the highest temperatures yielding the lowest concentrations. During the study, horseshoe crabs held in 18°C water yielded a 43.9% decrease in hemocyanin concentration, while those held in 23°C water showed a 69.3% decrease (Coates et al., 2012). Although the density of amebocytes decreased across all temperatures studied, the greatest decrease also occurred at the highest temperatures, with those held at 23°C yielding a decrease of 71.7%; which was also accompanied by notable morphological changes in the amebocytes.

Other, more nuanced behavioral changes brought about by the bleeding process have also been documented. Behavioral changes in horseshoe crabs have been observed for up to 2 weeks after harvesting (Anderson et al., 2013). The horseshoe crabs showed: slower walking; a 33–66% reduction in overall activity; and decreased expression of tidal rhythms, which dictate movement and spawning activity. Harvesting, in particular, may reduce spawning activity of females; which is especially problematic, since horseshoe crab harvest often takes place while spawning, when the crabs are easily accessible on the beach (Leschen and Correia, 2010). Upon habitat reintroduction, females have demonstrated markedly lethargic behavior and failed to spawn entirely (Anderson et al., 2013). This negative impact on the horseshoe crab population is further compounded by the high mortality rate of 30% following the bleeding of female horseshoe crabs regardless of pre- or post-spawning phases (Leschen and Correia, 2010); whereas, bleeding male horseshoe crabs has demonstrated a mortality rate of 8% (Walls and Berkson, 2003). In 2013, the reported mortality rate of horseshoe crabs harvested for solely biomedical purposes was 15%. However, when the number of crabs harvested, bled, sold by biomedical companies for bait, and counted against state bait quotas was factored in, the mortality rate jumped to 26% (Atlantic States Marine Fisheries Commission, 2013).

While research organizations continue to investigate the industry practices and associated effects of the horseshoe crab biomedical bleeding process, such studies have been largely dismissed or regarded as not following industry established Best Management Practices (BMP) in 2011 (Atlantic States Marine Fisheries Commission, 2014). Furthermore, some regulatory agencies have also asserted that such efforts would only be

scientifically valid if all protocols were independently reviewed and approved by an advisory panel (Atlantic States Marine Fisheries Commission, 2010a). Notably, the current elected panel is predominantly comprised of stakeholders representing the agencies that control horseshoe crab biomedical assay commerce (Atlantic States Marine Fisheries Commission, 2013), suggesting that effective management strategies may be compromised by conflicting economic and environmental considerations.

DECLINING POPULATIONS OF HORSESHOE CRABS

Atlantic States Marine Fisheries Commission reports on horseshoe crab harvest mortality date back to 2004. From 2004 to 2012, the number of crabs delivered to biomedical bleeding facilities increased from 343,126 to 611,827, or by about 78%; while total mortality correspondingly increased by 75% (Atlantic States Marine Fisheries Commission, 2013). The percentage of horseshoe crabs that died prior to being bled more than doubled from 2008 to 2012 (Atlantic States Marine Fisheries Commission, 2013), which may be attributed to deleterious harvest and transportation practices. The maximum harvest mortality limit of 57,500 set by the ASMFC (based on the 15% mortality allowance) has been exceeded at times by more than 20,000 horseshoe crabs every year since 2007 (Atlantic States Marine Fisheries Commission, 1998, 2013). More recently, ASMFC data has estimated the mortality of horseshoe crabs harvested for the biomedical industry to be 70,000 (with a range of 23,000–140,000; Atlantic States Marine Fisheries Commission, 2016).

The cumulative effects of horseshoe crab harvest have also been well documented. An especially compelling example has been observed near Cape Cod at Mashnee Dike, where the spawning horseshoe crab count dwindled from around 3,000 to 148, representing a 95.3% decline over a 15-year period (1984–1999). Mashnee Dike was brought under the protection of the United States Army Corps of Engineers, and no external development in that area has been permitted since that time. As researchers have observed availability of a consistent food supply for the crabs (Widener and Barlow, 1999), human predation appears to be the primary cause of this collapse. Albeit not as severe, a Long Island-based study monitoring 68 sites showed horseshoe crab populations decreased just over 10%, or roughly one percent per year from 2003 to 2014 (Tanacredi and Portilla, 2015).

Reports from Delaware Bay and a few additional sites have cited modest horseshoe crab recoveries, but such examples have been the exception and seem to have been more than offset by shifting commercial activity to other geographic regions (Smith et al., 2009). Stricter horseshoe crab regulations around the Delaware Bay/New Jersey coastlines have led to increased harvesting in New England, where continued population declines were noted in a 2009 survey (Atlantic States Marine Fisheries Commission, 2013). As a result, regional management plans require coordination to ensure that horseshoe crabs are protected throughout their purview (Berkson et al., 2009).

The increased demands of the U.S. population, which is growing by 2.6 million people each year, and rapidly growing medical device and vaccine industries (Gauvry, 2015; Central Intelligence Agency, 2016) may not bode well for horseshoe crab populations. Based on current rates of horseshoe crab mortality and related population trends, over the next two decades, demand for the LAL test is likely to reach unsustainable levels. While horseshoe crab populations have moderately stabilized in some regions of the Atlantic, increases have also not been observed, which may be a result of negative behavioral or reproduction changes once the animals are returned to the ocean (Anderson et al., 2013) as well as deteriorating coastlines.

Global endotoxin detection is also dependent upon the TAL (*Tachypleus* amebocyte lysate) test produced in China, which is derived from the amebocytes of *Tachypleus tridentatus* and *Tachypleus giga,* Asian horseshoe crab species. Because these horseshoe crabs are often secondarily sold for human consumption or for the production of chitin after biomedical bleeding, resulting in a 100% mortality rate, population decline of these two species is a serious concern (Gauvry, 2015). While specific survey data are not available as in the U.S., decreased harvest quantities suggest an 83% drop in abundance (Gauvry, 2015). Unless China begins to regulate the harvest of *T. tridentatus* and *T. giga*, declining availability of the TAL test would be expected to increase demand for the LAL test throughout Asia (Gauvry, 2015).

ENVIRONMENTAL CONSIDERATIONS

Sustaining the horseshoe crab population is also ecologically essential, as they play key roles as: bioturbators; hosts to a variety of epibionts on their shells; controllers of the population of many benthic invertebrates; and as a food source for a multitude of marine animals (**Figure 3**) (Botton and Haskin, 1984; Botton and Ropes, 1989; Walls et al., 2002; Botton, 2009). Barnacles, slipper limpets and blue mussels frequently live on the shells of horseshoe crabs, although the relationship is mostly neutral (Botton, 2009). Although they consume a broad, omnivorous diet, adult horseshoe crabs are important predators of benthic invertebrates, such as bivalves, polychaetes, crustaceans and gastropods, with a particular preference for thin-shelled bivalves, like small surf clams and blue mussels (Botton and Haskin, 1984; Botton and Ropes, 1989).

Shorebirds (e.g., red knots and ruddy turnstones), sand shrimp and fish (e.g., American eel, Atlantic silverside, catfish, devil ray, mullet, northern kingfish, silver perch, summer flounder, striped bass, swordfish, weakfish, white perch and winter flounder) consume horseshoe crab eggs and larvae (Warwell, 1897; Perry, 1931; Price, 1962; Spraker and Austin, 1997; Walls et al., 2002). In turn, crabs (e.g., blue, fiddler, green and hermit crabs) and pufferfish eat juvenile horseshoe crabs (Walls et al., 2002; Botton, 2009).

Mature horseshoe crabs are not significantly threatened by natural predators due to their large size and thick shell, but some have been identified (Walls and Berkson, 2003). For example, large American alligators have been observed eating adult

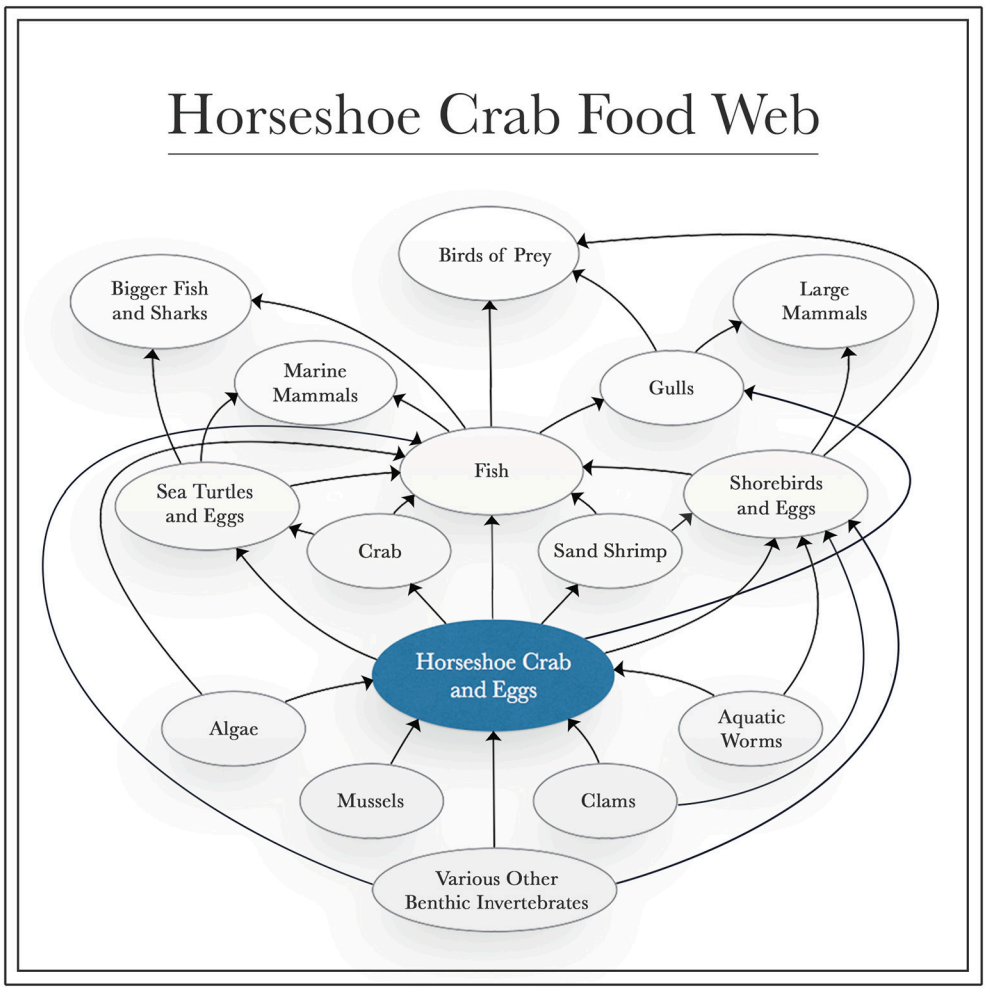

FIGURE 3 | Diagram of feeding interactions of the horseshoe crab. Directions of arrows indicate impacts of each group on others through feeding interactions.

horseshoe crabs in the Indian River Lagoon in Florida on several occasions, and leopard sharks have occasionally consumed them, as well (Reid and Bonde, 1990; Walls et al., 2002). Whenever adult horseshoe crabs are upturned and stranded on the beach, herring and black-backed gulls typically eat them, removing the gills and legs in order to access eggs and internal organs (Botton and Loveland, 1993; Walls et al., 2002). However, loggerhead turtles (listed as threatened by the U.S. Endangered Species Act) are the most common predator of mature horseshoe crabs, which have comprised a significant portion of these turtles' stomach contents when found in the lower Chesapeake Bay (Keinath, 2003).

That said, in the mid-1980s the diet of loggerhead turtles in Virginia was dominated by horseshoe crabs, before transitioning to blue crabs in the 1980s, and more recently to finfish (Seney and Musick, 2007). These shifts are believed to have been caused by the decline in horseshoe and blue crab populations. The drop in these two populations may also correlate to the overall decrease in the number of sea turtles in the Chesapeake Bay over the past few decades (Botton, 2009). In one survey, the sea turtle density in the lower Delaware Bay was comparable to the density of sea turtles in the lower Chesapeake Bay (Spotila et al., 2007), indicating the possibility that loggerheads in this area also feed on horseshoe crabs (Botton, 2009). While few such observations have been published to conclude whether many other species of sea turtles also consume horseshoe crabs, one of the most endangered, Kemp's Ridley turtles, have been observed eating them (Servis et al., 2015).

SHOREBIRDS AS BELLWETHERS

The spawning of horseshoe crabs in the Delaware Bay occurs between May and June, with 70% occurring during the first two spring tides in May (Smith and Michels, 2006). The migration of many shorebirds, such as the red knot (*Calidris canutus*), semipalmated sandpiper (*Calidris pusilla*), ruddy turnstone (*Arenaria interpres*), and sanderling (*Calidris alba*), correspond to horseshoe crab spawning (Clark et al., 1993). The rufa subspecies of the red knot (*Calidris canutus rufa*), for example, is a shorebird with one of the longest migrations in the animal kingdom, traveling up to 19,000 miles from its wintering regions

in the southeastern U.S., northeastern Gulf of Mexico, northern Brazil, or the southern tip of South America to its breeding ground in the Canadian Arctic (U.S. Fish and Wildlife Service, 2015).

Arguably the most important site in the red knot migration is the final stop in the Delaware Bay, where shorebirds feed on horseshoe crab eggs and rely heavily on the nutrition they receive to survive the final stretch of their journey to the frigid, unpredictable Canadian or Arctic tundra, which is often barren of further sustenance when they arrive (Baker et al., 2004; Mizrahi and Peters, 2009). Once in the Delaware Bay, red knots and other shorebirds feed predominantly on the eggs laid by spawning horseshoe crabs while they are available (Tsipoura and Burger, 1999; Mizrahi and Peters, 2009; McGowan et al., 2011; Smith et al., 2013). It is unlikely that other food sources found at shorebird stopover sites would be as widely available or as nutritionally dense as horseshoe crab eggs (Mizrahi and Peters, 2009).

Specifically, the high fatty acid content of these eggs makes them the ideal food source for migrating shorebirds that need to rapidly gain weight in order to ensure robust fitness, and consequently survival, on their journeys (Mizrahi and Peters, 2009). Survival and successful reproduction of the many migratory shorebirds that stop to feed at Delaware Bay are strongly linked to horseshoe crab reproduction. It is unlikely that these birds would be able to adjust their migration schedule over time (Baker et al., 2004; Mizrahi and Peters, 2009). From 1980 to 2014, red knot populations decreased by as much as 75% in some areas, largely due to the lack of horseshoe crab eggs in Delaware Bay (U.S. Fish and Wildlife Service, 2014).

Stable isotope tracking to analyze the diets of shorebirds revealed that free-ranging shorebirds possessed ^{15}N signatures identical to those of shorebirds raised in captivity and fed a diet solely comprised of horseshoe crab eggs (Haramis et al., 2007). An estimated 107 billion horseshoe crab eggs are necessary to support 423,000 shorebirds flying into Delaware Bay to feed before continuing on to their breeding grounds (U.S. Fish and Wildlife Service Shorebird Technical Committee, 2003). For example, when preparing for migration, sanderlings consume an average of 8,300 horseshoe crab eggs per day; ruddy turnstones, a daily average of 13,300 (with a peak daily consumption of 19,360 eggs); and red knots consume an average of 18,350 (with a peak consumption of 23,940 eggs/day) (Castro et al., 1989; Haramis et al., 2007).

Ensuring a consistent and sustainable supply of horseshoe crab eggs is of particular concern with respect to the red knot, which has been listed as a threatened species and may be one of most studied proxies for tracking horseshoe crab populations and viability (U.S. Fish and Wildlife Service, 2014). The current red knot population is estimated to require 15.4 billion eggs to obtain sufficient energy levels for migration, which is equal to the number of eggs laid by about 170,000 female horseshoe crabs (U.S. Fish and Wildlife Service Shorebird Technical Committee, 2003). To be healthy enough to complete their migration, red knots need to double their body mass (usually arriving at 90–120 g and departing at 180–220 g) before the entire flock departs Delaware Bay at month's end (Baker et al., 2004). In total, 1,890 kilojoules (kJ) of stored energy is necessary to successfully complete the 2,400-kilometer flight from Delaware Bay to the Arctic (Baker et al., 2004).

From 1997 to 2002, the number of red knots that reached their target weight decreased by 70%, possibly due to late arrival in the Delaware Bay, compounded by a shortage of horseshoe crab eggs. Average body mass upon departure showed significant decline from 1997 to 2002, going from 182.8 grams (±22.6 g) to 162.3 g (±24.5 g) (Baker et al., 2004). During these same 5 years, tagged survivors that made the journey back to Delaware Bay and were recaptured at least once were heavier than birds not seen again. Between May 2000 and May 2001, the number of returning red knots decreased by 47% (Baker et al., 2004). The trends in declining body mass and population of red knots in Delaware Bay have correlated to an increase in the harvest of horseshoe crabs. Beginning in 1990 and peaking in 1998, horseshoe crabs were used largely as bait for eel and whelk fisheries, further impacting the availability of eggs for shorebird consumption (Walls et al., 2002).

The size of the population of red knots in Tierra del Fuego, Argentina, also rapidly declined from 51,000 in 2000 to 27,000 in 2002 (Morrison et al., 2004). In January 2003, an aerial survey of red knot sites along the Patagonian coast known to be abundant in the 1980s, located only 560 red knots. Likewise, a survey in December 2003 of northern Brazil indicated an abnormally small population of birds, suggesting evidence of red knot mortality, rather than just redistribution (Baker et al., 2004).

The shorebirds' considerable nutritional requirements might appear to be decimating the horseshoe crab population. Although mortality in the early stages of life is a major impediment to horseshoe crab population growth (Sweka et al., 2007), shorebird predation on horseshoe crab eggs has not been found to reduce the size of the horseshoe crab population. Eggs brought to the surface by wave action (Nordstrom et al., 2006) or other spawning horseshoe crabs (Sweka et al., 2007) dry out and die if not consumed (Botton, 2009). In fact, the eggs most accessible to the birds in the upper 5 cm of the beach comprise about 10% or less of the total density of buried eggs (Smith, 2007; Botton, 2009).

Further, red knots and other shorebirds have long been of interest to recreational birdwatchers, and efforts in recent years have been made to ensure that their foraging goes undisturbed. A combination of specified viewing locations and enforcement of policies, such as keeping unleashed dogs from roaming the beaches, have greatly reduced interruptions of these pivotal refueling stops during their migration (Burger et al., 2004).

CONSERVATION EFFORTS

Regulatory efforts are underway to address dwindling horseshoe crab numbers. The Horseshoe Crab Management Board of the ASMFC approved the Horseshoe Crab Fishery Management Plan (FMP) in October 1998, which provided initial management of horseshoe crabs in and around Delaware Bay. However, conservation efforts in Delaware ultimately led to increased biomedical and bait harvesting in other areas, offsetting the

prospects for overall horseshoe crab population growth (Atlantic States Marine Fisheries Commission, 2013). Later addenda to the Horseshoe Crab FMP specified annual state-by-state landing quotas across the east coast and contributed to the establishment of the Carl N. Schuster Jr. Horseshoe Crab Reserve, a 1,500 square mile harvest-free zone (Atlantic States Marine Fisheries Commission, 2000). Historically, the most comprehensive data on horseshoe crab abundance has been based on the Benthic Trawl Survey conducted by Virginia Polytechnic Institute (VPI), but the survey faces inconsistent funding circumstances. Other studies including the Delaware Trawl Survey, the New Jersey Delaware Bay Trawl Survey, and the New Jersey Ocean Trawl Survey have been established and helped intermittent funding limitations (Smith et al., 2016). Funding for future years of the studies is undergoing evaluation.

Addendum IV of the FMP delayed harvest in Maryland and Virginia and restricted bait harvest in Delaware and New Jersey to 100,000 male-only crabs. It was approved in May 2006; and the addendum was extended through October 2013 (Atlantic States Marine Fisheries Commission, 2006) with the addition of seasonal harvest restrictions on all horseshoe crabs from January to June and on female horseshoe crabs from June to December in Delaware and New Jersey (Atlantic States Marine Fisheries Commission, 2010b).

An Adaptive Resource Management (ARM) framework, designed to account for populations of both red knots and horseshoe crabs when implementing regulations, was established by Addendum VII in 2012. The ARM framework uses models considering red knot stopovers in Delaware Bay to determine optimal horseshoe crab harvest; has shaped several initiatives that aid in the protection of both species; and is the basis for ongoing assessments to optimize management plans (Atlantic States Marine Fisheries Commission, 2012).

As a result of these efforts, the aggregate harvest of horseshoe crabs declined 70% from 1998 to 2006, with the greatest reductions occurring in Delaware Bay states (Smith et al., 2009). The focus for conservation has also gradually shifted specifically to the spawning locations of the horseshoe crabs. As most crabs bury their eggs approximately 15 cm from the surface and above the high tide line toward the shore (Weber and Carter, 2009), protection of coastlines in which the horseshoe crabs spawn has been vital in working to restore their numbers to previous levels (Berkson et al., 2009).

Actions have also been taken in the fishing industry to reduce harvesting of horseshoe crabs for use as bait. These include alternative baits (Ferrari and Targett, 2003; Fisher and Fisher, 2006; Atlantic States Marine Fisheries Commission, 2015) and using bait bags with improved efficiency that require as little as one-tenth of traditional quantities per bag (Atlantic States Marine Fisheries Commission, 2017). However, tensions persist due to the demand for horseshoe crab bait by both the eel and whelk fishing industries (Atlantic States Marine Fisheries Commission, 2013). Furthermore, in 2015, conservation groups listed the red knot as endangered in the U.S., which would result in increased protection for horseshoe crabs. Conversely, members of the fishing industry have also challenged quotas due to an apparent continuation in the decline of the red knot population, despite a 2-year ban on horseshoe crab harvest in 2006 and 2007 (Moore, 2008).

COMMERCIAL CONSIDERATIONS

Given mixed results from these conservation efforts and impact from unabated LAL testing demands and utilization as a bait in the fishing industry, more sustainable approaches to horseshoe crab management and harvesting practices are urgently needed for medical and environmental applications.

Before adoption of the LAL test, most research facilities, pharmaceutical and medical device companies used the United States Pharmacopeia (USP) rabbit pyrogen test to determine the presence of endotoxins (Pharmacopeial Forum, 1983). However, the method took significantly longer to obtain results, notwithstanding the inherent variability and ethical issues with the use of live rabbits. The World Health Organization (WHO) now recognizes several bacterial endotoxin test (BET) methods using amebocyte-derived LAL from the horseshoe crab, including measuring turbidity or chromophore release from the BET reaction; however, the preferred method is based on amebocyte lysate clotting upon exposure to endotoxins or β-glucans (World Health Organization, 2011). Notably, β-glucans can also be selectively "ignored" by removing the G factor responsible for the β-glucans clotting reaction.

To date, the LAL test has been the test of choice, despite a more recently uncertain supply of horseshoe crab blood. Fortunately, it has been possible to increase the hemolymph extraction volume from *L. polyphemus*, as more accurate techniques for measuring blood volume have been discovered. It was initially estimated that the blood volume of a horseshoe crab was 10% of its total body weight; however, more recent findings have shown that blood volume is actually closer to 25% of the animal's total weight (Hurton et al., 2005).

New endotoxin tests have been developed and may have the potential to replace or supplement the LAL test; and thus, reduce or eliminate the demand for wild horseshoe crab capture. The recombinant factor C (rFC) test, for example, uses a cloned rFC reagent extracted from the DNA of the Singapore horseshoe crab and thereby eliminates the need for repetitive bleeding (Ding et al., 1995). Like the LAL test, the rFC test triggers a pathway to coagulation when endotoxins come into contact with Factor C. The rFC molecule has multiple potential endotoxin binding sites, and as such, the rFC assay has been shown to be more sensitive and specific than the LAL test (Ding and Ho, 2001; Thorne et al., 2010). However, the rFC test is currently considered an "alternative assay" as outlined in the Pyrogen and Endotoxins Testing; Questions and Answers, released by the FDA in 2012 (U. S. Department of Health and Human Services, 2012), which also stipulates that manufacturers must provide method validation in compliance with requirements outlined in by United States Pharmacopeia (USP) section on Bacterial Endotoxin Testing (USP, Chapter 85).

Another "alternative assay" to the LAL test is the Monocyte Activation Test (MAT) (U. S. Department of Health and Human Services, 2012), the MAT uses the monocytes of humans to mimic

febrile reactions and thus requires no horseshoe crab byproducts, in contrast to the LAL and rFC assays (Stang et al., 2014). The MAT has been used reliably to resolve discrepancies between LAL test results; however, it has been shown to be ineffective in the presence of cytotoxic agents (Dobrovolskaia et al., 2014; Stang et al., 2014). The standard MAT procedure also lacks the sensitivity to detect the required amount of pyrogens on medical surfaces (which is also a limitation of the LAL assay). While the MAT has been optimized to detect such pyrogens, including the ability to ensure sensitivity by incubating test materials in the MAT, the modified version can take up to 20 h and is therefore too time-consuming for practical application in most settings (Stang et al., 2014).

Although the rFC and MAT methods produce results comparable to the LAL test (Alwis and Milton, 2006; Thorne et al., 2010; Hermanns et al., 2011) while conserving the horseshoe crab and surrounding ecosystems, the widespread adoption of these alternative tests may prove to be extremely challenging. The industry has been reluctant to transition to newer methods due to the complex validation procedure and subsequent redesign of the manufacturing processes that would necessarily accompany the change to procedures that have been established and followed for approximately 40 years (Cohen, 1979; U. S. Department of Health and Human Services, 2012).

In fact, revising the current system to improve efficiencies in horseshoe crab use may be more viable in the near term. Rather than adopting alternative tests, some biomedical companies have opted to make existing tests more sustainable. For example, LAL assays with specially designed cartridges have been developed to reliably screen for endotoxins, while also using one-twentieth of the raw horseshoe crab material required by conventional LAL tests (Wainwright, 2013).

Another alternative would be the use of a line of amebocytes that could be cultured *in vitro*. Research in this arena has yielded promising but inconsistent results (Joshi et al., 2002; Hurton et al., 2005); whereas, mounting pressures on the harvest of horseshoe crabs may yet help justify continued efforts and investment into this approach.

ALTERNATIVES TO CURRENT HORSESHOE CRAB HARVESTING PRACTICES

As more may be learned from further study, ranching of horseshoe crabs could be considered to help replenish populations. An instructive 56-day study of horseshoe crabs in captivity revealed decreases in body weight and deteriorating health, as reflected in various biological markers, including hemocyanin and amebocyte concentrations, which declined significantly (Coates et al., 2012). Although these changes occurred at all temperatures over time, horseshoe crabs held in higher temperatures (23°C) experienced the most significant decreases in these key metrics. To achieve the lowest horseshoe crab mortality and highest blood quality during biomedical bleeding, a more systematic understanding of the nuances of the optimal horseshoe crab environment, feeding and care would be required to pursue this alternative. Alternatively, if horseshoe crabs were allowed to reach maturity in the wild and transferred to native and protected estuary habitats with periodic monitoring, the species vitality could be improved and a better chance of survival might be achieved, as well as facilitate more controlled bleeding operations and schedules.

Notwithstanding the deleterious effects of wild capture and transport, the mechanism of blood harvest via a needle puncture to the arthrodial membrane could also cause unintended damage to the horseshoe crab circulatory system. No studies to date have systematically examined the effects of the puncture wound itself; however, anatomy of the area whereby the cardiac rhythm is controlled by ganglia suggests the potential for such punctures to interfere with normal function (Watson and Groome, 1989). Given a better understanding of the bleeding process, more advanced protocols could assess the potential for using indwelling catheters or alternative extraction sites.

Within this same paradigm, additional research focused on the optimization of bleeding volume and intervals could assess the potential to decrease horseshoe crab mortality and increase amebocyte yield. If bleeding horseshoe crabs in a controlled protocol (e.g., a temperature-controlled environment with the immediate return to their habitat) might correspond to human benefits from blood donation (Salonen et al., 1998), horseshoe crabs could potentially be bled more frequently with less trauma, while removing a smaller volume of blood per drawing. Also analogous to human plasmapheresis, the crab blood could be separated from the amebocytes and reinfused, or be replaced with a blood volume expander; this could alleviate hypovolemia while reducing stress and should allow for more rapid recovery.

Another important improvement to the bleeding process would be to minimize or prevent horseshoe crab hypoxia caused by extended periods outside the water. This could be accomplished by transporting the crabs in compatible tanks; employing wet covers, or towels, etc. (Novitsky, 2015). Less stressful transportation might also be achieved with temperature-controlled containers and/or by locating bleeding facilities closer to the harvest sites.

Further, using a formula to estimate the total hemolymph volume could help ensure that safer amounts of blood are extracted on an individual basis, rather than applying a broad standard to all crabs (Hurton et al., 2005). Well enforced restrictions on female horseshoe crab bleeding would also help mitigate any resulting behavioral changes; foster future spawning; and help stabilize egg production for migrating bird sustenance. Establishing optimal female to male bleeding ratios to manage commercial pressures associated with a greater yield from females would also help ensure necessary breeding ratios toward the species' long-term viability.

Improving the survival rate of horseshoe crab larvae into adulthood would likewise contribute toward replenishing the horseshoe crab population. With a characteristically high mortality rate the early stages of life for numerous marine species, approximately 0.001% of crabs survive through the first year (Carmichael et al., 2003; Sweka et al., 2007). Researchers have had some success from collecting horseshoe crab eggs, rearing them in a laboratory, and releasing the crabs as juveniles. However,

this approach has not been conducted on a large scale, and any objections to egg collection that might interfere with shorebird feeding would need to be addressed before advancing this notion to a broader initiative (Mishra, 2009; Schreibman and Zarnoch, 2009).

Finally, various steps could be employed to reduce fishing industry demands on wild horseshoe crab populations, such as alternative and/or synthetic baits for whelk and eel, which could be used in lieu of horseshoe crabs. Such alternatives utilizing reduced quantities of horseshoe crab have been researched and field-tested with encouraging results (Ferrari and Targett, 2003; Fisher and Fisher, 2006).

As horseshoe crabs harvested for bait have outnumbered biomedical counts in recent years (Atlantic States Marine Fisheries Commission, 2016), a reduction in bait harvest is vital for conservation of horseshoe crab populations. Yet, because biomedical harvesting does not typically result in immediate mortality, the full impact might be underestimated and unaccounted for once fatigued, traumatized, and sometimes dying horseshoe crabs are returned to their habitat. Given these factors, both bait and biomedical demands appear to pose unsustainable challenges to horseshoe crab populations. However, in the absence of a widely accepted alternative to the LAL test, and as it remains vital to global medicine, there are promising approaches that could be employed to lessen the impact and reduce the ultimate mortality from biomedical harvesting.

CONCLUSIONS

The unique characteristics of horseshoe crabs underpinning their irrefutable importance to medicine, environmental safety, and their role as a keystone species highlight an urgent and compelling need for conservation and sustainable practices. To date, horseshoe crab conservation has been largely unidimensional, with many of the regulations applying only to the commercial fishing industry (Berkson, 2009). However, ensuring the wellbeing of this enigmatic species—and those whose survival depends on it—requires a multi-faceted approach that combines informed and fair regulation; responsible and more innovative harvesting and bleeding practices; and a commitment to continued research in pursuit of viable alternatives to avert collapse, while working toward ultimately eliminating the demand for harvesting wild horseshoe crabs, entirely.

Moving forward, effective horseshoe crab management must also extend beyond traditional approaches (e.g., stock abundance, recruitment, and growth rates) and begin to incorporate interventional ecosystem strategies. New and improved operational protocols should be established scientifically and implemented universally. In October 2011, early steps were taken to establish a blueprint for Best Management Practices for the collection, bleeding and releasing of horseshoe crabs (Atlantic States Marine Fisheries Commission, 2011, 2013). The document was generated by the Horseshoe Crab Biomedical *ad hoc* Working Group and was comprised of experts from each of the key biomedical bleeding organizations. The early draft of the document showed promise, but it has yet to be updated based on further research, nor has it progressed beyond recommendations to enforcement. The ability to assess the value of a document has also been undermined by unpublished industry reporting, whereby horseshoe crab mortality is neither reported publicly nor tabulated empirically: it is merely assumed that 15% of the harvested crabs perish (Atlantic States Marine Fisheries Commisson, 2009).

While horseshoe crab populations have modestly stabilized in some regions (Smith et al., 2017), the International Union for Conservation of Nature (IUCN) has predicted declines of at least 30% over the next three generations (∼40 years) (Smith et al., 2016). In sharp contrast, the global demand for vaccines, pharmaceuticals and medical devices over approximately the same period will require an increasing supply of LAL. These dynamics pose significant uncertainties as to whether current harvesting levels can be sustained, much less meet projected demands. With particularly rapid development in vaccine production, global pharmaceutical and the U.S. medical device markets have already been trending toward 6–8% and 25% annual growth, respectively.

In addition to these projections, these challenges intensify if the Asian species continues to decline at its current rate (Gauvry, 2015). Utilized for analogous testing by Asian and Pacific-based pharmaceutical and medical device manufacturers in TAL assays, a shortfall in Asian horseshoe crabs could lead to a spike in the global demand for LAL or force adoption of costly, alternative testing methods. Dependent on these two species of horseshoe crabs that appear to be facing significant decline, the growth in vaccine production is especially problematic; whereby, a large percentage of endotoxin detection is also performed using TAL for vaccines destined for emerging markets (Gauvry, 2015). Despite recent, isolated recoveries, the IUCN forecast nonetheless suggests that the U.S. indigenous horseshoe crab biomass could not withstand the growth of the LAL market, much less absorb a shift from current TAL shares.

Thoughtful and conservative approaches are needed, but require a fair understanding of the threats. Regulators are faced with the paradox of managing a species to protect and maintain dependent shorebird populations; facilitate multibillion dollar eel, whelk and conch fisheries; and support the growing global dependence on an essential medical safety resource (LAL). These drivers are both environmental and economic. Indeed, progress and effective management will only be achieved once Best Management Practices are universally adopted and implemented; as public reporting is instituted; and as empirical data are gathered and tracked over time to inform industry and environmental regulatory oversight so as to ensure the viability of this ancient and essential species.

AUTHOR CONTRIBUTIONS

AD, TB, JK-G, and WA conceived of the presented idea. JK-G, WA, KD, RT-K, TB, and MG developed

the theory and performed primary research reviews. AD, KD, CK, and TB verified the materials and methods. TB encouraged AD, JK-G, KD, and WA to investigate the biomedical medial industry impact on horseshoe crabs. AD supervised the findings of this work. All authors prepared analysis of the primary research and contributed to the final review manuscript.

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Horseshoe Crab Aquaculture as a Sustainable Endotoxin Testing Source

*Rachel Tinker-Kulberg[1], Kristen Dellinger[1,2], Terry E. Brady[1], Lee Robertson[1], Jerrold H. Levy[3], Sarah K. Abood[4], Frank M. LaDuca[5], Christopher L. Kepley[1,2] and Anthony L. Dellinger[1,6]**

[1] Kepley Biosystems Incorporated, Greensboro, NC, United States, [2] Department of Nanoengineering, Joint School of Nanoscience and Nanoengineering, North Carolina A&T State University, Greensboro, NC, United States, [3] Department of Anesthesiology, Critical Care, and Surgery, Duke University School of Medicine, Durham, NC, United States, [4] Ontario Veterinary College, University of Guelph, Guelph, ON, Canada, [5] LADUCA Regulatory, Quality, Compliance and Scientific Advisors LLC, East Brunswick, NJ, United States, [6] Department of Nanoscience, Joint School of Nanoscience and Nanoengineering, University of North Carolina at Greensboro, Greensboro, NC, United States

Horseshoe crab (HSC) hemolymph is the source of Limulus amebocyte lysate (LAL), a critical component in sterility testing that ensures drug and medical device safety for millions of patients every year. Wild HSC populations have been declining as a result of its use as whelk and eel bait, environmental changes, and its capture and bleeding for hemolymph by the biomedical industry, thus posing significant risks to species viability and the LAL raw material supply chain. We designed a controlled aquaculture habitat to husband HSCs and evaluated the effects of captivity on health markers (e.g., amebocyte density, hemocyanin levels, and LAL activity). We found HSC aquaculture to be practicable, with routine hemolymph harvesting resulting in high LAL quality, while safeguarding animal well-being with 100% HSC survival. Further, low-impact hemolymph harvesting via an indwelling catheter revealed rapid amebocyte rebound kinetics after consecutive 10% hemolymph extractions. Sustainable supplies of LAL could also be adapted to address daunting trends in septicemia and antimicrobial resistance. LAL is uniquely sensitive and specific for gram-negative bacteria, which represent 70–80% of pathogens that typically lead to sepsis. However, erratic results associated with interfering substances plagued efforts to adapt LAL for clinical use in the past. We report the development of a new LAL-based assay that can detect gram-negative bacteria and endotoxins in human blood without interference using aquaculture-derived LAL. Based on this research, sustainable LAL production from aquaculture could potentially satisfy industry needs with a fraction of one year's current capture via year-round harvesting from a finite cohort of HSCs and expand raw materials supplies for potential future clinical applications.

Keywords: aquaculture, diagnostics, gram-negative bacteria, horseshoe crabs, Limulus amebocyte lysate, sepsis, sustainable

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***Correspondence:**
Anthony L. Dellinger
adellinger@kepleybiosystems.com; adellinger@gmail.com

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Abbreviations: ASMFC, Atlantic States Marine Fisheries Commission; BCA, Bicinchoninic acid assay; b/w, Body weight; CFU, Colony-forming unit; COCH, Cochlin; DO, Dissolved oxygen; HSC, Horseshoe crab; *E. coli, Escherichia coli*; EU, Endotoxin unit; g/mol, Grams per mole; kDa, kilodalton; kJ, kilojoules; Hc, Hemocyanin; LAL, Limulus amebocyte lysate; LB agar, Luria-Bertani agar; L-chain, Light chain; LPS, Lipopolysaccharide; mmol/L, Millimoles per liter; NaCl, Sodium chloride; nm, Nanometers; $N\text{-}NH_{3-}$, Ammonia nitrogen; Oxy-Hc, Oxyhemocyanin; PCR, Polymerase chain reaction; ppm, Parts per million; RAS, Recirculating aquaculture system; rcf, Relative centrifugal force; rFC, Recombinant Factor C; SOP, Standard operating procedure; Tris-HCl, Tris(hydroxymethyl)aminomethane hydrochloride; v/v, Volume/volume; w/v, Weight/volume.

INTRODUCTION

In 1983, the Limulus amebocyte lysate (LAL) assay was approved by the Food and Drug Administration (FDA) for pharmaceutical quality control testing and became the bacterial endotoxin test of choice for detection of gram-negative pathogens due to its simplicity, specificity, and sensitivity (Hochstein, 1990; Cooper, 2001). Early discoveries revealed instantaneous, rigid gel clot formation upon LAL exposure to lipopolysaccharides (LPS) at *parts per trillion* (Roslansky and Novitsky, 1991). The clotting cascade and LPS gel detection method are shown in **Figure 1**. As a highly toxic, heat-stable molecule, LPS comprises approximately 70% of the outer membranes of gram-negative bacteria. The unique sensitivity (0.05–50.0 EU/ml) and specificity of LAL characterize its potential for detecting LPS in human blood with important clinical indications as the most sensitive and rapid method for endotoxin detection (Food and Drug Administration [FDA], 1987; Hochstein, 1990) with specificity for up to 80% of pathogens that typically lead to sepsis (MacVane, 2017).

From a clinical perspective, a lack of tools for the early detection of blood-borne endotoxins, including LPS, often leads to inappropriate antibiotic administration (Baker et al., 2018). Antibiotics accelerate LPS shedding from the outer membrane of gram-negative bacteria and release inflammatory mediators (Jean-Baptiste, 2007). Early detection and timely, informed therapy could thus help decrease LPS levels during sepsis, potentially minimizing its toxic effects and improving patient survival rates (Berg and Gerlach, 2018); whereby, they increase by 7.6% per hour with appropriate antibiotic administration (Kumar et al., 2006). However, conventional laboratory methods for detecting bacteria in human blood have not advanced substantially since the advent of culture, which often requires 2–3 days for test results (Samuel, 2018).

Basal LPS levels are subject to significant variation due to an array of bacterial species and the preparation method used. Nonetheless, they are consistently higher in septic patients (0.96–3.45 EU/ml and 300 pg/ml) when compared to healthy subjects with ranges from 0.04 to 0.36 EU/ml and 5.1 pg/ml (Fleishman and Fowlkes, 1982; Casey et al., 1993; Bates et al., 1998; Ketchum et al., 1997; Strutz et al., 1999; Hurley et al., 2015). For FDA testing protocols, the acceptable safe (non-pyrogenic) endotoxin limit for injected and inhaled pharmaceutical products is ∼0.25–0.50 EU/ml (Williams, 2007; Hynes, 2016), which falls within the expected range of clinical insignificance.

However, LAL testing in human blood has been historically problematic, with considerable variability due to the presence of interfering substances that have either suppressed or spuriously activated the assay (Rowley et al., 1958; Keene et al., 1961; Oroszlam et al., 1966; Rudbach et al., 1966; Skarnes, 1966, 1968; Levin et al., 1970, 1971; Elin et al., 1975, 1976; Wardle, 1979; Freudenberg et al., 1980; Ulevitch et al., 1981; Yoshioka and Konno, 1984; Martel et al., 1985; Dawson, 1993; Elsbach and Weiss, 1993; Hurley, 1994, 1995; Marra et al., 1994; Gnauck et al., 2016). Early efforts to evaluate LAL for detection of endotoxins in patient blood were largely abandoned due to a lack of standardized procedures that often included extensive specimen manipulation (McCabe et al., 1972; Martinez et al., 1973; Stumacher et al., 1973; Feldman and Pearson, 1974; Elin et al., 1975; Zinner and McCabe, 1976; Gnauck et al., 2015). In particular, one published LAL method requires substantial sample dilution (up to 40-fold; Lonza Walkersville Inc, 2014), denaturing (heating at 70°C), and the addition of chemical inhibitors; whereas, dilution can limit sensitivity and overall complexity would lessen practicality as a rapid patient screening assay (Obayashi, 1984; Dawson, 2005; BioDtech Inc., 2014; Lonza Walkersville Inc, 2014). Anticoagulants, chemical inhibitors, and temperature-induced pH changes of the blood after preparation have also been shown to interfere with the reliability of the LAL assay (Gnauck et al., 2016). Further, the ratios and function of the clotting components in pooled LAL reagents from random wild horseshoe crab (HSC, *Limulus polyphemus*) harvesting are variable, which can affect reaction kinetics, LPS sensitivity, and result in batch-to-batch variation (Massignon et al., 1996).

From a conservation perspective, a clinical application for LAL may not be feasible, as the natural LAL resources are already strained by pharmaceutical sterility testing. Annually, some 600,000 HSCs are captured and bled to meet LAL demand, of which some 30% expire in the process (Atlantic States Marine Fisheries Commission [ASMFC], 2019). Current threats to wild HSC populations include not only biomedical harvesting but also habitat encroachment, global warming, and its use as bait for eel and whelk fisheries, which have negatively impacted population numbers (Krisfalusi-Gannon et al., 2018). The uncertain future of HSCs and the importance of LAL to modern medicine have prompted innovations for sustainable HSC cultivation (Krisfalusi-Gannon et al., 2018). As such, aquaculture has become a promising approach to restore dwindling populations. However, long-term efforts to husband adult HSCs have been largely unreported or unsuccessful for the last three decades (Carmichael and Brush, 2012). After 3–4 months of husbandry, research has indicated that hemolymph protein concentrations drop below reference ranges (3.4–11.8 mg/ml) and result in mortality, which is thought to be linked to nutritional deficiencies that give rise to panhypoproteinemia (Nolan and Smith, 2009).

The first objective of this study was to develop and optimize an indoor recirculating aquaculture system (RAS) for captive HSC husbandry that would facilitate repetitive LAL harvesting while maintaining animal wellbeing. A surgically implanted catheter was developed to achieve a low-impact, routine harvesting method. In this study, hemocyanin (Hc), amebocyte, and LAL activities were analyzed to examine the effect of aquaculture on adult HSCs. These health parameters play an essential role in immunity to defend against pathogens and ensure the overall health of the HSCs (Nagai et al., 2001). The rationale behind this objective was that controlled aquaculture could provide an alternative to current practices and foster conservation by establishing a more sustainable endotoxin testing resource from a finite cohort of captive HSCs. The expansion of LAL resources toward clinical medicine should also emphasize responsible methods of raw material collection. The establishment of an aquaculture model that could produce greater volumes of LAL reliably, sustainably, and economically would benefit nature and industry. Carefully controlled collection of LAL from monitored

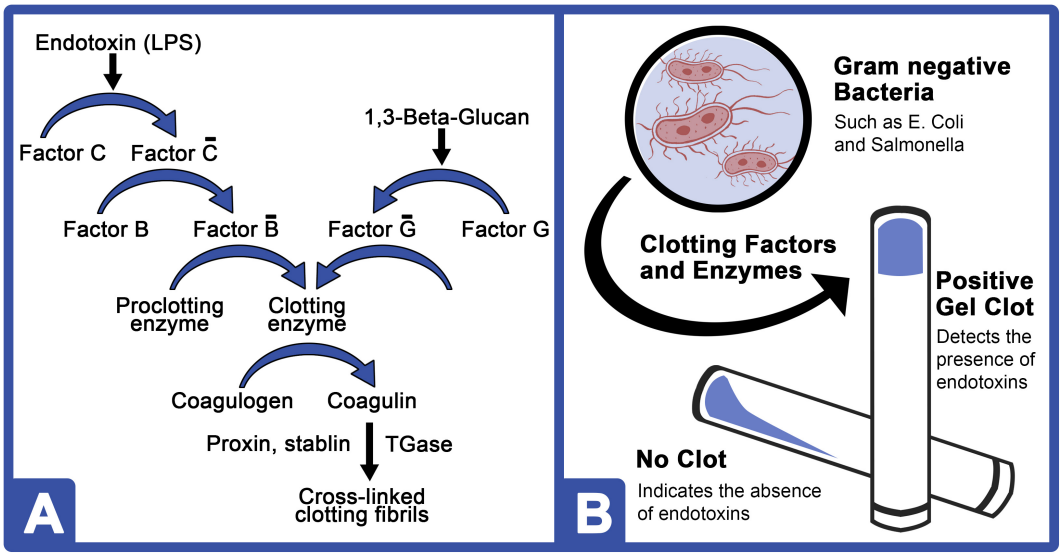

FIGURE 1 | LPS and 1,3-β-D-glucan clotting cascades of the HSC amebocyte lysate. **(A)** Bacterial endotoxins (LPS) activate Factor C (C-bar), which then activates Factor B (B-bar). Activated Factor B converts the proclotting enzyme to a clotting enzyme and cleaves two coagulogen peptide bonds to form an insoluble coagulin gel. A similar pathway is shown for 1,3-β-D-glucan, which comprises the cell wall of most invasive fungal pathogens. **(B)** Schematic of a typical LPS-mediated LAL gel clot assay.

and well-maintained HSCs in aquaculture could increase LAL supply quantities, ensure species viability, and allow for new clinical innovations. The second objective of this study was to demonstrate the potential use of aquaculture-derived LAL as a rapid, reliable, and cost-effective method for detection of LPS as a marker for gram-negative bacteria that overcomes erratic results heretofore characteristic of testing human blood with LAL assays.

MATERIALS AND METHODS

Husbandry in RAS

The indoor management and maintenance of HSCs was evaluated in a pair of RAS at a salinity of 19.5–22.0‰. Each RAS was equipped with two holding tanks (4 ft × 6 ft × 1 ft), a biofiltration tank with K1 media (Pentair, Minneapolis, MN, United States), and a clarifying (solids separation) tank (**Figure 2**). Ambient air supplied oxygen to the water via a piston air pump with supplemental air stones positioned in each tank (Nelson and Pade, Montello, WI, United States). Water was recirculated through biological filtration tanks, allowing for water purification, hygiene, and disease prevention. Water parameters were measured and adjusted as needed six times per week over the course of the study.

HSCs were stocked at a density of one animal per 3 ft^2. An isolated circular resin tank (diameter: 3.25 ft) equipped with a solids filter was used as a "hospital" tank to quarantine HSCs prior to RAS introduction and allow for recovery after catheter surgery (see Catheter Implantation section). HSCs (n = 24) were procured (Dynasty Marine Associates, Marathon, FL, United States) or acquired and maintained in accordance with collection and aquaculture operation permits (North Carolina Department of Environmental Quality, Division of Marine Fisheries; #1946771 and #1947050). A health assessment was recorded for each HSC upon intake (i.e., sex, weight, and carapace length/width, as well as appendage, carapace, eye, and mouth evaluation). HSCs sourced directly from the wild ("wild type") were used as controls for some studies, from which hemolymph extracts were collected within 1 h of habitat removal to establish baselines.

In order to ensure HSC well-being and optimal hemolymph parameters, diet and feeding rates were established over a trial period of 6 weeks prior to the initiation of the study. Feed

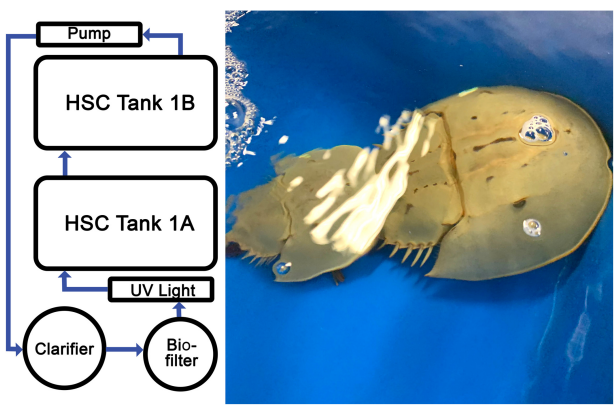

FIGURE 2 | Recirculating aquaculture system (RAS). Temperature-controlled, saltwater system optimized for HSC aquaculture comprising multiple tanks, filtration units, UV sterility lights, and aeration system. Photograph of two HSCs in the RAS engaging in typical breeding behaviors.

rates, dietary protein (DP), nutrient composition, and daily energy requirements were determined for natural feed sources and a commercially manufactured aquafeed (Classic Brood 46/12 Complete Feed, Skretting, Tooele, UT, United States). Initial observations revealed that HSCs fed at different rates per day (expressed as a percentage of the b/w of the HSC) depending on the diet (Tinker-Kulberg et al., under review). In this study, the nutrient composition of the selected commercial aquafeed after modification with gelatin (5% w/v) included protein (23%), lipids (6%), carbohydrates (2%), and moisture content (50%). Gelatin served as a binding agent to provide rigidity, extended water stability and nutrient retention; thus eliminating buoyancy (for sinking density) and affording HSCs time to forage on the tank floor to accommodate characteristic bottom scavenging. A daily feed rate of 3% of their b/w sustained (or increased) HSC weight (∼1.2% change on average) and serum protein levels (above 5.0 mg/ml) over the course of the study, with an associated survival rate of 100%. Any feed not consumed after 8 h was removed. Sodium bicarbonate was added to the RAS at 0.12 g per 1.0 g of feed to maintain alkalinity and pH.

Catheter Implantation

The catheter was composed of a radiopaque tube and a valve. For implantation, each HSC was gently immobilized, and the book gills were bathed with saltwater. Protective draping surrounded access to the pericardial membrane, and the region was prepped with 70% ethanol and betadine. An 18-gauge needle on the intravascular system was inserted at a 45° angle toward the prosoma (cephalothorax). Once the appearance of hemolymph was observed in the catheter, the guidewire was inserted and the device positioned. The needle was then removed; the site was cleaned; and a sterile, quick-drying topical tissue adhesive (Vetbond^{TM}, 3M, St. Paul, MN, United States) was applied. The catheter was capped with a sterile luer-lock, and it was affixed to the shell of the HSC in the dorsal prosoma region. Postoperative HSCs were monitored during recovery in the hospital tank. Using the implanted catheter, hemolymph was extracted for an initial health evaluation after 7 days, and the HSCs were reintegrated into the RAS cohort.

Hemolymph Collection

Hemolymph was collected via previously established methods for both the implanted and control cohorts (Coates et al., 2012; Anderson et al., 2013). Materials were rendered pyrogen-free by cleaning and overnight soaking in 1% E-Toxa-Clean(R) Solution (Sigma-Aldrich, St. Louis, MO, United States), heating at 200°C for 3 h, and rinsing with endotoxin-free water. For the control group without catheter insertion, the pericardial membrane was cleaned twice with 70% ethanol prior to hemolymph collection with a 22-gauge needle (Becton Dickson, NJ, United States) using a standard SOP (Armstrong and Conrad, 2008); for the implanted HSCs, it was collected using the catheters. An aliquot of the hemolymph was diluted (1:1) in a prechilled mixture of *N*-ethylmaleimide (0.125%), NaCl (3%), and Tris-HCl (0.5 M, pH 7.5) for amebocyte density analysis (Armstrong and Conrad, 2008; Coates et al., 2012; see Hemocyanin and Amebocyte Density section). Sterile tubes containing the balance of each harvest were centrifuged (1,000-rcf/5-min), and the supernatant was removed. Any samples that appeared clotted after centrifugation were not processed or analyzed. Amebocyte pellets were then washed with endotoxin-free NaCl (3% v/v), and both fractions were stored at −70°C pending analysis and LAL extraction.

Over the course of this research, to monitor the health of individual HSCs, 1 ml of hemolymph was extracted and analyzed for Hc concentration, amebocyte density, and LAL activity. For hemolymph extractions (e.g., 10%), blood volume was calculated as 25% (b/w), and the density was estimated at 0.00104 kg/ml (Hurton et al., 2005).

Hc and Amebocyte Density

Hc and amebocyte density were measured throughout the study as an indicator of HSC health. Total serum protein concentration was used as a proxy for Hc by diluting the hemolymph supernatant in Tris-HCl (0.1 M, pH 7.5) and measuring absorbance at 280 nm on a UV-Vis Spectrometer (Cary 6000i, Agilent Technologies). Dioxygen-bound Hc (Oxy-Hc) was likewise measured at 340 nm. The total protein concentration was calculated according to Nickerson and Van Holde (1971), using the absorption coefficient A_{280} = 1.39 mg^{-1} ml^{-1} cm^{-1} for Hc, and A_{350} = 0.223 mg^{-1} ml^{-1} cm^{-1} for Oxy-Hc. The percentage of Hc that was oxygen bound was determined using the ratio of Oxy-Hc (mg/ml) to Hc (mg/ml) × 100.

A 10 μl volume of the diluted aliquot was placed in a hemocytometer and analyzed microscopically. Since amebocytes are susceptible to rapid degranulation, a digital camera was used to record bright-field images from the hemocytometer, and counts were processed off-line using the ImageJ software (National Institutes of Health, Bethesda, MD, United States).

LAL Preparation and Analysis

LAL activity was assessed throughout the study as an indicator of HSC health and to investigate the effects of aquaculture on quality. LAL was prepared by thawing frozen amebocyte pellets on ice and then lysing them in pyrogen-free water at a 1:1 ratio of original hemolymph collection volume under gentle agitation over night at 4°C. Next, a chloroform extraction (1:1 v/v) of the lysate was performed for total protein measurement using a BCA kit (Pierce, Thermo Fisher Scientific, Waltham, MA, United States). All LAL extracts were aliquoted and frozen at −80°C pending analysis (no loss in LAL activity was observed in frozen samples for up to 6 months). Equal protein amounts of the LAL from aquaculture HSCs and commercial kits (E-Toxate^{TM}, Sigma-Aldrich, St. Louis, MO, United States) were diluted to a total volume of 100 μl and tested for their respective and relative activities. All evaluated LAL samples were subjected to a single freeze–thaw cycle to preserve activity.

LAL activity was evaluated turbidimetrically. The commercial kit served as a control, and assays were performed following the manufacturer's instructions (Sigma-Aldrich, St. Louis, MO, United States). Aquaculture and commercial LAL (100 μl) samples were gently mixed with 100 μl of standard endotoxin solution (final concentration range: 0–50 EU/ml) in a pyrogen-free, 96-well microplate and incubated at 37°C for 1 h. The

conversion of coagulogen to coagulin was measured at 340 nm on a microplate reader (BioTek 800™, BioTek Instruments, Winooski, VT, United States). The turbidity of the blank (endotoxin-free lysate) was subtracted from test values, and the relationship between endotoxin concentration and clot formation was measured against a standard curve derived from commercial LAL.

Equal amounts of amebocyte total protein isolated from the individual HSCs were pooled and analyzed for LAL activity in triplicate. A turbidimetric LAL assay was performed by incubating a standard endotoxin solution (50 EU/ml, Sigma Aldrich, St. Louis, MO, United States) with different concentrations of LAL (0–300 μg). Hemolymph extracted from wild-caught HSCs ("wild type") was collected within 1 h of capture, from which LAL was processed and frozen at −80°C pending analysis and then used as a control; results were also compared to a commercial LAL kit (E-Toxate™, Sigma Aldrich, St. Louis, MO, United States). Both controls were diluted to the same concentration as the aquaculture LAL.

Purified protein extracts (10 μg) from commercial, aquaculture, and wild-type LAL were subjected to SDS-PAGE in the presence of β-mercaptoethanol. The total protein concentration of each sample was determined with a BCA assay. Each sample was diluted in a 4 × NuPAGE™ loading buffer (Invitrogen, Carlsbad, CA, United States) containing 2.5% β-mercaptoethanol and heated to 85°C for 3 min. Electrophoresis was performed on 8% tris-glycine gels (Novec™ Wedge Well, Invitrogen, Carlsbad, CA, United States) and stained with Coomassie Brilliant Blue R-250 (Bio-Rad, Hercules, CA, United States). A SeeBlue® Plus2 Pre-stained Protein Standard (Invitrogen, Carlsbad, CA, United States) was used as a molecular weight marker.

Aquaculture LAL Assay of Human Blood Specimens

To test the ability for LAL to detect endotoxins (LPS) in human blood, presumably endotoxin-negative human blood was freshly drawn from healthy volunteers using citrated collection tubes (3.2% buffered sodium citrate to inhibit clot formation). Aliquots were then spiked with various concentrations of LPS (*Escherichia coli* 055:B5, World Health Organization [WHO], 2012) or left neat as negative controls and incubated at room temperature for 15 min (to allow unbound LPS to disperse so as to mimic circulating endotoxins in patient specimens). Before LAL analysis, the samples were centrifuged; aliquots were removed and chemically treated with a matching volume of endotoxin-free reagents (to that of the initial volume) to remove blood components known to interact with LPS and inhibit LAL activation. LAL assays were then performed as described above and demonstrated consistent spiked bacterial concentrations.

To evaluate detection of intact bacteria in human blood, exponential growth of *E. coli* (Turbo Competent) was selectively achieved in LB broth containing 100 μg/ml of ampicillin to an optical density of 0.35. The culture was centrifuged to pellet the bacteria and washed twice to remove unbound LPS. A sample containing only the LB growth medium in the absence of bacteria was also processed in parallel, to account for any LPS contamination (negative "broth" control). Samples were then resuspended in isotonic buffer (0.9% NaCl), and a bacterial count (CFU/ml) was performed by plating serial dilutions on LB agar plates containing 100 μg/ml of ampicillin. Blood samples were then spiked with equal volumes of bacteria at different concentrations or with the negative "broth" control and incubated at RT for 15 min. All samples were chemically treated as with the LPS-spiked blood samples and then frozen at −70°C for 1 h to destabilize the *E. coli* cellular membranes. Samples were then thawed at RT, and LAL assays were performed.

LPS-spiked blood (0–50 EU/ml), *E. coli*-spiked blood (0–5.0×10^5 CFU/ml), and controls were incubated with LAL at 37°C for 1 h at a 1:1 dilution, and clot formation was evaluated. A standard LAL gel clot assay with LPS (0–50 EU/ml) or *E. coli* (0–5.0×10^5 CFU/ml) was also performed as a positive control. A clot remaining at the bottom of the tube after a gentle 180° inversion was considered positive, whereas if the sample remained liquid and flowed down the tube, the assay result was considered negative. Clots were rated as follows: firm, opaque gel remaining stable after 180° inversion (++); soft gel with moderate to considerate opacity and tube adhesion when rotated 90°; weak gel with slight-to-moderate opacity and adhesion of starch-like floccules to sides of the tube when slanted (±); and no visible increase in viscosity or opacity (–) (Bishop and White, 1986).

Data Analysis

Individual HSC cohorts were used for each respective analysis. HSC Cohort A ($n = 6$) was used to measure differences in Oxy-Hc and amebocyte density over an 88-day period. HSC Cohort B ($n = 6$) was used to measure LAL activity in aquaculture HSCs (after 88 days of husbandry) in comparison to LAL from wild-type HSCs and a commercially available LAL kit (E-Toxate™, Sigma-Aldrich). HSC Cohort C ($n = 4$) was used to measure differences in amebocyte density, Hc concentrations, and LAL activity (after 6 months of husbandry) for the 10% bleed study. Cohort D ($n = 4$) served as a no-bleed control to the 10% bleed studies.

Individual HSC data (Oxy-Hc and amebocyte density) varied slightly from animal to animal but with a low standard deviation. The LAL activity derived from individual HSCs was also similar, with low standard deviation. For simplicity and assay accuracy, LAL activity was measured using pooled samples from the entire aquaculture cohort, composed of equal amounts of proteins from individual HSCs. The standard deviation reported for LAL activity represents each assay, performed in triplicate.

RESULTS

HSC Aquaculture Husbandry

The RAS used for this HSC study was designed for conventional aquaculture operations and scalability using standard equipment. Specifically, it was configured to house 24 HSCs, with six in each of four groups. The RAS design, methods, and HSC viability with respect to repetitive bleeding and catheter tolerance were established during the study period (**Figure 2**).

Determining and maintaining optimal water quality parameters were especially important in assessing the feasibility of an indwelling catheter (**Figure 3**). Water temperatures were between 17.1 and 21.7°C (desired range: 15.0–25.0°C); pH was maintained from 7.5 to 8.0 (desired range: 7.5–8.8); and dissolved oxygen (DO) remained within 6.3–11.1 mg/L (desired range: >3.0–5.0) across all tanks. The target ammonia concentration was established at <1.0–3.0 ppm, with the average calculated at 0.732 (± 0.206) ppm across the study; and it stayed within the determined cautionary range (Timmons, 1994; Schreibman and Zarnoch, 2009; Ebeling and Timmons, 2010; Shelley and Lovatelli, 2011).

All HSCs in the RAS thrived throughout a 12-month period, as measured by biologic markers, including LAL activity, bleeding frequency, and breeding behaviors (i.e., copulation and sperm and egg release) and remained healthy throughout the investigation (100% survival). Water parameters did not change significantly throughout the course of the study at the calculated HSC density and feeding rate. Immediate and long-term action plans for system perturbations were established and deemed convenient and effective. The results indicated that HSC RAS husbandry is practicable, that management protocols can be easily executed using conventional equipment, and that available feed inputs are nutritionally robust and affordable (feed cost per pound: $0.78).

HSC Health Assessment After Catheter Implantation

Intravascular catheters were inserted into the respective pericardial membranes of the HSCs, which allowed for routine hemolymph collection up to three times per month (see HSC Health Assessment After Repetitive Hemolymph Collection section; **Figure 7**) without repetitive membrane punctures (**Figure 4**). The procedure and device design were optimized over time and well tolerated by the HSCs, as all survived catheter implantation and continued to demonstrate robust health after surgery, as reflected in subsequent testing. The following parameters were analyzed at predetermined intervals (see HSC Health Assessment After Repetitive Hemolymph Collection section) throughout the study: total serum protein concentration; amebocyte concentration (cells/ml); and the LAL activity of amebocyte extracts in response to LPS.

Hemocyanin concentration was examined over the course of 88 days (HSC Cohort A; n = 6). The proportion of Hc with dioxygen-bound Hc (i.e., Oxy-Hc) is an indicator of health status, and it remained within a desirable range of 85–95% across all HSCs (**Figure 5A**). Amebocyte concentration was used to assess immune function and as an indication of well-being versus stress (Coates et al., 2012). The HSC amebocyte density levels remained high throughout the study and correlated with optimal vitality (**Figure 5B**; Day 0: 1.806E^{+07}; Day 88: 2.093E^{+07}).

The aquaculture LAL (HSC Cohort B; n = 6) had similar activity to that of the wild HSCs, and both extracts revealed slightly increased activity compared with the lyophilized and preserved LAL from the commercial kit (although values were

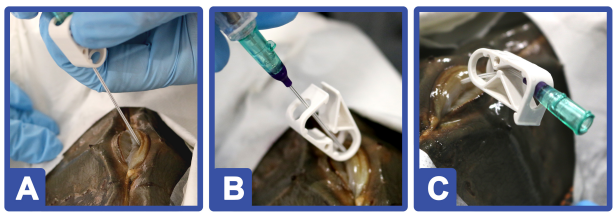

FIGURE 4 | HSC catheter implantation: **(A)** An 18-gauge needle on an intravascular catheter is inserted into the pericardial membrane toward the prosoma. **(B)** Once hemolymph was observed in the catheter tubing, a guidewire was deployed. **(C)** After the catheter was positioned, the needle was retracted; the insertion site cleaned; and a topical adhesive applied. HSCs (n = 12) were monitored in a "hospital" tank for 7 days before returning to the RAS.

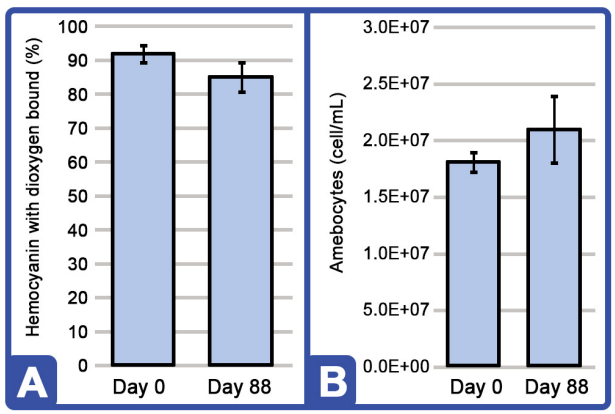

FIGURE 5 | HSC dioxygen-bound hemocyanin (Oxy-Hc) and amebocyte density stability throughout the 88 days study. **(A)** Percentage of Oxy-Hc from aquaculture HSC hemolymph RAS. **(B)** Aquaculture HSC amebocyte density concentration. Results are represented as mean ± SD between individual HSCs in Cohort A (n = 6).

Water Parameters	Safe	Indoor
Ammonia, Total (ppm; Daily)	> 1 - 3	0.73
Carbon Dioxide (ppm; Daily)	< 10	0
Dissolved Oxygen (mg/L; Daily)	> 3 - 5	8.30†
pH (Daily)	7.5 - 8.8	7.64
Water Temperature (C; Daily)	15 - 25	19.9‡
Nitrate (ppm; Bi-weekly)	15 - 150	40.6
Nitrite (ppm; Bi-weekly)	< 2	5.5
Alkalinity (ppm; Monthly)	75 - 175	120-160
Calcium (ppm: Monthly)	25 - 100	207
Hardness (ppm; Monthly)	40 - 150	120
Salinity (ppt; Monthly)	15 - 35	20.55

FIGURE 3 | RAS water quality parameters and testing. Measurements were performed daily, weekly, biweekly, or monthly, as indicated. †Average dissolved oxygen range: 6.3–11.1 mg/L. ‡Average temperature range: 17.1–21.7°C.

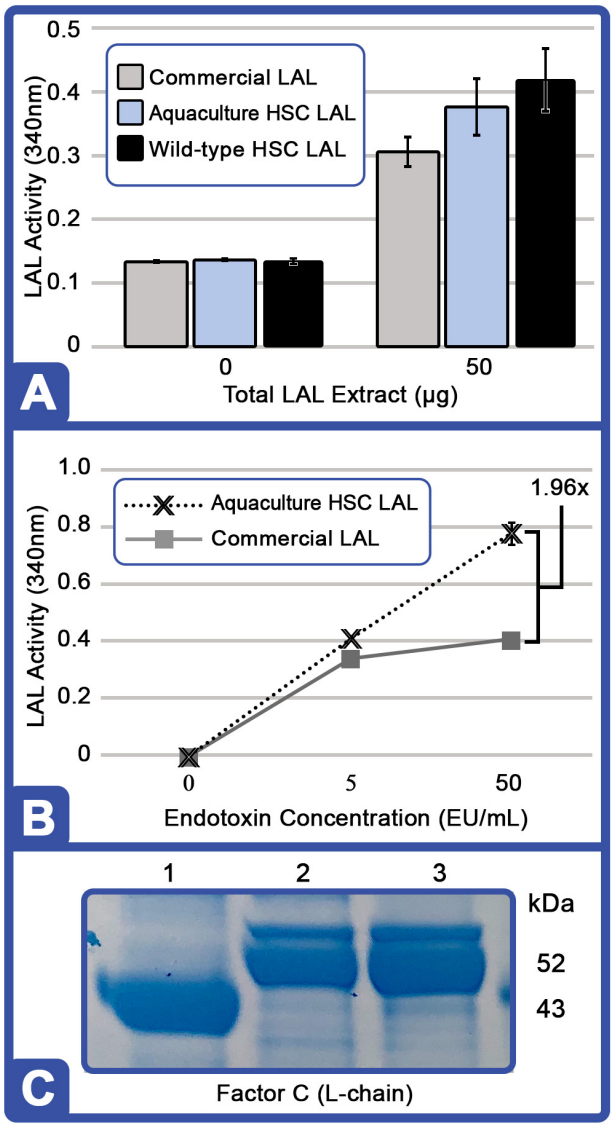

FIGURE 6 | Evaluation and protein analysis of aquaculture-derived LAL at Day 88. **(A)** Degree of gel clot formation (measured at 340 nm) based on the total protein content of amebocyte extracts (50 µg) using lysates prepared from aquaculture HSC (equal amounts of protein pooled from individual HSC in Cohort B; n = 6); freshly removed from wild-captured HSC (wild-type); or a commercial kit (Sigma E-Toxate™). The total LPS concentration used for each was 50 EU/ml. **(B)** Degree of clot formation (measured at 340 nm) of aquaculture LAL (150 µg total protein, equal amounts of protein pooled from individual HSC in Cohort B; n = 6) compared to a commercial kit (150 µg protein) at different endotoxin concentrations: 0, 5.0, and 50.0 EU/ml. **(C)** Coomassie blue staining of crude protein lysate (10 µg per well): commercial LAL (Lane 1), aquaculture LAL (Lane 2), and wild-type LAL (Lane 3). Aquaculture LAL is representative of equal amounts of protein pooled from individual HSC in Cohort B (n = 6). Molecular weight marker (SeeBlue™ Plus2; Invitrogen, Thermo Fisher Scientific) is indicated.

not determined to be statistically significant; **Figure 6A**). The activity of aquaculture LAL remained constant throughout the 88-day culture period (data not shown). At higher concentrations (150 µg), the enzymatic activity of aquaculture LAL was twofold higher than that of the commercial LAL at an LPS concentration of 50 EU/ml (**Figure 6B**). These findings suggest that fresh aquaculture lysates may contain a greater abundance of clotting factors per microgram of total extract than that contained by commercial sources, as evidenced by clot formation after 1 h of incubation and as LPS concentrations become saturated, typical of a zero-order kinetic reaction.

Crude LAL derived from the aquaculture cohort (HSC Cohort B; n = 6) and the wild control demonstrated similar protein banding; however, different patterns were also observed with the commercial LAL using Coomassie-stained tris-glycine denaturing gel (**Figure 6C**). One protein band, identified as the L-chain of Factor C, migrated faster in the commercial LAL (∼43 kDa) than in the aquaculture LAL (∼52 kDa), which was confirmed via Western blotting with rabbit polyclonal antibody (COCH, ABclonal, Inc., Woburn, MA, United States; data not shown). Factor C typically generates a 43 kDa subunit (L-chain) under denaturing conditions (Shibata et al., 2018), and thus, the aquaculture-derived Factor C L-chain subunit could migrate at a higher molecular weight due to posttranslational modifications. As lysates prepared from freshly harvested wild HSCs revealed, this higher molecular weight form of Factor C does not appear to be a consequence of aquaculture but rather an effect of commercial LAL preparation (**Figure 6C**).

HSC Health Assessment After Repetitive Hemolymph Collection

The effects of aquaculture on Hc and amebocyte rebound kinetics were evaluated after repetitive HSC harvests (10% of total hemolymph volume). Hemolymph was drawn at three intervals: Bleed 1 (10% hemolymph volume at time = 0); Bleed 2 (10% hemolymph volume at time = 16 days); and Bleed 3 (1 ml of hemolymph at time = 23 days, health assessment; **Figure 7**). HSC amebocyte density, total serum protein concentration, and LAL activity were thus measured to assess the impact of consecutive bleeding at t_0 = 0 days, t_1 = 16 days, and t_3 = 23 days (n = 4; **Figure 7**).

Average serum protein levels recovered after each successive 10% hemolymph extraction (t_0 vs. t_1 = 10.2% Δ; t_1 vs. t_2 = 6.7% Δ; **Figure 7A**). Amebocyte density decreased slightly (−10.7% Δ) after the first collection (t_0 vs. t_1) and rebounded above baseline after the second (12.8% increase between t_1 and t_2, however; this increase was associated with a higher standard deviation among individual HSCs). LAL activity was notably higher after the second instance over the 7-day recovery period (t_0 vs. t_1 = −14.9% Δ and t_1 vs. t_2 = + 5.0% Δ; **Figure 7C**). As a "no-bleed" control, a health assessment specimen (1.0 ml) from a second HSC cohort (n = 4) was taken at the same time and analyzed in parallel, with no significant change in hemolymph parameters (data not shown).

Detection of Endotoxins and Gram-Negative Bacteria in Human Blood

Aquaculture LAL was evaluated for detection of endotoxins (LPS-spiked) in chemically treated blood samples and compared

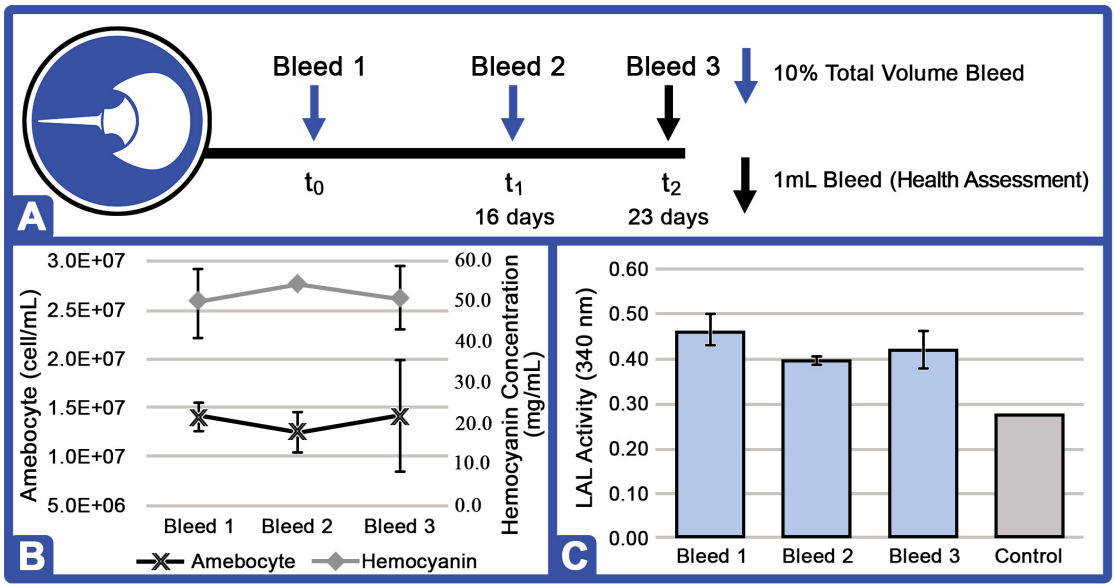

FIGURE 7 | Amebocyte density, hemocyanin concentration, and LAL activity of aquaculture HSC after repeat hemolymph collection. **(A)** After 6 months of aquaculture, each HSC (n = 4) was bled at t_0 and t_1 (16 days after t_0) for 10% total hemolymph volume and returned to the RAS, and a final health assessment sampling (1 ml bleed) was performed at t_3 (23 days after t_0). **(B)** Representative HSC amebocyte density and hemocyanin concentration. **(C)** Aquaculture HSC LAL activity after bleeds compared to control (commercial kit LAL; Sigma E-Toxate™) at the same protein concentration (300 μg protein) exposed to 50 EU/ml of LPS.

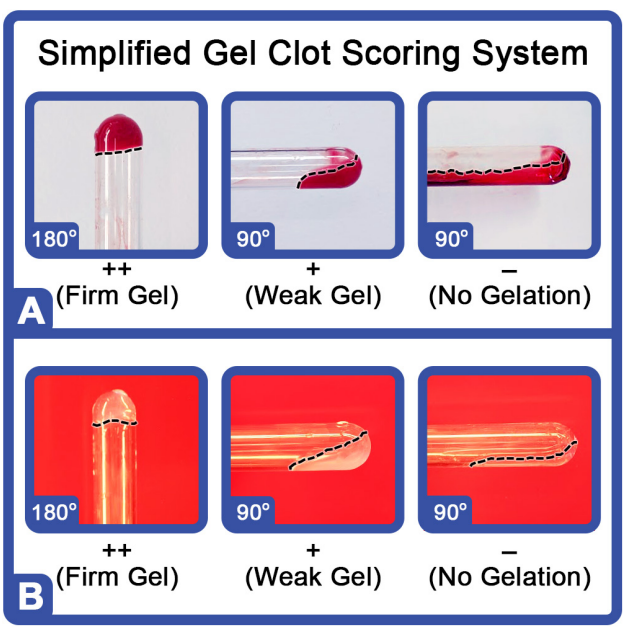

FIGURE 8 | Visual scoring of gelation of LAL in the presence of LPS or bacteria. Representative results of LAL in the presence of LPS or bacteria in **(A)** human blood or **(B)** a standard control (endotoxin-free water). The gel clot detection range of 0.05–50.0 EU/ml was used in the test. Clot ratings: firm, opaque gel remaining stable after 180° inversion (++); soft gel with moderate to considerate opacity and tube adhesion when rotated 90° (+); weak gel with slight-to-moderate opacity and adhesion of starch-like floccules to sides of the tube when slanted (±); and no visible increase in viscosity or opacity (–) (Bishop and White, 1986).

to commercial standards. The previously described method (Bishop and White, 1986) to evaluate clot integrity was followed [**Figure 8**: firm gels (++); soft gels (+); weak clots (±); and no gelation (–)]. LPS-spiked blood samples and LAL commercial standards yielded comparable degrees of gelation from 0.5 to 50.0 EU/ml (**Table 1**). Strong clots (++) formed at endotoxin levels of 12.5–50 EU/ml; soft gel clots (+) formed at 3.125–6.25 EU/ml, and weak gel clots (±) formed at 0.5 EU/ml in both LPS-spiked blood samples and LAL commercial standards, while at the lowest concentration of 0.05 EU/ml, no gel clots were discernible [(–) clot rating; **Table 1**]. The neat (negative) blood samples remained viscous yet revealed no gelation (–).

Human blood with and without anticoagulant (heparin) demonstrated similar results: chemical treatment of the blood was required in order to detect LPS; whereas gel clots

TABLE 1 | Summary of gel clot assays for LPS detection in human blood.

LPS concentration	Clot rating
	Human blood
50.000 EU/ml	++
25.000 EU/ml	++
12.500 EU/ml	++
6.250 EU/ml	+
3.125 EU/ml	+
0.500 EU/ml	+/–
0.050 EU/ml	–
0 EU/ml	–

TABLE 2 | Summary of gel clot assays for *Escherichia coli* detection in human blood.

***E. coli* concentration**	**Clot rating**	
	Human blood	**Control**
5×10^5 CFU/ml	++	++
5×10^4 CFU/ml	++	++
5×10^3 CFU/ml	++	–
5×10^2 CFU/ml	++	–
50 CFU/ml	++	–
5 CFU/ml	++	–
1 CFU/ml	++	–
0 CFU/ml	–	–
Broth alone	–	–

did not form in untreated blood samples (with or without anticoagulant) across the range of endotoxin concentrations (up to 50 EU/ml). This was consistent with previous studies showing that human blood inhibits LAL reactions (Martel et al., 1985; Lonza Walkersville Inc, 2014).

When *E. coli* was added to *treated* blood at concentrations between 0.5 and 500,000 CFU/mL, a firm gel clot formed [(++) clot rating; **Table 2**]. Interestingly, bacterial concentrations lower than 5,000 CFU/ml were not detectable in the absence of treated blood. To validate whether the treatment altered LPS activation of LAL, the LPS (0.05–50 EU/ml) was also added *following* chemical treatment, and clot formation was consistent with that of LPS spiked-blood samples prior to treatment (data not shown). Blood spiked with both free LPS and bacteria (exponential-phase) activated LAL clot formation (**Tables 1**, **2**) with no false positives or false negatives observed. The "broth" control (no bacteria) did not form a gel clot, also suggesting that the bacterial growth medium was sufficiently washed away from the bacteria in the procedure and did not cause a false positive in the bacteria-spiked samples (**Table 2**).

DISCUSSION

Current wild harvest practices typically correspond with the HSC spawning season (May and June), when they swarm beaches and allow for easy collection, despite subsequent exposure to the attendant temperature and potential hypoxia variables associated with boat and truck transport to and from bleeding facilities. This period represents an especially vulnerable metabolic phase for the HSCs, whereby coastal migration and breeding activities (laying and subsequently fertilizing millions of eggs) are energy intensive (Sullivan and Watson, 1974; Leschen and Correia, 2010; Malkoski, 2010; James-Pirri, 2012; Anderson et al., 2013; Owings et al., 2019). These factors combine at the most perilous time to subject HSCs to bleeding and hemolymph depletion from a migratory, reproductive, and ultimately, LAL resource quality perspective. They further underscore the rationale for end-users and regulators to require all commercial LAL to be sustainably sourced from aquaculture by a date certain upon scale-up for the sector.

Our results demonstrate the potential to practicably harvest hemolymph from aquaculture HSCs (two distinct 10% extractions per month or potentially 12–24 times annually), in contrast to singular, commercial harvest requiring wild capture, extended habitat extraction, puncture and bleeding (typically 30% of hemolymph volume), and transport back to the shore during spawning season. Every HSC received ample nutrition before and after *in situ* hemolymph harvest that was completed within minutes, rather than days outside the water, allowing for a more rapid recovery while eliminating hypoxia and myriad transport and habitat extraction risks.

HSC vitality was demonstrated, as reflected by stable Hc levels, amebocyte density, and aquaculture LAL activity (**Figures 5**, **6**) over the course of the study. Both diet and water quality in indoor aquaculture were shown to be controllable and optimized while yielding well-fed, vigorous HSCs. Moreover, aquaculture demonstrated enhanced reactivity when compared to that in commercial kits, which may be an indicator of healthier and less stressed animals in an optimized environment. Aquaculture also enabled repetitive, low-impact bleeding via an indwelling catheter proximal to the water, thereby eliminating the risks of repeat membrane puncture, transport, and lengthy extraction periods likely to account for the current loss of up to 30% of the harvested wild crabs each year (Hurton and Berkson, 2006; Leschen and Correia, 2010).

Determination of optimal hemolymph collection intervals and volumes was essential for rapid recovery, highly reactive LAL production, and maintenance of animal health. Previous reports showed that 4 months was required to reestablish baseline amebocyte counts after a 30% bleed with "well-fed" captive HSCs in laboratory settings and outdoor tanks (Rudloe, 1983; Novitsky, 1984; Anderson et al., 2013); whereas, just 60% of baseline Hc concentrations were restored after 6 weeks (Coates et al., 2012; Anderson et al., 2013). In contrast, we found that HSCs bled at 10% recovered serum protein after 16 days, and a subsequent (second) 10% harvest appeared to trigger an increase in amebocyte counts after 7 days (**Figure 7B**). The reactivity of the LAL was notably higher after the second instance over the 7-day recovery period (**Figure 7C**). This rapid proliferation of circulating amebocytes may be a physiologic response to the initial bleeding (Hufgard, 2012).

HSC aquaculture has disruptive potential, especially given the threatened collapse of Asian populations (Akbar John et al., 2018), consequent pressure on Atlantic HSCs, and the self-evident global conservation benefits from species recovery in North America and preservation in Asia. HSC aquaculture could match industry needs for several years with the equivalent of 5–10% of *one year's* annual catch, leaving nearly 600,000 HSCs in the wild each year thereafter. In fact, these findings suggest that ∼60,000 aquaculture HSCs could be sustainably bled 12–24 times annually and *exceed* current biomedical LAL demand. Notably, such a finite cohort would likewise amount to a fraction of the

30% of the harvested HSCs that would otherwise *perish* every year (Atlantic States Marine Fisheries Commission [ASMFC], 2019). Estimated average life spans of up to 20 years (Shuster, 1958) could further position HSCs as a highly productive aquaculture species with competitive economic potential.

This study also demonstrated the potential for a rapid, reliable, and cost-effective method for detection of LPS as a marker for gram-negative bacteria that overcomes erratic results heretofore characteristic of testing human blood with LAL assays. Separation of specific blood components and a proprietary chemical treatment were shown to isolate LPS and therefore gram-negative bacteria from inhibitors without heating or dilution, thereby allowing for low levels of detection using a gel clot assay (**Figure 8**).

In patients experiencing sepsis, blood concentrations of LPS as high as 3.5 EU/ml, or 300 ng/ml, and above, have been documented, although specific levels may vary with the type of bacteria (Brandtzaeg et al., 1989). Nonetheless, recovery of spiked LPS and bacteria from modified blood was achieved, even at low concentrations. The specimen preparation method resulted in LPS detection in clinically relevant ranges (3.125 EU/ml of free LPS and 1.0–500,000 CFU/ml of gram-negative bacteria; **Tables 1**, **2**), which would be suitable for early screening of patients at risk for septicemia, even if asymptomatic, to inform appropriate clinical management. While this assay would screen for gram-negative pathogens, it could save valuable time from earlier initiation and results from cultures for identification and susceptibility given the length of time required to perform them (2–3 days). Ultimately, LAL clinical endotoxin screening could outperform current culture and PCR-based methods in speed, labor, and cost.

In addition to human diagnostic potential, a robust, sustainable source of LAL could play a vital role in other sectors. In the agricultural arena, nearly 80% of antibiotics in the US are sold for use in livestock feed (Martin et al., 2015). Intensive agriculture operations are used to raise large numbers of livestock in confined facilities, whereby animals are kept in close proximity. Identifying, isolating, and treating sick animals can prevent disease spread, avoid unnecessary antibiotic administration to healthy animals, reduce risks of antibiotic resistance, protect food supplies, and improve animal welfare. The factors driving research in this space include food security, global demand for animal-derived food products, and increasing incidence of zoonotic diseases at these facilities (Krehbiel, 2013). The same platform could also help ensure safety for agricultural personnel routinely exposed to such pathogens.

Other clinical and diagnostic applications for sustainable, abundant LAL-based assays could include helping municipal shelters in housing large populations of at-risk companion animals to reduce disease transmission, while save associated veterinary resources, and ultimately maximize adoption rates of healthy rescues. And food security applications could be used to prevent harmful pathogens from entering the supply chain, reduce costly management of food-borne illness, and protect public safety as well as food producers' reputations.

CONCLUSION

HSC aquaculture with year-round, low-impact harvesting could supply current biomedical LAL demand from a fraction of the harvested HSCs that would otherwise *perish* every year (Atlantic States Marine Fisheries Commission [ASMFC], 2019), while eliminating the potentially deleterious effects of current practices. At scale, these findings suggest that ∼60,000 aquaculture HSCs (equivalent of 5–10% of *one year's* annual catch) could be sustainably bled 12–24 times annually and *exceed* current biomedical LAL demand. Estimated average life spans of up to 20 years (Shuster, 1958) could further position HSCs as a highly productive aquaculture species with competitive economic potential.

Controlled-environment aquaculture and hemolymph collection could therefore alter the practice of wild harvest, expand the global LAL supply, and avert related viability threats, while building an irrefutable case for species conservation. Innovations like recombinant Factor C (rFC), an alternative to LAL assays, have emerged to address declining HSC populations (Bolden, 2019), with recent FDA approval for use of rFC in Eli Lilly's Emgality™ (galcanezumab). However, additional capital resources for equipment and end-user validation are also required to adopt such alternatives, which would be anticipated as significant drivers of industry demand and rapid acceptance of assays using abundant, *sustainable*, aquaculture-derived LAL.

These findings have demonstrated the potential for HSC aquaculture to alter the way that the current LAL industry operates and serve as a source of abundant, sustainable, and highly reactive LAL – with global conservation benefits from species recovery in North America and preservation in Asia. In turn, an expanded global supply of sustainable LAL raw material would provide a compelling rationale to develop new clinical, agricultural, veterinary, and food safety applications. Given the specter of antimicrobial resistance, these advances would address the urgent need for a new paradigm that moves from widespread antibiotic prophylaxis to dispositive, timely therapeutic administration for gram-negative pathogens consistent with human and veterinary disease management standards.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/supplementary material.

ETHICS STATEMENT

The research on the HSCs (Limulus polyphemus) involved in this study did not require approval. HSCs are classified as an exempt invertebrate species. All HSC collection and aquaculture techniques and methods were reviewed by the

North Carolina Department of Environmental Quality, Division of Marine Fisheries (Collection permit #1946771 and Aquaculture operation permit #1947050).

AUTHOR CONTRIBUTIONS

RT-K, KD, TB, and AD conceived the research hypothesis and developed the research objectives and experimental plans. RT-K, KD, CK, and TB verified the materials and methods. RT-K, KD, LR, and AD conducted the research experiments. AD supervised the findings of this work. All authors prepared analysis of the primary research and contributed to the final writing of the manuscript. SA was a DVM and Ph.D. in Veterinary Nutrition with 25+ years of clinical nutrition experience and provided nutritional guidance. FL was an expert in blood coagulation, regulatory affairs, and bedside testing and provided diagnostic expertise for the analysis of clinical specimens in the LAL experimentation. JL was a critical care physician and cardiac anesthesiologist engaged in sepsis treatment, transfusion, and ECMO and provided experimental guidance on developing a LAL-based blood diagnostics for patient care.

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Conflict of Interest: AD, RT-K, TB, and LR were employed by Kepley BioSystems, Inc. KD and CK were full-time professors at the Joint School of Nanoscience and Nanoengineering. JL was a practicing critical care physician and cardiac anesthesiologist; he was also a Professor of Anesthesiology and Surgery (Cardiothoracic), and Co-Director of the Cardiothoracic Surgical Intensive Care Unit at Duke University Medical Center; and JL serves as the head of the advisory board for Kepley Biosystems, Inc. FL was President and Owner of LADUCA RCA LLC in New Jersey, an independent consultant and adviser to the medical industry, and serves on the advisory board at Kepley BioSystems, Inc. SA was an Assistant Professor of Clinical Nutrition at the Ontario Veterinary College, University of Guelph, and Owner of Sit, Stay, Speak Nutrition, LLC; SA specializes in nutritional coaching and serves as a scientific advisor for Kepley Biosystems, Inc. The authors alone are responsible for the content and writing of the article.

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A Novel Index of the Performance of *Mytilus galloprovincialis* to Improve Commercial Exploitation in Aquaculture

Jose M. F. Babarro[1], Ramón Filgueira[2], Xosé A. Padín[1] and M. Angeles Longa Portabales[3]*

[1] Instituto de Investigaciones Marinas, IIM-CSIC, Vigo, Spain, [2] Marine Affairs Program, Dalhousie University, Halifax, NS, Canada, [3] Departamento de I+D, Consello Regulador Mexillón de Galicia, Vilagarcía de Arousa, Spain

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***Correspondence:**
Jose M. F. Babarro
jbabarro@iim.csic.es

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Estuarine environments are highly heterogeneous habitats where numerous organisms interact with each other. Aquaculture systems encompass such interactions, and the eventual yields depend on how the cultivated species respond to the environmental heterogeneity. Marine mussels are calcifying organisms that rely on calcium carbonate shell and byssus filaments secreted during their lifetime to protect soft vital tissues against aggressive (abiotic and biotic) environments. Nevertheless, these protective structures can be energetically costly, depending on the environment, thus affecting the energy allocation patterns in the organism. Consequently, other important fitness parameters for the aquaculture industry, such as soft tissue condition and market value, may also be affected. Here, we present a spatial and temporal analysis of the protective and soft tissues responses in the mussel *Mytilus galloprovincialis* with the aim of obtaining a better understanding of the inter-location variability in individual's performance and the corresponding main environmental drivers. Local environment with regard to food availability and hydrodynamics impact very distinctly mussel tenacity and shell thickening, e.g., higher-energy environments at the outer exposed zones caused individuals secreted stronger byssus and thicker shells. By contrast, the state of soft tissues (condition index) was similar between very distinct and distant outer exposed and inner sheltered zones due to differences in both environmental drivers. A novel, intuitive ecological index that measures the impact of both protective and soft tissues was developed and is discussed in relation to cultivation timing. The data presented provides the basis for understanding the natural variability in energetic requirements for different vital tissues in bivalve mollusk that support survival and growth. We report the scientific basis for management actions aimed to shortening the cultivation cycle in the aquaculture sector. These actions are based on the combined use of the PROFIT index and other aquaculture practices (e.g., modifying density). PROFIT helps to identify *when* the quality of the product, understood as PROtection and FITness strategies, would be best suited for the market.

Keywords: *Mytilus galloprovincialis*, ecophysiological index, aquaculture, environmental drivers, industry

HIGHLIGHTS

- PROFIT is a novel, simple eco-physiological index for use in the cultivation of marine mussels.
- Consideration of protective tissues and yield in cultivated marine bivalves is needed for better comprehension of energetic *trade-offs* during the life cycle.
- Innovations in aquaculture practices, here with the marine mussel *Mytilus galloprovincialis* as a case study, should consider new indicators like PROFIT (and others) for better management of the natural resource and for differentiating production areas or exploitation methods.

INTRODUCTION

The morphology and performance of bivalve mollusks in general, and marine mussels in particular, are known to vary in relation to key environmental factors for different species and habitats (Bergström and Lindegarth, 2016; Kroeker et al., 2016; Telesca et al., 2018). Like many other marine invertebrates, mussels secrete exoskeletons that provide a physical barrier that protects against abiotic and biotic aggressors (Burnett and Belk, 2018). Calcified shells therefore play a primary ecological role, reducing predation impacts on mussels, protecting them from intense wave action and providing mechanical strength for natural clustering in beds, cultivation ropes or matrices (Briones et al., 2014 and references therein).

As well as producing calcified shells, many marine bivalves (especially those in the family Mytilidae) secrete byssus, a non-calcified extracellular tissue. This attachment-related tissue confers the individuals the ability to maintain a relatively sessile life mode and hence, contributes to their successful cultivation around the world. Byssus filaments must be replaced continuously as the threads decay over time (approximately 4–6 weeks; Carrington, 2002). Secretion is thus dynamic rather than static and is also affected by various environmental parameters, seasonality and the endogenous rhythms of the organisms (Carrington, 2002; Moeser and Carrington, 2006; Zardi et al., 2006). As animals can modify energy allocation to protective tissues, energetic *trade-offs* between soft and hard (byssus filaments and shell) tissues production can occasionally occur (Carrington, 2002; Moeser et al., 2006; Zardi et al., 2007; Babarro and Carrington, 2011). Therefore, for a comprehensive analysis of the fitness of an organism in a particular environment, the energetic requirements for the secretion of these protective structures must be considered. Byssus and shell secretion may represent up to 8–15% and 25–50% respectively of the total energy expenditure in mussels (Hawkins and Bayne, 1985; Gardner and Thomas, 1987; Steffani and Branch, 2003).

The shell represents a barrier that protects soft tissues being influenced by multiple environmental and endogenous factors and is generally linked to specific life-habitat and ecological niches of the organisms (Zuschin and Stanton, 2001; Zuschin et al., 2003). Shell thickness has recently been described as a proxy for the health status of shellfish as well as an indicator of environmental change, including climate change (Martinez et al., 2018). The aforementioned study reported shell thickness as "*a new reliable indicator when informing the Marine Strategy Framework Directive (MSFD) (2008/56/CE),*" in order to achieve *"good ecological status (GES)"* in European Marine Waters by 2020 (see Martinez et al., 2018). A clear link between shell thickness and strength has been established previously (Nagarajan et al., 2006) although other factors like shell shape, mineralogy local environment or genotypes may also impact shell performance (Penney et al., 2007; Fitzer et al., 2014). Shell fragility is not only important for specimens living in high-energy environments (see Kidwell and Bosence, 1991 for a review; Beadman et al., 2003; Steffani and Branch, 2003; Babarro and Carrington, 2011), but also for those inhabiting low-energy habitats where biogenic interactions can lead to shell breakage (Powell et al., 1989). Overall, shell tissues have been used to explore the intraspecific variations in different environments and its implications in aquaculture activities have been considered (Lewis and Magnuson, 1999; Beadman et al., 2003; Nagarajan et al., 2006).

Similarly, byssus failure, understood as lack of primary physiological response to attach optimally on substrates, has been used to explain dislodgement and biomass loss in aquaculture (Babarro et al., 2019). According to this study, the local environment in terms of hydrodynamics and food availability helped to minimize dislodgement from ropes through optimal byssus attachment strength. In the specific case of farmed mussels, operational mechanical procedures such as separation of clusters, removal of the byssus and cleaning attached fouling from the shell may lead to loss of marketable product due to shell breakage. Some examples of these types of actions affect approximately 5–15% of the whole annual production (G. Sarà, personal communication in Martinez et al., 2018). Cracked mussels are unwanted products representing production loss, and factors affecting the resistance of the shell to breakage other than ecological aspects are therefore also of great concern in the aquaculture sector (Penney et al., 2007).

Coastal embayments (rias) in areas characterized by upwelling systems, such as the Atlantic Galician coast (NW Iberian Peninsula), are heterogeneous ecosystems due to the mixing of salt and fresh water. They are also very productive in relation to nutrient and phytoplankton availability, which affect both farmed and wild bivalve species. These aspects are very important for rope-grown mussel cultivation. The heterogeneous environments may significantly affect the energetics and allocation patterns for investment in protective and vital tissues, with significant consequences for survival, biomass maintenance on cultivation structures and yield at harvest. Furthermore, the outcomes of this investment may also be affected by growth. As mussel growth involves continued byssus secretion, increased shell area and thickening, juvenile mussels will have thinner and weaker shells that can be more easily attacked and broken by predatory or mechanical actions; by contrast, adults will attach less strongly to substrates (e.g., littoral coastline in intertidal environments) as a consequence of lower byssus tenacity (Babarro and Carrington, 2013). The energetic expenditure may significantly affect the performance of individual mussels as the *trade-off* of energy allocation to byssus secretion in relation to

growth and reproduction may be critical for individual fitness (Carrington et al., 2015). The ratio between soft tissue weight and shell weight (i.e., the condition index) may represent a proxy for product quality (Watanabe and Katayama, 2010), which is of great interest for harvesting purposes, e.g., to indicate the market value (Martinez et al., 2018). The physiological implications of mussel size on shell thickening or byssus attachment strength, together with the ecological implications driven by site-specific heterogeneity, will therefore play a key role in mussel performance in a widely exploited area such as the Ría de Arousa (NW Iberian Peninsula). To our knowledge, this study represents the first attempt to investigate how both byssus and shell secretion are affected by different natural environments in aquaculture systems, and how the potential energetic *trade-offs* affect mussel fitness, survival and growth performance. Field responses were combined in a novel and intuitive eco-physiological index that could be used to identify *how* the local environment may determine aspects that matter for aquaculture activities, i.e., biomass maintenance through attachment, structural integrity of the shell and yield or market value, and also *when* these characteristics are optimal for harvest within the usual cultivation period. Therefore, hypotheses planned in this survey can be summarized as follows: (i) local environment within a single embayment like Ría de Arousa (NW Iberian Peninsula) would modify significantly the energy allocated to distinct hard and soft tissues that matter for the life cycle of the mussels and, (ii) apart from individual size effect associated to organism's growth, food availability resources and hydrodynamic profiles, e.g., wave height together with salinity variability during the cultivation cycle would explain differences in mussels performance in the aquaculture system. In accordance with the importance of these protective and soft tissue structures in relation to aquaculture practices and markets, an integral index of mussel eco-physiology is proposed for evaluating mussel status with the aim of minimizing losses in biomass and better practices.

MATERIALS AND METHODS

Description of the Study Area

The coastline of Galicia (NW Iberian Peninsula) represents the northern limit of the North Atlantic Upwelling System. The area is one of the world's major upwelling zones and is therefore highly productive. The northerly component of shelf wind stress on the Galician coast drives the surface layer offshore, inducing the cooler and nutrient-rich sub-surface waters close to the coast to rise the surface (Arístegui et al., 2009; Álvarez-Salgado et al., 2010). These oceanographic characteristics are modulated by the seasonal cycle of the wind direction, which is dominated by northeast winds between May and October (Wooster et al., 1976; Pardo et al., 2011). The highest mussel growth rates worldwide have been reported in the four large coastal embayments on the Galician coast (Pérez-Camacho et al., 1995; Fernández Reiriz et al., 1996), collectively known as the Rías Baixas. The Ría de Arousa is the largest of these embayments, which occupy an area of 245 km^2 (**Figure 1**). Mussels are cultivated in the area on floating rafts (20 m × 25 m), each of which supports 500 ropes each of length 12 m. The annual mussel production in Galicia is approximately 250,000 t, representing around 12% of the worldwide production (FAO, 2018).

Experimental Design and Sampling

Four different mussel farm sites were chosen for study in the Ría de Arousa (NW Spain; **Figure 1**), covering the outer exposed vs. inner sheltered spatial gradient (OuN and OuS at the northern and southern edges of the outer mouth of the ría, MidW in the middle section and InC in the innermost zone). Standard handling and cultivation techniques were used. Juvenile mussels of shell length approximately 20 mm were collected for use as seed from an intertidal environment on the Galician coastline. In all locations, the cultures were initiated with 900 mussels per m of rope at the first stage, from seeding to thinning out, and the density at the beginning of the second phase, from thinning out to harvest was 800 mussels per m of rope. Special caution was taken to ensure a similar seeding density in all ropes, as any differences could significantly affect mussel growth and physiological performance (Fréchette et al., 1996; Cubillo et al., 2012). The culture ropes were suspended on the rafts between February 2015 and August 2016 to encompass the two cultivation phases. Six ropes each of length 12 m (replicates), separated by a distance of 1 m, were hung from the aft of each raft (i.e., opposite to where the mussel raft is anchored with an iron chain, at the bow). Sampling was carried out approximately once a month. At each sampling time, 60 individual mussels (n = 240 for all cultivation sites) were dislodged from the mussel clusters along the length of the rope for estimation of byssal attachment force and tenacity (see below). The mussels used in the dislodgement tests occupied the outer positions of the cluster along the rope, as this provided easier access to each individual. Mussels were sampled at different depths along the rope (1, 3, 5, 7, 9, and 11 m) (n = 10 mussels per depth) with the aim to monitor several mussel responses and obtain sufficient biological material along the rope. The mussels dislodged from different sections of the ropes were also used to determine the shell thickness index (see below). Despite the main goal of sampling at different depths was to collect enough biological material, the data was pooled into two main groups. This allowed us to collect sufficient biological material, but also test the general effects of depth. The selection 1–5 and 7–11 m was made based on previous results in the Galician mussel cultivation for the importance of depth (Fuentes et al., 2000) and the empirical knowledge of the farmers with regard to both water sections depth and mussel performance.

A second group of mussels was sampled at the same depths as before to measure tenacity. Entire sections of the rope (inner and outer sections of the cluster) of known length were extracted. The maximum length, height and width of the shell were measured as the anterior-posterior, dorsal-ventral and lateral axes, respectively, in a minimum of 200 individuals. These mussels were also used to determine the condition index.

Environmental Conditions

A network of 38 oceanographic stations has been maintained by a local government agency in Galicia (Instituto Tecnolóxico

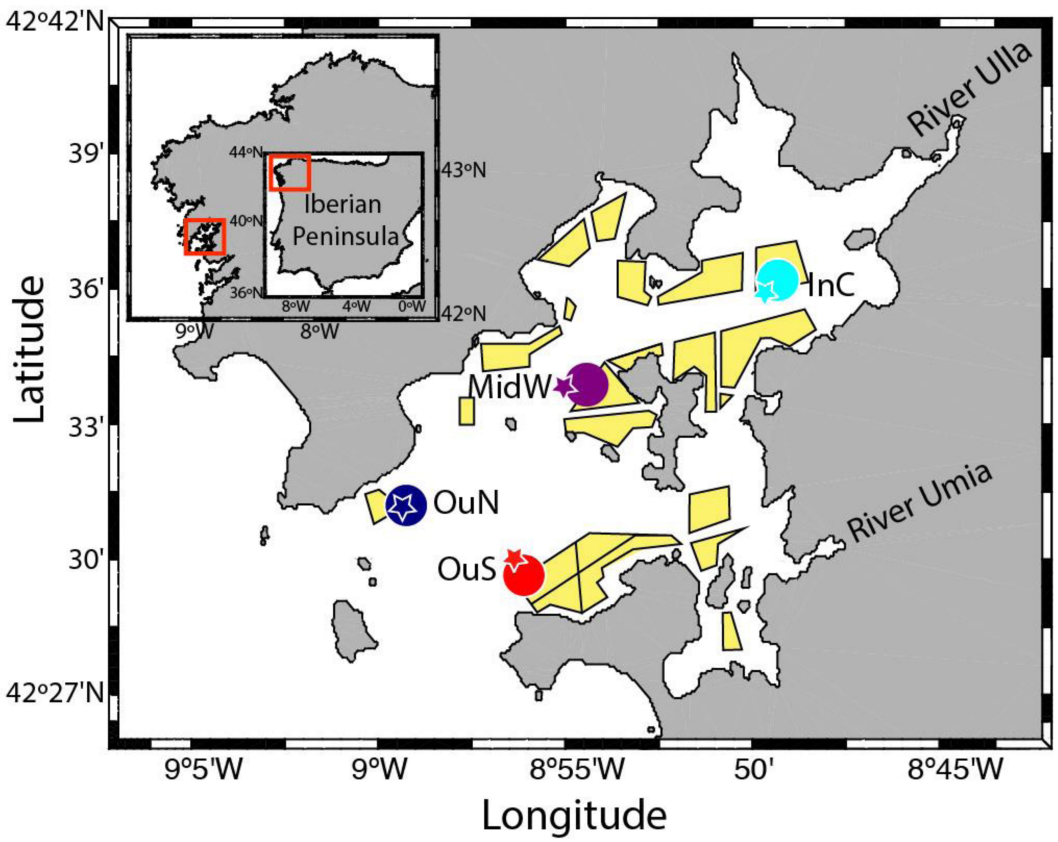

FIGURE 1 | The experimental mussel cultivation sites and nearby environmental monitoring stations in the Ría de Arousa (NW Spain).

para o Control do Medio Mariño[1]) since 1992 (11 of them at Ría de Arousa), providing weekly hydrographic data. Data on the water column from the four stations closest to the mussel experimental rafts in the Ría de Arousa was obtained for a total of 147 vertical profiles, between February 2015 and August 2016 (**Figure 1**). Vertical hydrographic profiles were obtained throughout the water column, from the sea surface to the bottom at the four stations, with an SBE 25 Sealogger CTD (Sea-Bird Scientific). Chlorophyll *a* (Chl*a*) was sampled at three depth intervals (0–5 m; 5–10 m; and 10–15 m) according to the Lindhal technique (Sutherland et al., 1992). Chlorophyll *a* concentration was measured by spectrofluorometric analysis (Zapata et al., 1994).

Current data were obtained from the output of a numerical configuration of the Regional Ocean Modelling System (ROMS) model (Schepetkin and McWilliams, 2005), for the three southernmost Galician Rías: Vigo, Pontevedra and Arousa. This configuration was established by a system of nested configurations used to describe the two-way exchange between the rías and the coastal ocean. Circulation in the rías is measured at a spatial resolution of 1/450° (about 190 m) and includes wind-induced currents, tides and estuarine circulation due to inflowing fresh water. The results were validated with Intecmar data[1] and ADCP sampling of currents in the Ribeira buoy, also managed by Intecmar. The configuration is forced by wind stress, extracted from the Meteogalicia weather service through the threaded web server mandeo.meteogalicia.es, with a resolution of 4 km. River inflow from the tributary rivers in the Rías was downloaded from the measurements of gauging stations closer to the mouths of the Umia and Ulla rivers distributed by Meteogalicia[2].

Wave parameters were calculated from the forecast power spectra of the SWAN model, developed at Delft University of Technology. The model computes random, short-crested wind-generated waves on a curvilinear grid on the Galician coast and was forced by Meteogalicia operational WW3 model. The output of the model estimated with an hourly frequency was averaged daily.

Mussel Tenacity

The attachment strength of mussels aggregated in clusters was measured by connecting a single mussel to a spring scale (Digital Force Gauge DN431, 0.01 N resolution) with the aid of custom-made forceps (see Babarro and Comeau, 2014; Babarro et al., 2019 for details of the procedure). Care was taken to

[1]http://www.intecmar.gal

[2]https://www.meteogalicia.gal

avoid disturbing neighboring mussels when dislodging individual specimens. Individuals immediately adjacent to those selected for dislodgement were not considered in the trials if they had interconnected byssus threads. Individual mussel attachment strength in the field can be overestimated as a consequence of the dense network of fibers that several individuals secrete within the cluster. However, this functional value is valid for comparisons as it represents the force required to pull an individual from the cultivation ropes as done by currents or predators (Babarro et al., 2019). Dislodgement measurements were made with wet mussels to prevent modification of the mechanical properties of the byssus. Attachment force *(F)* was normalized by mussel size in order to calculate tenacity (TEN, N m^{-2}), as follows: TEN = *F*/AP, where AP is the projected area of the individuals pulled for dislodgement, approximately an ellipse obtained by the product of width and height values of shells (Bell and Gosline, 1997).

Shell Thickness Index

After dislodgement of mussels, the anterior-posterior (length), dorso-ventral (height) and lateral axis (width) of the shell were measured (n = 60 per rope) with the aid of Vernier calipers (±0.1 mm). Live weight of the whole individual and dry weight of the shells were also recorded. Shell thickness index (STI) values were obtained, according to the following formula:

STI = 1000 × dry shell wt / $[L(H^2 + W^2)^{0.5} \times \pi/2]$ (1) where L, H and W are respectively the length, height and width of the shell (Freeman and Byers, 2006; Freeman et al., 2009). To measure shell weight (wt), mussels were sacrificed, the tissue was dissected, and the shells were patted dry with paper towels and weighed on a Sartorius digital balance (±0.01 mg). Organic residues were removed from the shell surfaces and the shells were dried in a muffle furnace at 40°C for 24 h.

Shell Length Growth and Condition Index

The second group of mussels sampled was used to determine growth and condition index. Mussels of different sizes can co-exist within clusters on the ropes. Therefore, once the individual size (shell length) of all mussels from the cluster was determined, and with the aim of establishing the total weight of tissues (soft and shell dry weights), mussels assigned to specific interquartile range, e.g., 25, 25–50%, 50, 50–75%, and 75% of the latter size-frequency range of the whole cluster were considered.

Shell length growth curves were fitted to Gompertz models: $L_t = L_\infty(e^{(-e(-k(t-t')))})$ where L_t is the shell length (mm) at time t (months), L_∞ the maximum size, k is the growth parameter indicator of the speed at which maximum size is attained, and t' is the inflexion point of the curve (Ratkoskwy, 1990). The Gompertz model parameters were estimated by non-linear regression, using the Levenberge-Marquardt algorithm and least squares as loss function.

The condition index (CI) of the mussels was calculated as follows: CI = $(DW_{tissue}/DW_{shell}) \times 100$, where DW_{tissue} is the dry weight of the soft tissue and DW_{shell} is the dry shell weight (Freeman, 1974). Both shell and soft tissues were dried at 110°C for 48 h before being weighed.

Statistical Analysis

Mussel tenacity (TEN) and shell thickness index (STI) values obtained for the whole mussel cultivation period were analyzed using allometric functions in relation to individual shell length, SL (TEN or STI = aSL^b). ANCOVA was used to explore the effect of both cultivation site and depth as independent factors, and mussel size was considered a co-variable for TEN and STI variability.

TEN and STI were standardized to 60 mm shell length in order to determine the temporal variability throughout the cultivation cycle, allowing for site comparisons and seasonal patterns. For this purpose, specific allometric functions (TEN or STI = aSL^b) obtained for each cultivation site and depth were used to standardize the size effect.

Gompertz parameters of mussel growth were compared using a set of pairwise contrast and F statistical tests, according to Peteiro et al. (2006). Condition index variability was examined by ANCOVA, to test the effect of both cultivation site and depth as independent factors and mussel size as a co-variable.

Considering the previously described individual responses, two distinct eco-physiological strategies were explored for inclusion in the new PROFIT (PROtective – FITness) index: (i) shell thickness index (STI) and byssus functionality (TEN) as primary protective responses to aggressive abiotic (hydrodynamic) and biotic (predatory actions, inter-specific competition) factors, and (ii) condition index (CI), which as an indicator of soft tissue state provides an idea of the potential market value. The PROFIT index thus integrates both the quality of the shell (fragility) and attachment strength (maintenance on substrate), together with the marketable product (meat yield). Responses of a standard 60 mm-SL mussel were used to determine the PROFIT index and considering specific percentage values that each metabolic response represents in the energetic expenditure rates of the whole organism, as follows: an average value of 10% for the energy fraction represented by byssus secretion (see Griffiths and King, 1979; Hawkins and Bayne, 1985), 40% for the energy fraction represented by the shell tissue thickening (Gardner and Thomas, 1987) and 50% for mean somatic and reserve tissues (Fuentes-Santos et al., 2019). PROFIT is expressed as a percentage, and each component of the index (TEN_i, STI_i, and CI_i) for a site i (OuN, OuS, MidW, and InC) was corrected to the mean value for the four experimental sites and the whole cultivation period, as follows:

$$PROFIT(\%) = [(TEN_i - TEN_{all})/TEN_{all} \times 10] +$$

$$[(STI_i - STI_{all})/STI_{all}) \times 40] +$$

$$[(CI_i - CI_{all})/CI_{all}) \times 50]$$

ANOVA was used to test the effects of both cultivation site and depth on the variability in the PROFIT index.

Normality and homogeneity of variances were examined by the Shapiro–Wilk *W*-test and Levene's test, respectively. All data were log-transformed when necessary, and rank

transformation was applied when the heterogeneity persisted (Conover, 2012). Significant differences between experimental groups were identified by a posteriori tests (Tukey and Bonferroni). All analyses were performed using SPSS Statistics 23 (IBM España S.A., Madrid, Spain) and STATISTICA 7.0 software (TIBCO Software Inc., Palo Alto, CA, United States). All data are reported as means ± SD.

RESULTS

Environment

The sampling period, between March 2015 and August 2016, in the Ría de Arousa was characterized by alternating periods dominated by northerly and southerly winds. However, northerly winds prevailed during spring and summer (**Table 1**), while southerly winds clearly dominated in the winter of 2016, with a mean value of -832 m^3 km^{-1} h^{-1} and a daily maximum value of -3808 m^3 km^{-1} s^{-1} in January (**Figure 2a**). During the period dominated by southerly winds, the wave height increased greatly, especially in the outer areas of the Ría de Arousa, reaching 4 m at the end of December 2015 (**Figure 2b**). Propagation of the waves along the Ría de Arousa attenuated the wave height, which never exceeded 2 m, in the inner waters. The waves were lowest in summer, of average height less than 1 m (**Table 1**).

The discharges from the Rivers Ulla and Umia were significant throughout the winter and spring of 2016 (**Table 1**) and occurred in three pulses that reached maximum continental inputs of around 400 m^3 s^{-1} in February (**Figure 2a**). However, accumulation of fresh water in the estuary, with minimum salinity values close to 18 psu in the surface waters, was observed in the interior of the Ría de Arousa in January 2016 (**Figure 2e**). The clear signal in the variability of salinity occurred during piling up of the offshore surface waters toward the coast as a result of the southern winds, as shown by the strong negative Iw values (**Figure 2a**). Similar continental inputs in February and April did not cause such low salinity values as they occurred during null or positive values of upwelling index and thus with a net offshore transport of surface waters. Despite these differences, the large freshwater inputs caused notable salinity gradients in the surface waters, with lowest values at InC and OuN sites and maximum salinity at OuS (**Figure 2e**). The variability in salinity observed in the upper layer at the Ría de Arousa was greatly reduced at depths between 6 and 12 m, in which the salinity ranged between 31 and 36 psu during the study period (**Figure 2f**). Both surface and subsurface waters were very homogeneous by the end of summer, with values around 35.5 psu (**Figures 2e,f**). Similarly, the temperature of the surface waters also varied greatly, ranging from 11 to 20°C, in comparison to the temperature span of subsurface waters, which ranged between 12 and 19°C (**Figures 2c,d**). The temperatures were characterized by an obvious seasonal cycle repeatedly broken by the entry of deeper colder waters during upwelling at the mouth of the estuary and less often, the presence of warmer waters coming from offshore under southerly wind domain. The arrival of cold water to the exterior sites during the upwelling episodes warmed the interior waters, which are also shallower in the area of the Ría de Arousa, especially in summer.

The food availability (FA), a proxy for available food supply for the mussels, was estimated as the product of the chlorophyll *a* (Chl-*a*) concentration and current velocity. The FA indicated strong short-term variability, with intense phytoplankton proliferation pulses at all experimental sites and a period where there was almost no food available for mussels, between December 2015 and January 2016 (**Figures 2g,h** and **Table 1**). The FA values were generally higher in the surface waters at the outer exposed northern OuN site where high current velocity and maximum Chl-*a* values coincided. The FA values were lowest in the southern outer site (OuS) in the Ría de Arousa. The values estimated for the area along the estuary between 6 and 12 m depth were lower than those found at the surface, but were more homogeneously distributed due to the higher concentration of chlorophyll in inland waters, as observed at the MidW and InC sites (**Table 1**).

Mussel Tenacity (TEN)

The variability in mussel tenacity in relation to individual length in the selected experimental sites and for different sections along the ropes is shown in **Figures 3A,B**. Byssus tenacity was inversely related to the increase in mussel size according to negative allometric functions (see equations in **Figure 3**). This relationship is a consequence of the relatively lower magnitude of the increase in byssus attachment force relative to shell area during growth. Therefore, older and larger mussels are relatively weaker in terms of tenacity than juveniles (**Figures 3A,B**). ANCOVA applied to TEN variability reflected the size effect as a co-variable ($p < 0.001$) and also the impact of cultivation site ($p < 0.001$) and depth ($p < 0.05$) (**Tables 2**, **3**). The TEN values were higher for the northern outer exposed site (OuN) followed by the southern outer site (OuS), and finally the weakest tenacity was observed for mussels in the middle and most inner sheltered sites (MidW-InC), with no differences between these sites (**Figures 3A,B** and **Tables 2**, **3**). Cultivation depth had a minor (residual) impact on TEN, which was slightly lower (4.5%) in the deeper section (6–12 m) than in the upper section (0–6 m) of the water column at all sites; no effect was noted for the site × depth interaction term (**Figures 3A,B** and **Table 2**).

Spatial and temporal analyses of the standardized TEN (60 mm-SL) confirmed the main effect of the cultivation site (see **Figure 3C** with average TEN values for both depth intervals). This finding highlights the stronger byssus tenacity in mussels from the northern and southern outer exposed sites (OuN followed by OuS) than in mussels from the innermost locations, whereas seasonality was represented by greater TEN through the autumn-winter period than in the spring-summer period (**Figure 3C**).

Shell Thickness Index (STI)

The variability in STI for the cultivated mussels is represented in **Figure 4**. Cultivation site was the only factor that significantly affected STI (**Tables 2**, **3**), apart from the individual size effect, as illustrated by the allometric functions obtained (**Figures 4A,B**). Standardized STI (i.e., 60 mm-SL) is shown to represent the

TABLE 1 | Variability of the environmental parameters temperature (°C), salinity, chl-*a* (mg m^{-3}), food availability (FA, mg m^{-2} s^{-1}), wave height (m), upwelling index (10^3 m^3 km^{-1} s^{-1}) and river input (m^3 s^{-1}) in the different sites of cultivation and as a function of experimental time.

	Region	T °C	S psu	chla mg m^{-3}	FA mg m^{-2} s^{-1}	WH m	Iw river
Spring 2015 March–May, 2015	**OuN**	**13.7 ± 1.3**	**34.5 ± 0.8**	**6.0 ± 4.2**	**1.0 ± 0.7**	1.3 ± 0.4	
		13.6 ± 1.4	*34.9 ± 0.4*	*3.1 ± 1.9*	*0.5 ± 0.3*		
	OuS	**13.7 + 1.2**	**35.0 ± 0.5**	**4.9 ± 3.1**	**0.8 ± 0.6**	1.2 ± 0.3	**234**
		13.4 ± 1.3	*35.2 ± 0.3*	*3.8 ± 2.4*	*0.5 ± 0.3*		
	MidW	**13.6 ± 1.3**	**34.2 ± 1.1**	**2.6 ± 1.8**	**0.4 ± 0.3**	0.7 ± 0.2	58
		13.3 ± 1.3	*35.2 ± 0.3*	*5.1 ± 2.0*	*0.7 ± 0.4*		
	InC	**14.2 ± 1.4**	**33.0 ± 0.8**	**3.0 ± 1.5**	**0.4 ± 0.2**	0.6 ± 0.2	
		13.3 ± 1.3	*35.0 ± 0.5*	*4.0 ± 2.0*	*0.5 ± 0.2*		
Summer 2015 June–August, 2015	**OuN**	**16.3 ± 1.3**	**35.4 ± 0.1**	**3.5 ± 1.8**	**0.6 ± 0.4**	0.9 ± 0.3	
		15.2 ± 0.6	*35.5 ± 0.1*	*3.5 ± 1.6*	*0.5 ± 0.3*		
	OuS	**16.3 ± 1.2**	**35.5 ± 0.1**	**3.9 ± 1.9**	**0.6 ± 0.3**	0.9 ± 0.3	**252**
		15.3 ± 0.9	*35.5 ± 0.1*	*5.0 ± 1.8*	*0.7 ± 0.3*		
	MidW	**16.6 ± 1.5**	**35.4 ± 0.1**	**2.4 ± 1.3**	**0.4 ± 0.2**	0.5 ± 0.1	17
		15.2 ± 1.0	*35.5 ± 0.1*	3.6 ± 1.8	*0.4 ± 0.2*		
	InC	**17.0 ± 1.3**	**35.1 ± 0.1**	**3.6 ± 2.1**	**0.4 ± 0.3**	0.4 ± 0.1	
		14.9 ± 1.2	*35.4 ± 0.1*	*4.3 ± 2.0*	*0.5 ± 0.3*		
Autumn 2015 September–November, 2015	**OuN**	**16.0 ± 0.6**	**34.8 ± 0.6**	**9.1 ± 2.8**	**1.5 ± 0.7**	1.3 ± 0.7	
		15.8 ± 0.6	*35.1 ± 0.4*	*5.0 ± 2.0*	*0.7 ± 0.3*		
	OuS	**16.0 ± 0.6**	**35.0 ± 0.5**	**7.5 ± 3.1**	**1.0 ± 0.4**	1.1 ± 0.5	**−10**
		15.8 ± 0.8	*35.2 ± 0.4*	*3.5 ± 2.1*	*0.5 ± 0.3*		
	MidW	**16.0 ± 0.5**	**34.9 ± 0.5**	**4.1 ± 2.7**	**0.6 ± 0.4**	0.6 ± 0.4	21
		15.9 ± 0.6	*35.1 ± 0.3*	*7.0 ± 2.4*	*0.9 ± 0.3*		
	InC	**16.1 ± 0.3**	**34.6 ± 0.7**	**2.9 ± 1.6**	**0.3 ± 0.2**	0.6 ± 0.4	
		15.7 ± 0.6	*35.1 ± 0.4*	*3.8 ± 1.5*	*0.4 ± 0.2*		
Winter 2016 December, 2015–March, 2016	**OuN**	**12.9 ± 0.6**	**28.2 ± 4.7**	**1.3 ± 0.8**	**0.3 ± 0.1**	2.5 ± 0.7	
		13.8 ± 0.3	*33.5 ± 1.5*	*0.8 ± 0.4*	*0.1 ± 0.1*		
	OuS	**13.6 ± 0.6**	**30.6 ± 3.5**	**0.8 ± 0.5**	**0.1 ± 0.1**	2.1 ± 0.7	**-832**
		14.2 ± 0.2	*34.7 ± 0.7*	*0.7 ± 0.4*	*0.1 ± 0.1*		
	MidW	**13.6 ± 0.4**	**31.8 ± 2.1**	**0.7 ± 0.4**	**0.1 ± 0.1**	1.3 ± 0.5	125
		14.1 ± 0.1	*34.5 ± 0.8*	*1.4 ± 0.7*	*0.2 ± 0.1*		
	InC	**13.2 ± 0.8**	**27.4 ± 5.7**	**0.7 ± 0.5**	**1.0 ± 0.1**	1.2 ± 0.4	
		14.0 ± 0.2	33.7 ± 1.3	*0.8 ± 0.4*	*0.1 ± 0.1*		
Spring 2016 March–May, 2016	**OuN**	**13.3 ± 1.0**	**32.3 ± 2.2**	**5.0 ± 2.1**	**1.0 ± 0.5**	1.4 ± 0.6	
		13.1 ± 0.8	*34.4 ± 0.7*	*4.0 ± 1.5*	*0.6 ± 0.3*		
	OuS	**13.4 ± 0.9**	**33.8 ± 0.7**	**4.1 ± 1.8**	**0.7 ± 0.3**	1.2 ± 0.4	**50**
		13.1 ± 0.7	*34.7 ± 0.5*	*2.7 ± 1.1*	*0.4 ± 0.1*		
	MidW	**13.4 ± 0.9**	**33.3 ± 1.0**	**2.8 ± 1.2**	**0.5 ± 0.2**	0.7 ± 0.3	115
		13.0 ± 0.7	*34.6 ± 0.5*	*7.7 ± 4.1*	*1.0 ± 0.7*		
	InC	**13.7 ± 1.0**	**31.8 ± 2.0**	**2.2 ± 1.2**	**0.3 ± 0.2**	0.7 ± 0.3	
		13.1 ± 0.6	*34.5 ± 0.6*	*5.0 ± 1.8*	*0.5 ± 0.2*		
Summer 2016 June–August, 2016	**OuN**	**15.9 ± 1.0**	**35.2 ± 0.5**	**7.8 ± 6.3**	**1.3 ± 1.1**	0.8 ± 0.3	
		14.8 ± 0.7	*35.4 ± 0.3*	3.3 ± 2.3	*0.5 ± 0.4*		
	OuS	**15.5 ± 1.0**	**35.2 ± 0.7**	**8.5 ± 5.9**	**1.3 ± 1.0**	0.8 ± 0.3	**362**
		14.4 ± 0.8	*35.6 ± 0.4*	*4.8 ± 3.2*	*0.6 ± 0.4*		
	MidW	**16.1 ± 1.2**	**35.0 ± 1.1**	**2.4 ± 1.5**	**0.4 ± 0.3**	0.5 ± 0.1	32
		14.6 ± 0.9	*35.5 ± 0.4*	*4.4 ± 2.4*	*0.5 ± 0.3*		
	InC	**16.8 ± 1.6**	**34.4 ± 1.7**	**3.2 ± 2.2**	**0.4 ± 0.3**	0.4 ± 0.1	
		14.3 ± 1.3	*33.6 ± 2.9*	*5.1 ± 3.5*	*0.5 ± 0.4*		
Autumn 2016 September, 2016	**OuN**	**15.5 ± 0.4**	**35.4 ± 0.2**	**7.3 ± 2.5**	**1.2 ± 0.5**	1.2 ± 0.2	
		14.9 ± 0.4	*35.5 ± 0.1*	*5.8 ± 4.0*	*0.9 ± 0.7*		
	OuS	**15.6 ± 0.7**	**35.8 ± 0.3**	**8.7 ± 2.5**	**1.4 ± 0.6**	1.1 ± .0.2	**252**
		14.8 ± 0.3	*35.9 ± 0.3*	*6.5 ± 5.2*	*0.8 ± 0.6*		
	MidW	**16.4 ± 1.1**	**35.7 ± 0.3**	**5.3 ± 3.8**	**0.5 ± 0.4**	0.6 ± 0.1	12
		15.2 ± 0.6	*35.8 ± 0.3*	*7.2 ± 7.1*	*0.9 ± 1.0*		
	InC	**16.9 ± 1.2**	**35.3 ± 0.2**	**4.9 ± 3.5**	**0.6 ± 0.5**	0.5 ± 0.1	
		15.0 ± 0.6	*35.7 ± 0.4*	*6.6 ± 5.2*	*0.8 ± 0.7*		

Bold and italic numbers represent values for the water sections 0–6 and 6–12 m, respectively. Iw (bold) and river inputs are shown as single value for the whole experimental area (Ria de Arousa).

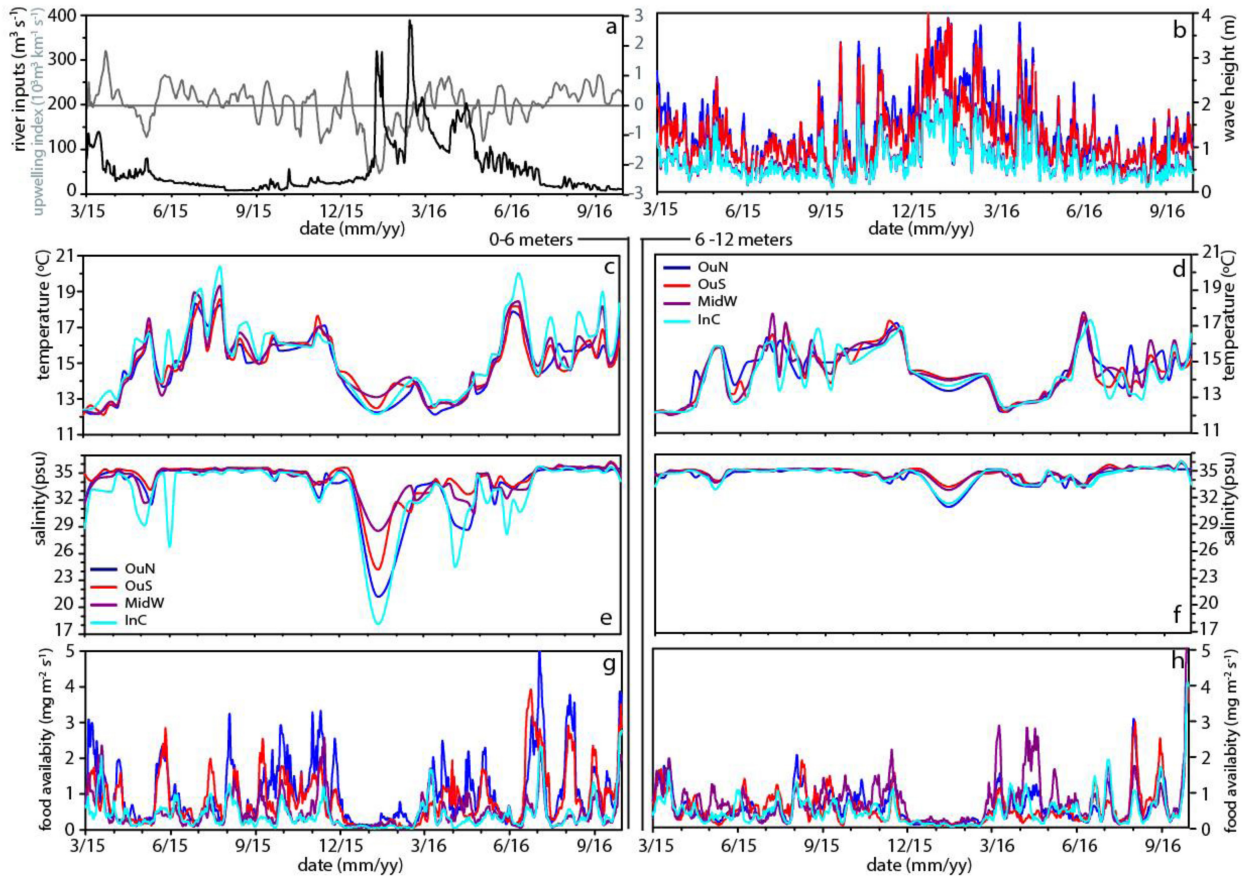

FIGURE 2 | Variability of the environmental parameters river discharge (m^3 s^{-1}; **a**) and moving average of upwelling index with window size of 15 days (10^3 m^3 km^{-1} s^{-1}; **a**), wave height (m; **b**), temperature for 0–6 m (°C; **c**) and 6–12 m (°C; **d**), salinity for 0–6 m **(e)** and 6–12 m **(f)**, total food availability for 0–6 m (mg m^{-2} s^{-1}; **g**) and 6–12 m (mg m^{-2} s^{-1}; **h**).

temporal variability in the index (**Figure 4C**) after removing the impact of mussel size. Two different aspects can be highlighted: the seasonal effect of the standardized STI, which was significantly higher (by approximately 15%) between autumn 2015 and winter 2016 (September–February) than in the summer period (May–August) for all cultivation sites (**Figure 4C**). On the other hand, inter-site variability showed a significant effect of culture location on STI variability ($p < 0.001$) (**Figure 4** and **Tables 2**, **3**). Mussels from the most inner and sheltered site (InC) had much thinner shells than mussels from the other locations with the thickest shells being obtained for OuS mussels followed by OuN and MidW mussels (**Figures 4A,B**). No differences were observed in relation to depth of water (**Table 2**).

Condition Index (CI) and Mussel Growth

The values of the condition index (CI), i.e., the ratio between soft and shell tissue weight, for the different cultivation sites and depths are shown in **Figures 5A,B**. The ANCOVA showed that CI was significantly influenced by both site and depth of cultivation ($p < 0.01$), together with the co-variable mussel size ($p < 0.001$) (**Tables 2**, **3**). The main difference for the inter-site comparison corresponded to both outer sites ($p < 0.01$), the northern (OuN) and the southern (OuS) sites, with highest and lowest CI values, respectively (**Figures 5A,B**). All other comparisons between sites were not significant with the exception of the minor effect noted for the comparison OuN *versus* MidW (**Table 3**). Interestingly, the CI values were similar in very different sites in terms of hydrodynamics, food availability and salinity regime, like OuN and InC (i.e., in the outermost exposed and inner sheltered areas) (**Figures 5A,B**). Regarding cultivation depth, CI was significantly lower in deeper waters (6–12 m) than in the surface waters (0–6 m), approximately 10% higher on average for all sites, as the interaction term site x depth was not significant (**Figures 5A,B** and **Table 2**).

Shell length growth curves for the mussels cultivated in the different experimental sites are shown in **Figure 6**. Values of the growth-related parameters of the Gompertz models used to analyze these curves are shown in **Table 4**. Significant differences between mussel populations referred to the maximum shell length obtained, i.e., L_∞ but also for the slope of growth vs. time regressions (-k) and the inflecion point of the curve (t') (**Table 4**). Asymptotic length (84–88 mm SL: considering the entire length of the ropes as the depth factor was not significant) was higher in mussels cultivated in the outer exposed northern site (OuN)

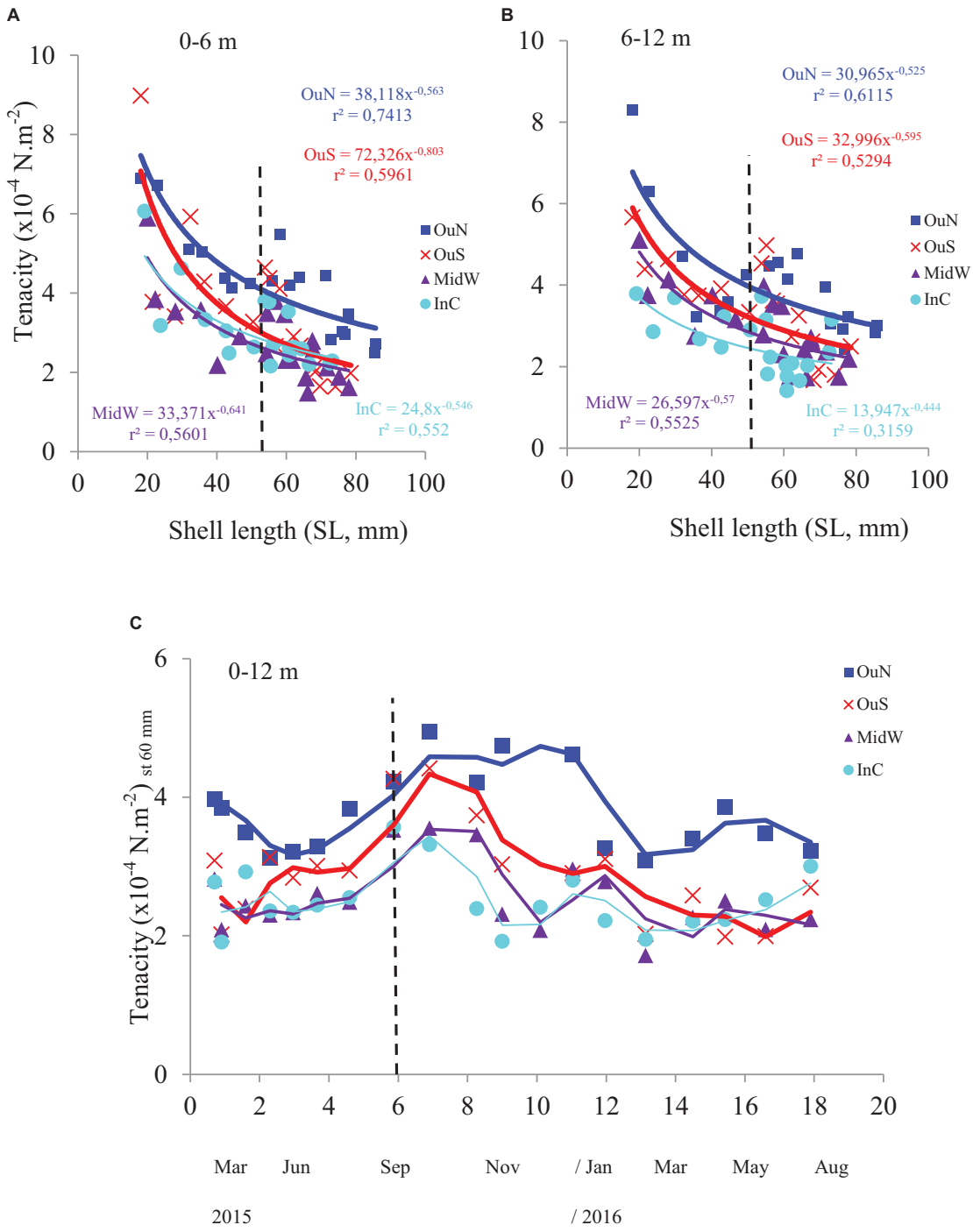

FIGURE 3 | Byssus tenacity in the mussels cultivated at the four distinct locations in the Ría de Arousa, at depths of **(A)** 0–6 and **(B)** 6–12 m in relation to mussel size (SL, mm). Variability in byssus tenacity (as mean values for the entire length of the cultivation rope; moving average) in relation to **(C)** cultivation time once standardized to a common mussel size of 60 mm shell length. The dashed line represents the timing of the thinning out process usually carried out between seeding and harvest.

than in mussels from the other sites, which did not differ significantly among them (67–73 mm SL) (**Figure 6** and **Table 4**). This represented mean values of 14–22% greater shell length growth for OuN mussels relative to the other cultivation sites (**Figure 6**). Differences in the other two regression parameters (k and t') are a consequence of the lengths obtained throughout the experimental period, highlighting the greater growth of mussels in the outer exposed OuN site (**Figure 6**). As water depth did

TABLE 2 | Results of ANCOVAs to determine the effect of cultivation site (si) and depth (z) on mussel tenacity (TEN), shell thickness index (STI), and condition index (CI) variability.

	Factor	df	*F*	*P*
ANCOVA, mussel tenacity (TEN)				
	SL	1	181.675	**<0.001**
	site (si)	3	29.765	**<0.001**
	depth (z)	1	4.768	**<0.05**
	si × z	3	1.008	ns
	Error	139		
ANCOVA, Shell thickness index (STI)				
	SL	1	3086.121	**<0.001**
	site (si)	3	6.385	**<0.001**
	depth (z)	1	0.007	ns
	Si × z	3	0.154	ns
	Error	137		
ANCOVA, Condition index (CI)				
	SL	1	15.763	**<0.001**
	site (si)	3	5.283	**<0.01**
	depth (z)	1	8.755	**<0.01**
	si × z	3	2.156	ns
	Error	139		

Shell length (SL) of the mussels was used as co-variable. Values in bold are statistically significant ($p < 0.05$); ns: not significant. For analysis assumptions, see section "Materials and Methods."

TABLE 3 | Statistical output of the statistical analyses *post hoc* for the four experimental localities selected for the experiment.

Muessel Tenacity		**OuN**	**OuS**	**MidW**	**InC**
ANCOVA	OuN	-----			
	OuS	**0.0027**	-----		
	MidW	**8×10^{-6}**	**0.0049**	-----	
	InC	**8×10^{-6}**	**0.0009**	ns	-----

STI		**OuN**	**OuS**	**MidW**	**InC**
ANCOVA	OuN	-----			
	OuS	**8×10^{-6}**	-----		
	MidW	**2.4×10^{-6}**	ns	-----	
	InC	**8×10^{-6}**	**0.0018**	**1.2×10^{-5}**	-----

CI		**OuN**	**OuS**	**MidW**	**InC**
ANCOVA	OuN	-----			
	OuS	**0.0001**	-----		
	MidW	**0.0182**	**0.0049**	-----	
	InC	ns	ns	ns	-----

Values in bold are statistically significant ($p < 0.05$); ns: not significant. For analysis assumptions, see section "Materials and Methods."

not significantly affect the shell growth parameters, the plotted values in **Figure 6** represent mean values for the entire length of the rope (0–12 m).

Integrating PROtective vs. FITness Responses: PROFIT Index

The PROFIT index was developed to illustrate the impact of local environmental conditions and how mussels respond with actions that are important for both survival (byssus tenacity and shell thickening) and yield (i.e., CI) with a simple ratio. The variability in this ecological indicator as a function of position along the Ría de Arousa is shown in **Figure 7**. ANOVA applied to the variability in PROFIT revealed a significant impact of the cultivation site, but no effect of depth or the interaction term (site × depth) (**Table 5**). Then, the PROFIT values shown in **Figure 7** corresponded to the entire length of the rope (0–12 m).

In general, the PROFIT values were highest ($p < 0.01$) for mussels from the outer exposed northern site (OuN) throughout the cultivation period (**Figure 7**). Differences between mussels from the other locations (OuS-MidW-InC) and differences in relation to depth were not significant (**Table 5**). Regarding the timing of harvest, for both outer and more exposed sites (OuN and OuS), PROFIT values were highest after 8 months of cultivation when the shell length reached approximately 60 mm (maximum PROFIT values of 33 and 12% for OuN and OuS, respetively). For the OuN site, where the mussels were largest (86 mm SL), maximun PROFIT values were only comparable to that reported at the end of the experiment (31%; after 18 months). For the OuS site, PROFIT

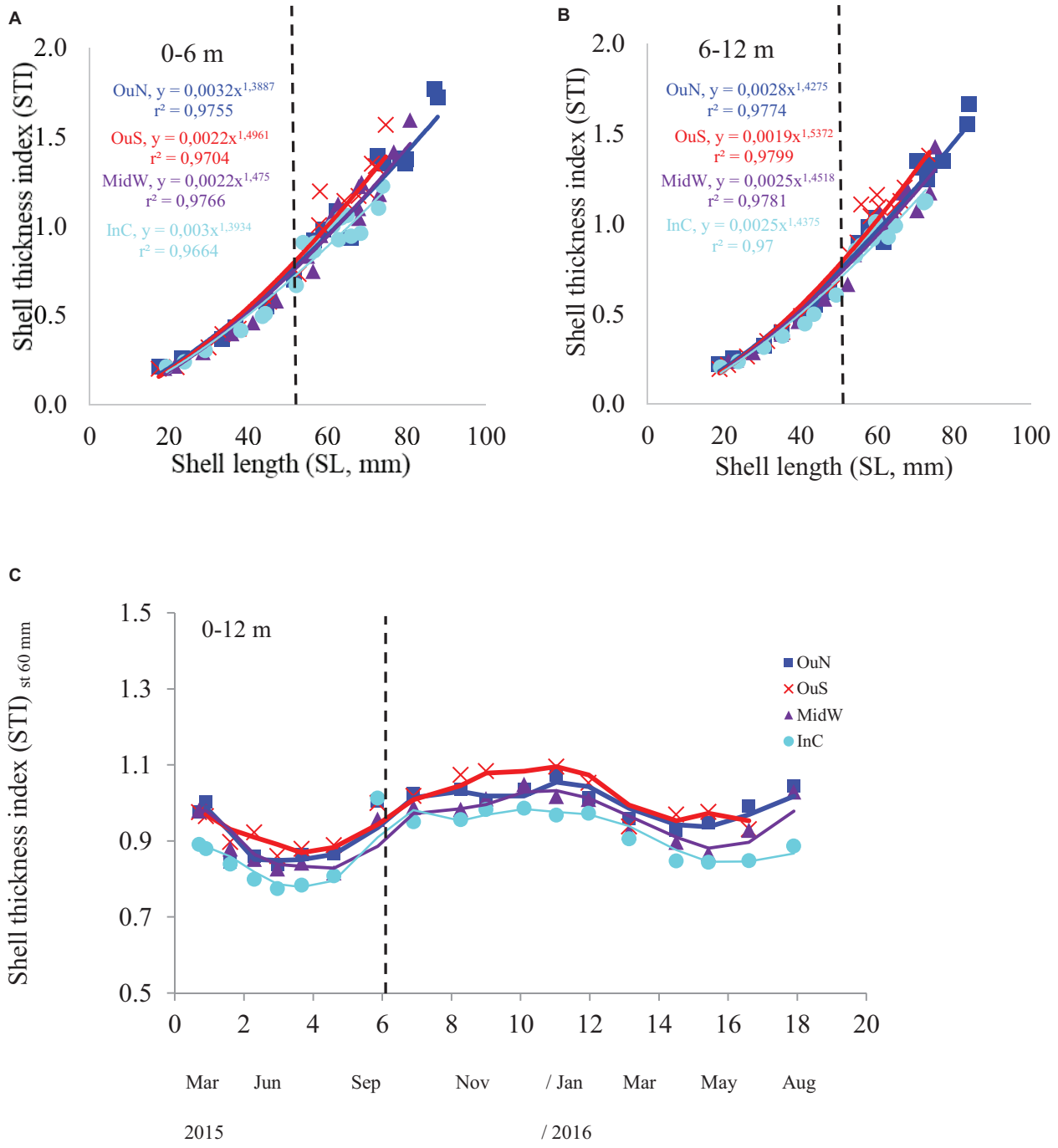

FIGURE 4 | (A) Shell thickness index (STI) of the mussels cultivated at the four different locations in the Ría de Arousa at depths of **(A)** 0–6 and **(B)** 6–12 m in relation to mussel size (SL, mm). Variability in STI in relation to **(C)** cultivation time once standardized to a common mussel size of 60 mm shell length. The dashed line represents the timing of the thinning out process usually carried out between seeding and harvest.

values at the end of the experiment were still lower than those observed at 8 months (**Figure 7**). For the innermost sites, MidW and InC, the PROFIT values were highest (14–18%) at the end of the experimental period (18 months) (**Figure 7**), revealing a clear difference between outer and inner estuarine areas.

DISCUSSION

The aim of this study was to relate a number of field responses in the mussel *Mytilus galloprovincialis*, linking individual performance, understood as the capacity to withstand natural threats (flow currents, waves, physical disturbance by

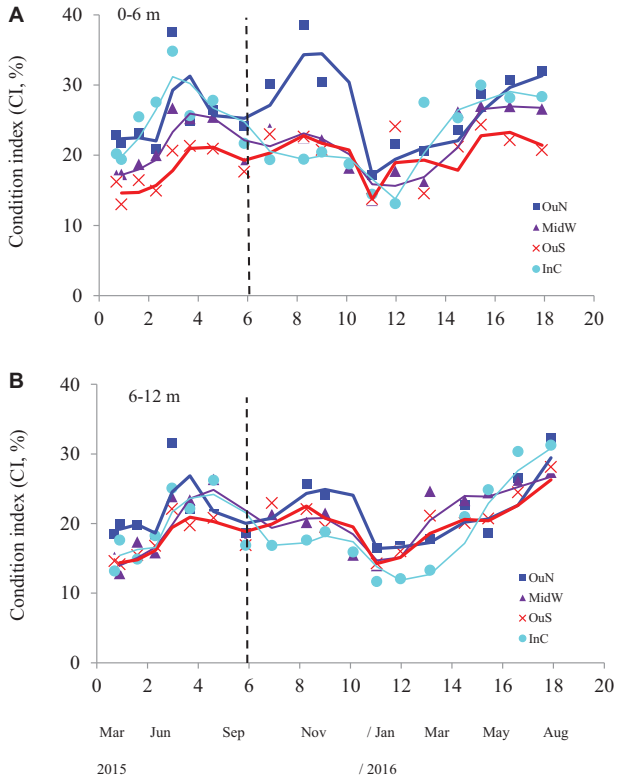

FIGURE 5 | Condition index of the cultivated mussels at the four different locations in the Ría de Arousa throughout the whole cultivation cycle (moving average) and at two depth intervals: **(A)** 0–6 m **(A)** and **(B)** 6–12 m. The dashed line represents the timing of the thinning out process usually carried out between seeding and harvest.

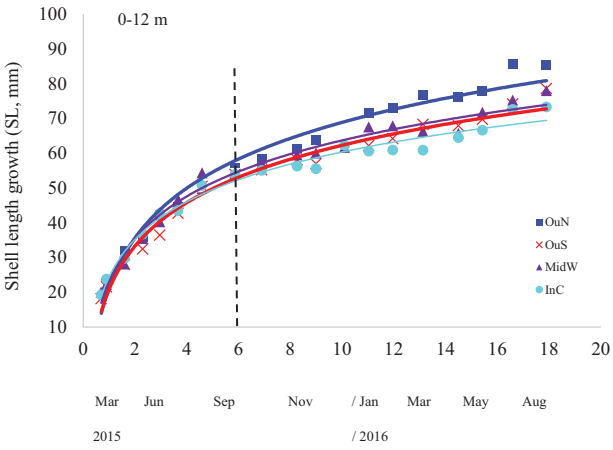

FIGURE 6 | Shell length growth curves for the cultivated mussels at the distinct experimental sites of the Ría de Arousa and considering the whole length of the cultivation rope (0–12 m). The dashed line represents the timing of the thinning out process usually carried out between seeding and harvest.

competition etc.), to condition index and the associated market value. For this purpose, byssus tenacity and shell thickening responses were considered proxies for the ability of mussels to attach to the substrate and resist hydrodynamic forces and predatory or competitive actions, respectively though the latter factor (biological pressure) would be of lower magnitude in the cultivation system as compared to rocky shore assemblages. As such actions may affect the commercial value by making the shell more fragile, the condition index was considered as proxy for the market value. The complementary analysis of the strategies used by the mussels during their life cycle enabled us to develop a novel, intuitive index integrating both energetic expenditure routes balancing protection/survival and fitness.

Protection and Fitness Along an Environmental Gradient

Differences in shell morphology, shape or thickness of bivalve mollusks are influenced by key environmental parameters such as food competition, substrate type, crowding, temperature, wave impact and predatory actions, among others (see Alunno-Bruscia et al., 2001; Beadman et al., 2003; Steffani and Branch, 2003; Valladares et al., 2010). In the present study, considering the spatial gradient between outer exposed and inner sheltered waters, we observed a significant decrease in shell thickness of mussels cultivated in the sheltered site (thinner and weaker shells). The same pattern was also observed for other protective tissue like byssus (weaker attachment). As well as being thinner, the shells of the mussels in the most inner and sheltered site (InC) were also more elongated, although shell height did not differ (see **Supplementary Appendix A**). Regarding the environment, the different patterns in shell shape were closely associated with the lower (two-fold) hydrodynamic values (wave height) in the most inner-sheltered site, more than any other parameter. Temperature and salinity can affect shell development

TABLE 4 | Estimated parameters and coefficients of the growth curves for cultivated mussels according to Gompertz model [$L_t = L_\infty\, e^{(-e(-k(time-t')))}$] for the four cultivation sites under study, March 2015 to July 2016.

Sites	Parameters			
	L_∞	k	t'	r^2
OuN, 0–12 m	86.233^{a} (3.171)	0.191^{a} (0.023)	1.910^{a} (0.260)	0.979
OuS, 0–12 m	73.381^{b} (2.463)	$0.233^{a,b}$ (0.031)	$1.504^{b,c}$ (0.259)	0.967
MidW, 0–12 m	$72.030^{b,c}$ (2.075)	0.271^{b} (0.036)	$1.203^{b,c,d}$ (0.244)	0.961
InC, 0–12 m	67.386^{c} (2.079)	0.271^{b} (0.043)	0.825^{d} (0.291)	0.948

All parameters are statistically significant ($p < 0.001$). Distinct letters and numbers identify significant differences for growth parameters.

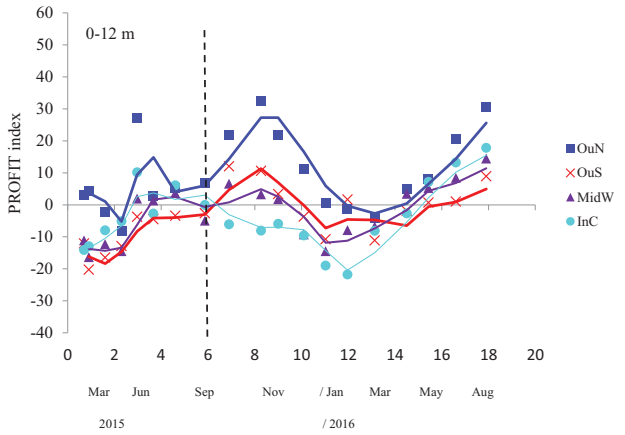

FIGURE 7 | PROFIT index variability throughout the whole cultivation cycle (moving average) of the mussels at the four experimental locations of the Ría de Arousa. Values shown correspond to the entire length of the rope (0–12 m) because of the non-significant differences by water depth. The dashed line represents the timing of the thinning out process usually carried out between seeding and harvest. Since each component of the PROFIT (expressed as a percentage) was corrected to the mean value for the four experimental sites and the whole cultivation period, positive and higher values represent better performance of the mussels.

TABLE 5 | Results of ANOVA to determine the effect of cultivation site (si) and depth (z) on the new index PROFIT variability for the whole mussel cultivation period.

Factor	df	F	P
Profit index			
Site (si)	3	13.0955	**<0.001**
Depth (z)	1	0.0005	ns
si × z	3	2.4172	ns
Error	144		

Values in bold are statistically significant ($p < 0.05$); ns, not significant. For analysis assumptions, see section "Materials and Methods."

(Kautsky et al., 1990; Nagarajan et al., 2006; Martinez et al., 2018; Telesca et al., 2018), but their impact, especially for temperature, can be considered minor in this study as the greatest differences between the most separated sites (inner InC and outer OuN-OuS) were reached in summer and winter periods respectively, with differences of 0.57°C and 2.67 psu, for temperature and salinity, respectively. For the case of salinity, it is known that low values of this factor are accompanied by lower pH and $CaCO_3$ saturation states, reducing the availability of calcium ions and dissolved inorganic carbon for calcification (Miller et al., 2009). However, the manuscript also focused on the need and importance of integrated approaches to the new ecological PROFIT index reported here which integrates different energetic aspect that are important for the survival and fitness of organism. Since salinity variation is relatively high at the most inner and sheltered InC site which in turn must be accompanied by supplementary energetic cost for intracellular osmotic pressure adjustments (Neufeld and Wright, 1996), this factor must be considered further for testing the effect of these punctual salinity decreases. Furthermore, the hydrodynamics appear to be more important for explaining the differences in the protective structures since salinity and temperature were similar in the middle site (MidW) and the most exposed sites (OuN-OuS). Similar results were obtained for shell thickness, byssus tenacity and condition index in mussels from the MidW and inner InC sites, with comparable hydrodynamics.

For most mussels in inner sheltered sites, thinner and weaker shells will increase shell fragility (see Penney et al., 2007). Both individual size (e.g., refugee size) and shell thickness are key factors regarding the vulnerability of the mussels as targets for predators (Leonard et al., 1999; Smith and Jennings, 2000; Zuschin and Stanton, 2001; Zuschin et al., 2003). Shell thickening is a well-known protective strategy in bivalves and is probably influenced by the evolutionary history of interactions between species (Freeman and Byers, 2006). Shell fragility is also important in aquaculture as the mechanical process of breaking the mussel clusters apart to separate individuals can lead to a significant loss of biomass (broken shells). Furthermore, factors such as shell appearance and integrity are also important, especially for the fresh market as broken shells can lead to rejection of the product for commercial purposes. Between 5 and 15% of the whole annual production may be lost due to broken shells being considered undesirable products (G. Sarà, personal communication, see Martinez et al., 2018). In Galicia, it is commonly known that mussels from outer/exposed sites are mainly destined for the fresh market, in which the appearance of the (more robust) shell is very important; mussels from the innermost sheltered sites are used in the canning industry or in processed products for which only the soft tissues are used. Consistently, in the present study, both strength of attachment and shell thickness were greater in outer and exposed sites (especially OuN) than in the inner areas. Future studies should address the biomass losses associated with shell failure or fragility as the spatial differences in both environmental factors and shell/byssus responses in the mussels from the Ría de Arousa are well known.

Shell length growth of the mussels differed significantly depending on the location. Growth was higher in the most outer and exposed (northern) site (OuN) than in the other sites, particularly the inner-sheltered InC, in which growth in length was lowest at the end of the experimental period, regardless water depth. This finding is consistent with those of previous studies in the same embayment regarding the spatial patterns of growth, i.e., greater mussel growth and productivity for the northern outer site (OuN) (Pérez-Camacho et al., 2014). Similarly to growth parameters, STI was less affected by water depth, and only byssus TEN (residually) and CI (10%) decreased in deeper waters probably in association with lower availability of natural resources and the lower level of movement at depth than in surface waters. In contrast to shell-related parameters (thickening and growth), the variations in CI were very different. Highest and lowest CI values were obtained in the outer exposed northern (OuN) and southern (OuS) sites, respectively. Beside, no differences were noted between mussels grown at the OuN site and those cultivated at the inner cand calmer InC site throughout most of the study period, except in autumn 2015 and in the surface waters (0–6 m). Mussels growing in the southern exposed site (OuS) had to develop high-standard protective tissues (TEN

and STI) as a consequence of the high-energy environment, e.g., hydrodynamics. However, the availability of natural food resources was lower in the southern site than in the northern site (with comparable hydrodynamics) and similar to that in the other inner, calmer locations. This energetic *trade-off* may have occurred at OuS where limited food resources available needed to be allocated to stronger byssus attachment and shell at the cost of soft tissue development, e.g., CI (see also Carrington, 2002; Moeser and Carrington, 2006; Babarro and Carrington, 2011). Following previous reasoning in regard to energy allocation patterns, mussels in the innermost sheltered site (InC) may have taken advantage of the less energy-demanding environment and in consequence, the energy not required for strong attachment or shell thickening may have been used for soft tissue growth.

Although the possibility that differences encountered in this study with regard to byssus tenacity and shell thickness index can partly reflect a genetic component in terms of divergence or selection cannot be completely ruled out without experimentation supporting that, it can be proposed for further research with complementary experimental design. High density of mussels in the rías and their spawning events in the previous spring-summer period are responsible of the seed attached along the rocky shore forming a relatively mixed genetic pool. Mussel seed used in our survey was obtained from the same intertidal rocky shore location of the Galician Coastline; consequently same origin was chosen to start mussel cultivation at the four different sites along the Ría de Arousa, which allow us to assume that variability shown in the mussel responses for the present study were due to phenotypic plasticity and adaptation patterns to key environmental parameters like food resources available, hydrodynamic, salinity etc. that characterized each zone of the ría. A detailed phylogeographic study to investigate genetic diversity in the Galician Rías showed that inter-population differences were half of that from other Iberian Atlantic samples using the same loci (Diz and Presa, 2009). Both large-scale (among five different Galician Rías) and short-scale (comparing inner and outer zones of single ría as performed in our own study) analyses showed a relatively weak divergence in genes which can be interpreted as not clear genetic differentiation (Diz and Presa, 2009).

The new ecological PROFIT index reported here integrates different energetic aspects that are important for the survival and fitness of organisms. This non-dimensional ratio may provide accurate information about the magnitude of certain energy allocation patterns that depend on widely heterogeneous environments in a given geographical area. PROFIT is a good indicator of the specific habitat conditions in terms of food availability, considering both natural seston load and hydrodynamics. Indeed, the northern exposed zone (OuN), according to food availability resources monitored, was identified as an environment where mussels may develop key metabolic processes that matter for the life cycle, with enough energetic resources to get high-standard protection through a strong byssus attachment and shell thickening, and high growth performance in relation to shell length and soft tissue production. Differences in the PROFIT index between the other experimental sites (OuS-MidW-InC) were of minor interest as was the effect of water depth. The fact that PROFIT values for mussels growing in the latter sites did not reach similar values in relation to commercial size and timing of cultivation period was a consequence of limiting energetic resources that could not meet the requirements for both protective (byssus and shell) and soft tissues equally. PROFIT index reported here can be easily updated in the near future with extra information about health of sampled mussels as well as environmental status of interest in mussel farming areas such as water pollution (D'Agata et al., 2014; Cappello et al., 2018), using the role of multi-marker approach to monitor pollution exposure or anthropogenic effects on immune system of the mussels (Giannetto et al., 2017; Caricato et al., 2019; Parrino et al., 2019).

Implications for Aquaculture

The most important finding for the aquaculture industry is that irrespective of the energy distribution between protective tissues and yield for a given environment, the PROFIT index will reflect both the importance of the spatial analysis and the optimal timing for harvesting. The timing is determined by the size of the mussel and the soft tissue yield (CI) as well as by the capacity of the mussels to remain attached to the cultivation rope (TEN) and to have a shell that can support the handling process without breakages (STI). The variability in TEN and STI in the sub-tidal mussels showed that juveniles were more strongly attached than adults (exponential decrease in TEN with size) whereas adults had stronger shells than juveniles (exponential increase in STI with size), as previously reported for intertidal individuals attach on rocky shore (see Babarro and Carrington, 2013). Thus, a general scheme can be considered, in which juveniles are more prone to being predated (thinner and weaker shells) and adults are more prone to being dislodged (weaker byssus tenacity) (see **Supplementary Appendix B**). The use of TEN and STI to calculate PROFIT enables integration of these often- overlooked characteristics, which vary throughout the life cycle of the mussel and are critical for maximizing the quality of the product depending on local environment.

The current perception of Spanish mussel industry is that market interests have shifted to smaller sizes of the cultivated product, as in France and Italy in recent years (see fluctuations in the listed price change for the period 2004–2012, Monfort, 2014). Accordingly, the percentage of large mussels obtained at harvest decreased significantly, from 24 to 8%, in the last 15 years, and the opposite was observed for the proportion of small sized mussels harvested, which increased from 51 to 68% (Aquaculture YearBook[3]). Indeed, the minimum commercial size for mussels was 70 mm shell length a few decades ago, whereas the corresponding value for Spanish mussel cultivation is now 50 mm (Pérez-Camacho et al., 2013), which is similar to the size demanded by the market. In 2018, the European Commission endorsed the inclusion of small sized mussels in the Protected Designation of Origin (PDO) scheme used in Galicia for mussel certification with the aim of satisfying the European demands, while always preserving high quality.

[3]https://www.pescadegalicia.gal/gl/publicacions

The study findings show that the PROFIT index, which standardizes all aspects that matter for the survival and yield of the cultivated product, reached the maximum value in the outer sites after 8 months (winter), although with significant differences between the northern (OuN) and southern (OuS) sites according to food availability resources. The PROFIT value then remained lower, only reaching values close to the maximum at the end of the experimental period (18 months). Regarding mussel size, the maximum PROFIT value was reached at 60 mm shell length after 8 months, which is lower than the size above which a negative effect of high density on size/weight growth was reported for typical farming practices in Spain, 66 mm (Cubillo et al., 2012). This information can be used to offer a set of recommendations regarding the shortest time within which individuals in these locations can be harvested. New expectations and market tendencies can be taken into account, to prevent loss of market quality (CI variability) and to decrease the risk of dislodgement (based on byssus tenacity) during an extended cultivation period. By contrast, for mussels cultivated in inner and calmer sites, the PROFIT index reached the maximum value at the end of the cultivation period (18 months), possibly suggesting that the best compromise between optimal meat yield and strength of attachment and shell thickness is obtained much later than in outer and more expose sites due to lower availability of food resources. Consequently, depending on the cultivation site, harvesting could be managed according to the market interests for the specific size of mussels demanded and the appropriate cultivation timing, according to PROFIT, in order to reduce both total cultivation time and labor costs. Shortening the cultivation time by aiming for smaller mussels at harvest would allow for high culture densities and the thinning process to be omitted, which would represent a significant improvement in cultivated biomass and yield (Pérez-Camacho et al., 2013). This strategy would maximize growth and minimize dislodgement, given the limited intraspecific competition (Cubillo et al., 2012) and high tenacity (this study) of small mussels. Here, we complement this innovation strategy (Pérez-Camacho et al., 2013) with the underlying eco-physiological mechanisms to support any potential shift in the classical development of mussel raft cultivation, as a novel and integral management tool to valorize different production zones or exploitation methods. PROFIT index can be used to optimize the timing of harvest and minimize dislodgements and consequently also minimize the negative effects of biomass on the sea floor.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

JB and XP conceptualized and performed the experiment. ML helped in the field sampling organising trips and monitoring mussel responses on the raft. RF supported with data analysis, discussing, writing and editing the manuscript. JB wrote the first draft of the article. All authors added valuable comments on the writing the first draft and contributed to manuscript revisions and read and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2020.00719/full#supplementary-material

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Atlantic Horseshoe Crabs and Endotoxin Testing: Perspectives on Alternatives, Sustainable Methods, and the 3Rs (Replacement, Reduction, and Refinement)

*Richard Gorman**

Department of Geography, University of Exeter, Exeter, United Kingdom

Endotoxin testing is a vital part of quality and safety control in pharmaceutical production. The primary method for this testing in North America and Europe is the limulus amebocyte lysate (LAL) test, a critical component of which is the blood of Atlantic horseshoe crabs (*Limulus polyphemus*). Procuring blood for LAL testing involves capturing and bleeding over 500,000 crabs from wild marine populations each year. Whilst efforts are made by manufacturers to return crabs to the sea following the collection of blood, there is a level of mortality and sub-lethal impact involved, prompting increasing discussions about welfare and ethics. The 3Rs – the ambition to where possible, replace, reduce, and refine the use of animals – are established and accepted worldwide as the best framework for governing animal-dependent science. However, the biomedical utilization of horseshoe crabs to produce the LAL test has rarely been viewed through a 3Rs framework. More recently, there has been a renewed attention on sustainable methods and alternatives to the LAL test. Drawing on in-depth qualitative interviews, this article examines stakeholder perspectives on opportunities for thinking with the 3Rs, considering current appetites to replace, refine, and reduce contemporary biomedical reliance on horseshoe crabs. The shape of conversations about the biomedical utilization of horseshoe crabs has shifted significantly in recent years, and the 3Rs are an important driver of change, offering the potential to advance the use of more sustainable methods, and realize the welfare considerations increasingly expected across science and society.

Keywords: limulus amebocyte lysate, horseshoe crab (*Limulus polyphemus*), recombinant factor C, 3Rs (replacement, reduction, refinement), alternatives to animal testing, social science and humanities

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Nicholas Santangelo, Hofstra University, United States
Christopher C. Chabot, Plymouth State University, United States

***Correspondence:**
Richard Gorman
r.gorman@exeter.ac.uk

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INTRODUCTION

Atlantic horseshoe crabs (*Limulus polyphemus*) – though classed as 'vulnerable' (Smith et al., 2016) – play a vital role in enabling global public health. These blue-blooded marine invertebrates are, at least currently, used by humans to ensure that the vaccines, injectable medicines, and medical devices utilized in human and veterinary medicine are safe from contamination by endotoxins. Endotoxins, synonymously known as lipopolysaccharides (LPS), are molecules found as part of

the outer membrane of Gram-negative bacteria, released upon the disruption of intact bacteria (through death or cell lysis). If released into the mammalian blood stream endotoxins are capable of producing a complex pattern of systemic toxicity, ranging from fever through to life threatening effects such as hypotension and shock (Levin et al., 2003). Endotoxins are highly heat-stable, and thus not destroyed as part of conventional sterilizing or heat-treating protocols. Indeed, sterilizing a product to kill any bacteria present can actually result in the release of LPS, and thus an essential – and regulatory mandated – part of quality and safety control in pharmaceutical production involves testing injectable drugs and medical devices for contamination by endotoxin (Joiner et al., 2002).

In North America and Europe the primary method for endotoxin testing is the limulus amebocyte lysate (LAL) test.[1] This utilizes the coagulative properties of the blood of Atlantic horseshoe crabs to detect endotoxins, linking this immunologically unique and ancient species to the global supply chains of modern health and medicine. LAL is commonly understood and positioned as a 'replacement' itself, an alternative to the *in vivo* Rabbit Pyrogen Test (RPT), the previous regulatory standard for endotoxin testing, and thus development of the LAL test ostensibly led to a significant reduction in harm to animals (Hartung, 2001).

However, procuring blood for LAL testing involves capturing and bleeding over 500,000 crabs from wild populations each year, many of which die (Atlantic States Marine Fisheries Commission, 2019). The Atlantic States Marine Fishery Commission (ASMFC, the regulatory body around the horseshoe crab) assumes a 15% mortality rate for bled and released crabs. This 15% rate is highly debated and contested, ranging widely within published literature – Hurton and Berkson's (2006) experiments resulted in a post-bleeding mortality rate of 8.3%, whilst Leschen and Correia's (2010) study concluded a post-bleeding mortality rate as high as 29.8%.

The continued use – and potential rise in use given increasing global demand for pharmaceuticals – of horseshoe crabs is prompting growing questions around welfare and sustainability (Krisfalusi-Gannon et al., 2018). As alluded to earlier, the Atlantic horseshoe crab has been classed as 'vulnerable' by the IUCN, with populations trending to 'decreasing' (Smith et al., 2016), although the extent to which the biomedical use of crabs has an impact on population numbers is hotly debated and contested (Dawson and Hoffmeister, 2019). Whilst most crabs are returned to the sea following the collection of their blood, and some view the bleeding process as harmless, there are increasing discussions about the impact that capture and bleeding can have on crab health and mortality (Krisfalusi-Gannon et al., 2018; Maloney et al., 2018; Atlantic States Marine Fisheries Commission, 2019). Anderson et al. (2013) found that bled horseshoe crabs, post-release, suffered from significant changes in activity levels and behavioral rhythms, reductions in hemocyanin levels (potentially affecting immune function), and decreased reproductive fitness (prompting concerns about longer-term population impacts). Similarly, Owings et al. (2019) found that bled animals approached mating beaches less than control animals during the first week after release. Relatedly, there are growing debates around *Limulus*' sentience and capacity to suffer (John et al., 2018), with animal protection groups posing welfare and ethical questions about the continued use of horseshoe crabs within the pharmaceutical industry.

Despite the global reliance on this immunologically unique and ancient species and its centrality and indispensability amongst the supply chains of modern health and medicine, the pharmaceutical utilization of horseshoe crabs to produce the LAL test is rarely viewed through a 3Rs framework. The 3Rs – the ambition to where possible reduce, refine, and, replace the use of animals – are established and accepted worldwide as the best framework for governing animal-dependent science, playing an integral role in ensuring high standards of ethical consideration whilst also maximizing the potential for high-quality science (Kirk, 2017). The 3Rs, first formulated by Russell and Burch (1959) in their book 'The Principles of Humane Experimental Technique' have become central to how the use of animals in scientific procedures is socially understood, politically imagined, and (inter)nationally regulated (Davies et al., 2018). They exist as a means of weaving together 'good science, good care, and socially acceptable practices' (Davies et al., 2018, p. 607), providing a pathway to aligning both 'moral and scientific values within a pragmatic ethical framework' (Kirk, 2017, p. 21).

At present, horseshoe crabs are outside of the scope of most formal legislation regulating animal use; not considered a 'protected' animal. Horseshoe crabs are a wild invertebrate, managed as a fishery, and bled through a process widely imagined as both non-invasive and a force for conservation, in order to produce a reagent that is readily positioned as an *in vitro* alternative in the first place (see also, Gorman, 2020). Hence, thinking about horseshoe crabs as a 3Rs issue has rarely been a priority to date.

Yet, there is growing interest amongst stakeholders in more fully engaging with a 3Rs framework (Bolden and Smith, 2017; Marius et al., 2020; Piehler et al., 2020). Bringing conversations about horseshoe crabs into connection with wider discussions about the 3Rs is seen as offering valuable opportunities for restructuring debates about biomedical horseshoe crab use, highlighting the efforts made within industry to improve practices. However, different stakeholders see different value and possibilities in each of the individual 'Rs', to the point

[1]Global endotoxin detection is also dependent upon the Tachypleus amebocyte lysate (TAL) test, produced in China, and utilized for analogous testing by Asian and Pacific-based pharmaceutical manufacturers (Krisfalusi-Gannon et al., 2018). However, this involves a complex geography, as many multi-national pharmaceutical companies that manufacture products in China ultimately test their end products with LAL as they are selling worldwide (Gauvry, 2015). This involves the utilization of *Tachypleus tridentatus* (commonly known as the Chinese horseshoe crab, Japanese horseshoe crab, or tri-spine horseshoe crab), a species which is listed as endangered (Laurie et al., 2018). With the population of *T. tridentatus* declining drastically there is speculation that this could lead to a compensatory spike in the global demand for LAL (Krisfalusi-Gannon et al., 2018). There is concern that 'regulations to protect the harvest of crabs for biomedicine are not as successful in Asia as in the United States,' and that bleeding practices often involve 'draining to death,' rather than the catch and release fisheries practiced in America (Moore, 2017, p. 120; Laurie et al., 2018). Thus, although this article focusses specifically on the Atlantic horseshoe crab (*Limulus polyphemus*) and the Limulus Amebocyte Lysate (LAL) test, it will be increasingly prudent to look at the 3Rs and issues of animal welfare across all species of horseshoe crabs and forms of endotoxin detection solutions.

of substantial friction between those who advocate focus on 'replacement' above 'reduction.' Ideas about replacement have tended to dominate discussions, a focus that has detracted from equally important efforts toward developing alternative approaches involving reduction and refinement.

As its central research question, this article queries what role the 3Rs – the ambition to replace, reduce, and refine the use of animals – might play in driving more sustainable methods within the biomedical utilization of horseshoe crabs? Additionally, the article considers what a social science perspective might add to discussions about the biomedical use of horseshoe crabs, and what might be learnt by bringing discussions about horseshoe crabs into conversation with the wider literature around the 3Rs? Thus, in what follows, the article moves to assemble stakeholder perspectives on opportunities for thinking with the 3Rs, considering current appetites for the development, promotion, and implementation of more sustainable methods for, and alternatives to, the use of horseshoe crabs for biomedical purposes.

SOCIAL SCIENTIFIC METHODS FOR INVESTIGATING PERSPECTIVES ON ALTERNATIVES AND THE 3Rs

Social science is, broadly, the study of human society and social relationships. Social scientists explore how people interact with one another, how shared cultures develop, and how people influence the world. Social science can help explain how society works, what different groups expect, and inform governance and policy. Social scientists working within the broad field of 'science and technology studies' seek to understand how scientific research and technological innovation are affected by, and affect in turn, society, politics, and culture. This involves exploring the different ways that scientific knowledge is produced and contested, and how different 'claims' to evidence, truth, and ethics are made and invoked in ways that influence practice (Cassidy, 2019). More recently, social scientists have become interested in how the use of animals in biomedicine is impacted by social, cultural, and political relations, and particularly, the contemporary challenges emerging as scientific practices and social expectations change (Davies et al., 2020). This involves examining how different interests are spoken for, and decisions made, around the biomedical use of animals.

The use of horseshoe crabs in endotoxin testing engages a complex range of stakeholder perspectives and involves multiple 'epistemic communities' each with their own shared beliefs, working practices, and criteria for assessing validity that lead them to form different understandings. Discussions about alternatives to horseshoe crab derived reagents involve considerable uncertainties and diverse views, with discussions becoming increasingly polarized (Guest, 2019). Krisfalusi-Gannon et al. (2018, p. 10) suggest that the drivers for horseshoe crab protection 'are both environmental and economic.' This may be true, but they are also social and cultural. As Davies et al. (2016) argue, social science research can make a significant difference to laboratory policy and practice, opening up understandings of the social, economic and cultural processes that influence practices surrounding the scientific use of animals, and the wider social contracts that enable public acceptance of the scientific use of animals. Social science can help understand the 'shape of the conversation.' This involves considering who is included in a conversation about the use of horseshoe crabs and how it is framed, focusing on understanding the various perspectives, positions, and sides of the debate in order to try and move discussions forward in productive ways (Cassidy, 2019). This is less about definitively identifying what is factually or morally 'right' and instead exploring what different stakeholders believe, and why they believe the things that they do (Cassidy, 2019).

In practice, this involved conducting qualitative interviews as a means of exploring perspectives on alternatives, sustainable methods, and the 3Rs. As Tiller et al. (2016, p. 4) suggest, when thinking about the management of marine resources, 'there is a strong motive for engaging with stakeholders in order to access the expertise that they possess (i.e., "knowledge-base" data), which is characteristically strongly qualitative.' Interviewing is one of the most commonly used qualitative research methods in the social sciences. In-depth interviews enable researchers to learn from interviewees' perspectives, their situated and contextual experiences, and their attitudes and feelings toward – in this case – horseshoe crabs and endotoxin testing. Thirteen interviewees were selected from across the broad spectrum of groups with a stake in the biomedical use of horseshoe crabs: manufacturers, biotechnology companies, regulators, pharmaceutical scientists, conservationists, animal-welfare groups, academic researchers. This involved a level of purposive sampling – a commonly used sampling strategy in qualitative studies where respondents are selected to enable a subject to be studied in depth. These individuals were located across the United Kingdom, Europe, and North America. This approach enabled a narrow but deep focus. Interviews with these stakeholders explored their perspectives and concerns relating to the current and future roles of horseshoe crabs within practices of endotoxin testing. These interviews provide an opportunity to understand the priorities of stakeholders. Interviews lasted for an hour, on average, and were conducted via phone or online video-call. The research was assessed and approved by the University of Exeter's Ethics Committee. The 'semi-structured' nature of the interviews allowed participants to focus on areas they felt were most important about their contextual – and uniquely situated – perspectives and concerns relating to the current and future roles of horseshoe crabs – and alternatives to crab-derived products – within practices of endotoxin testing. Semi-structured interviews involve a pre-prepared schedule of questions, however, they are also characterized by their flexibility, discursiveness, and open-ended nature, allowing the researcher to explore emergent ideas as the conversation progresses (Bryman, 2001). Some questions were asked to all interviewees, such as "How do you feel the landscape of endotoxin testing, and the use of LAL, has changed in recent years?" Whilst other questions were shaped dependent on the interviewee's role regarding horseshoe crabs and endotoxin testing, with some specific to their sector (i.e., interviewees in the pharmaceutical sector were asked "What

would motivate you to change to an alternative (non-animal) method of endotoxin detection").

All of these conversations were recorded, with interviewee's consent, and transcribed to allow analysis. Participants received copies of their transcripts to review to enable accuracy and clarification. It is common practice within social scientific research to anonymize participants for reasons of confidentiality and ethical research practice. This is particularly the case here, given the sensitive nature of animals' involvement in testing, along with the need for sensitivity around commercial interests. As such, all interviewees have been assigned attributions based on their broad sector of work. These have been broadly grouped and defined as the 'biotechnology sector' (n = 5) (stakeholders involved in producing endotoxin testing solutions, either LAL or alternative/recombinant methods)[2], the 'pharmaceutical sector' (n = 3) (stakeholders involved in utilizing and conducting endotoxin testing as part of pharmaceutical production), the 'conservation sector' (n = 3) (stakeholders involved in advocacy, research, and practices aimed at conserving horseshoe crab populations), the 'regulatory sector' (n = 1) (stakeholders involved in setting and enforcing safety and standards), and the 'communications and media' sector (n = 1) (stakeholders involved in discussions about the use of horseshoe crabs from their perspectives as journalists and/or multimedia producers). However, it is particularly important to stress here that amidst these broad groupings, it is not possible to ascribe a singular view or sentiment to these categories – for example, as will be shown, whilst some within the pharmaceutical sector were positive about recombinant alternatives, others were still unconvinced.

Analysis followed a thematic analysis approach, utilizing NVIVO (a qualitative analysis software package). Thematic analysis is 'a method for identifying, analyzing and reporting patterns (themes) within data' (Braun and Clarke, 2006, p. 79). This involved comparing themes across different perspectives in order to understand what is important to different people, and identifying where there are differences and similarities between different groups. Through analyzing and examining different perspectives about evidence, validity, risk, we can begin to understand how different discourses about alternatives are forming, intersecting, and impacting practices.

PERSPECTIVES ON REDUCING BIOMEDICAL HORSESHOE CRAB USE

> *"If you can replace 95% of your tests with a method that uses 99% less LAL, your impact is – I won't go into the math – but it felt that it wasn't unreasonable that a significant reduction could lead to a massive impact for the better."*
>
> Interviewee, pharmaceutical sector

In biomedical research, reduction usually refers to ensuring that the minimum number of animals is used to answer the scientific question, using effective experimental design and statistical analysis to optimize numbers and avoid wasting animals. In the context of this use of the horseshoe crab, reduction can involve minimizing the number of animals that are required to be caught – or minimizing the amount of animal derived material used in a given method or process. Across stakeholder interviews, reduction was felt to be, as one interviewee from the pharmaceutical sector described, '*a big quick win, the sort of thing we're looking at all the time.*' Reduction was perhaps the most palatable of the 3Rs, with the qualitative analysis showing that a majority of interviewees' responses reflected a sentiment generally supportive of reduction (in principle), across the spectrum of stakeholders. Though the extent to which reduction could be achieved, how it could be achieved, and when it could be achieved, varied greatly. However, reduction is a framework which offers a progressive route for alleviating the burden placed on horseshoe crabs, but without requiring radical reconfiguration of existing practices. Reduction is increasingly framed as a process in contrast to replacement, for this exact reason, as Krisfalusi-Gannon et al. (2018, p. 9) argue, 'revising the current system to improve efficiencies in horseshoe crab use may be more viable in the near term.'

The number of crabs utilized for biomedical purposes prior to 2004 are uncertain, since there was no standardized reporting, though the ASMFC estimate that medical usage increased from 130,000 crabs in 1989 to 260,000 in 1997 (2019, 35). Biomedical use has continued to grow, with the number of crabs delivered to biomedical facilities increasing from 335,501 crabs in 2004 to 575,760 crabs in 2017 (peaking with 622,098 in 2012). The ASMFC (2019, ii) suggests that biomedical use is now 'fairly stable.' However, there is concern that growing global demand (and emerging markets) for vaccines, pharmaceuticals and medical devices will require an increasing supply of LAL (Krisfalusi-Gannon et al., 2018). Whilst additionally, the rise of personalized medicine could necessitate individualized product testing on a per use basis, adding further pressure (Porzio, 2018). More recently, there is also concern that the COVID-19 pandemic will increase the need for endotoxin testing, particularly in the face of vaccine production[3].

The numbers of crabs collected for biomedical purposes are frequently contrasted against the earlier fertilizer industry – in 1880 over 4 million crabs were harvested from Delaware Bay alone (Kreamer and Michels, 2009), as well as the contemporary bait fishery, which utilizes horseshoe crabs as bait to catch eel and conch (whelk) – a process that involves a 100% mortality rate. Harvests here peaked in 1999 at 2.6 million horseshoe crabs, with 994,491 harvested in 2017) (Atlantic States Marine Fisheries Commission, 2019). This use of the animals was viewed as much more of a priority for replacement, reduction, and refinement, and 3 out of the 5 biotechnology respondents reported feeling aggrieved by what they described as 'the vastly uneven attention' placed on LAL manufacture, a view also shared by some pharmaceutical stakeholders:

[2]Some biotechnology companies have a portfolio that includes manufacturing both LAL and rFC endotoxin testing solutions.

[3]Whilst this has been widely speculated, in interviews with Arnold (2020) in *National Geographic*, one manufacturer is quoted as explaining "that to make five billion doses of the COVID-19 vaccine, 600,000 tests will be performed, which will use the amount of lysate created in a single day," something they go on to argue would place no undue burden on either the lysate supply chain or horseshoe crab populations. Regardless, the pandemic has brought renewed attention to the many animals that are entangled in ensuring human health.

"And the bait industry doesn't get any media attention, that takes a million crabs and chops them up every year. It's like can the bait industry reduce their reliance first?"

Interviewee, pharmaceutical sector

However, as Bolden (2019, p. 504) summarizes, it is 'unproductive to pit the bait fishery take against the biomedical take.' Indeed, as Davies (2019) has explored in the context of laboratory animals more broadly, drawing comparisons to the high numbers of animals used in other sectors does not reassure nor create a strong basis from which to argue an ethical point. However, interviewees felt that engaging with the specific numbers of crabs used biomedically did offer a route to creating a localized culture of care and interest in the 3Rs within endotoxin testing:

"When you translate it [reduction] back to crabs – very approximately because there's so much variability – but when you can convert number of test vials and lysate for the crab, people are keen to hear that [. . .] we had challenges, and I said "just remember the drivers for change here, this is your forecasted burden reduction on the crabs and I understand this is difficult but if we can do this quicker, the impact is there" and that worked."

Interviewee, pharmaceutical sector

Creating this engagement is important as the number of crabs bled is ultimately linked to demand and use, and thus the largest opportunities for reduction occur further down the supply-chain. There is huge scope – though presently, little awareness – for end-users in laboratories around the globe to effect reduction and significantly decrease the amount of crab blood used. AstraZeneca's 2018 Sustainability report describes how as part of their 'Culture of Care' and focus on high standards of animal welfare they were keen to reduce the use of horseshoe crabs in testing, and have invested in new technology 'which will see a reduction in annual lysate (crab blood reagent) consumption from approximately 7.5 litres to just a few hundred millilitres' (AstraZeneca, 2018, 48).

Technological fixes are regularly viewed as the way forward in terms of reduction. For example, one manufacturer of LAL has developed new technologies that allow the use of less raw material than traditional endotoxin testing methods. Charles River Laboratories argue that 'if all tests were performed using cartridge technology, today's entire worldwide LAL demand could be met with less blood than from Charles River's current annual quota' (Charles River Laboratories, 2020). Additionally, Guest (2019) advocates for the automation of endotoxin testing, suggesting it would result in a significant reduction in waste and in invalid tests that need repeating, along with the streamlining of testing plans to increase the number of tests per run, thus reducing total lysate used. Marketing for automation argues that 'the most expensive LAL test is the one that must be repeated because of invalidity' (Charles River Laboratories, 2020) – and this is also true in terms of the burden placed on crabs by testing errors.

There were also suggestions that optimizing the welfare of crabs might enable the collection of higher quality raw material, and thus offering pathways to reducing the number of crabs required to sustain the industry. This begins to blur the lines between reduction and refinement.

"The LAL that we're getting in the wild probably isn't the best LAL that could be available if the organism was fed appropriately and sufficiently, and managed in a temperature that was controlled, an environment that was controlled. So you can use less LAL, because – and it's not even just an engineering function where you make the assay smaller – but because your source material is better. It can be higher in reactivity or activity and it can be better, so you can start to reduce the amount that you need and those things can start going toward the reduction component, if you're making a better reagent in higher quantity, then that translates to needing less."

Interviewee, biotechnology sector

This, however, is not straightforward, efforts to rear and maintain *Limulus* are wrought with welfare and economic challenges – as will be discussed more specifically in the later section on efforts for aquaculture-based refinements. However, even if a variety of these methods can be used to reduce the number of animals required, as an interviewee from the pharmaceutical sector reported, ultimately "*some people don't believe that reduction's enough, they want replacement, the ethical quandary of fishing these creatures doesn't sit well with some people.*" Yet perspectives on replacement are even more complex and contentious.

PERSPECTIVES ON REPLACING BIOMEDICAL HORSESHOE CRAB USE

"Because of the 3Rs, there's a move in the pharmaceutical industry to get animal sources of raw material, out of any raw material they use."

Interviewee, biotechnology sector

Replacement refers to replacing animals with non-animal methods wherever feasible. A synthetic substitute to horseshoe crab blood was introduced in 2001 – laboratory-synthesized genetically engineered recombinant factor C (rFC) (Ding and Ho, 2001), becoming commercially available in 2003 (Bolden, 2019). Factor C here being 'the endotoxin-sensitive serine protease that initiates the coagulation cascade' in horseshoe crab blood (Ding and Ho, 2001, p. 277). However, initial uptake of this replacement was extremely limited due to the availability and market-dominance of the LAL test, combined with concerns about a single-source and supply of the synthetic, cautions over the validation of the alternative, and a lack of regulatory requirements to consider alternatives to testing in non-vertebrates.

More recently, there has been a renewed attention on replacements to the LAL test, emerging as a result of concerns relating to the sustainability of horseshoe crab populations and as recombinant reagents have become commercially available from multiple manufacturers (Bolden and Smith, 2017). One review of the performance of rFC as an endotoxin detection method suggested it is equivalent to, or better than, LAL in terms of the ability to detect and quantifiably measure bacterial endotoxin (Maloney et al., 2018). However, others have been less positive

about the potential to move to this alternative on a routine or commercial basis, citing concerns about the current ability of the alternative to achieve adequate specificity (Dubczak, 2018). The topic of alternatives here has generated much discussion, with debates becoming increasingly polarized. Indeed, Guest (2019) (a scientist working on drug safety) describes inquiring about the technologies available for endotoxin testing, and receiving a 'completely polarized message.' Guest's article goes on to explain, "in all honesty, with such a conflicting view, it was hard to trust the information" (Guest, 2019). This split was evident within stakeholder interviews too, with a divide between a belief that:

> *"Conversion to rFC would result in a 90% reduction in the demand for LAL, which means that mortality resulting from bleeding would decrease by an estimated 100,000 horseshoe crabs annually."*
> Interviewee, conservation sector

And the contrasting idea that:

> *"Recombinant factor C is not going to "save" the horseshoe crab."*
> Interviewee, biotechnology sector

Whilst scientific consensus over whether current replacement technologies are fit for purpose is still playing out, there is no question that their existence has changed the very shape of discourse around alternatives here:

> *"Before you couldn't say that, now you can say it, particularly since there is an alternative, so it's just changed the whole conversation. So now if the alternative is no good, that's a different conversation, let's talk about the efficacy of the alternative, but there* ***is*** *an alternative."*
> Interviewee, conservation sector

Further, what we can see is the way in which the 3Rs framework is positioned as a driver for this move to seek a replacement. Bolden and Smith (2017, p. 405), note that 'replacing the animal-derived LAL with rFC for endotoxin testing is consistent with the 3 R principles (replacement, reduction, refinement) for more ethical and sustainable use of animals for testing.' They go on to explicitly link their interest in using recombinant factor C to replace LAL for endotoxin testing to their company's 'commitment to animal welfare and conservation.' This link to animal welfare is particularly novel in terms of discussions of *Limulus*[4]. Though others questioned whether this turn to welfare and the 3Rs is an attempt to capture market-share through a cynical branding move:

> *"These companies have an alternative and they can market it to their marketing people as how it saves horseshoe crabs and all that, you wonder if they really, at the end of the day with all the marketing people aside, do they really believe it?"*
> Interviewee, conservation sector

One particular challenge here is that the shape of the conversation about replacement is focused on the idea of 'saving' the horseshoe crab – as can be seen in several quotes above. As such, discussions are mired in an unconstructive rhetoric that leads to defensive comparisons with other aspects affecting crab population vulnerability:

> *"So let's not talk about the biomedical industry, let's talk about erosion, let's talk about development, let's talk about all of these things in terms of protecting the horseshoe crab population. I'm willing to accept that the biomedical industry does have some horse in that race but I'm also convinced that they're not the culprit here."*
> Interviewee, regulatory sector

Whilst these are all valid concerns, and the horseshoe crab is indeed threatened by multiple compounding factors, this obscures arguments for replacement that are about reducing suffering to individual animals or improving animal welfare. Discussions about replacements for horseshoe crab blood would be better to frame themselves in terms of how they are restructuring the harm-benefit equations involved in the biomedical use of these animals (Davies, 2018).

There is also concern that a turn to synthetic alternatives might actually result in more harm to horseshoe crab populations; rather than being a high-value 'catch and release' asset within the biomedical economy, the rise of alternatives may shift the crab's status as a commodity solely to that of fishing bait. For example, Charles River Laboratories, a manufacturer of LAL, suggest on their website that:

> *"Many companies think that moving away from LAL to reduce the use of the HSC will help them comply with 3R principles. This idea should be carefully evaluated, as moving to rFC could produce counterproductive effects by endangering the conservation efforts [. . .] Without the need for LAL in biomedical use, the legal protection of the horseshoe crab is not guaranteed in the future, and they would again fall prey to overfishing and use as bait."*
> Charles River Laboratories, 2020.

Conservation is positioned here as a way of practicing care, performing stewardship, and offsetting harms to some crabs through providing affordances to the species at large. However, the idea that horseshoe crabs are only afforded protection and conservation by an ongoing exploitation of the species is one that did not appeal to everyone, and adds another level of complexity and contestation around the replaceability of horseshoe crabs. The likelihood of a rise in the bait fishery as a result of biomedical reduction or replacement is debated, given that there are already strict quotas on the bait industry.

In highly regulated areas, like the pharmaceutical industry, transforming the 3Rs from principles into practice can be a challenge (Törnqvist et al., 2014). In the case of rFC, regulatory approval has been slow to emerge. The FDA issued guidance allowing for the use of recombinant factor C instead of LAL-based assays in 2012, though this was still considered an 'alternative test' and subject to validation requirements, rather than considered a fully equable replacement (Levin, 2019). This was followed by revisions to the European Pharmacopoeia in 2016, which included recombinant factor C (rFC) as an alternative method, again subject to validation requirements. However, this amendment specifically attested that 'the use of alternative reagents such as recombinant factor C as a

[4]Whilst some would argue that from the outset 'LAL manufacturers were concerned about the welfare of the horseshoe crab' (Novitsky, 2002, p. 79), this often appears conflated with ideas of conservation. In such that the focus is themed around preservation, survival, and preventing '*Limulus polyphemus* from going the way of the passenger pigeon and the dodo bird (Galler, 1979, p. 108), rather than welfare as a concept focused on the quality of life of animals (Fraser et al., 1997).

replacement to the amebocyte lysate eliminates the use of a reagent extracted from live animals.'

However, there is concern amongst pharmaceutical scientists (66% of those interviewed) that the additional validation involved in using the replacement requires a considerable amount of additional time and expense. As one interviewee from the pharmaceutical sector explained, '*the likelihood of any company doing a validated alternative is not great because of the amount of validation that is required*' and that '*unless it was included in a pharmacopeial method*' they were unlikely to consider replacing their LAL use. Others argued that whilst the validation process was something of a hurdle, the extent of this had been greatly inflated, and could be negated over time through experience and a corporate commitment to animal welfare above what was easy.

> *"A lot of the other companies are out there marketing against it saying, "you're going to have to spend all this money revalidating your methods," and the reality is we can knock out a method validation in 2 days, instead of the 1 day that it takes. It's four experiments instead of one, right? It's not a huge amount."*
>
> Interviewee, pharmaceutical sector

Whilst scientific and industry consensus over rFC is still playing out, in November 2019 the European Pharmacopoeia (Ph.Eur) Commission designated the 'test for bacterial endotoxins using recombinant factor C (rFC)' as a compendial method, effective as of July 2020 (Edqm, 2019). This will, at least within Europe, put the replacement test on an equal footing with crab-blood tests. However, pharmaceutical manufacturers operate in a globalized market, and without harmonization across the various Pharmacopoeias, there is still a long road for the alternative to gain industry confidence and uptake.

> *"So that specifically will help, if you're a small European based manufacturer and you only sold in Europe. You could immediately switch to that and that would be great. Directionally, it's great, it's awesome and we're very supportive. However, it's tough for us because we operate globally."*
>
> Interviewee, pharmaceutical sector

Concerns over patient safety were for many the bottom line. In a conservative, risk-averse sector, whilst many were encouraged by the promise of replacement, there was a desire for more data to emerge before people would feel confident to make this transition[5].

> *"I can't emphasize enough how much that patient centric approach is personally for me, it's critical, I don't want to have to question myself that I got it wrong. I'm sure it's fine! But I'd like to see more data on it and I think there will be some more data coming out."*
>
> Interviewee, pharmaceutical sector

Questions remain as to what level of evidence is required to achieve this confidence, and how to achieve industry acceptance. Some argued that much of the desired evidence is already available, and thus, the focus may need to be on education, improving access to existing evidence, and better communication of data.

> *"That's where the effort needs to go and we think there's an overwhelming amount of data that supports it, it's just overcoming some of the political realities I think now, and just get in there [. . .] There's actually a good deal of data out there and so we're just trying to hope to direct people to that body of work, to show them there has been a lot of data out there and published."*
>
> Interviewee, pharmaceutical sector

Ultimately, replacing LAL will take time, confidence, and data. For that reason, there are huge opportunities for refinement to the process, as one interviewee from the conservation sector concluded, '*as we are going to continue to bleed animals, as we make this migration from LAL to rFC, we should do it as humanely as possible.'*

PERSPECTIVES ON REFINING BIOMEDICAL HORSESHOE CRAB USE

> *"What they've been looking at is a way of finding a less invasive way of taking blood from the animal and also they wanted to look at the stressors that the animal goes under through the entire process, from being harvested to being bled to being released, I know there's been a lot of papers done on that but nobody's really gotten into the changes that are going on within the animal itself."*
>
> Interviewee, conservation sector

Refinement involves reducing suffering and improving welfare throughout animals' lives, including during and after procedures, and within transport, housing, handling, husbandry and care. Because of the multiple stages involved in procuring horseshoe crab blood – collection, transportation, storage, bleeding, re-release – there is considerable scope for refinement of processes. Krisfalusi-Gannon et al. (2018, p. 5) note that the percentage of horseshoe crabs that died prior to being bled more than doubled between 2008 to 2012, and suggest that a culture of deleterious harvest and transportation practices exists. Recently, Owings et al. (2020, p. 225) have argued that 'if biomedical facilities take sex differences and seasonal fluctuations in HCY (hemocyanin) levels into account, they might be able to reduce some of the deleterious effects of the bleeding process.'

Much of the work refining processes regarding the biomedical use of horseshoe crabs has gone quietly unnoticed, thanks to the industry's tendency toward secrecy. However, the ASMFC's 'best management practices' introduced in 2011 represent a significant step-change in how the welfare of individual crabs was considered at each stage within the collection, bleeding, and release of crabs collected for biomedical purposes.

> *"I go out with our fishermen and I audit their practices. In our contract with them, we have it specified as per the best practices document and so it's actually in our contracts with our fishermen on how they are to handle the horseshoe crabs. They're treated very gently and they're brought back to the same spot where they were taken, within 24 h, the shells are marked so they're not re-bled in the same year."*
>
> Interviewee, biotechnology sector

[5]Some interviewees drew parallels to the original transition from the Rabbit Pyrogens Test to LAL: "when you look at it, it was probably a good 15 or more years before the LAL test was really accepted as a substitute for the rabbit pyrogen test."

Whilst some were reassured by the stated efforts toward refinement, and the acts of care they involved, this was not shared by everyone. As one interviewee from the conservation sector remarked, *'you can find the guidelines and stuff, the recommendations, the best practices but that's not to say that they're actually following those.'* Several manufacturers commented that they are routinely audited and inspected by regulators, with strict mandates and conditions of operation imposed at State levels. However, at a broader (public) level, opportunities for witnessing refinement are limited, and with little openness in the sector, much has to be taken on good faith that moves toward more refined, less harmful, methods are taking place. Future work might involve an independent and public assessment of the extent of the implementation of these best management practices. Indeed, at present, there is a hesitancy to explore refinement in case this implied an admittance or acceptance that current standards and practices were not adequate at safeguarding animal welfare.

> *"That's a hard thing to get them to swallow, to change their operational position and that would further have to make them kind of suggest that their processes, to some extent, are deleterious to the species. And can they say that?"*
>
> Interviewee, biotechnology sector

Krisfalusi-Gannon et al. (2018, p. 4) suggest that 'improved harvest practices have the potential to reduce mortality rates during biomedical harvest by more than half.' Krisfalusi-Gannon et al.'s (2018) paper is a highly novel investigation into opportunities for implementing sustainability and welfare considerations into the supply chains of *Limulus* blood. They question a range of possibilities from removing a smaller volume of blood per drawing, to the scope for using indwelling catheters, and even the potential to develop processes of plasmapheresis and reinfusing crabs. However, it is still early days for refinement in this area. Krisfalusi-Gannon et al. (2018, p. 9) go on to argue that 'to achieve the lowest horseshoe crab mortality and highest blood quality during biomedical bleeding, a more systematic understanding of the nuances of the optimal horseshoe crab environment, feeding and care would be required.'

Other movements toward refinement in this area have involved exploring the potentials of aquaculture and the maintenance of captive populations. There are relatively few published studies that discuss husbandry conditions for horseshoe crabs, and many researchers consider 'captive rearing to be difficult, time consuming and impractical' (Carmichael and Brush, 2012, p. 39). To date, most conceptualizations of horseshoe crab aquaculture have been framed as stock enhancement through the release of juveniles into the wild (Schreibman and Zarnoch, 2009). Crabs are slow to reach maturity, and many studies report chronic mortality after 6 months in captivity (thought to be linked to diet) (Carmichael and Brush, 2012). Aside from the obvious implications on animal welfare, these challenges also pose significant economic barriers. Despite these challenges, there are aspirations that creating what might be understood as a laboratory 'strain' of horseshoe crab, as opposed to the current wild usage, might offer opportunities for greater care and welfare.

> *"We started to aquaculture horseshoe crabs, provide them an optimized management and optimized feed, with the hopes of going a low impact resource harvesting and we have some interesting ideas, that fall in probably the first or second R, it's not necessarily in that Replacement R.[. . .] That's the luxury we have here because after [bleeding], we then have the opportunity to be three steps from them and care for them and then feed them, whereas opposed to just saying 'you're on your own now'."*
>
> Interviewee, biotechnology sector

Aquaculturing might offer opportunities to remove the stresses induced by capture and transport of wild stock. Similarly, Tinker-Kulberg et al. (2020) suggest that aquaculture would allow for taking smaller amounts of blood, more frequently, rather than the current system that can involve collecting 'between 25 and 40% of a crab's blood (Moore, 2017, p. 112). The effect that the amount of blood extracted has on mortality rates, and what the upper limits of this should be for 'best practice,' remains a contested area (Atlantic States Marine Fisheries Commission, 2019). Whilst taking less more frequently, may be appealing at first, there are also ethical concerns about the cumulative effect on potential animal suffering, something which must be considered in any harm-benefit analysis of 'refinements' (Davies, 2018). Despite this, Tinker-Kulberg et al.'s (2020, p. 9) experiments found that aquaculture approaches seemed to indicate 'healthier and less stressed animals,' resulting from an optimized environment. Rather than a brief and extractive relationship, aquaculturing crabs might allow for more caring relationships with crabs. One that is less 'parasitic,' as Moore (2017) suggests of contemporary practices. Of course, other ethical arguments might question what freedoms are being lost through an enforced captivity.

Some expressed a concern too that large-scale efforts at refinement, like aquaculture, detracted from smaller, quieter, efforts that might improve horseshoe crab welfare, efforts such as training or auditing, that might contribute more to the establishment of a culture of care for crabs. Ultimately however, whilst there are burgeoning efforts toward refinement, stakeholders were also downcast about the possibilities of these refinements being taken up within industry at large, particularly from the perspective of the additional costs associated with aquaculture compared to wild-catch:

> *"I don't necessarily think that we can change the way that the biomedical industry is harvesting and I think that that's a tall order, when they're paying fishermen a little bit of money to go collect them and put them in the vehicles and bring them back, it's basically a free resource for them so if we were to go in and say, "invest money and do all of these things, it will improve your image and it will safeguard the future of your business." I personally don't necessarily think that it's a feasible task to get them to change their mind but maybe we can get the people that buy it to change their mind on where they buy from, to where the animal is at least treated ethically throughout their donation process."*
>
> Interviewee, biotechnology sector

As the above quote draws attention to, it is easy to place all of the burden of welfare on the manufactures of LAL who bleed the crabs, however, everyone within the pharmaceutical supply chain who uses LAL is implicated in contributing to horseshoe crab welfare. This may involve developing communication

strategies that highlight that LAL is derived from a living animal, taking steps to ensure efficient and non-wasteful use, exploring opportunities to replace, reduce, or refine use, and questioning and holding suppliers to account about how welfare considerations are implemented in their manufacture of LAL.

DISCUSSION

Creating more ethical and sustainable futures for humans and horseshoe crabs alike will require changing the shape of conversations about horseshoe crabs. The use of existing ethical, regulatory, and conceptual frameworks like the 3Rs offers huge potential to reframe discussions and find ways to talk about the biomedical use of horseshoe crabs that avoid the growing polarization, whilst introducing a means of extending – and conveying – the welfare considerations that are increasingly expected across science and society. Importantly, this should be viewed as the 3Rs in concert, as one interviewee from the biotechnology sector concluded:

> *"I like your 3Rs because I'm involved in all of them. They're all important and the thing is that everybody has to recognize that all of them are important and they all interact."*
>
> Interviewee, biotechnology sector

As Prior et al. (2019, p. 11) conclude, 'it is clear that there are still many opportunities to replace, refine or reduce the use of animals in pharmaceutical safety evaluation.' Endotoxin testing, and its reliance on horseshoe crabs is no exception. There are exciting efforts emerging to replace, refine, and reduce contemporary biomedical reliance on horseshoe crabs. The extent to which a turn to welfare and the 3Rs exists as an attempt to capture market-share may be debated. Certainly, the choice of 'R' here is often heavily influenced by economic interests, with existing manufacturers keen to innovate technological fixes that move toward some semblance of reduction, but ultimately, maintain the *status quo* of crab-bleeding, whilst the replacement products serve to create a pathway for new players to enter a market reportedly valued at $462.38 million in 2014 (Moore, 2017, p. 119). However, it remains the case that efforts to innovate here do offer the potential for improving the level of humane care deployed in the biomedical utilization of horseshoe crabs – something that is increasingly expected by publics. These expectations of 'good' care will remain on the agenda as the knowledge of the use of these ancient animals grows within public understandings of, and engagements with, science.

Luo et al. (2020, p. 11) suggest that 'we need additional cooperation to discuss and improve horseshoe crab research.' This cooperation should increasingly be interdisciplinary. This research has demonstrated the value that a social science perspective can bring to understanding perceptions about the development, promotion, and implementation of more sustainable methods for, and alternatives to, the use of horseshoe crabs for biomedical purposes. Whilst this research has taken a narrow and deeply focused approach, working with key stakeholders, to understand the 'anatomy of arguments' around horseshoe crab use, there is great potential for future work to incorporate a mixed methods approach, including quantitative analysis of responses to map and poll attitudes more widely. By unpacking what the meaningful questions may be to ask, this research sets the stage for future, more quantitative, work. Further work to include a focus more inclusive of the TAL sector too would help. Better understanding how different stakeholders perceive, and make value judgments about, horseshoe crabs – as strange, distant, invertebrates – is necessary to create more sustainable futures. The use of horseshoe crabs in endotoxin testing is a complex scientific and societal issue, situated at the interface of human, animal, and environmental health. Addressing emerging questions in global public health, which intersect with ecological concerns and ethical issues, requires novel interdisciplinary collaborations involving social science.

As discussed earlier, although scientific consensus over whether current replacement technologies are fit for purpose is still playing out, there is no question that their existence has changed the very shape of discourse around alternatives here:

> *"I think what's been really good for the industry over the past few years is that the discussion is there on the table, which it wasn't prior to 2016, everyone was just sort of taking it for granted. There was a bit but it was just people went merrily on their way so I think it's good that we've got the discussion on the table."*
>
> Interviewee, pharmaceutical sector

It highlights how conversations about sustainability, care, welfare, and replacing, reducing, and refining the current use of horseshoe crabs are here to stay. Requests for more data about the efficacy of recombinant factor C, along with a desire to await the development of more complex alternatives that involve recombinant formulations of the other factors involved in the clotting cascade within 'natural' horseshoe crab blood (such as Factor B and pro-clotting enzymes) will no doubt continue to shape discussions. However, this demonstrates the direction that the industry is moving – ultimately, toward more sustainable methods. Questions are increasingly less about could, or should, horseshoe crab blood be replaced, but more about when, and what the threshold of confidence, data, and trust, might be to do this. This discursive move is a significant achievement for all of those concerned about the animal welfare and environmental sustainability impacts of current LAL testing.

There is still a long road for alternatives and replacements to gain industry confidence and uptake, but being a 'compendial test' in Europe represents a significant milestone in the use of non-animal methods. The European decision is a positive result for a marine species afforded little protection or welfare considerations, despite – as social media reactions to articles about horseshoe crab use regularly demonstrate – a public desire to see more care expressed in the biomedical use of animals. Importantly, this social expectation of care is not just for those animals we find deeply familiar or appealing, but also for enigmatic invertebrates like horseshoe crabs.

DATA AVAILABILITY STATEMENT

The datasets generated during the study are being prepared for deposit to the UK Data Archive at the end of the project and are not currently publicly available. Anonymized interview

transcripts from participants who consented to data sharing are available from the corresponding author, subject to reasonable request. Requests to access the datasets should be directed to "r.gorman@exeter.ac.uk."

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the University of Exeter's Geography Ethics Committee. Participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable data included in this article.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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frontiers in Marine Science

DATA REPORT
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Transcriptome and miRNA Profiles of Black Tiger Shrimp, *Penaeus monodon*, Under Different Salinity Conditions

*Yundong Li, Falin Zhou, Jianhua Huang, Lishi Yang, Song Jiang, Qibin Yang and Shigui Jiang**

Key Laboratory of South China Sea Fishery Resources Exploitation and Utilization, Ministry of Agriculture and Rural Affairs, South China Sea Fisheries Research Institute, Chinese Academy of Fishery Science, Guangzhou, China

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Beibu Gulf University, China

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Jianguo He,
Sun Yat-sen University, China

****Correspondence:***
Shigui Jiang
jiangsg@21cn.com

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INTRODUCTION

The black tiger shrimp (*Penaeus monodon*) has high economic and nutritional value, and is the second widely cultured penaeid shrimp species worldwide (FAO, 2018). Extensive researches and development programs of the black tiger shrimp have focused on understanding its various biological processes, improving farming practices, and advancing selective breeding programs to achieve the sustainable shrimp farming industry. Through the genetic breeding research conducted by our team on *P. monodon* in China, we have cultivated two new varieties of *P. monodon*, "Nanhai No. 1" and "Nanhai No. 2," which have solved problems related to their growth and survival rates. However, despite breakthroughs in shrimp breeding, salinity remains an impeding factor for the development of shrimp aquaculture.

Salinity is an important environmental factor related to the physiological function, growth, and survival of shrimp (Li et al., 2017). *P. monodon* is primarily cultured in coastal areas with estuarine and oceanic water, while the production of euryhaline shrimp has expanded to inland recently, where have the advantage of the economic development and clean environment with sufficient groundwater supply (Roy et al., 2010). *P. monodon* is to be a promising aquaculture species for low salinity farming in inland worldwide. However, long-term low salinity cultivation is associated with multiple problems, including slow growth and increased susceptibility to pathogens. These problems restrict the large-scale promotion of *P. monodon* cultures in low salinity areas.

Previous studies on the responses of shrimp to salinity stress have primarily been concentrated on several important genes (Shekhar et al., 2013; Zhao et al., 2015; Xu et al., 2016), but the molecular mechanisms underlying the responses of shrimp to salinity stress, in relation to other salinity-regulated genes, remains unclear. Recently, a transcriptome regulation mechanism of the rapid and reversible post-transcription, via the action of non-coding RNA molecules, has been reported to be involved in environmental stress survival responses (Ebert and Sharp, 2012; Biggar and Storey, 2015). Various studies have shown that miRNAs were involved in many biochemical and physiological processes and played important roles in stress responses in aquatic organisms (Burgos-Aceves et al., 2018; Wang et al., 2018, 2020). However, to the best of our knowledge, few studies focused on stress-responsive miRNAs in *P. monodon*.

Transcriptomic profiling using next-generation sequencing technologies addresses this gap by providing a new approach to study stress response mechanisms in shrimp. For example, the transcriptome analysis of three different salinities cultivation conditions for *Litopenaeus vannamei* revealed numerous potential genes related to the responses of osmose (Zhang et al., 2016). In a transcriptome analysis of *L. vannamei* under chronic low-salinity stress, 855 genes showed

significant expression changes, and these were enriched in lipid metabolism, the phosphonate and phosphinate metabolism pathways, and steroid hormone biosynthesis (Chen et al., 2015). Transcriptomic profiling has also elucidated potentially novel miRNAs or stress-related miRNAs. There were 4,524 known miRNAs and 73 novel miRNAs reported to be significantly differentially expressed after copper exposure by small RNA sequencing analysis in shrimp under copper stress (Guo et al., 2018). A miRNA analysis of the hepatopancreas of *L. vannamei* using high-throughput sequencing, examined the potential regulatory mechanisms of cold adaptation and revealed 68 known mature miRNAs, 47 novel miRNAs, and 34 miRNAs that were related to cold adaptation (He et al., 2018). Overall, these transcriptomic and miRNA analyses of stress responses in shrimp allow us to understand their molecular mechanisms and identify novel genes or miRNAs related to environmental stress.

Here, we performed RNA sequencing analysis and detected differential expression genes and miRNAs in the gills of *P. monodon* at different groups with high, medium, and low salinity culture conditions, respectively. The quality control of sequencing data was conducted to present a high-quality dataset. We also performed an integrative comparative analysis of mRNA and miRNA at three salinities, to screen for key genes and miRNAs associated with salinity stress. These transcriptomic and miRNA data could provide a valuable resource for further shrimp studies on screening genes related to salinity stress responses and understanding the molecular mechanism of salinity stress.

METHODS

Ethics Statement

The study was approved by the Animal Care and Use Committee of South China Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences (No. SCSFRI96-253). Sampling and all experiments strictly complied with the guidelines and regulations established by the committee.

Shrimp Rearing and Stress Treatment

In the present study, healthy black tiger shrimp with 7.25 ± 0.26 g weights were collected from the experimental base of the South China Sea Fisheries Research Institute (SCSFRI, Shenzhen, China). All shrimp were cultured in laboratory tanks for 1 week and fed by shrimp diet for four times a day. The laboratory tanks were filled with filtered seawater (pH, 7.8–8.2; temperature, 27–29°C; salinity, 18 psu) and aerated constantly. To maintain water quality, we changed about 1/3 of the tanks' water daily. Then, all individuals were randomly divided into three groups which would be cultivated in three different environments for 30 days: high salinity [HC, 30 psu (practical salinity units)], medium salinity (MC, 18 psu), and low salinity (LC, 3 psu). Before the start of the experiment, the salinity of tanks' water was changed gradually (3 psu per day) to make the shrimp acclimate the 3 psu and 30 psu. A total of three thanks (500 L per tank) with 40 shrimp per tank for each group, and the gill tissues of the shrimp were collected from each tank and stored in liquid nitrogen at −80°C immediately until further RNA extraction. A total of nine samples (HC1, HC2, HC3; MC1, MC2, MC3; and LC1, LC2, LC3) were used for the subsequent transcriptome and small RNA sequencing (**Tables 1**, **2**). The experimental design and analysis pipeline was shown in **Supplementary Figure 1**.

RNA Extraction, Library Construction, and Sequencing

The TRIzol reagent (Invitrogen, Shanghai, China) was used to perform the total RNA extraction, according to the manufacturer's instructions. The 0.8% agarose gel electrophoresis and NanoDrop (Thermo Fisher Scientific, Waltham, MA, USA) were used to assess the RNA integrity and concentration, repetitively. There were three biological replicates for each group, and the construction of mRNA and small RNA were performed for all samples by Shanghai OE Biotech (Shanghai, China). Based on the protocol, the TruSeq Stranded mRNA LT Sample Prep Kit (Illumina) and TruSeq Small RNA Library Prep Kit (Illumina) was used to construct mRNA and small RNA libraries, respectively. To construct the Small RNA sequencing libraries, the 3′ end of the miRNA was specifically ligated with 3′ SR Adaptor. The excess 3′ SR Adaptor was hybridized with SR RT Primer, and the M-MuLV Reverse Transcriptase (Shanghai Sangon Biotech, China) was used to synthesis the first strand cDNA. It was used for PCR amplification with index (X) primer, SR Primer (Illumina) and LongAmp Taq 2× Master Mix. The DNA fragments within 140–150 bp of these amplification products were recovered by denaturing 12% polyacrylamide gel electrophoresis. To ensure the corresponding relationship between the mRNA and miRNA, we simultaneously constructed the transcriptome and mRNA libraries for each sample. All libraries were sequenced using Illumina HiseqTM 2500 instruments (Illumina).

Quality Validation, Data Cleaning, and Normalization

The quality of the raw sequencing data was validated by FastQC software (Andrews, 2010) (**Table 1**). Next, we used the paired-end splicing method in the Trinity (Grabherr et al., 2011) software to obtain the transcript sequence and designated the longest sequence as the unigene according to the sequence similarity and length. We then used the TGICL (Pertea et al., 2003) software to cluster and extend redundantly, to obtain a set of final unigenes, that served as the reference sequence for the subsequent analysis (**Supplementary Figures 2C,D**). For the small RNA sequencing data, the primer and adapter sequences were removed from the original data, and a sequenced fragment with reliable quality was selected after quality inspection and length screening of the bases. The adapter sequence was removed using cutadapt (version 1.14) software (Marcel, 2011), and the sequence with a length of 15–41 bp was retained. Reads containing N bases were filtered out using the NGSQCToolkit (version 2.3.3) software (Patel and Jain, 2012). The clean reads with high quality were finally obtained for the subsequent analysis (**Table 2**, **Supplementary Figure 3A**). The statistics summary of the sequencing data is presented in **Table 2**. The FPKM was

TABLE 1 | The statistics summary for the RNA-seq data of the nine samples.

Sample name	RNA-seq						
	Raw reads	**Raw bases**	**Clean reads**	**Clean bases**	**Valid bases %**	**Q30 %**	**GC %**
HC1	50738124	7610718600	49236624	7382338470	97.00	94.45	45.50
HC2	50070750	7510612500	48478994	7268894580	96.78	94.50	45.00
HC3	50471830	7570774500	48743030	7308267352	96.53	94.10	44.00
MC1	49304948	7395742200	47468446	7117119054	96.23	94.01	45.50
MC2	50386986	7558047900	48525278	7275715614	96.26	94.11	45.50
MC3	50217760	7532664000	48485970	7269993126	96.51	94.34	45.00
LC1	49842448	7476367200	48220504	7230152087	96.71	94.36	45.00
LC2	49281406	7392210900	47663910	7146623289	96.68	94.24	46.00
LC3	49409288	7411393200	47972088	7192992271	97.05	94.61	45.00

TABLE 2 | The statistics summary for the smallRNA-seq data of the nine samples.

Sample name	smallRNA-seq						
	Raw_reads	**Raw_adaptor**	**Reads_length**	**Reads_Q20**	**Reads_N**	**Clean_reads**	**Reads_uniq**
HC1	26632177	26480814	22634737	22609297	22604781	22604781	947640
HC2	28092509	27999363	18768214	18747278	18743733	18743733	1463712
HC3	22561374	22405977	18798221	18777805	18774099	18774099	981886
MC1	22489386	22170711	18483943	18465031	18461764	18461764	732662
MC2	19659523	19383377	16305671	16287547	16284314	16284314	772175
MC3	22483298	22105766	18566741	18548077	18544654	18544654	866788
LC1	25565542	25425400	22987325	22962409	22957776	22957776	1001894
LC2	25570719	25348867	21662368	21639070	21635076	21635076	972856
LC3	25364789	25226008	22699354	22675224	22670827	22670827	863254

(raw_adaptor, number of reads after removal of adaptor; reads_length, number of reads in the range of 15–41 bp; reads_Q20, number of reads with a Q20 percentage >80%; reads_N, number of reads after removing N from the sequence; clean_reads, reads used for analysis after sequence removal and quality control; reads_uniq, number of clean read types).

calculated to assess the expression levels of the unigenes, and the miRNA expression was normalized to transcripts per million.

Downstream Analysis

The differential expression analysis was conducted by the DESeq2 package (Anders and Huber, 2010), and the genes (or miRNA) with fold changes (FC) $\geq$ 2 ($|\log2 \text{ ratio}| \geq 1$) and adjusted $p < 0.05$ were considered as differentially expressed genes (DEGs) or differentially expressed miRNAs (DEMs). Bowtie software was used to perform a mismatch-free alignment with the mature sequences of the miRNAs in miRBase (version 21.0) (Griffiths-Jones et al., 2006), and the sequences on the alignment were considered to be known miRNAs (**Supplementary Figure 3B**). Novel miRNAs with a sequence length of at least 18 bp were identified using Mirdeep2 software and were mapped onto the reference genome (Friedlaender et al., 2012). The secondary structure of the novel miRNAs was predicted by RNAfold software (Denman, 1993), and the target genes of the identified miRNAs were performed by MiRanda with the parameters S $\geq$ 150 and Δ G $\leq$-30 kcal/mol (John et al., 2004). The analysis workflow was shown in **Supplementary Figure 1**.

Analysis of Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Twelve DEGs and six DEMs were randomly selected from the HC and LC groups, and the qRT-PCR was performed using the extracted total RNA to validate the selected DEGs and DEMs. The LightCycler® 480 II Real-time PCR Instrument (Roche, Basel, Switzerland) was used for the qRT-PCR reactions. The miRNA expression level was determined by a 10-μL PCR mixture which was mixed of 1 μL of cDNA, 0.2 μL of miRNA-specific primer, 0.2 μL of universal primer (Qiagen), 5 μL of 2 × QuantiFast® SYBR® Green PCR Master Mix (Qiagen, Hilden, Germany), and 3.6 μL of nuclease-free water. The mixture was incubated in a 384-well optical plate (Roche) at 95°C for 5 min, and then performed 95°C for 10 s with 40 cycles, followed by at 60°C for 30 s. For mRNA expression, the PCR mixtures, including 2 mL of real-time PCR-ready cDNA, 0.5 mL of specific primer (10 mM), 6.3 mL of SYBR Premix Ex Taq (2 ×), and 2.7 mL of ddH_2O, for a total volume of 12 mL, were performed 30 s denaturation at 95°C, followed by 95°C for 5 s with 40 cycles, and 62°C for 20 s. The **Supplementary Table 1** shown the primers used for these genes and miRNAs. The internal control for normalization was defined by Elongation factor 1a (EF-1a), and the $2^{-\Delta\Delta CT}$ method was performed to calculated the expression levels of relative genes (Livak and Schmittgen, 2001).

Technical Validation

For the importance of the total RNA quality in the libraries construction and the downstream analyses, the 260:280 ratios of all extracted RNA samples used in our study were ≥2, and the RNA integrity number (RIN) ≥7.0. We totally constructed nine mRNA and nine miRNA libraries for three groups (HC, MC, and LC) in which three biological replicates for each group (**Supplementary Figure 1**).

RESULTS

The obtained data were tested for contamination. For comparison, the most suitable result with coverage >80% and an *e*-value <1e−10 was obtained. The top 10 species in each sample were either the same species or related species between samples, and it was preliminarily judged that the samples were contamination-free. The top five species were *P. monodon* (50,946 reads; 62.14%), *L. vannamei* (8,870 reads; 10.82%), *Cyprinus carpio* (3,759 reads; 4.59%), *Fenneropenaeus* chinensis (3,032 reads; 3.7%), and *Marsupenaeus japomicus* (1,569 reads; 1.91%) (**Supplementary Figure 2A**). By applying FastQC to the entire dataset, we successfully obtained high-quality clean data with 94% of the bases scoring Q30 and above (**Table 1**). Then, a total of 64,475 unigenes were assembled. The range of these unigenes was from 301 bp to 38,289 bp with 1,275 bp of the average length. The length of most transcripts was between 300 to 600 bp, and 11,771 transcripts were larger than 2,000 bp (**Supplementary Figure 2D**). We identified 20,990 (32.56%), 16,505 (25.60%), 15,488 (24.02%) 14,284 (22.15%), and 7,508 (11.64%) genes homologous to the sequences in the Nr, Swiss-Prot, GO, KOG, and KEGG databases, respectively. All unigenes were functionally annotated, with 6,281 (9.74%) unigenes could match to all databases, and 21,997 (34.12%) could match to at least one database (**Supplementary Figure 2B**). The distribution of GC content frequency of the assembled unigenes was shown in **Supplementary Figure 2C**. We identified 2,671, 1,317, and 2,062 DEGs and 50, 69, and 55 DEMs, respectively, in three pairwise comparisons (HC vs. LC, MC vs. LC, and HC vs. MC) (**Supplementary Figures 4A,C**). These DEGs were mainly involved in energy metabolism, immune system, transport and signal transduction, and genetic information process. We totally identified 1,740 known miRNAs and predicted 111 novel miRNAs. These miRNAs greatly enriched the miRNA database of *P. monodon*, and more research are needed to verify and explore its functions. The data on these miRNAs were deposited in figshare. Although the $\log_2$fold change values of the sequencing data analysis were different from the qPCR analysis, the expression levels of these selected DEGs and DEMs were highly consistent between these two analyses (**Supplementary Figures 4B,D**). These DEGs and DEMs were valuable for understanding the molecular mechanism of shrimp response to salinity stress.

In conclusion, our findings provide a high-quality transcriptome and miRNA dataset characterizing how *P. monodon* responds to long-term salinity stress. Additionally, we screened multiple genes and miRNAs related to the salinity stress response. The dataset provides a valuable resource for screening genes related to salinity stress responses, which will contribute to improve our understanding of the molecular mechanisms of salinity stress in shrimp.

CODE AVAILABILITY

The software utilized in this study, including the specific versions used, were described in the part of methods and the following list:

1. FastQC version0.11.6 was used for the quality assessment of the FASTQ data: http://www.bioinformatics.babraham.ac.uk/projects/fastqc/
2. Trinity software versionr20131110 was used to assemble the transcript sequence: https://sourceforge.net/projects/trinityrnaseq/
3. TGICL17 software version2.1 was used to cluster and extend redundantly in order to obtain a set of final unigenes: https://sourceforge.net/projects/tgicl/files/
4. Cutadapt version1.14 was used to remove the adapter sequence: http://code.google.com/p/cutadapt/
5. NGSQCToolkit version2.3.3 was used to filter out reads containing N bases: http://www.nipgr.res.in/ngsqctoolkit.html
6. Mirdeep2 software was used to identify novel miRNAs: https://github.com/rajewsky-lab/mirdeep2
7. MiRanda software was used to predict the target genes of the identified miRNAs: http://www.microrna.org/microrna/getDownloads.do
8. DESeq2 package v1.8.2 was used for the differential expression analysis: https://www.rdocumentation.org/packages/DESeq2.

DATA AVAILABILITY STATEMENT

The raw FASTQ files for this study can be found in the NCBI Sequence Read Archive (SRA) [http://identifiers.org/ncbi/insdc.sra:SRP262105] with accession number SRP262105 (Bioproject ID: PRJNA631809) (NCBI Sequence Read Archive., 2020). The annotation information of the transcripts in this study was deposited in figshare (Li, 2020a). In addition, the quality assessment data of raw reads and differentially expressed genes were deposited in figshare (Li, 2020b,c). The differentially expressed miRNAs, predicted novel miRNAs, and corresponding heatmaps were deposited in figshare (Li, 2020d,e). The assembled unigenes, novel miRNA structures, and predicted target genes were also deposited in figshare (Li, 2020f).

ETHICS STATEMENT

The animal study was reviewed and approved by Animal Care and Use Committee of South China Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences (No. SCSFRI96-253).

AUTHOR CONTRIBUTIONS

ShJ conceived the project and supervised the work. YL performed the bioinformatics analysis and prepared the manuscript. FZ and JH conducted the experiment. LY, SoJ, and QY collected the samples and performed sequencing. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2020.579381/full#supplementary-material

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Evaluation of Indoor and Outdoor Aquaculture Systems as Alternatives to Harvesting Hemolymph From Random Wild Capture of Horseshoe Crabs

Rachel Tinker-Kulberg[1], Anthony L. Dellinger[1,2], Lisa C. Gentit[3], Bryan A. Fluech[3], Cole A. Wilder[3], Ivy L. Spratling[3], David J. Stasek[4], Christopher L. Kepley[1,2], Lee Robertson[1], Melinda K. Goddard[5], Terry E. Brady[1], Lance Tōland[1] and Kristen Dellinger[6]**

[1] Kepley BioSystems Incorporated, Greensboro, NC, United States, [2] Department of Nanoscience, Joint School of Nanoscience and Nanoengineering, University of North Carolina at Greensboro, Greensboro, NC, United States, [3] Marine Extension and Georgia Sea Grant, University of Georgia, Brunswick, GA, United States, [4] Department of Biology, College of Coastal Georgia, Brunswick, GA, United States, [5] ClienTell® Consulting, LLC, The Valley, Anguilla, [6] Department of Nanoengineering, Joint School of Nanoscience and Nanoengineering, North Carolina A&T State University, Greensboro, NC, United States

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Edited by:
Morten Omholt Alver,
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Aarhus University, Denmark

***Correspondence:**
Kristen Dellinger
kdellinger@ncat.edu
Anthony L. Dellinger
adellinger@kepleybiosystems.com;
adellinger@gmail.com

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This study evaluated two approaches to the aquaculture of *Limulus polyphemus* with the ultimate goal of harvesting Limulus amebocyte lysate (LAL) at an industrial scale. To monitor Horseshoe crabs (HSCs), a combination of physical, biochemical and cellular components were examined for HSC cohorts in an indoor recirculating aquaculture system (RAS) and an outdoor on-bottom pen culture system (PCS) over a 6-month period. The metrics included body weight, hemocyanin (Hc) concentration, amebocyte density, and LAL reactivity. In addition, a simulated biomedical bleeding event (extracting 30% of the total hemolymph volume) was performed to assess the impact on physiochemical properties of the hemolymph and amebocytes. Overall, the HSCs fared better in the RAS compared to the PCS, with higher rebound kinetics with respect to Hc, amebocyte density, LAL reactivity, and with 100% survival in the RAS cohort. Further, hemolymph reinfusion (after amebocyte removal) was shown to improve HSC recovery time. In summary, outcomes of this research show that a RAS, coupled with adequate nutrition and monitoring can provide HSCs with a suitable environment for sustainable hemolymph extraction and year-round LAL production.

Keywords: horseshoe crab (*Limulus polyphemus*), hemocyanin, pen culture system, recirculating aquaculture system, Limulus amebocyte lysate

Abbreviations: ‰, Parts per thousand; BET, Bacterial endotoxin test;°C, Celsius; ANOVA, Analysis of variance; DO, Dissolved oxygen; HSC, Horseshoe crab; EC, Electric conductivity; EU, Endotoxin unit; FDA, Food and Drug Administration; ft, Feet; Hc, Hemocyanin; LAL, Limulus amebocyte lysate; lb, pound; LPS, Lipopolysaccharide; NaCl, Sodium Chloride; NEM, N-ethylmalemide; nm, Nanometer; OD, Optical density; Oxy-Hc, Oxyhemocyanin; PCS, Pen culture system; ppm, Parts per million; PTFE, polytetrafluoroethylene; RAS, Recirculating aquaculture system; rcf, Relative centrifugal force; rFC, Recombinat Factor C; SD, Standard deviation; SE, Standard Error; SOP, Standard operating procedure; Tris–HCl, Tris(hydroxymethyl)aminomethane hydrochloride; µ, Micro; UV-Vis, Ultraviolet-visible; v/v, Volume/volume.

INTRODUCTION

The horseshoe crab (HSC) belongs to the order of arthropods, xiphosurans, dating back to the Ordovician period and are believed to be morphologically unchanged for some 148 million years (Rudkin and Young, 2009; Blazejowski, 2015; Manca et al., 2016; Akbar-John et al., 2018; Bicknell and Pates, 2020). Annually, several hundred thousand Atlantic HSCs (*Limulus polyphemus*) are collected from the wild along the Eastern shore of the United States for production of biomedical industry quality control tests. Upon harvesting amebocyte cells from HSC hemolymph or "blue blood," a vital extract called Limulus amebocyte lysate (LAL) is used to ensure sterility of pharmaceutical and medical devices, worldwide. When combined with demand for HSCs as whelk and eel fishing bait, as well as climate change and consequent habitat erosion also threatening numerous migrating shorebird species, compounded by corresponding threats to Asian HSCs, alternative strategies to ensure sustainable supplies of LAL are increasingly vital.

Notwithstanding their distinction as "living fossils," HSCs are of interest to the medical device and pharmaceutical industries given the importance of their blood (hemolymph), which contains a specialized cell, or amebocyte (Mürer et al., 1975; Van Holde and Miller, 1995; Rudkin et al., 2008). LAL extracted from the amebocytes contain clotting factors which catalyze a highly defined and immediate coagulation cascade when exposed to lipopolysaccharides (LPS) in the outer membranes of gram-negative bacterial endotoxins and/or fungal beta-glucans (Tai et al., 1977; Liu et al., 1979; Krisfalusi-Gannon et al., 2018). HSCs evolved with a form of "innate immunity" characterized by these granular amebocytes that can trigger extracellular clot formation (a polymer of protein coagulin) to act as an anti-microbial and anti-fungal barrier for the neutralization and immobilization of infectious pathogens (Shuster, 1990; Iwanaga et al., 1998; Medzhitov and Janeway, 2000; Iwanaga, 2002; Isakova and Armstrong, 2003). This immediate response to LPS and subsequent gel-clot formation has been employed in an assay using LAL, which has been considered the gold standard for quality assurance against pyrogens in the pharmaceutical and medical device industry since the 1970's (Mikkelsen, 1988).

Currently, to obtain amebocytes for LAL extraction, HSCs are removed from their habitats, transported to secondary locations, bled, and then returned to the water. Given increasing international demand for LAL for the testing of biomedical products sold in markets requiring FDA compliance, these practices pose considerable uncertainties as to whether current harvesting levels are sustainable while meeting future requirements (Krisfalusi-Gannon et al., 2018). This demand has been fueled by rapid global growth in vaccines, pharmaceuticals and United States medical device markets (trending at 6–8% and 25% per annum, respectively; Novitsky, 2015; Smith et al., 2017). Bled for Asian and Pacific biomedical testing reagents, increasing threats to the Asian HSC species could also lead to increased international demand for American LAL or require industry use of other methods (Akbar-John et al., 2018). While testing alternatives such as recombinant factor C (rFC) have been introduced, the LAL assay has remained the most reproducible, sensitive, rapid, and accurate FDA-approved method for the detection and quantitation of LPS. Although the European health authorities have approved rFC as an alternative to the LAL assay, the United States Pharmacopeia has recommended more validation studies would be necessary before rFC can be considered equivalent to the LAL test. To date the FDA has denied approval of this alternative Bacterial Endotoxin Test (BET) as a standalone compendial standard. The LAL assay market has been entrenched by specifications in FDA guidance documents for nearly four decades, and quality standards for existing products have been based on the LAL test with rare exceptions (Hochstein, 1990). Accordingly, additional resources would be required for existing LAL users to adopt alternative methods. These hurdles represent a significant driver for industry to source LAL derived from an abundant, sustainable approach.

Beyond such demands, an immediate environmental consideration is the viability of various species of birds that depend on HSC eggs for fuel and nutrition during migration (Botton et al., 1994; Gillings et al., 2007; Krisfalusi-Gannon et al., 2018). Thus, the overall aim of this work was to develop a sustainable approach to address LAL supply by cultivating HSCs in captivity as an alternative to annual wild capture and the consequent ecological threats to HSCs and dependent species. Past captivity research was dissuasive and cited numerous reasons why the species would languish and die in captivity (Walls et al., 2002). However, such reports seemed counterintuitive, since HSCs have survived and adapted over millions of years in an almost unchanged form and appear readily adaptable (Kin and Blazejowski, 2014; Blazejowski, 2015; Bicknell et al., 2019). Nevertheless, the literature suggested that a natural habitat would satisfy the environmental needs of this species (Botton, 1984); therefore, a saltwater pond in Coastal Georgia was selected as an experimental location for an outdoor HSC habitat. A second cohort of animals were subsequently tested in an indoor aquaculture facility to compare the two cultivation models with respect to feasibility and practicability.

In summary, two approaches to HSC husbandry were evaluated by designing an indoor recirculating aquaculture system (RAS) and an outdoor on-bottom pen culture system (PCS). Water quality in both systems, as well as HSC health parameters were monitored [i.e., weight, hemocyanin (Hc) levels, amebocytes concentration, LAL reactivity, and survival]. Tests were performed to analyze the rebound kinetics of amebocytes and Hc levels after hemolymph extraction to help maximize LAL yield and maintain HSC vigor, allowing for more frequent, sustainable harvesting over the 6-month study.

MATERIALS AND METHODS

Horseshoe crabs (n = 64) used in this study were procured manually, as bycatch from regional shrimpers, or purchased commercially from a sea life supplier (Dynasty Marine Associates, Marathon, FL, United States; NC Division of Marine Fisheries; and Georgia Department of Natural Resources) in accordance with an aquaculture operation permit (North Carolina Department of Environmental Quality, Division of

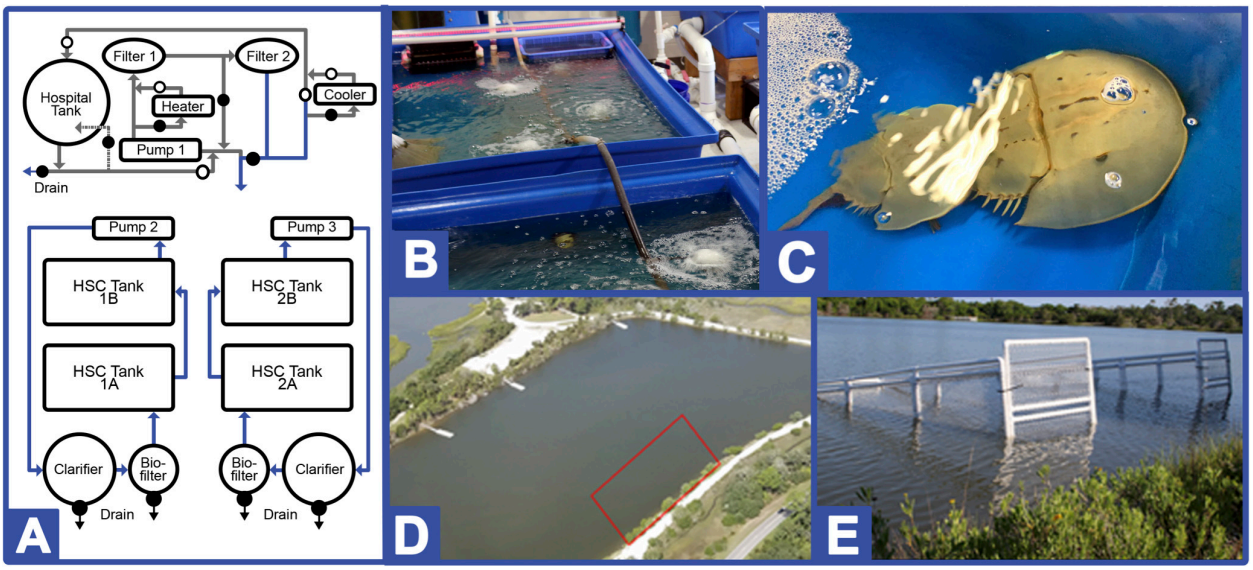

FIGURE 1 | Indoor and Outdoor HSC culturing systems. **(A)** Recirculating aquaculture system (RAS; Greensboro, NC, United States). The temperature-controlled system is comprised of two flow-through saltwater aquaculture systems, each containing two raft tanks (one pair is outfitted with UV lights for sterility), and a hospital tank for extraction recovery. Open circles represent open valves, closed circles represent closed valves during normal operational procedures. Closed valves can be open for clean-out or for water temperature control. Photographs of **(B)** installed system and **(C)** HSCs in place. Tidelands pond pen culture system (PCS; Jekyll Island, GA, United States). Photographs of **(D)** enclosure location and **(E)** two of four pens positioned in Jekyll Island.

Marine Fisheries #1946771 and #1947050; Georgia Department of Natural Resources LOP20180194). Mature HSCs used in this study on average weighed 1.77 ± 0.77 kg (3.91 ± 1.58 pounds). The salinity of both the indoor and outdoor culturing systems was consistent with the ambient salinities of where the HSCs were originally collected and/or maintained.

Recirculating Aquaculture System

The HSC studies were conducted in a RAS (Clear Flow Aquaponic System, Nelson and Pade, Montello, WI, United States) as previously described (Tinker-Kulberg et al., 2020a,b; **Figures 1A–C**). The tanks were brought to a salinity of 20 to 22‰ using a commercial sea salt preparation (Crystal Sea®, Marine Enterprise International, Baltimore, MD, United States) and weekly saltwater exchanges were performed between 1 and 2%.

Target water-quality parameters were determined based on standard saltwater aquaculture methods, and comprehensive daily, weekly and monthly analyses were conducted as previously described (Timmons, 1994; Nolan and Smith, 2009; Schreibman and Zarnoch, 2009; Ebeling and Timmons, 2010; Shelley and Lovatelli, 2011; Tinker-Kulberg et al., 2020a,b). Briefly, daily water conditions were optimized as shown in **Table 1** for: temperature (18.5–20°C); salinity (20–22‰); pH (7.5–8.2); dissolved oxygen (6–9 mgL^{-1}); photoperiod (12-h of natural light); ammonia (0–1.5 ppm); nitrates (< 150 ppm); nitrites (2–5 ppm), and alkalinity (90–150 ppm). Water parameters were measured and adjusted as needed six times per week and did not vary significantly throughout the course of the study.

In the indoor system, HSCs (n = 24) were provided a natural feed (e.g., glass minnows and krill; Aylesworth's Fish and Bait, St. Petersburg, FL, United States) *ad libitum* and maintained as described previously (Tinker-Kulberg et al., 2020a). A health assessment was recorded for each HSC upon intake (i.e., sex, weight, carapace length/width, as well as an appendage, carapace, eye, and mouth evaluation). HSCs were stocked at a density of 0.454-kg (1.0-lb) per square foot, resulting in approximately one HSC per 4-ft^3 of tank space. Each cohort (n = 6 per indoor tank) had the same number of males and female HSCs to avoid differences in feeding behavior or data bias.

Pen Culture System

An HSC outdoor PCS was designed using PVC pipe and crab trap netting and was located in a semi-tidal saltwater pond (Tidelands Pond, Jekyll Island, GA; 31° 2′ 19.2048″ N81° 25′ 12.8496″ W; ∼10.2 hectares). Designed to allow HSCs (n = 40) to naturally forage along the bottom of the salt marsh, four (4) outdoor PCS enclosures (15 ft long × 5 ft high × 5 ft wide) were erected to house HSCs at a density of 1 HSC per 6 ft^2 (**Figures 1D,E**). Pens were positioned proximal to the shore, and they extended into the water to a minimum depth of 4 ft and staked to the bottom along the perimeter. The pens were separated by approximately 4 ft to support the availability of natural food and exchange of materials with the bottom, as well as adequate water flow through the cages to avoid formation of "dead zones" along the bottom. To decrease build-up of sediment pollution, one pen was left empty, and each week one HSC cohort was moved in a clockwise manner into the adjacent empty pen in succession, allowing the pen that previously held the HSCs to remain vacant over the ensuing week. The HSC cohorts in the outdoor PCS were fed *ad libitum* once per week on the same diet used in the indoor RAS study (e.g., glass minnows and krill; Aylesworth's Fish and Bait, St. Petersburg, FL,

TABLE 1 | HSC water quality and nutrient parameters comparisons between indoor (RAS) and outdoor (PCS) systems (value ranges throughout study).

Water quality parameters	Ideal	Caution	Danger	RAS	PCS
Ammonia, total (ppm; Daily)	<1–3	2–4	>4	<1–3	0
Carbon dioxide (ppm; Daily)	<10	>10–40	>60	0	0
Dissolved oxygen (mg/L; Daily)	>3–5	1–3	<1	8.30	5.51
pH (Daily)	7.5–8.8	6.5–6.9	<6.5; >9.0	7.64	7.61
Secchi disk (cm; Daily – Pond)	15–25	<30	<12; >80	–	59
Water Temperature (°C; Daily)	15–25	29–32	<15; >32	19.9	32.2
Nitrate (ppm; Bi-weekly)	15–150	200–400	>400	40.6	0
Nitrite (ppm; Bi-weekly)	0.5–10	>10	15–300	5.5	0.76
Alkalinity (ppm; Monthly)	75–175	25–50; >200	<25; >300	150	90
Calcium (ppm; Monthly)	25–100	>600	<10; >250	207	234
Hardness (ppm; Monthly)	<3,200	<40	<20; >3,200	2,500	3,570
Salinity (ppt; Monthly)	15–35	<15; >35	–	20–22	19–23
Nutrient parameters	**RAS**	**PCS**			
Aluminum (ppm)	0	0			
Bicarbonate (mEq/L)	2.21	1.80			
Boron (ppm)	3.56	3.02			
Carbonate (mEq/L)	0	0			
Chlorine (ppm)	13,400	12,800			
Copper (ppm)	0.02	0			
EC (mS/cm)	41.10	38.40			
Iron (ppm)	0.02	0			
Magnesium (ppm)	0.05	0			
Manganese (ppm)	636	729			
Nitrogen (ppm)	12.40	1.65			
Phosphorus (ppm)	1.34	0.01			
Potassium (ppm)	218	226			
Sodium (ppm)	8,550	7,640			
Sodium absorption ratio	66.60	54.30			
Sulfur (ppm)	726	596			
Zinc	0.01	0			

United States). Due to the natural environmental limitations and in compliance with the governing ecological authorities, feeding was performed by hand outside of the PCS. These limitations represented a challenge with the experimental design, and the impact of such restrictions should be considered throughout the comparative analysis. Each cohort (n = 13–14 per outdoor enclosure) had the same number of males and female HSCs to avoid differences in feeding behavior or data bias.

The pH, ammonia, temperature, alkalinity, and carbon dioxide were recorded daily, while the remaining water parameters (i.e., nitrate, nitrite, calcium, hardness, and salinity) were conducted monthly at both aquaculture sites as shown in **Table 1**. Prior to hemolymph collection and periodically throughout the study, HSCs were weighed, measured (prosomal width and length from the tip to the tip of the telson) and inspected physically to determine whether the animals had sustained any injuries (i.e., to carapace, joints, eyes, and book gills), infections or other changes over time (Nolan and Smith, 2009).

The invertebrate benthic diversity of Tideland Pond was sampled from April-October 2019. Seven sites were sampled along the eastern shore of the pond near the HSC enclosures. Two of the sampling sites were culverts which connect the pond to the ocean. Kick sampling was conducted parallel to the eastern shore for 5 m in the direction of the current. One researcher held a D-net with 500 micron mesh against the bottom of the pond while another researcher dislodged benthic animals by shuffling their feet while moving toward the net. Specimens were preserved in 95% ethanol and identified to species or morphospecies if identification to species was not possible. Sampling was conducted every-other week or as often as weather conditions permitted. Alpha diversity was calculated using Simpson's diversity index to give weight to more common species by using the formula Diversity = $1/\Sigma(\text{pi})^2$ where pi is the relative abundance of the ith species.

Hemolymph Collection

Hemolymph was collected for amebocyte counting and for supernatant and amebocyte collection as described previously (Tinker-Kulberg et al., 2020a,b).

Hemolymph extraction for HSC "no bleed controls" (n = 4) and post-bleed health assessments to monitor recovery was

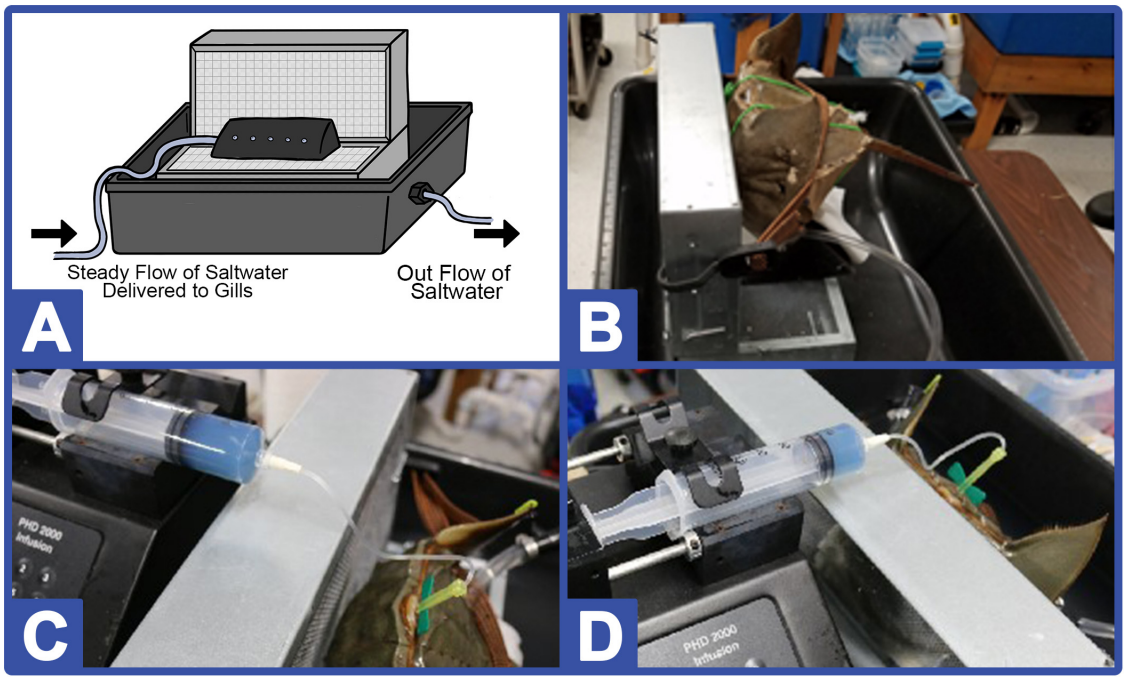

FIGURE 2 | HSC reinfusion and immobilizing station. **(A)** The immobilizing block is comprised of two ventilated chambers attached perpendicularly and fitted with foam padding in a 24-gallon reinforced PTFE (polytetrafluoroethylene) tub with pipe to allow saltwater flow outward. A separate tube is positioned beneath the foam padding to allow flow over the HSC book gills (maintaining respiration rates) during the biomedical bleeding procedure. **(B)** Side-view of system displaying internal saltwater flow tubing to the HSC. Two photographs **(C, D)** of the hemolymph reinfusion process.

carried out by removing 2.5 mL of hemolymph for analysis. To simulate a biomedical bleeding event, hemolymph volumes equivalent to a total of 30% of the total hemolymph were removed from HSCs (n = 4) and collected via syringe-free drip method directly into a pyrogen-free vial. Hemolymph volume of the HSCs was calculated as a percentage of wet body weight with 25 ± 2.2% for males and 25 ± 5.1% (mean ± SD) for females with the density of hemolymph estimated at 1.04 g/mL (0.0023-lbs per mL; Hurton et al., 2005).

Hemolymph Reinfusion

For comparison, a subset of the HSCs (n = 4) were bled at 30%, amebocytes were pelleted and removed from the hemolymph by centrifugation (e.g., 1000-rcf for 5 min), and the amebocyte-free hemolymph (referred to as autologous supernatant, hereafter) was reinfused at different volumes back into the HSC. To do this, the autologous supernatant was transferred into an endotoxin-free 60 mL syringe (to maintain a closed system for reinfusion), and a standard evacuated tube/straight needle connected to tubing was then attached to the end of the syringe. The syringe was then placed in a calibrated pump, and the needle at the other end of the sterile tubing was inserted into the sterilized HSC pericardial membrane. The autologous supernatant was then reinfused into the HSC at a rate of 5,000 μL/min. Given the time and precision required for reinfusion, an apparatus was engineered that secured the HSC in place and gently pumped saltwater across its book gills throughout the procedure; within the inert polyethylene retention tub, an immobilizing block was affixed to prevent movement during reinfusion and to facilitate pericardial membrane access. A schematic and photographs of the reinfusion system are shown in **Figure 2**.

Hemocyanin Concentration and Amebocyte Density

Total serum protein concentration was determined as described previously (Tinker-Kulberg et al., 2020a,b) via UV-Vis Spectrometer (Cary 6000i, Agilent Technologies, Santa Clara, CA, United States) and measured at 280 nm. Dioxygen-bound Hc (Oxy-Hc) was likewise measured at 340 nm. The total protein concentration was calculated according to Nickerson and Van Holde (1971), using the absorption coefficient A280 = 1.39 mg^{-1} ml^{-1} cm^{-1} for Hc and A350 = 0.223 mg^{-1} ml^{-1} cm^{-1} for Oxy-Hc. Amebocyte density was calculated as described previously (Tinker-Kulberg et al., 2020a,b).

LAL Reactivity

To determine LAL reactivity to endotoxin, LAL was purified as described previously (Tinker-Kulberg et al., 2020a,b) and fractions were then tested for LAL reactivity against a commercial endotoxin standard (Escherichia *coli* 055:B5 lipopolysaccharide, Sigma Aldrich, St. Louis, MO, United States). Aliquots that had been frozen and thawed only once were used for comparison assays. Four different batches of commercial LAL (E-Toxate™, Sigma Aldrich, St. Louis, MO, United States) were evaluated,

yielding reproducible results likewise from freshly thawed commercial lysates in all LAL experiments.

A gel clot assay was performed by incubating a standard endotoxin solution (50 EU/mL) with varying concentrations of the LAL extracts (ranging from 25 to 75 μg) in a 96-well plate. The gel-clot was allowed to form for 1 h at 37°C. Results were measured using a microplate reader (BioTek 800, BioTek Instruments, Winooski, VT, United States) at 340 nm, and the relative absorbance readings between experiments were reported.

Data Analysis

Data from 24 HSCs (procured from Georgia) were included in this study with 12 each in the respective indoor and outdoor systems. Data from indoor HSCs ($n = 12$) procured from Florida were also collected to serve as a control. Three groups of 4 HSCs served as: "No Bleed" controls, as "30% Bleed" controls, and "30% Bleed + Reinfusion" controls in each system. Each data point reported represents the combined data from four of the HSCs in each system (indoor versus outdoor); thus, the unit is equivalent to the average data collected from each HSC cohort and not the individual crabs, unless otherwise noted. All data points represent the mean ± standard error, where n equals the number of HSCs in each data subset. Data were subjected to a one-way analysis of variance (ANOVA) to determine significant differences among groups. If a difference was determined, it was compared by a Student's *t*-test, assuming equal variance, with a significant difference set at $p \leq 0.05$.

RESULTS

Water Quality

As shown in **Table 1**, both the indoor and outdoor systems revealed similar water quality parameters, with only temperature and DO (dissolved oxygen) differing over the course of the study in the outdoor system (**Figure 3**). In the indoor RAS, water temperature varied between 17.1–21.7°C and DO between 6.3–11.1 mgL^{-1} across all tank systems using supplemental aeration, without discernable trends appearing over time (**Figures 3A,C**). The temperature in the outdoor system increased steadily (∼15°C) over the course of the study due to seasonal fluctuations and consequently, the DO varied between 3.7–7.6 mgL^{-1} (supplemental aeration was not utilized in the outdoor system), with a subtle, yet concurrent, decrease over time (**Figures 3C,D**).

The indoor system yielded greater viability with survival of all HSCs over the course of the study. The outdoor system, however, recorded a loss of 15% of the HSCs over a 3-month period, which was concurrent with high water temperatures and lower levels of DO, with an initial loss of the outdoor HSCs occurring prior to any non-health bleeding events (**Figures 3C,D**).

Physical Parameters and Cohort Survival

All HSCs were physically inspected prior to introduction into the study and at various intervals over the course of this research. HSCs included in both the indoor and outdoor systems were deemed acceptable for inclusion in the study based on physical characteristics (free of crush fractures or open wounds); and any arthropod (e.g., barnacles) or mollusk growth was carefully removed using a hard bristle brush without damaging the shell. For all animals included in the RAS cohort, any cosmetic observations noted at the beginning of the study were monitored and remained unchanged over the course of the study. However, HSCs in the PCS accumulated a layer of biofilm and discoloration on their shells, without apparently affecting their health.

All HSCs were mature and no longer prone to molting, and thus their growth rate was consistent over the course of the study (data not shown). The weight of HSCs not subjected to a bleeding regimen was recorded and also remained constant in both the indoor and outdoor cohorts (**Table 2**).

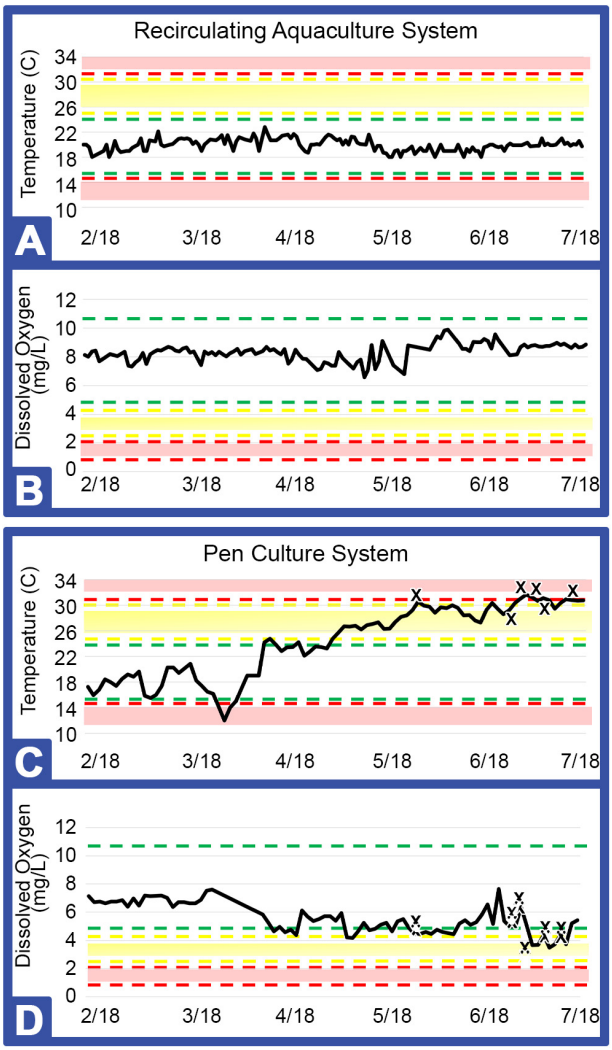

FIGURE 3 | Key metrics for RAS (Greensboro, NC) and PCS (Jekyll Island, GA). Areas within the green bars indicate safe ranges for temperature (°C) and dissolved oxygen (mgL^{-1}), yellow or red bars and shading indicate caution or danger levels, respectively (**Table 1**). Graphs represent temperature and dissolved oxygen for RAS **(A, B)** and for PCS **(C, D)** installations. **X:** Indicates each horseseho crab (HSC) that expired during the study.

TABLE 2 | HSC weight of all experimental groups reared in RAS and PCS.

System	Day 0	Day 47	Day 88
Recirculating aquaculture system	3.35 ± 0.45	3.43 ± 0.97	3.32 ± 0.98
Pen culture system	3.00 ± 0.34	3.05 ± 0.49	3.02 ± 0.53

ANOVA analysis revealed no statistically significant differences in either group during the study.

TABLE 3 | Dates and values of Simpson's diversity index for the PCS region.

Date	Simpson's diversity index
April 5, 2019	3.895978
April 19, 2019	5.244538
May 3, 2019	4.538752
May 18, 2019	4.878173
June 4, 2019	1
June 28, 2019	1.742268
August 3, 2019	1.623608
September 13, 2019	3.571429
September 27, 2019	2.159551
October 12, 2019	1.8

Although HSC weight did not vary throughout the study in the RAS or PCS groups (**Table 2**), mortality occurred in the outdoor PCS cohort with a recorded loss of 15%. Behavior and health parameters of indoor HSCs (n = 12) procured from Florida were indistinguishable from that of HSCs procured from Georgia, and therefore data from the latter were only reported in this study.

The following invertebrate species identified as potential natural HSC feed sources in Tideland pond were indexed: *Cancer irroratus (Atlantic rock crab); Dosinia discus clam; Geukensia demissa (ribbed mussel); Mnemiopsis leidyi (sea walnut comb jelly); Molgula manhattensis (sea grape tunicate); Neopetrolisthes maculatus (spotted porcelain crab); Palaemonetes pugio (grass shrimp); Penaeus setiferus (white shrimp); Petrolisthes armatus (porcelain crab); Phrontis vibex (Bruised nassa snail); Styela plicata (rough tunicate); Tritia obsolete (Eastern mud snail); Tritia trivittata (threeline mudsnail); and Tubeworms (species unknown)*. Invertebrate diversity decreased over the summer as shown in **Table 3**. The diversity index takes into account the number of species in a habitat but also the relative abundance of each species (i.e., how many of each species are sampled). The higher the index, the greater the diversity of species were collected.

Hemolymph Parameters

Hemocyanin concentration was tracked in HSCs maintained in both the PCS and the RAS. Results shown in **Figure 4** revealed that Hc concentrations remained stable in the HSC "No Bleed" RAS cohort, with a slight, but not statistically significant decrease over time (48.9 ± 11.13 mg/mL to 37.1 ± 9.85 mg/mL). The mean concentration observed in this group was within the range of values observed in wild caught HSCs from past studies (i.e., 32–97 mg/mL; Smith et al., 2002; James-Pirri et al., 2012). In contrast, a significant decrease was observed in HSCs in the "No Bleed" control group maintained in the outdoor environment, with an

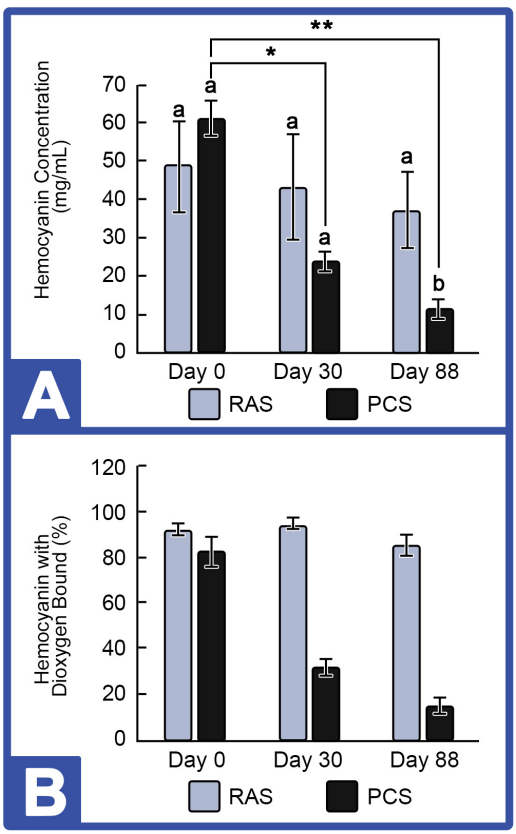

FIGURE 4 | Hemocyanin concentrations in the HSC "No Bleed" control group cultured in RAS and PCS. **(A)** HSC Hemocyanin (Hc) Concentrations in RAS and PCS. Hc concentrations were unchanged in the RAS but decreased over time in PCS cohorts. **(B)** Percent Hc with Dioxygen Bound (Oxy-Hc) in RAS and PCS. Hc concentration and Oxy-Hc remained stable in HSCs in the RAS but decreased over time for those in the PCS. Results are represented as mean ± S.E. (n = 4); bars sharing different letters in each index are significantly different ($P < 0.05$). *, ** represent significance ($p < 0.05$) between groups.

absolute decrease from 61.1 ± 9.45 mg/mL to 11.5 ± 9.45 mg/mL at the culmination of this study. Similarly, the proportion of dioxygen bound Hc (i.e., Oxy-Hc), an indicator of health status, ranged from 60 to 76% across all assays in both the indoor and outdoor systems. Statistically significant effects were not observed in either control group.

Amebocyte concentrations in hemolymph from HSCs in both systems were monitored to assess immune function, as decreases had been observed among stressed HSCs (Coates et al., 2012). Using a standard hemocytometer and cell counting technique, results showed that amebocyte density remained consistent in "No Bleed" HSCs maintained in the RAS; whereas, amebocytes decreased slightly over time in HSCs maintained in the outdoor PCS (**Figure 5**).

Changes in LAL reactivity to endotoxins from the respective aquaculture settings were directly analyzed. Pooled LAL extracts from 4 individual HSCs in the "No Bleed" control groups reared in either the PCS or RAS were sampled for LAL reactivity (**Figure 6**). Results showed that gel-clot formation increased as

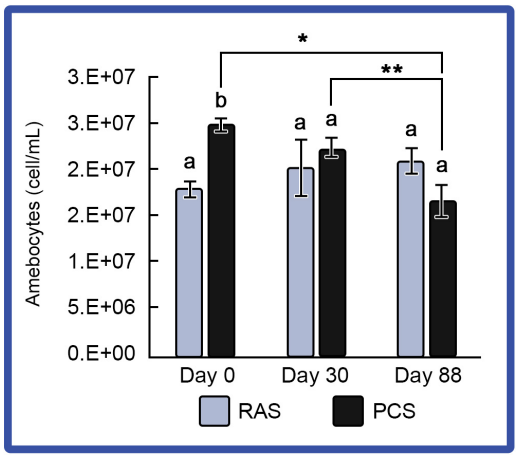

FIGURE 5 | Amebocyte density in the HSCs "No Bleed" control group in RAS and PCS. Amebocyte concentrations were stable for the RAS cohort, but decreased over time in the PCS HSCs. Results are represented as mean ± S.E. (n = 4); bars sharing different letters in each index are significantly different ($P < 0.05$). *, ** represent significance ($p < 0.05$) between groups.

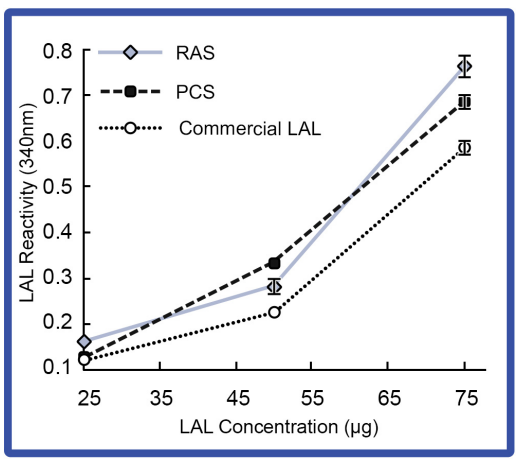

FIGURE 6 | LAL reactivity of HSCs in the "No Bleed" control group in RAS and PCS. LAL reactivity of the RAS and PCS HSC cohorts compared to control (commercial kit LAL; E-Toxate™, Sigma Aldrich, St. Louis, MO, United States) at increasing LAL concentrations (25–75 µg protein) exposed to 50 EU/mL of LPS. Reactivity of LAL derived from RAS and PCS HSCs were consistent with commercial LAL (E-Toxate™, Sigma Aldrich, St. Louis, MO, United States). Results represented as mean ± S.E. (n = 4).

LAL concentrations increased in both the indoor and outdoor cohorts, which was consistent with the commercial control (E-Toxate™, Sigma Aldrich, St. Louis, MO, United States). Additionally, LAL extracts from both the indoor and outdoor cohorts, exposed to higher endotoxin concentrations (e.g., 25 and 75 µg), had higher levels of reactivity when compared with that of the commercial control (E-Toxate™, Sigma Aldrich, St. Louis, MO, United States).

Simulated Biomedical Bleeding Response

To determine if hemolymph rebound rates after simulated biomedical bleeding differed in indoor and outdoor systems, HSCs were separated into a control (No Bleed) group; those from which 30% of the hemolymph was extracted (30% bleed); and those with 30% extracted, followed by reinfusion of autologous supernatant (i.e., amebocyte-free hemolymph; 30% bleed + reinfusion). Hc concentration and amebocyte densities were assessed just prior to bleeding, 72 h after, and 7 days following the procedure.

Notably, the weight of all HSCs decreased after a 30% hemolymph extraction (without reinfusion) and recovered to baseline within 72 h. As expected, serum protein concentrations and amebocyte density decreased initially during this period, presumably due to a dilution effect caused by the animal replacing its lost hemolymph with interstitial fluid. As expected, Hc concentrations were more stable after 30% of the hemolymph was removed when followed by reinfusion of autologous supernatant (i.e., Hc restored); a decrease in amebocyte density was also seen due to the dilution effect during this 72-h window (data not shown). Results, presented as a function of the percent change before and after bleeding, showed that HSCs reared in the indoor environment showed improved rebounding based on Hc (**Figures 7A,C**; 30% Bleed) and amebocyte concentrations (**Figures 7B,D**; 30% Bleed) 7 days after the 30% bleed compared to the outdoor cohort.

However, 7 days after HSCs were bled at 30% and immediately reinfused with a portion of autologous supernatant, Hc concentration rose (∼10%) above pre-bleed baselines (**Figure 7A**). After this 7-day recovery period, amebocyte density increased ∼31% after reinfusion of the RAS cohort (**Figure 7B**). Results from the outdoor study were similar; with reinfusion resulting in a higher rebound of Hc (∼4.5%; **Figure 7C**) and circulating amebocytes surging almost 25% following reinfusion at 7 days post-recovery (**Figure 7D**).

With respect to LAL reactivity before and after bleeding, reinfusion also appeared to have enhanced LAL reactivity when compared to that of HSCs that were not reinfused (**Figure 8**). However, after hemolymph removal with or without reinfusion, the average LAL reactivity across the indoor HSC groups decreased below baselines, despite increasing amebocyte counts. All HSCs tolerated bleeding in both the RAS and PCS groups.

DISCUSSION

In the wild, HSCs consume a diverse array of prey in their natural habitat (Botton, 1984; Botton and Ropes, 1989; Hu et al., 2013; Kin and Blazejowski, 2014). Although information concerning the natural diet of HSCs has been reported (Botton, 1984), dietary requirements under natural pond-farming systems and how natural food supplies impact overall health have not been previously established. While macronutrient and caloric densities of feed for aquatic species can be matched in captivity, compositional disparities exist, which was illustrated in bottlenose dolphin research showing a lack of diversity in the diet

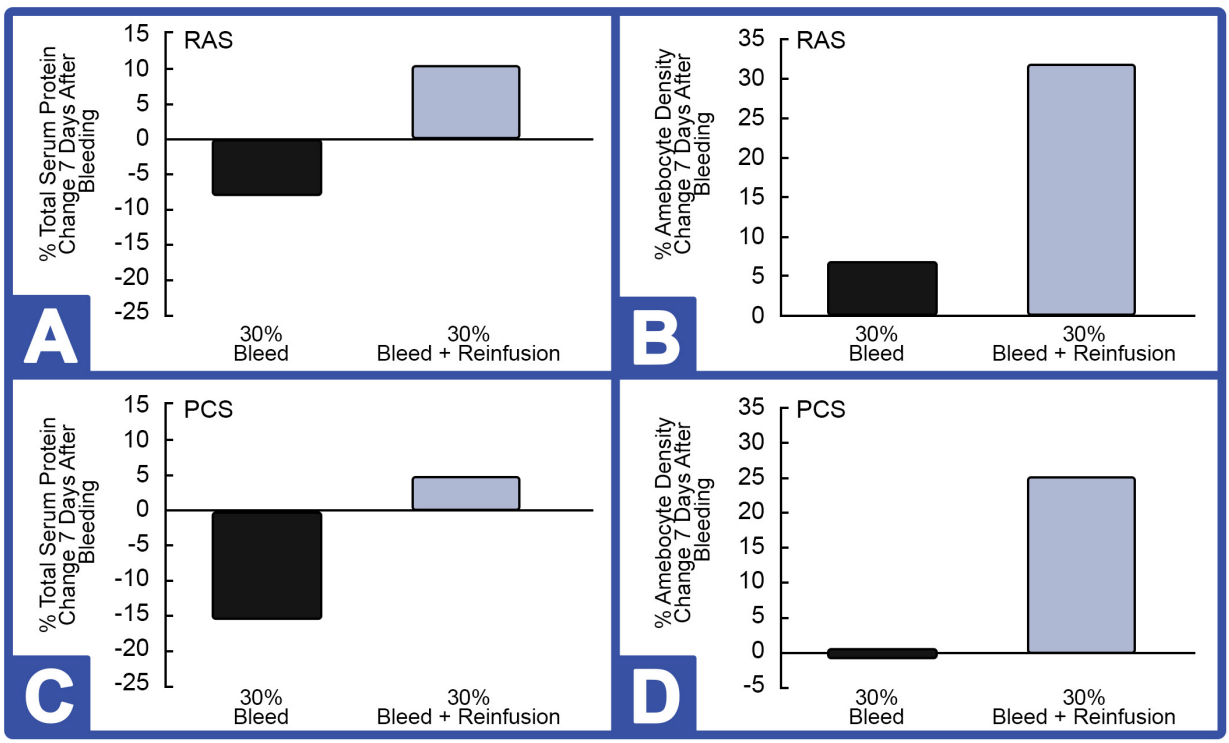

FIGURE 7 | Effect of 30% hemolymph extraction and autologous supernatant reinfusion on hemocyanin (Hc) and amebocyte density in HSCs in RAS and PCS. Note: black bars = with reinfusion; blue bars = without reinfusion. RAS: **(A)** The percent change in serum protein concentration, measured by Hc and **(B)** in amebocyte density, 7 days after 30% bleed (n = 4) with and without hemolymph reinfusion. PCS: **(C)** Percent change in Hc and **(D)** amebocyte density, 7 days after 30% bleed (n = 4) with and without reinfusion.

contributes to poor nutrition and health (Barros and Wells, 1998; Cunningham-Smith et al., 2006; Berens-McCabe et al., 2010; Powell and Wells, 2011). In the case of HSCs, the most abundant food source in the wild may not be the most suitable feed source for aquaculture (Botton, 1984; Hu et al., 2013). Likewise, the nutritional requirements of HSCs in captivity based on controlled feeding trials have not yet been defined (Botton, 1984; Carmichael and Brush, 2012). Inadequate nutrition and poor water quality (due to high stocking density) can also contribute to HSC stress, increasing susceptibility to pathogens and vulnerability to captivity-induced disease (Kautsky et al., 2000; Carmichael et al., 2003; Smith and Berkson, 2005; Defoirdt et al., 2007; Nolan and Smith, 2009; Schreibman and Zarnoch, 2009; Kwan et al., 2014).

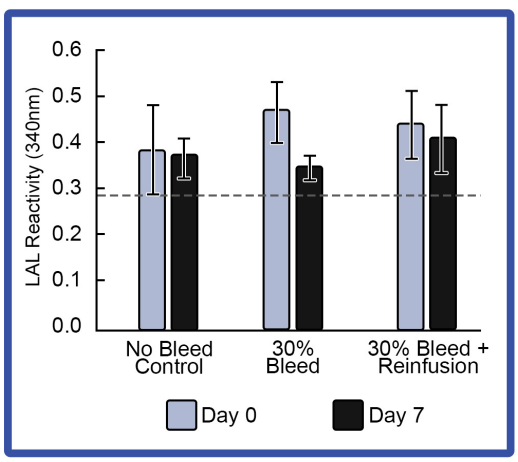

FIGURE 8 | Effect of 30% hemolymph extraction and autologous supernatant reinfusion on LAL reactivity. Average LAL reactivity from amebocytes extracted at Day 0 and a week after 30% bleed (Day 7). Reactivity of a commercial LAL control (E-Toxate™, Sigma Aldrich, St. Louis, MO, United States) is represented by a dashed line. Results are represented as mean ± S.E. (n = 4).

The objective of this research was aimed at comparing an indoor RAS and an outdoor PCS for sustainable LAL production. The initial hypothesis was that HSCs raised in an outdoor PCS with access to natural sources of feed, as well as their exposure to tidal rhythms and circadian light cycles approximating their wild habitat, would fare better than HSCs reared in an indoor RAS (Shuster, 1982; Barlow et al., 1986; Botton and Ropes, 1989). However, the results of the studies suggest that indoor RAS cohorts demonstrated more vigor across all health parameters, with significant monitoring, access and management advantages over the PCS approach.

Nutrition and farm maintenance strategies will undoubtedly play a critical role in HSC immunocompetence and disease resistance. For outdoor farming strategies, HSCs need to forage along the bottom of the pond; therefore, PCS enclosures could not float and instead needed to be fixed in place. This presented

two major health problems with respect to depletion of natural feed sources in confined bottom surface areas and waste build-up, as well as difficulty in animal collection. The labor involved in HSC collection was complicated by the depth and murkiness of the water which made it difficult to find and retrieve the animals, especially considering their ability to burrow into the sediment that created a suction-like force or vacuum around the shell and the bottom of the sea floor. The unexpected difficulty in retrieving HSCs from such outdoor PCS traps for frequent bleeding and routine health maintenance checks made this an unsuitable aquaculture approach for LAL production. In addition, outdoor (PCS) approaches between different farming operations could vary depending on where and how HSC are maintained in the outdoor facility in relation to water quality, culture density, feed availability, natural waste build-up and removal strategies, and other abiotic environmental variables such as seasonal temperatures. In fact, earlier research showed batch-to-batch seasonal variations in LAL prepared from wild harvested populations in their sensitivity to LPS, which may be related to HSC health and diet at varying times of the year (i.e., after, during, or before spawning), as well as conditions in the surrounding ecosystem (Lindberg et al., 1972). Thus, HSCs cultured in an indoor RAS controlled diets and environmental conditions should reduce these batch-to-batch variations that might be expected in HSCs cultured in outdoor PCS or retrieved from the wild. HSCs maintained in RAS after the bleeding process may also provide more protection during this recovery period; whereas, bled HSCs returned to the wild may be more susceptible to disease from external influences related to poor water quality and diet (Rudloe, 1983; Hurton et al., 2009; Anderson et al., 2013).

Due to existing nature conservation, water policy/legislation and environmental constraints on the selected outdoor study site, the ability to match the feeding regimen of the indoor system was limited. Alterations to the habitat were also restricted; thus, additional aeration was not incorporated. The mortality in the outdoor system was attributed to diurnal and seasonal fluctuations in water temperatures over the hotter summer months (June-July). This was consistent with past results showing that higher temperatures result in poorer HSC hemolymph quality and lower survival rates (Coates et al., 2012). A lack of feed diversity in the diet during the summer months (June-Aug; **Table 3**) may have also contributed to poor nutrition and consequent mortality. Although the HSCs in the PCS were given supplemental feed weekly, the loss of 6 HSCs was not attributed to starvation because the HSCs in that cohort rejected more frequent feeding events (>1X per week when fed *ad libitum*). However, the actual available feed intake, caloric density, and nutrient complexity of the natural feed was not examined at the pond foraging HSC site and should be considered further when evaluating differences between the two systems.

Evidence from this study suggest that differences in environmental conditions between outdoor PCS and indoor RAS bring about major changes in terms of HSC health performance and vitality. Further studies and research would be required to determine whether supplemental feed and presentation strategies could help address the nutritional needs and overall health of pond-cultured HSCs without deleterious effects on water quality (i.e., waste build-up). However, other uncontrollable environmental factors such as fluctuating seasonal temperatures can also directly influence oxygen and food consumption, as well as natural feed (invertebrates) populations, affecting overall HSC health. Appropriate site selection for pond-based PCS farms would need to be evaluated to ensure that optimal seasonal water and feed conditions could offer economic advantages over environmentally controlled indoor RAS practices (e.g., reduced feed, infrastructure, and operational costs). Further, hemolymph quality, as assessed by Hc concentration and amebocyte density, was shown to be consistent over the course of the 6-month study in HSCs maintained in the RAS. In contrast, the HSC mortality, coupled with declining Hc and amebocyte concentrations in the outdoor study, suggested that this approach was suboptimal for husbandry and therefore, LAL production.

LAL reactivity to an endotoxin standard (measured by the opacity of gel-clot) was similar in the indoor and outdoor HSC "No Bleed" test cohorts. LAL reactivity was also higher in LAL prepared from husbanded HSCs compared to commercially available LAL preparations (E-Toxate^{TM}, Sigma Aldrich, St. Louis, MO, United States); although additional studies would be needed to identify and assess possible variables in reagent preparation versus the harvest, freezing and single-thaw methods herein (**Figure 6**). These results also indicate that husbandry systems and nutrition influence the ratio and/or biochemical properties of LAL protein factors (Tinker-Kulberg et al., 2020a,b).

The impact and long-lasting effects of biomedical bleeding and handling stress on hemolymph properties of HSCs has not been fully characterized. The traditional LAL extraction procedure involves capturing HSCs during the spawning season, ambient exposure during transport and containment of the animal up to 72 h at the biomedical facility, and substantial blood loss before HSCs are returned to the point of capture, resulting in 15–30% mortality or long-term health sequalae (Atlantic States Marine Fisheries Commission (ASMFC), 1998; Leschen and Correia, 2010; Anderson et al., 2013). Past studies addressing rebound kinetics of blood components after extensive bleeding have shown that HSCs regain their blood volume within 1–3 days after a 30% bleeding extraction, but hemolymph protein concentration (e.g., 90% being Hc) takes 2–6 weeks to return to normal; whereas, restoration of amebocytes takes up to 4 months (Rudloe, 1983; Novitsky, 1984; James-Pirri et al., 2012). In comparison, Hufgard (2012) showed that amebocyte density can be recovered after 2 weeks within ten percent of the baseline value following significant hemolymph removal, and that the new amebocytes are smaller than fully mature and degranulating amebocytes.

The effect of biomedical bleeding of 30% of total HSC hemolymph volume was studied in both RAS and PCS to determine the rebound kinetics of Hc and amebocyte concentration after a 7-day recovery period (**Figure 7**). In contrast to prior studies, HSCs immediately regained their weight within hours after a 30% bleed extraction presumably through water sequestration from the recovery tank, a normal phenomenon used by aquatic animals to regulate their volume and ion concentrations (Rudloe, 1983; Novitsky, 1984; James-Pirri et al., 2012). As expected, Hc levels were not fully recovered within 7 days after being bled in the absence

of supernatant reinfusion, however, in comparison to prior studies the amebocyte counts were fully restored within this 1-week period. HSCs that received reinfusion of the autologous supernatant (amebocyte-free hemolymph) showed increased serum protein levels and amebocyte counts above baseline levels (**Figure 7**). Further studies have shown similar results when two successive 10% bleeds, separated by a 1-week recovery period, were performed on indoor cultured HSCs (Tinker-Kulberg et al., 2020b). This rapid upregulation of Hc and circulating amebocytes above baseline levels may be a physiologic response to compensate for natural blood loss and injury (Hufgard, 2012).

Notably, although amebocyte levels rebounded 1 week after the 30% bleeding, LAL reactivity did not, with or without reinfusion. Thus, during amebocyte biosynthesis and regeneration, immature amebocytes may contain lower concentrations of LAL components (i.e., lower reactivity per μg of LAL); and as ameboctyes mature, maximum LAL reactivity would presumably be reached. Although HSCs maintained in the RAS and PCS demonstrated similar Hc and amebocyte rebound kinetics, a slight improvement was observed in RAS HSCs. In summary, reinfusion appears to enhance post-bleed recovery, which would allow captive HSCs to be bled more often if routinely reinfused, and high LAL yields may be obtained if newly formed amebocytes are allowed to mature.

Reinfusion of supernatant after hemolymph collection and amebocyte extraction appeared to be not only salutary for HSC recovery, but it also establishes an entirely new method in HSC bleeding and management. The commercially vital component of HSC hemolymph comprises 5–10% of overall hemolymph volume, leaving some 90% as waste in current industry practices, and its loss may contribute to subsequent mortality and deleterious effects after being returned to the water (Anderson et al., 2013). That is, reinfusion to restore hemolymph volume and oxygen transfer could prove beneficial to the HSCs by reducing the risks of hypoxia while accelerating recovery of metabolic homeostasis and amebocyte rebound; and therefore, allow for more frequent hemolymph collection. Further validation of HSC aquaculture could yield significant industry disruption of current practices, given that a small, captive cohort of HSCs could be sustainably bled up 12 times per year with 100% survival in an industry with 30% mortality resulting from random wild capture and bleeding just once per year. Further studies to determine the precise relationship between maximum threshold levels of bleeding and recovery time required for optimal amebocyte and Hc rebound kinetics will be particular useful to safeguard against such post-bleeding mortality in HSCs.

Finally, with respect to outdoor aquaculture as a sustainable means of producing LAL, and notwithstanding these findings, the labor and logistics associated with accessing the outdoor cultivated HSCs for bleeds and health assessments proved a significant challenge throughout the course of this work. Despite the custom design of an inshore pen system, the labor and time associated with swimming/wading to retrieve the HSCs (via tags affixed to the HSC shell), were especially problematic aspects of the outdoor system. The indoor system, on the other hand, proved to be conducive to regular handling of the HSCs, with the potential for more frequent, low-impact hemolymph harvesting. Outdoor pond-culturing systems would also be highly variable in abiotic and biotic factors. While seasonal changes in salinity and temperature in an outdoor system are inevitable, there is a greater degree of control in the indoor system and therefore, reproducibility. Thus, while there were indeed slight differences in specific water quality parameters between our indoor and outdoor system (**Table 1**), this study was aiming to assess how those differences might affect HSC health and hemolymph quality. As such, irrespective of the outdoor limitations, indoor RAS husbandry provided greater environmental control (e.g., DO, temperature, and water chemistry) and ease of operation that would be more practicable for year-round LAL production.

The effects and optimization of diet and different bleeding volumes and regimens, coupled with optimal recovery times, on HSC health and LAL production from HSCs raised in an indoor RAS have been recently reported (Tinker-Kulberg et al., 2020a). The use of intravascular catheters surgically implanted in the respective pericardial membranes of the HSCs, which allowed for routine hemolymph collection without repetitive membrane punctures, has also been recently investigated (Tinker-Kulberg et al., 2020b). Notably, these additional strategies would help to ensure wellbeing of HSC in captivity for optimal LAL production in indoor culturing systems.

CONCLUSION

During this study, HSC culture conditions, health parameters, collection and processing SOPs for both indoor and outdoor aquaculture systems were established. The process was designed to establish an alternative, sustainable LAL source to address current and projected issues that face wild HSC populations, the global biomedical industry demands, and the patients that depend on safe products. Indoor HSC aquaculture was proven practicable by demonstrating that indicators of well-being, including weight, Hc concentrations, amebocyte levels, and LAL reactivity remained constant; however, the outdoor PCS in this study posed overarching regulatory, environmental and accessibility challenges and yielded inferior results across all parameters, including HSC survival. Additional application of the indoor RAS method and HSC catheterization, combined with nutritional optimization could therefore serve to *enhance* HSC wellbeing and allow for sustainable, year-round harvesting of LAL, a wholly unique and globally vital resource. Once thoughtful innovation and stewardship are amalgamated into LAL production, the discussion should move toward integrated aquaculture of the species to ensure sustainability for both HSC and human medicine.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The research on the HSCs (*Limulus polyphemus*) involved in this study did not require approval, as HSCs are classified as an exempt invertebrate species. For RAS experiments (Greensboro, NC), all HSC collection and aquaculture techniques and methods were reviewed and approved by the North Carolina Department of Environmental Quality, Division of Marine Fisheries (Collection permit #1946771 and Aquaculture operation permit #1947050). For PCS experiments (Brunswick and Jekyll Island, GA), all HSC collection and aquaculture techniques were performed by University of Georgia Marine Extension and Georgia Sea Grant with approval from the Jekyll Island Authority Committee Members, Director of Conservation, and Georgia Department of Natural Resources (Georgia Department of Natural Resources LOP20180194).

AUTHOR CONTRIBUTIONS

RT-K, KD, TB, MG, CK, LT, and AD conceived the research hypothesis. RT-K, KD, TB, MG, CK, LT, and AD developed the research objectives and experimental plans. RT-K, KD, and TB verified the materials, methods and design of the RAS. RT-K, KD, LR, and AD conducted the RAS research experiments. RT-K, KD, BF, and LG designed and deployed the PCS system. BF, LG, IS, and CW collected, maintained, monitored HSCs, and performed the outdoor studies on Jekyll Island, with oversight from RT-K and KD. DS collected the data on the group composition of invertebrate samples from Tideland Pond. KD supervised all of findings of this work. MG and AD performed the final editing of the manuscript for publication. All authors prepared analysis of the primary research and contributed to the final writing of the manuscript.

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Effects of Diet on the Biochemical Properties of Limulus Amebocyte Lysate From Horseshoe Crabs in an Aquaculture Setting

Rachel Tinker-Kulberg[1], Anthony Dellinger[2], Terry E. Brady[1], Lee Robertson[1], Melinda K. M. Goddard[1,3], John Bowzer[4], Sarah K. Abood[5], Christopher Kepley[1,2] and Kristen Dellinger[1,6]**

[1] Kepley Biosystems Incorporated, Greensboro, NC, United States, [2] Department of Nanoscience, Joint School of Nanoscience and Nanoengineering, University of North Carolina at Greensboro, Greensboro, NC, United States, [3] ClienTell® Consulting, LLC, The Valley, Anguilla, [4] Archer Daniels Midland Company, Decatur, IL, United States, [5] Ontario Veterinary College, University of Guelph, Guelph, ON, Canada, [6] Department of Nanoengineering, Joint School of Nanoscience and Nanoengineering, North Carolina A&T State University, Greensboro, NC, United States

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Marty Riche,
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University of New Hampshire,
United States

***Correspondence:**
Kristen Dellinger
kdellinger@ncat.edu
Anthony Dellinger
adellinger@kepleybiosystems.com;
adellinger@gmail.com

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The Limulus amebocyte lysate (LAL) isolated from cells in the horseshoe crab (HSC) hemolymph is a critical resource for global biomedical and pharmaceutical quality control and sterility testing. Given the necessity of and limitations associated with wild capture, a conservational approach to LAL harvesting would benefit the medical community that relies on the raw material while helping ensure species viability. We posited that aquaculture and year-round collection represented a sustainable alternative for the production of LAL from a finite HSC cohort, thereby averting the impact of current practices on wild populations. Given the specter of captivity diseases linked to diet, such as panhypoproteinemia, this work, at the outset, focused on optimizing a feed formulation to ensure animal vitality. In turn, each preparation required evaluation with respect to effects on LAL, as well as vital HSC health markers, so as to meet or exceed industry requirements and establish a new supply chain paradigm. In this controlled husbandry study, we conducted three 8-week feeding trials and demonstrated a ∼7-fold LAL reactivity range among the HSC feed groups. Relative protein abundance patterns of HSC amebocyte clotting factors (i.e., Factor C, Factor B, and proclotting enzyme) were influenced by diet in particular, and the up-regulation of specific LAL factors correlated with enhanced reactivity. These results also cite the discovery that coagulation Factor C, the LPS-sensitive serine protease proenzyme, may be a phosphoprotein.

Keywords: amebocyte, aquaculture, horseshoe crab, Limulus amebocyte lysate, nutrition, protein expression, sustainable

Abbreviations: Δ, delta; ‰, parts per thousand; A-chain, alpha-chain; AF, As fed; AP, alkaline phosphatase; ALP, aquaculture lysate pool; ASMFC, Atlantic States Marine Fisheries Commission; β, beta; B-chain, beta-chain; BET, bacterial endotoxin test; BCA, bicinchoninic acid assay; b/w, body weight; BSA, bovine serum albumin; Ca^{++}, calcium; $CaCl_2$, calcium chloride; °C, celsius; COCH, cochlin; dL, deciliter; DM, dry matter; CP, crude protein; HSC, Horseshoe crab; EC, electric conductivity; EU, endotoxin unit; ft, feet; g/mol, grams per moles; kDa, kilodalton; kJ, kilojoules; kJ/g, kilojoules per gram; H-chain, heavy chain; Hc, hemocyanin; LAL, Limulus amebocyte lysate; lb, pound; L-chain, light chain; LPS, lipopolysaccharide; $MgCl_2$, magnesium chloride; mmol/L, millimoles per liter; NaCl, sodium chloride; nm, nanometer; OD, optical density; Oxy-Hc, oxyhemocyanin; g/mol, molar mass; GPCR, G-Protein-Coupled Receptor; PNGase, glycopeptidase F; ppm, parts per million; RAS, recirculating aquaculture system; rcf, relative centrifugal force; SOP, standard operating procedure; TGase, transglutaminase; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; μ, micro; UV-Vis, ultraviolet-visible; v/v, volume/volume; w/v, weight/volume.

INTRODUCTION

Marine pharmacognosy focuses on the development of clinically significant products obtained from marine species (Malve, 2016). A common limitation of this sector is the lack of viable supplies of unique compounds (Lindequist, 2016). In recent decades, aquaculture has advanced significantly and currently plays a prominent role in global food and environmental sustainability. Similarly, aquaculture represents an opportunity to establish a reliable source for production of marine-derived biomedical compounds.

The HSC, *Limulus polyphemus*, is one such marine species that has been utilized by pharmaceutical and device manufacturers, worldwide (Krisfalusi-Gannon et al., 2018; Bolden, 2019). Annually, some 600,000 Atlantic HSCs are captured and bled to meet the demand for Limulus amebocyte lysate (LAL) testing (Atlantic States Marine Fisheries Commission, 2019). The assay is the primary FDA-approved method for bacterial endotoxin testing (BET) of biomedical products and devices (Wachtel and Tsuji, 1977; Hochstein, 1990; Bryans et al., 2004; Novitsky, 2009; Food and Drug Administration, 2014). Aquaculture husbandry represents a sustainable alternative to wild-capture bleeding practices associated with an estimated 30% mortality rate (Anderson et al., 2013). Establishing a finite cohort (∼5–10% of one year's catch) of carefully monitored and consistently nourished HSCs in managed habitats would allow year-round, low-impact harvesting to meet current LAL demand for the majority of the typical HSC life expectancy of up to ∼20 years while helping to replenish wild populations (Grady and Valiela, 2006).

Derived from HSC blood cells (amebocytes), LAL is exceptionally sensitive (*parts per trillion*) to lipopolysaccharides (LPS). Also known as endotoxin, LPS is a constituent of the cell wall of gram-negative bacteria (Roslansky and Novitsky, 1991). Activation of the LAL pathway results in cleavage of three serine protease zymogens: Factor C, Factor B and proclotting enzyme, as well as the secretion of transglutaminase (TGase) from the amebocyte cytosol (Osaki et al., 2002; Osaki and Kawabata, 2004). Endotoxins trigger autocatalytic activation of Factor C zymogen into its active form, which in turn activates Factor B in an LPS-dependent manner, converting the proclotting enzyme to the clotting enzyme that cleaves coagulogen (Iwanaga et al., 1992, 1998; Muta and Iwanaga, 1996; Kawabata and Muta, 2010; Kobayashi et al., 2015). The endpoint is the conversion of coagulogen into coagulin, an altered fibrin-like protein gel (Iwanaga, 2007). In a Ca^{++}-dependent pathway, TGase helps to stabilize the clot by cross-linking coagulin with two other proteins, proxin and stablin (Osaki et al., 2002; Matsuda et al., 2007).

Previous studies have determined the diet and food preferences of HSCs in their natural habitat, but there is relatively little information available regarding the diet of HSCs in aquaculture, with most captive diets composed of a natural feed sources, such as shrimp, krill, mussels, sprat, sand eels, squid, mackerel, and trash fish (Botton, 1984). Three decades of adult HSC aquaculture have yet to define optimal parameters required for long-term management in captivity (Carmichael and Brush, 2012). Sustained aquaculture (exceeding 6 months) has been associated with low survival rates, principally linked to poor water quality (due to high stocking density) and inadequate nutrition, both of which place the HSCs under stress and increase their susceptibility to pathogens and vulnerability to captivity-induced disease (Kautsky, 1982; Smith and Berkson, 2005; Defoirdt et al., 2007; Carmichael et al., 2009; Nolan and Smith, 2009; Schreibman and Zarnoch, 2009; Kwan et al., 2014). Specifically, the most notable malady of captive adult HSCs has been panhypoproteinemia; whereby, hemolymph protein concentrations drop below 3.4–11.7 mg/mL after 3–4 months (Nolan and Smith, 2009). This syndrome is thought to be caused by nutritional deficiencies and results in protein-losing enteropathy and nephropathy, as well as hepatic insufficiency (Nolan and Smith, 2009).

Studies of HSC feed formulations have also been largely lacking, defaulting to costly natural resources (e.g., fish, crustaceans, and mollusks) for short-term indoor research. Many factors affect the nutrient composition of natural feeds, further complicating nutritional parameters and requiring frequent adjustment. In the wild, most aquatic animals are opportunistic and consume a diverse array of prey. However, captive diets are typically limited in complexity due to species availability and consist of frozen and thawed bivalves, cephalopods, or pelagic fish, often exposed to prolonged storage. Freezing and thawing natural feeds can also cause water-soluble vitamin loss through diffusion at erratic, non-linear rates (Rigdon and Drager, 1955; Dierenfeld et al., 1991).

Alternatively, synthetic options provide affordable and nutritionally defined aquaculture feedstocks. Manufactured feed is typically formulated for the type and life stage of each species. To date, however, HSC-specific commercial feed has not been developed, and the suitability of adapting current feedstocks for captive HSCs has not been extensively studied. Given that such products are typically intended to stimulate consumption and rapid growth, they nonetheless may not be appropriate for long-term HSC maintenance. Current aquatic feed trends have also transitioned to alternative, terrestrial sources of proteins and lipids to keep prices competitive and growth rates high, whereas an optimal HSC product would center on animal vigor and cellular health.

One circulating cell type, the amebocyte, confers HSC immunity. In response to microbial exposure, amebocytes initiate formation of coagulin, a polymer of protein, to envelop endotoxins (LPS) and prevent systemic infection. This single-cell simplicity of the HSC hemolymph (copper-rich blood) offers a unique model to investigate the impact of various diets at the cellular level, not feasible in most species.

Ultimately, this aspect of our work focused on the effects of diet on HSC wellbeing, cellular health, and LAL quality in the greater context of establishing prototypical nutritional and aquaculture standards for adult HSC husbandry and year-round LAL production (Tinker-Kulberg et al., 2020a,b). Specifically, these efforts concentrated on developing feed formulations enriched with natural sources of vitamins, minerals, and probiotics to support long-term HSC aquaculture management and establish a new, sustainable supply paradigm for a critical

industrial raw material (LAL). This work may also provide a foundation for applications in a variety of sectors.

MATERIALS AND METHODS

Recirculating Aquaculture System for HSC Husbandry

The HSC studies were conducted in a recirculating aquaculture system (RAS; Clear Flow Aquaponic System, Nelson and Pade, Montello, WI, United States) equipped with four holding tanks (4′ × 6′ × 1′); a biofiltration tank with K1 medium (Pentair, Minneapolis, MN, United States); and a clarifying (solids separation) tank (**Figure 1**). Ambient air supplied oxygen to the water via a piston pump with supplemental air stones positioned in each tank (Nelson and Pade, Montello, WI, United States). Water was recirculated through biological filtration tanks, allowing for purification, hygiene and disease prevention. The tanks were brought to a salinity of 20–22‰ using a commercial sea salt preparation (Crystal Sea®, Marine Enterprise International, Baltimore, MD, United States), an aquatic salinity refractometer (AEROway, Imagitarium™, San Diego, CA, United States), and an electric conductivity (EC) meter (Bluelab Corporation, San Dimas, CA, United States). Weekly saltwater exchanges were performed between 1 and 2%.

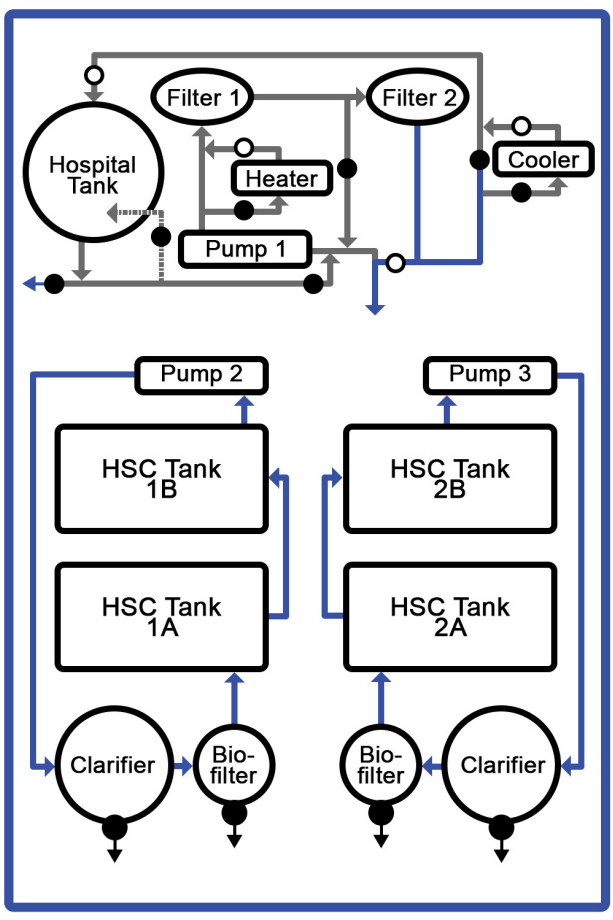

FIGURE 1 | Recirculating aquaculture system (RAS). Temperature-controlled, saltwater system optimized for HSC comprising: four discrete tanks, filtration units, and aeration system. Respective feed formulations were provided to specific tanks with 6 HSCs each.

Target water-quality parameters were determined based on standard saltwater aquaculture methods (Timmons, 1994; Schreibman and Zarnoch, 2009; Ebeling and Timmons, 2010; Shelley and Lovatelli, 2011), and comprehensive daily, weekly and monthly analyses were conducted. Daily water conditions were optimized for: temperature (18.5–20°C); salinity (20–22‰); pH (7.5–8.2); dissolved oxygen (6–9 mgL^{-1}); photoperiod (12-h of natural light); ammonia (0–1.5 ppm); nitrates (<150 ppm); nitrites (2–5 ppm), and alkalinity (90–150 ppm). Water parameters were measured and adjusted as needed six times per week and did not vary significantly throughout the course of the study.

HSCs (n = 24) were procured (Dynasty Marine Associates, Marathon, FL, United States) or obtained via a collection permit and then maintained in accordance with an aquaculture operation permit (North Carolina Department of Environmental Quality, Division of Marine Fisheries; #1946771 and #1947050, respectively). A health assessment was recorded for each HSC upon intake (i.e., sex, weight, carapace length/width, as well as an appendage, carapace, eye, and mouth evaluation). HSCs were stocked at a density of 1.0-lb per square foot, resulting in approximately one HSC per 4-ft^3 of tank space.

Feed Studies, Sources and Key Parameters

For the feed studies, ∼6 adult HSCs were held in each tank, such that their combined weight was similar between the four cohorts. Mature HSCs used in this study on average weighed 1.77 ± 0.77 kg (3.91 ± 1.58 pounds). Each cohort had the same number of males and female HSCs to avoid differences in feeding behavior or data bias. To ensure HSC wellbeing and avoid captive-induced panhypoproteinemia, diet and feeding rates were established over a 6-week period prior to initiation of the trials. The feed rates, crude protein (CP) intake, nutrient composition, and daily energy requirements were determined for natural feed preparations and a commercial aquaculture feed (**Tables 1–3**). Initial observations revealed that HSCs fed at different rates per day (expressed as the percent of b/w of the HSC) depending on the diet. Feed rates were calculated as a percentage of HSC body weight eaten within 8 h (any feed not consumed was removed from the tanks), with a total daily feed rate of 3%, consistent with Carmichael and Brush (2012). We found that at this rate, feed was in slight excess and not limiting, and thus HSCs were more or less feeding at *ad libitum* throughout the day. The average gross energy of each diet was calculated by summing the content of its protein (23.6 kJ/g), lipid (39.5 kJ/g), and carbohydrate (1.2 kJ/g) components (National Research Council, 1993). The nutrient content of natural sourced diets (Diets B–D; **Tables 2**, **3**) was estimated from published references (McRoberts Sales Co. Inc, 2014; Food and Agriculture Organization, 2019; United States Department of Agriculture, 2019). Chemical

TABLE 1 | Feed trial and respective cohort diets.

HSC Cohort	Stocking	Feed Rate†	Feed Trial 1	Feed Trial 2	Feed Trial 3
1	6 HSC/tank	3%	A	A	A
2	6 HSC/tank	3%	B	C	D
3	6 HSC/tank	3%	E	E	E
4	6 HSC/tank	3%	–	F	F

†*Feed was provided as a percentage of HSC body weight.*

TABLE 2 | Composition and costs of feed trial diets.

Diet	Type	Composition	Cost / lb
A†	Manufactured Feed	Beef Gelatin; Blood Meal; Corn Gluten Meal; Feather Meal; Fish Meal; Fish Oil; Poultry By-product Meal; Soybean Meal; Wheat Flour; Wheat Middlings; Synthetic Supplements (15 Vitamins and 9 Minerals)	$0.78
B	Natural Feed (Low Complexity)	Chicken Hearts; Shrimp	$5.00 – $6.00‡
C	Natural Feed (Mild Complexity)	Chicken Hearts and Livers; Earthworms; Fish Remnants; Mussels; Shrimp	$5.00 – $6.00‡
D	Natural Feed (High Complexity)	Anchovies; Chicken Hearts and Livers; Earthworms; Fish Remnants; Glass Minnows; Krill; Mussels; Scallops; Shrimp; Squid; Whole Fish	$5.00 – $6.00‡
E	Experimental Feed 1	Albumin; Beef Gelatin; Cholesterol; Enzymes; Fatty Acids; Fish (Several Species); Macro- and Microalgae; Prebiotics; Probiotics; Whey Proteins; Natural and Synthetic Supplements (20 Amino Acids; 17 Minerals; and 13 Vitamins)	$0.89
F	Experimental Feed 2	Beef Gelatin and Collagen; Poultry By-products; Natural Supplements (20 Amino Acids; 10 Minerals; and 6 Vitamins)	$2.00

†*Commercial Feed – Skretting Classic Brood 46/12 Complete Feed.* ‡*Average. Costs reflect the finished feed.*

TABLE 3 | Nutrient content of feed trial diets.

		% Nutrient Content (As Fed)				
Diet	Gross Energy*	Moisture	Protein	Lipids	Fiber	Ash
A	244	50.0	23.0	6.0	2.0	n.d.
B†	187	77.5‡	18.0	5.0	< 1.0	n.d.
C†	161	77.5‡	16.0	4.0	< 1.0	n.d.
D†	161	77.5‡	16.0	4.0	< 1.0	n.d.
E	196	66.0‡	16.2‡	6.9‡	< 1.0	5.8‡
F	153	66.0‡	11.8‡	5.9‡	< 1.0	1.15‡

DM, *dry matter.* **Gross Energy = Kilojoules/kg of HSC/day.* †*Natural diets estimated from literature.* ‡*Average.* ***n.d.,*** *not determined.*

analyses of experimental gelatin-based diets were performed by an external independent laboratory (i.e., dry matter, DM; Carolina Analytical Services, LLC, Bear Creek, NC, United States). Dry weight was determined by comparison before and after dehydration of feeds at 125–140°C for 48 h. The nutrient content of the wet feed (AF, As Fed) used in the feeding trials was determined by multiplying the DM nutrient content by the DM ratio (dried/wet feed weight).

Six feed sources were combined into the three evaluations, including: natural sources (three variations); a commercial hard pellet feed (Classic Brood 46/12 Complete Feed, Skretting, Tooele, UT, United States); and two experimental formulations shown in **Table 2** [amino acid composition (%); **Supplementary Table 1**]. The commercial and experimental feeds were modified with gelatin (5% w/v) to retain moisture, limit nutrient leaching and facilitate sinking to allow characteristic HSC bottom foraging while providing the same stability between feeds. The gelatin-based matrix conferred an ability to easily and economically encapsulate the nutritional inputs, while readily retaining palatability, consistency, and stability (in storage, as well as in the aquaculture system). Natural, frozen feeds were purchased commercially (Aylesworth's Fish and Bait, St Petersburg, FL, United States) or sourced locally and fed directly. Except for natural feeds that were given whole, the experimental feeds were processed through a meat grinder, mixed with hot gelatin (65–70°C) and allowed to solidify at room temperature. They were measured for each cohort based on daily consumption rates and then frozen at −20°C to preserve freshness. Feed was thawed only once and fed daily to each cohort. Fresh batches were made at the beginning of each trial to safeguard vitamins that are susceptible to oxidation during prolonged frozen storage. Diets were optimized to ensure that macronutrients (protein and lipids) and micronutrients (Vitamins B, C, E, and K, copper, etc.) were available for hemocyanin production, as hemocyanin loss initially follows hemolymph harvest. Some feed formulations (Diets B–F, **Table 2**) naturally contained or were enriched with probiotics and prebiotics from natural sources to ensure healthy gut microbiota and improve digestion and extraction of energy and nutrients from the feed (Abelli et al., 2009; Qi et al., 2009; Fukuda and Ohno, 2014; Torrecillas et al., 2014; Carnevali et al., 2017; Gibson et al., 2017; Egerton et al., 2018).

Respective feed trials, HSC cohorts, and diet compositions are shown in **Tables 1**, **2**. The feeds were dispersed in individual

tanks, and each group received the same feed at ∼3% of the total body weight per tank each day throughout the 8-week periods, with the exception of Cohort 2 (**Table 1**). The variety of natural feed sources was increased with each successive trial for the latter cohort (**Tables 1**, **2**) to investigate dietary complexity and diversity. After each study, HSCs were also physically examined with respect to the carapace, joints, eyes, and book gills, as well as weight and size. Total serum protein concentration, amebocyte density (cells/mL), and LAL reactivity were also analyzed at the beginning and conclusion of each feed trial.

Hemolymph Collection and Analysis

Hemolymph was collected at the start and culmination of each feed study via previously established methods (Coates et al., 2012; Anderson et al., 2013). The pericardial membrane was cleaned twice with 70% ethanol prior to hemolymph collection with a 22-gauge needle (Becton Dickinson, NJ) using a standard SOP (Armstrong and Conrad, 2008). An aliquot of the hemolymph was diluted (1:1) in a pre-chilled anticoagulant mixture (pH 7.3) of *N*-ethylmaleimide (NEM; 0.125%), NaCl (3%), and Tris-HCl (0.5M, pH 7.5) for amebocyte density analysis. Sterile tubes containing the balance of each harvest were centrifuged (1000 rcf/5 min), and the supernatant was removed and stored at −70°C pending hemocyanin analysis. Any samples that appeared clotted after centrifugation were discarded. Amebocyte pellets were washed with endotoxin-free NaCl (3% v/v) and then stored at -70°C pending LAL extraction.

Hemocyanin and amebocyte density were measured throughout the study as HSC health indicators (Nagai et al., 2001). Total serum protein concentration was used as a proxy for hemocyanin (Hc) by diluting the hemolymph supernatant in 0.1M Tris-HCl (pH 7.5) and measuring absorbance at 280nm (UV-Vis Spectrometer; Cary 6000i, Agilent Technologies, Santa Clara, CA, United States). Dioxygen-bound Hc (Oxy-Hc) was likewise measured at 340nm. The total protein concentration was calculated according to Nickerson and Van Holde (1971), using the absorption coefficient $A_{280} = 1.39\ mg^{-1}mLcm^{-1}$ for hemocyanin.

Amebocyte density was calculated by adding 10 μL of the NEM-stabilized cells to a hemocytometer and analyzed microscopically. Since amebocytes are susceptible to rapid degranulation, a digital camera was used to record bright field images from the hemocytometer, and counts were processed offline (ImageJ Software, NIH, Bethesda, MD, United States).

LAL Preparation and Reactivity

Limulus amebocyte lysate reactivity was also evaluated throughout the study as an HSC health indicator and to investigate the effects of aquaculture diets on quality. LAL was prepared by thawing frozen amebocyte pellets on ice and then lysing them in pyrogen-free water at a 1:1 ratio to original hemolymph collection volume under gentle agitation overnight at 4°C. Next, a chloroform extraction (1:1 v/v) of the lysate was performed, and the aqueous phase was measured for total protein using a BCA kit (Pierce, Thermo Fisher Scientific, Waltham, MA, United States). All LAL extracts were aliquoted and frozen at −80°C pending analysis (consistent LAL reactivity was observed in frozen samples for up to 6-months). LAL samples were also limited to a single freeze-thaw cycle to preserve reactivity.

Limulus amebocyte lysate from HSCs fed different diets was evaluated turbidimetrically. Equal protein amounts of amebocyte lysate (LAL) isolated from individual HSCs in each feed cohort (n = 6) were pooled and analyzed for their relative reactivity. LAL from commercial kits (E-Toxate™, Sigma Aldrich, St. Louis, MO, United States) served as a reference. For each LAL assay, an equal amount of protein from pooled aquaculture lysates and reference LAL were brought to a total volume of 100 μL using endotoxin-free water; these were mixed gently with 100 μL of endotoxin solution (final concentration: 0 – 50 EU/mL) in a pyrogen-free, 96-well microplate and incubated at 37°C for 1 h. The conversion of coagulogen to coagulin was measured at 340 nm on a microplate reader (BioTek 800, BioTek Instruments, Winooski, VT, United States). The turbidity of the blank (endotoxin-free lysates) was subtracted from test values, and the relationship between endotoxin concentration and clot formation was measured. The reactivity of the aquaculture LAL was normalized to the reference LAL, and relative absorbance was measured.

Protein Analyses

Purified protein extracts (10 μg) from each feed group were subjected to SDS-PAGE in the presence of β-mercaptoethanol. Each sample was diluted in a loading buffer (NuPage™, Invitrogen, Carlsbad, CA, United States) containing 2.5% β-mercaptoethanol and heated to 85°C for 3 min. Electrophoresis was performed on 8% Tris-Glycine gels (Novec™ Wedge Well, Invitrogen, Carlsbad, CA, United States). A pre-stained protein standard (SeeBlue® Plus2, Invitrogen, Carlsbad, CA, United States) was used as a molecular weight marker, and commercial LAL was used as a reference (E-Toxate™, Sigma-Aldrich, St. Louis, MO, United States). The gel was either stained (Coomassie Brilliant Blue R-250; Bio-Rad, Hercules, CA, United States) or transferred to a polyvinylidene fluoride (PVDF) membrane (Invitrogen, Carlsbad, CA, United States) for Western blot analysis.

For Western blotting, the PVDF membranes were blocked with PBS (pH 7.4) containing 5% BSA (or 5% non-fat milk) and 0.1% Tween 20 for 1 h at room temperature. Primary rabbit polyclonal antibodies [COCH (A6562), Factor IX (A1578) and KLKB1 (A5318)] were used for the detection of LAL clotting factors (ABclonal Inc., Woburn, MA) by overnight incubation at 4°C (1:1,000 – 1:3,000; determined experimentally). Membranes were washed 3X with PBS/Tween 20 (0.1%) for 7 min with agitation and then incubated with a goat anti-rabbit secondary antibody (1:10,000 dilution) labeled with horseradish-peroxidase (HRP; ABclonal Inc, Woburn, MA, United States) for 1 h at room temperature. Residual secondary antibody was removed by washing, as described above. The membranes were incubated with ECL reagent (Thermo Fisher Scientific, Waltham, MA, United States) for 5 min at room temperature, and consequent target protein luminescence was measured (Amersham™ Imager 600; GE Life Sciences, Piscataway, NJ,

United States). Relative protein abundance within the linear dynamic range was quantified using densitometry.

Post-translational protein modification of the LAL extracts was measured by Coomassie staining or Western blot, as described above. Prior to electrophoresis, LAL extracts were treated with either glycopeptidase F (PNGase F; ∼36 kDa; New England BioLabs, Ipswich, MA, United States), under denaturing conditions at a final concentration of ∼0.4 units of enzyme (20 ng/μg of lysate) or alkaline phosphatase (calf intestine AP; ∼54 kDa; Roche, Indianapolis, IN, United States) at a final concentration of 2 units of enzyme (2 μg/μg of lysate) according to the manufacturer's instructions. Protein deglycosylation and dephosphorylation were assessed by analyzing mobility shifts (Amersham^{TM} Imager 600, Buckinghamshire, United Kingdom).

Data Analysis

Individual HSC cohorts were used for each respective analysis (**Table 4**). Standard deviation between individual HSCs (n = 6) within each cohort was calculated. For simplicity and assay accuracy, LAL reactivity was measured using pooled samples from HSCs within each feeding cohort (n = 6), comprised of equal amounts of proteins from each one. The standard deviation of LAL reactivity represents each assay performed in triplicate. LAL reactivity derived from individual HSCs was close to the mean of pooled samples from each cohort. All trials represent the mean ± standard deviation, where n equals the number of HSCs per cohort. If a difference was determined, a Student's t-test was used for comparison, assuming equal variance, with a significant difference set at $p \leq 0.05$.

RESULTS

Feed Rate Determination

Three separate 8-week feed trials were conducted to investigate the effect of diet on health parameters of respective HSC cohorts (n = 6 per study) as described in **Table 1**. The nutritional composition and economic analyses were determined for each experimental feed (**Tables 2**, **3**). CP intake for adult HSCs ranged between 3.6 and 6.9 mg per gram of HSC b/w per day [(CP (mg)/(HSC body weight (g) per day)] when fed an 11–23% protein diet at a 3% daily feed rate. Experimental feeds were developed to provide the target energy requirements (Joules) from 153 to 244 kJ [HSC body weight (kg) per day] determined in the pre-trial feed studies (**Table 3**). These values are aligned with growth trials conducted with juvenile HSCs that were shown to require 224 kJ of digestible energy and 8.7 mg of digestible protein for maintenance per gram b/w per day (Tzafrir-Prag et al., 2010).

Irrespective of feed type, adult HSCs consumed similar amounts of food energy per day across all groups (i.e., mean = 150 kJ per kg of HSC b/w per day), and the previously cited 3% feed rate was thus validated and employed (Carmichael and Brush, 2012; **Table 1**). Maintenance levels of CP for adult HSCs were found to be ∼5.25 mg per gram b/w per day when fed an 11–23% protein diet, which aligned with various fish, shrimp, and juvenile HSC aquaculture studies (Kuresh and Davis, 2000; Blanc and Margraf, 2002; Lupatsch et al., 2008; Berkson et al., 2009; Tzafrir-Prag et al., 2010). The daily feed rate and diet formulations sustained HSC weight (±1.2%) and serum protein levels (>5.0 mg/mL) over the course of the feed study with a 100% survival rate (**Supplementary Table 2**). All HSCs were mature and no longer prone to molting; thus, their growth rate did not increase over the course of the study.

TABLE 4 | Effect of diet on aquaculture LAL vs. Reference LAL reactivity.

		LAL Reactivity (Compared to Reference LAL)		
HSC Cohort	**Diet Group**	**Feed Trial 1**	**Feed Trial 2**	**Feed Trial 3**
1	A	1.9X	1.5X	1.0X
2†	B	1.1X	–	–
2†	C	–	1.4X	–
2†	D	–	–	1.6X
3	E	2.1X	1.4X	0.4X
4	F	–	1.6X	2.9X

†*HSC Cohort 2 was provided increased dietary complexity in each successive feed trial.*

Feed Trial 1: Effect of Diet on LAL Reactivity and Protein Levels

Horseshoe crabs were provided three different diets over a period of 8 weeks (Diet A, Diet B and Diet E; **Tables 1–3**). At the outset of Feed Trial 1, the aquaculture HSC serum protein baselines (hemocyanin concentration, Hc) ranged from 44.49 to 65.15 mg/mL. Upon trial conclusion, HSCs fed Diet E had the highest LAL reactivity (2.1X higher than reference LAL) but showed the lowest Hc concentration (Diet E = 21.2 + 1.22 mg/mL vs. Diet A and B = 43.6 + 8.9 and 65.2 + 11.8 mg/mL, respectively); whereas, amebocyte density remained constant across the three groups, ranging between $\sim 2 \times 10^7$ to 3×10^7 cells/mL.

The reactivity of LAL extracts (150 μg) for each feed group and a reference LAL are shown in **Figure 2A**. LAL incubation with low concentrations of LPS (0.5 EU/mL) revealed similar reactivity (clot formation) for aquaculture-derived LAL from HSCs fed Diet E and reference LAL as a standard control (E-Toxate^{TM}; Sigma-Aldrich, St. Louis, MO, United States). However, the LAL reactivity derived from HSCs fed Diets A and E was ∼2X more responsive than reference LAL at high concentrations (saturation levels) of LPS (50 EU/mL) (**Figure 2B**). Diet B, the least-complex feed (**Table 2**) showed the lowest reactivity at 0.5 and 5.0 EU/mL concentrations of LPS, but it was equivalent to that of reference LAL at 50 EU/mL.

The reactivity of LAL from the Diet E cohort and the reference LAL were evaluated at increasing protein concentrations (0, 10, 25, 50, and 75 μg, **Figure 2C**) to assess gelation (ΔOD) between LAL extracts in the presence of saturation levels of LPS (50 EU/mL). Both extracts required at least 50 μg of total protein (or 250 μg/mL) for absorbance to increase 0.200 units above the

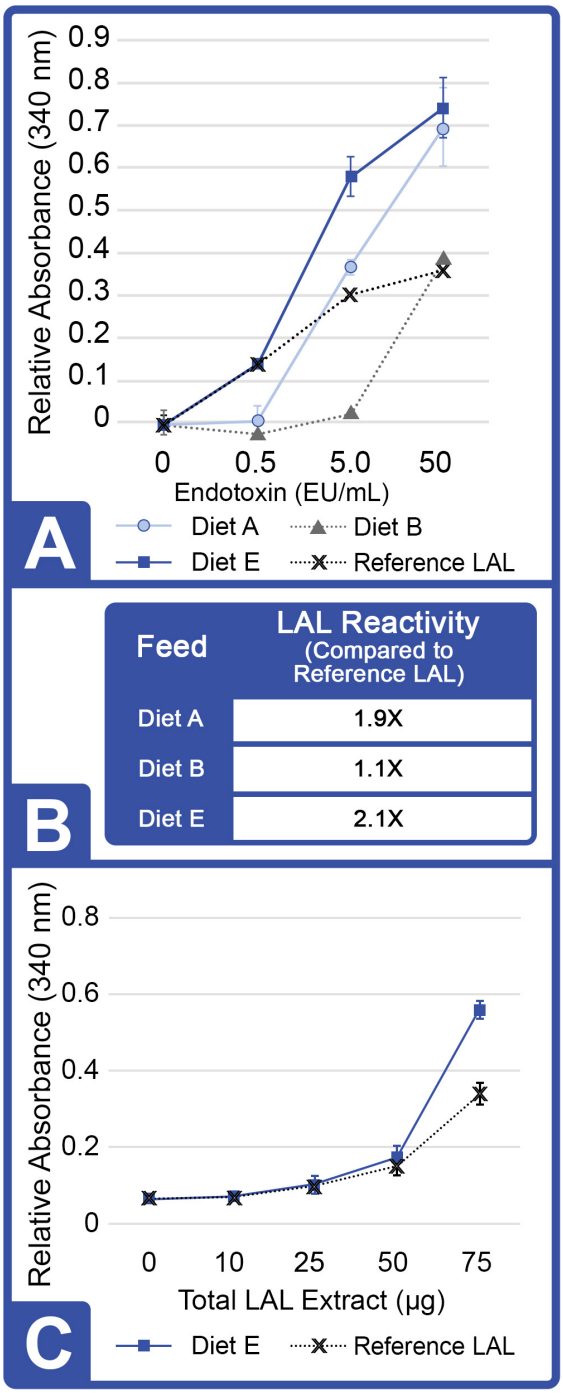

FIGURE 2 | Effect of diet on LAL reactivity to endotoxins (Feed Study 1). **(A)** Gel formation measured by absorbance at 340 nm. Hundred and fifty µg of aquaculture LAL was evaluated at a range of endotoxin concentrations: 0, 0.5, 5.0, and 50.0 EU/mL with a reference LAL. Results are represented as mean ± SD between individual LAL assays performed in triplicate. **(B)** LAL reactivity to endotoxin from each diet cohort was normalized to reference LAL reactivity at 50 EU/mL of LPS. **(C)** Reactivity (gelation) of increasing concentrations of LAL (0, 10 25, 50, and 75 µg total protein) from HSCs fed Diet E vs. reference LAL at 50 EU/mL of LPS measured by absorbance at 340 nm. Results are represented as mean ± SD between individual LAL assays performed in triplicate.

background (indicative of a positive reaction/gel formation). At 75 µg of LAL protein, the Diet E cohort also showed greater reactivity (2.1X higher) per µg of total protein compared to control LAL after 1 h of incubation with a saturation-level (50 EU/mL) of the LPS substrate.

Feed Trial 2: Effect of Increased Diet Diversity on LAL Reactivity

In the first feed trial, HSCs in Cohort 2 were provided a natural feed diet (Diet B; **Tables 1**, **2**) as previously reported (Carmichael and Brush, 2012), and their LAL demonstrated the lowest reactivity (**Figures 2A,B**). During the second feed trial, the nutrient complexity and diversity of the natural feed sources were increased by adding a greater variety of marine-derived proteins [**Table 2**: Diet B (2 sources) compared to Diet C (6 sources)]. Additionally, a new experimental feed (Diet F) was developed with different sources of proteins and lipids than those used for Diet E (**Table 1**), in an effort to avoid declining Hc (21.2 mg/mL) values observed in Trial 1 for Diet E. In Feed Trial 2, HSC Cohorts 1 and 3 were provided the same diets as in Trial 1 (**Table 1**: Diets A and E, respectively).

At the conclusion of Feed Trial 2, hemolymph was harvested from each HSC cohort, and LAL reactivity was measured. A titration was performed with increasing concentrations of LAL protein (37.5, 75.0, and 150 µg) derived from each diet and a reference LAL (E-Toxate™, Sigma-Aldrich, St. Louis, MO, United States) incubated with saturation levels of LPS at 50 EU/mL (**Figure 3A**). Similar to Feed Study 1, the aquaculture LAL had slightly greater reactivity per µg of total protein compared to the reference LAL (1.4–1.6X higher; **Figure 3B**). The degree of nutrient diversity affected LAL: Diet C (Feed Trial 2; 6 sources) outperformed Diet B (Feed Trial 1; 2 sources) in terms of reactivity (Diet B = 1.1X versus Diet C = 1.4X more sensitive when normalized to the control LAL).

Feed Trial 3: Effect of Diet on LAL Reactivity, Hc Concentrations and Amebocyte Density

In Feed Trial 3, a significantly more diverse natural feed diet (Diet D; 12 ingredients; **Table 2**) was compared with Diets B and C (2 and 6 ingredients, respectively). HSCs fed diet D yielded more reactive LAL than HSCs fed the other natural diets: 45% greater than diet B (the lowest-complexity diet) and 14% greater than diet C (moderately complex diet) (**Table 4**).

Overall, the HSC cohort fed Diet F over an 8-week period appeared to outperform all feeds with 2.9X greater LAL reactivity over the control; and across two successive feed trials (16 weeks), sensitivity increased (**Table 4**). Notably, the overall LAL reactivity of extracts derived from HSCs fed Diet F was 7.25X greater than those given Diet E (**Figure 4A**).

In contrast, the LAL reactivity of the HSC cohorts fed Diets A and E in all three trials (24 weeks) declined over time (**Table 4**). In Feed Trial 1, Diet E resulted in a 2.1X increase over baseline (**Figure 4**), which fell to 1.4- and 0.4X in Feed Trials 2 and 3, respectively. The LAL sensitivity from HSCs fed Diet E dropped

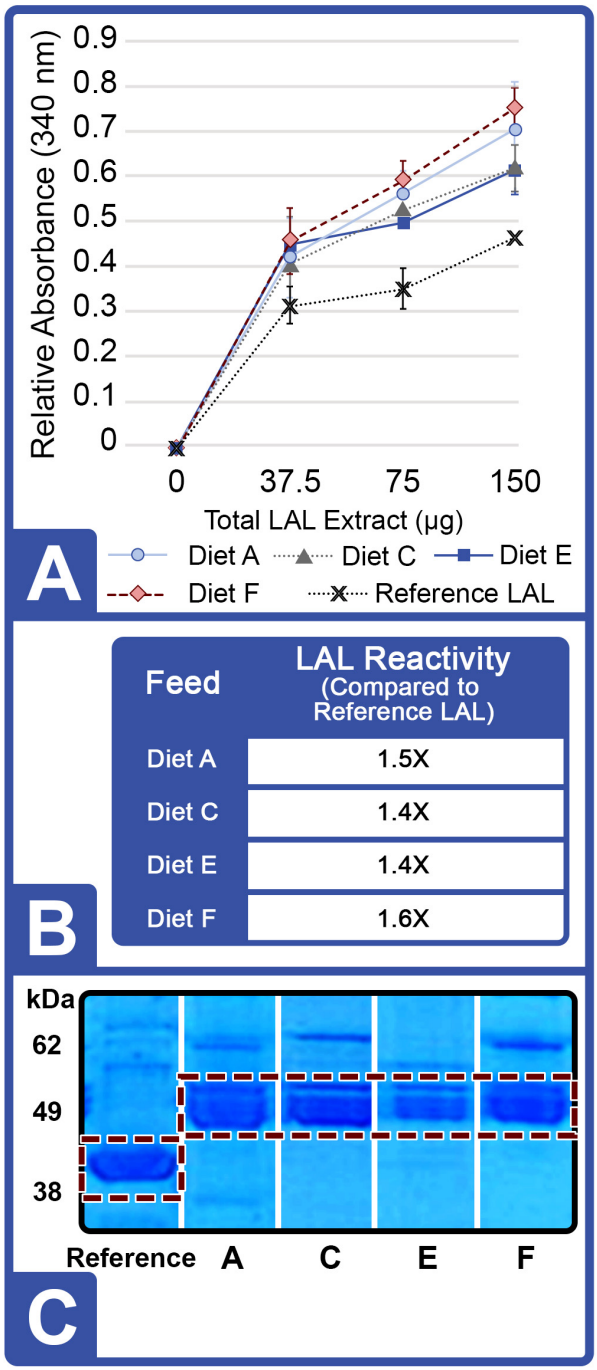

FIGURE 3 | Effect of diet on LAL reactivity to endotoxins (Feed Study 2). **(A)** Standard curves of aquaculture LAL reactivity to endotoxins by diet vs. reference LAL with 50 EU/mL LPS measured by absorbance at 340 nm. Results are represented as mean ± SD between individual LAL assays performed in triplicate. **(B)** Aquaculture LAL from each diet was normalized to reference LAL reactivity in the presence of 50 EU/mL of LPS. **(C)** SDS-PAGE of crude amebocyte lysates from aquaculture HSCs (Lanes 3 – 6) compared to reference LAL. Each sample was loaded at 5 µg protein per lane on an 8% Tris-Glycine gel and stained with Coomassie blue; the See Blue+2 molecular weight marker was loaded as a ladder. Factor C (L-chain) is highlighted in white and appeared as a well-defined band at ~43 kDa in reference LAL and at ~52 kDa in the aquaculture LAL.

33% after the second 8-week trial and 71% after the third 8-week trial.

Horseshoe crabs from each cohort demonstrated consistent total amebocyte counts (**Figure 4C**). The low LAL reactivity observed in HSCs fed Diet E was thus not related to lower amebocyte concentrations. Rather, in Feed Trial 3, it correlated with low Hc levels (**Figure 4D**). HSCs offered Diet F also had the highest Hc levels and produced the most reactive LAL. Conversely, Diet E resulted in the lowest Hc and LAL response among all evaluated feed cohorts.

In Feed Trial 3, the LAL reactivity varied between the different cohorts more than observed in the previous studies (e.g., **Figures 3A** vs. **4A**). Equal amounts of LAL protein (µg) pooled from each group (aquaculture LAL pool; ALP) had ~1.6X higher reactivity than reference LAL (**Figure 4B**). This reactivity is approximately equivalent to the average of the LAL response derived from individual diet cohorts (~1.5X). Therefore, pooling the LAL protein had no effect on reactivity. The LAL sensitivity of each HSC cohort relative to the reference LAL is shown in **Table 4**.

Effects of Diet on LAL Clotting Factors

LAL Factor C protein characteristically generates an 80 kDa (H-chain) and a 43 kDa subunit (L-chain) under denaturing electrophoresis (**Figure 5A**). LPS activation of Factor C results in the conversion of the L-chain into the A (9 kDa) and B-chain (34 kDa) fragments, thereby initiating the reaction cascade (**Figure 5B**). The Factor C L-chain subunit in the aquaculture-derived LAL extracts notably migrated as a higher molecular weight species compared to that of reference LAL (**Figures 3C**, **6A,C**).

Gel electrophoresis of crude LAL from the different feed groups also revealed distinct differences in Factor C patterns relative to other protein bands with Western blot testing (**Figure 6**). In fact, Western blot analysis of Factor C suggested that relative abundance of this protein correlated to LAL reactivity.

For example, the Diet E cohort yielded 40% lower LAL reactivity and 50% less Factor C than reference LAL per µg of total protein; whereas, Factor C protein levels and LAL reactivity were equivalent to the reference (**Figures 4**, **6A,B**). The HSCs fed Diet D showed 1.2X higher Factor C levels and 1.6X greater LAL reactivity compared to the reference; while the Diet F cohort had 1.5X higher Factor C protein levels and 2.9X greater LAL response (**Figures 4**, **6A,B**).

Western blot analysis with polyclonal antibodies specific to Limulus Factor B (**Figure 7**) and proclotting enzyme (**Figure 8**) was also conducted to determine the relative concentrations of these clotting factors. The single-chain form of Factor B (**Figure 5A**) was observed only in the aquaculture LAL (**Figure 7A**, L- + H-chain). The total amount of Factor B in the LAL of HSCs fed Diet E was 2.4X higher than reference LAL, despite this lysate revealing 40% lower LAL reactivity (**Figures 4B**, **7B** and **Table 5**). Factor B protein abundance was between 2 and 4X higher in all LAL derived from the aquaculture cohorts in comparison to the reference. In contrast to Factor B, the relative protein levels (**Figure 8**) of the proclotting enzymes

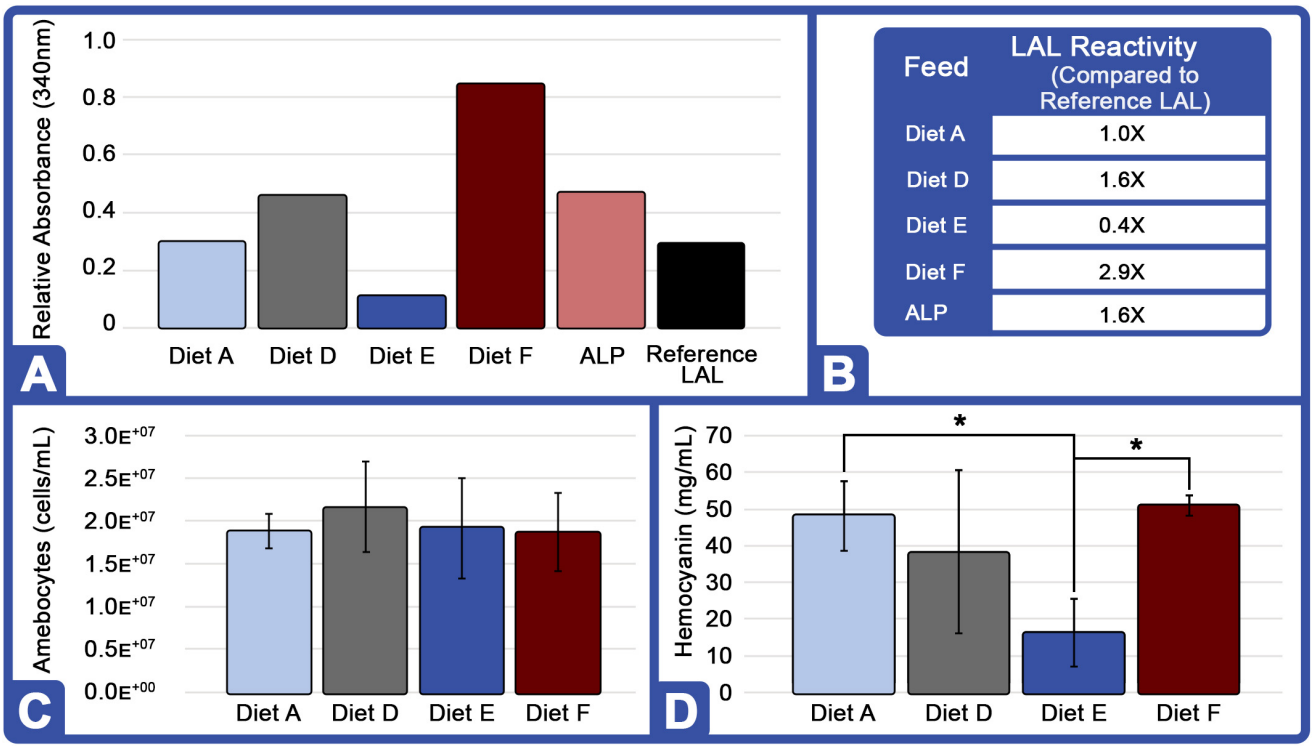

FIGURE 4 | Effect of diet on LAL reactivity, amebocyte density, and hemocyanin concentration (Feed Study 3): **(A)** Aquaculture LAL reactivity from four diets over 8-week period vs. reference LAL at 50 EU/mL LPS concentration. Equal protein amounts from each Diet (75 μg; total = 300 μg) were pooled and tested for clotting [ALP: Aquaculture Lysate Pool (Diet A, D, E, F)] and reactivity to endotoxins was measured by absorbance at 340 nm. **(B)** Aquaculture LAL reactivity to endotoxins vs. reference LAL. **(C)** Amebocyte density of aquaculture HSC determined microscopically on a hemocytometer. **(D)** Hc concentration of aquaculture HSC hemolymph measured by UV spectrophotometry (280 nm). Results are represented as mean ± SD between individual HSCs in each cohort ($n = 6$). *$p < 0.05$ compared to Diet E.

corresponded to the observed LAL reactivity of the respective groups (**Figures 4B**, **8B** and **Table 5**). However, Western blotting showed variations in the molecular weights of the proclotting enzyme(s) among the diet groups.

The highest LAL reactivity observed in the HSCs fed Diet F (2.9-fold; **Figure 4B** and **Table 4**) also correlated to the greatest abundance of clotting factor proteins relative to the reference LAL baseline [**Table 5**: Factor C: 1.5X (**Figure 6B**); Factor B: 3.8X (**Figure 7B**); and proclotting enzyme: 2X (**Figure 8B**)]. HSCs provided Diet E produced the least reactive LAL (0.4X; **Figure 4B** and **Table 5**); had the lowest protein expression for Factor C (0.5X; **Figure 6B** and **Table 5**), and of proclotting enzyme (0.7X; **Figure 8B**). However, Factor B in this cohort had 2.4X higher protein levels (**Figure 7B** and **Table 5**) compared to reference LAL. In fact, comparison of the highest (Diet F) and lowest (Diet E) LAL reactivity revealed that Factor B may not be the limiting component of the coagulation cascade.

Additionally, the Diet A cohort had similar LAL response (**Figure 4B**), levels of Factor C protein expression (**Figure 6B**), and levels/banding patterns of proclotting enzyme (**Figure 8B**) as the reference LAL. However, this group (Diet A) had 2X higher levels of Factor B protein (**Figure 7B**) than reference LAL, further signifying that Factor B is not the limiting component. In Diet D-derived LAL, Factor C and proclotting enzyme protein levels were directly related to LAL response.

In all feed groups, Factor C and proclotting enzyme protein levels correlated more closely to LAL reactivity; whereas, the relative abundance of Factor B did not.

Protein Analysis: Glycosylation and Phosphorylation of Clotting Factor Subunits

The most abundant protein bands produced by the crude LAL derived from all aquaculture cohorts were similar, but they differed from the reference LAL as shown via Coomassie gel staining (**Figure 3C**). Specifically, the L-chain of Factor C (subsequently determined via Western blot in **Figure 7**) migrated faster (∼43 kDa) in the reference LAL than in the aquaculture LAL (∼52 kDa) (**Figure 3C**). Both subunits of Factor C have multiple glycosylation sites. The glycosylation sites of the Factor C A-chain subunit have been shown to be partially modified; whereas, the B-chain has two potential glycosylation sites (**Figure 5A**).

The shift in mobility of the Factor C L-chain subunit between aquaculture and reference LAL was evaluated to determine if the difference in migration was related to glycosylation. Equal

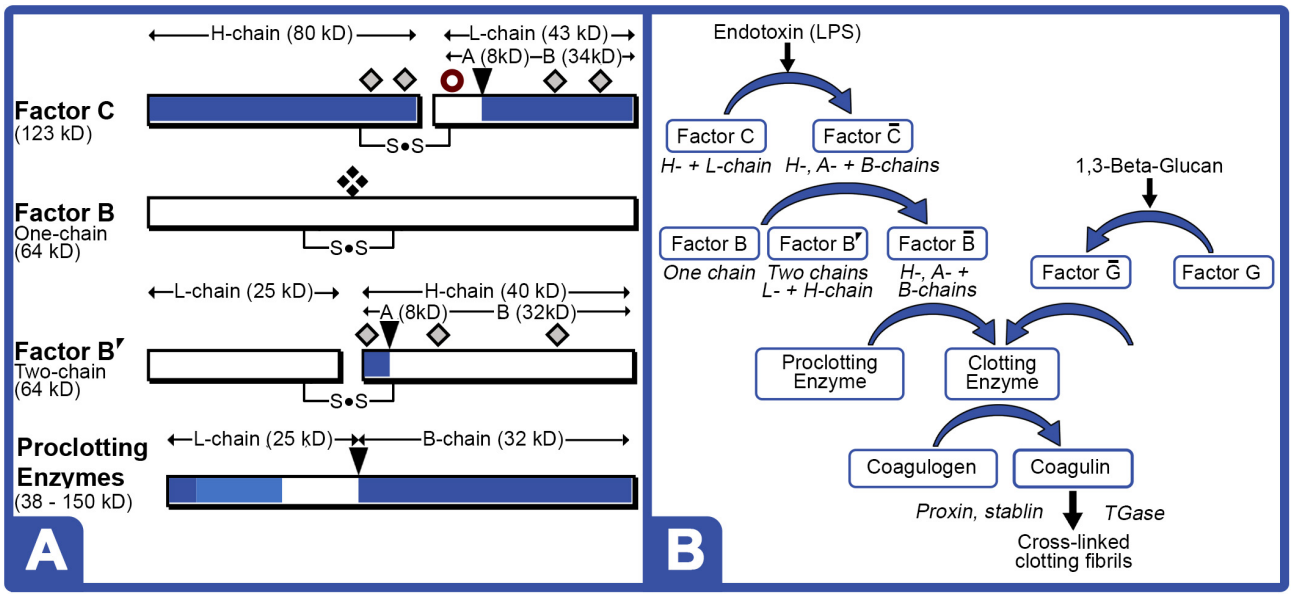

FIGURE 5 | LAL clotting factors structures and coagulation cascade. **(A)** Cleavage sites [▼] for activation; [❖] nicking site; [◆] potential glycosylation sites; and [O] newly proposed phosphorylation site in the protein zymogen. Zymogen Factor B is a single-chain form; whereas, "nicked" double-chain form is composed of L- and H-chains, both containing internal disulfide bond. **(B)** Schematic of LPS and 1,3-Beta-Glucan LAL coagulation cascade. LPS converts phosphorylated or glycosylated Factor C zymogen (H- + L-chain subunits) into activated Factor $\overline{C}$ enzyme (A- + B-chain subunits). Activated Factor C nicks single-chain Factor B zymogen into Factor B′ (H-chain) glycosylated precursor that is converted to activated Factor $\overline{B}$ enzyme (B-chain). Activated Factor B and activated (1–3)-D-glucan binding factor G, initiate cleavage of various proclotting enzymes to form the clotting enzyme, which hydrolyzes coagulogen into coagulin, resulting in insoluble clot.

amounts (μg) of aquaculture LAL protein (pooled from all diet cohorts; ALP) and the reference LAL were treated with a glycopeptidase (peptide:*N*-glycosidase F; PNGase) to remove N-linked glycans from glycoproteins. PNGase-treated LAL was then analyzed using Coomassie staining (**Figure 6C**). Both ALP and the reference LAL revealed a mobility shift in the H-chain subunit of Factor C, thus verifying glycosylation (**Figure 6C**). The PNGase-induced mobility shift caused an increase in migration that aligned with the expected 80 kDa protein subunit size. Western blotting with an anti-Factor C antibody revealed similar results (data not shown). PNGase treatment of reference LAL also caused an unexpected upward shift in the mobility of the Factor C L-chain subunit after removal of the *N*-glucan (**Figure 6C**), and the Factor C L-chain of PNGase-treated reference LAL continued to migrate faster than the ALP Factor C L-chain.

To test whether the higher molecular weight of the Factor C L-chain in aquaculture-derived LAL relative to reference LAL was caused by post-translational phosphorylation, LAL was treated with alkaline phosphatase (AP). As shown in **Figure 6D**, Western blot analysis revealed that the Factor C L-chain was phosphorylated in the aquaculture LAL; as treatment with AP resulted in a migration pattern of the Factor C L-chain subunit that aligned with reference LAL (∼43 kDa). The mobility of the B-chain subunit of activated aquaculture LAL Factor C (**Figure 6D**) was unaffected by AP treatment. Factor C in the reference LAL was also unaffected by AP treatment.

Western blotting of residual Factor C B-chain in LAL preparations, presumably generated from LPS activation of Factor C, showed that this segment was not glycosylated or phosphorylated, since PNGase and AP treatment did not change band migration (data not shown). Low levels of this "activated" Factor C B-chain subunit were found in both ALP and reference LAL and may be present naturally in HSC hemolymph (**Figure 6A**).

As shown in **Figure 5A**, Factor B and the proclotting enzyme also have characteristic glycosylation sites. Factor B is known to contain two molecular species: a single-chain form (64 kDa; L- + H-chain) and a "nicked" double-chain form (40 and 25 kDa) tethered by a disulfide bond (**Figure 6**). The reference LAL contained the double-chain but not the single-chain form of Factor B (L- + H-chain) found in aquaculture LAL (**Figures 5B**, **8A**). Glycosylation of the Factor B H-chain was present in both the aquaculture and reference LAL; however, the single-chain form of Factor B (L- + H-chain) was not sensitive to PNGAse treatment (**Figure 7A**). The banding patterns of the proclotting enzyme(s) were also not affected by PGNase treatment (data not shown). Lastly, both Factor B and proclotting enzyme were insensitive to AP treatment.

Effect of Divalent Cations on LAL Reactivity

The inclusion of divalent cations ($MgCl_2$ and $CaCl_2$) has reportedly enhanced LAL reactivity (Sullivan and Watson, 1974, 1978). Given that LAL preparation methods by commercial sources are proprietary, the inclusion of divalent cations were evaluated to determine the potential influence of processing techniques on the LAL reaction. Addition of $MgCl_2$ at 10 and

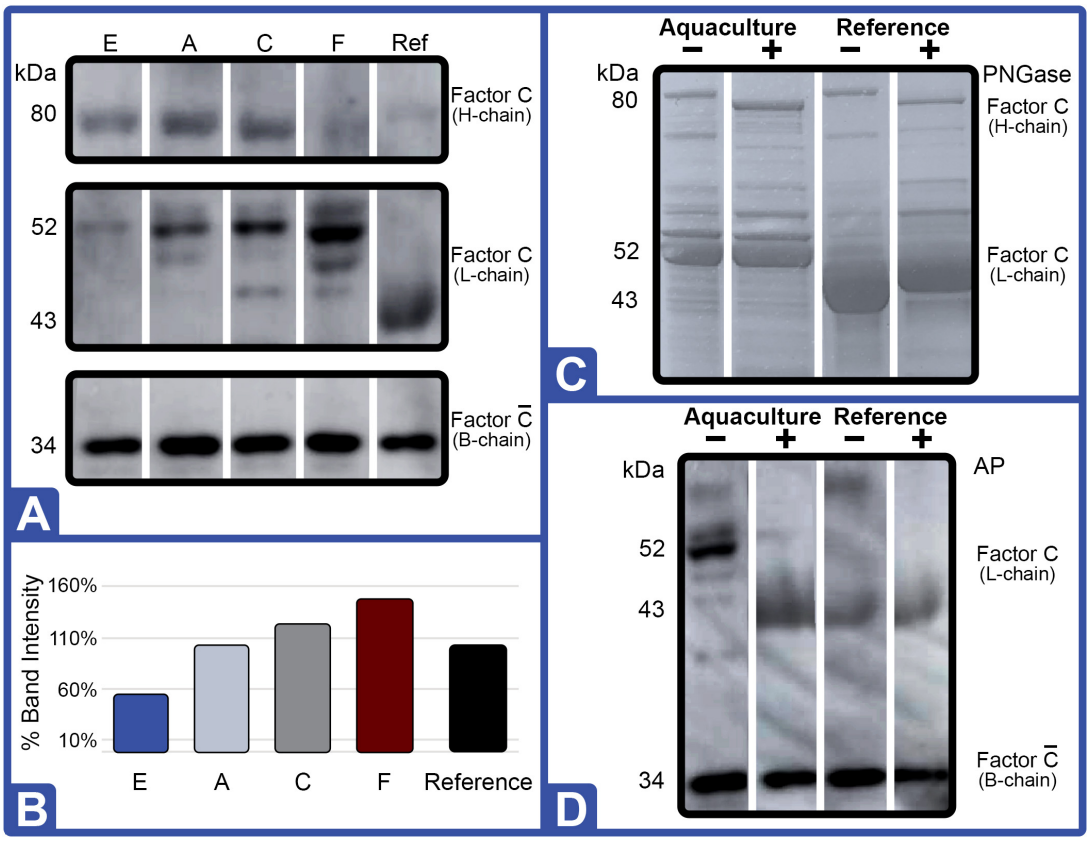

FIGURE 6 | Effect of diet on Factor C protein expression (Feed Study 3). **(A)** Western blot of crude lysates (5 μg) of LAL from each diet vs. reference LAL. Extracts from Diet E (Lane 1), Diet A (Lane 2), Diet C (Lane 3), Diet F (Lane 4), and reference LAL (Ref; Lane 5) with See Blue+2 molecular weight marker. Each sample was resolved across 8.0% Tris-Glycine gel and transferred to PVDF membrane. Proteins analyzed by membrane incubation with anti-Factor C polyclonal rabbit antibodies (COCH). **(B)** Total Factor C protein abundance (% band intensity) quantified on an imaging device. Zymogen and activated Factor C were observed. **(C)** Coomassie-blue staining of equal protein amounts from each diet were pooled [ALP: Aquaculture Lysate Pool (Diet A, D, E, and F)]; treated with (+) or without (–) PNGase. **(D)** Western blot of crude LAL lysate (ALP) and reference LAL treated with (+) or without (–) alkaline phosphatase (AP). Ref: Reference LAL.

25 mM to the assay using aquaculture LAL (Diet F) reduced reactivity (−32% and −30%, respectively; **Figure 9A**); whereas, the inclusion of 2 mM $CaCl_2$ revealed a modest increase (5.8%), but blunted response (−7.8%) at 10 mM (**Figure 9A**). In contrast, the addition of $MgCl_2$ (10 and 25 mM) and $CaCl_2$ (2 and 10 mM) in reference LAL increased sensitivity at all concentrations (**Figure 9A**). As shown in **Figure 9B**, aquaculture LAL revealed greater response than reference LAL preparations under optimal salt conditions for each lysate of 2 mM $CaCl_2$ and 25 mM $MgCl_2$, respectively.

Experimentally derived concentrations of divalent cation effects were also investigated across a range of endotoxin concentrations (0.5 – 50 EU/mL). Incubation of aquaculture LAL with divalent cations increased sensitivity at low endotoxin concentrations (0.5 EU/mL; **Figure 10**). As presented in **Figure 9A**, aquaculture LAL reactivity to moderate LPS levels (5.0 EU/mL) was also amplified by $CaCl_2$ (2 mM); but it was reduced with $MgCl_2$ (10 and 25 mM), as well as $CaCl_2$ (10 mM). Notably, the addition of divalent cations at both concentrations reduced LAL reactivity at high levels of LPS (50 EU/mL; **Figure 10**). While slight improvements were shown at lower levels of LPS with 2 mM Ca^{++}, inclusion of divalent cations did not significantly enhance aquaculture LAL assay performance.

DISCUSSION

Globally, four HSC species have suffered population declines as a result of increased harvesting for biomedical sterility testing and bait, commercial fishing bycatch, loss of spawning habitat, pollution, and declining oceanic ecosystems (Berkson et al., 2009; Gauvry, 2015; Krisfalusi-Gannon et al., 2018). Furthermore, commercial LAL reactivity (from various suppliers) has been shown to vary from batch to batch due to spawning-induced stress and diet limitations that reduce sensitivity (Sullivan and Watson, 1974; Owings et al., 2019). Sustainable HSC aquaculture could afford a more consistent LAL supply, eliminate HSC mortality associated with wild-capture bleeding practices (10–30%), and provide the potential to enhance reagent quality and reactivity. This study suggests that highly reactive LAL can be produced from HSC aquaculture on a year-round basis. The HSCs have continued to thrive in aquaculture after completion

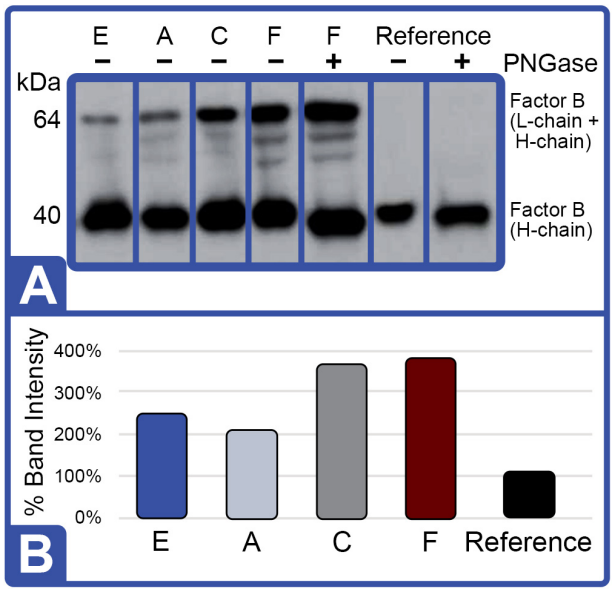

FIGURE 7 | Effect of diet on Factor B protein expression (Feed Study 3). **(A)** Western blot of crude lysates (5 μg) of LAL from each diet analyzed vs. reference LAL. Extracts from Diet E (Lane 1), Diet A (Lane 2), Diet C (Lane 3), Diet F (Lane 4), Diet F + PNGase (Lane 5), reference LAL (Lane 6), and reference LAL + PNGase (control; Lane 7) with See Blue+2 molecular weight marker. Each sample was resolved across 8.0% Tris-Glycine gel and transferred to PVDF membrane. Proteins analyzed by membrane incubation with anti-Factor B polyclonal rabbit antibodies (KLKB1). Both aquaculture (Diet F) and reference LAL revealed Factor B H-chain glycosylation as shown by slight molecular weight shift in Lanes 4 and 5 of aquaculture LAL and Lanes 6 and 7 in reference LAL. Single-chain Factor B (L- + H-chain) was resolved only in the aquaculture LAL (Lanes 1–5 vs. Lanes 6 and 7); it was not sensitive to PNGAse treatment (Lane 4 vs. 5). Activated Factor $\overline{B}$ (A- and B-chains) was not observed. **(B)** Total Factor B protein abundance (% band intensity) quantified on an imaging device. Ref, reference.

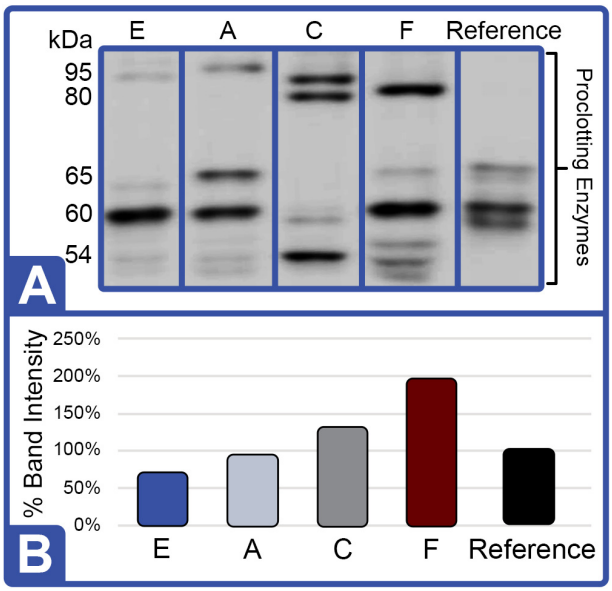

FIGURE 8 | Effect of Diet on Proclotting Enzyme Protein Expression (Feed Study 3). **(A)** Western blot of crude lysates (5 μg) of LAL from each diet analyzed vs. reference LAL. Extracts from Diet E (Lane 1), Diet A (Lane 2), Diet C (Lane 3), Diet F (Lane 4), and reference LAL (Lane 5) with See Blue+2 molecular weight marker. Each sample was resolved across 8.0% Tris-Glycine gel and transferred to PVDF membrane. Proteins analyzed by membrane incubation with anti-proclotting enzyme polyclonal rabbit antibodies (Factor IX). **(B)** Total proclotting enzyme protein abundance (% band intensity) quantified on an imaging device.

of this feed evaluation and after more than 1 year of captivity on routine feedings of Diet F. All HSCs have maintained their weight (+3.2% change) and the LAL isolated from this cohort remains 2.36 ± 0.44 times higher than reference LAL. To date, a key barrier to successful husbandry has been ascribed to nutritional imbalances, dietary deficiencies, captivity-induced diseases, such as panhypoproteinemia, and increased mortality rates over time (Smith and Berkson, 2005; Nolan and Smith, 2009; Carmichael and Brush, 2012). These feed studies suggest that diet directly affects LAL sensitivity and amebocyte protein production and/or prevention of breakdown.

In the wild, most aquatic animals are opportunistic feeders and consume a diverse array of prey; whereas, captive animal diets characteristically lack relative diversity and quality of feed products and can affect animal health through digestive microbiota. Captive adult HSCs reportedly have been provided natural sources of feed to mimic wild foraging; however, such diets can be costly, seasonally limited, and yield variable nutrient quality and composition due to storage and other variables. Specifically, in this study a comprehensive strategy was developed to leverage affordable, sustainable, and nutritionally diverse feed sources to satisfy metabolic requirements, promote cellular health, aid in harvest recovery, and support long-term husbandry. Wherein, some experimental diets promoted overall cellular health throughout the course of the trial by maintaining sufficient HSC hemocyanin levels and LAL sensitivity, both of

TABLE 5 | Effect of diet on aquaculture LAL reactivity and protein expression vs. reference LAL.

		Feed Trial 3 (Compared to Reference LAL)			
HSC Cohort	**Diet Group**	**LAL Reactivity**	**Factor C**	**Factor B**	**Proclotting Factor**
1	A	1.0X	1.0X	1.0X	0.9X
2	D	1.6X	1.2X	3.6X	1.3X
3	E	0.4X	0.5X	2.4X	0.7X
4	F	2.9X	1.5X	3.8X	2.0X

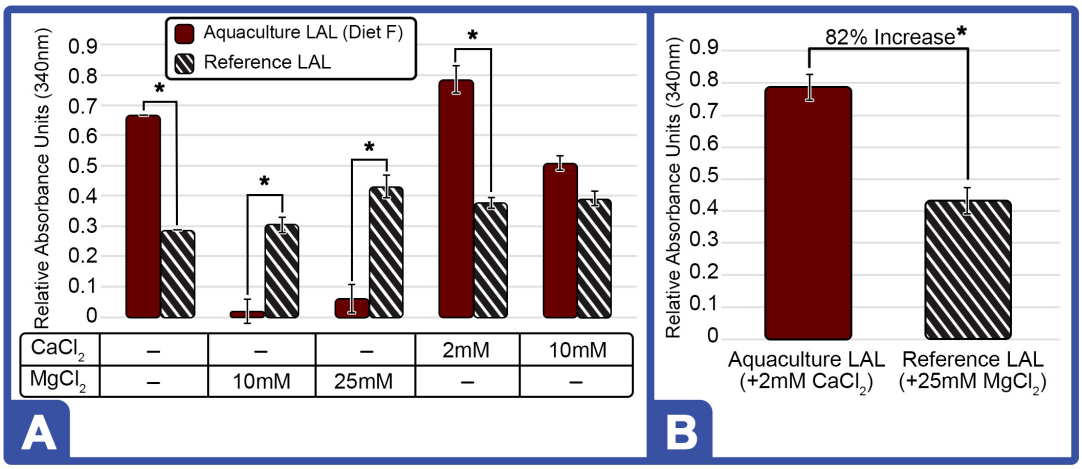

FIGURE 9 | Effect of Different Cations on LAL-LPS Reactivity of Aquaculture and Reference LAL. **(A)** Effect of $MgCl_2$ and $CaCl_2$ addition on LAL (300 μg) from HSCs fed Diet F in Feed Study 3 vs. reference LAL at 5.0 EU/mL of LPS. LAL reactivity to endotoxins evaluated for aquaculture (Solid Bars) and reference (Striped Bars) at 340 nm on microplate reader after 1-h incubation at 37°C **(B)** Maximum LAL reactivity of aquaculture LAL (Diet F; Feed Study 3) vs. reference LAL at 2 mM $CaCl_2$ and 25 mM $MgCl_2$, respectively. Results are represented as mean ± SD between individual LAL assays performed in triplicate. *$p < 0.05$ of aquaculture LAL compared to the reference LAL for the respective cation conditions evaluated.

which play a central role in immunity to defend against pathogens (Nagai et al., 2001).

Conversely, manufactured feeds are typically economical, abundant and shelf-stable; however, they are generally formulated to foster rapid growth rates in lieu of animal vitality, *per se*. As illustrated in this study, LAL reactivity from HSCs given manufactured feed declined over the three 8-week feeding trials. Another recent aquaculture study has also validated the link between diet and adult HSC hemolymph enzyme activity, indicating that serum hemocyanin levels increased at a faster rate with low levels of copper than when higher levels were added to feed formulations (Xu et al., 2020).

The feeding rationale for this study focused on economical, diverse sources that met the nutritional requirements of other aquatic invertebrates with respect to maintenance, digestibility, and utilization (i.e., 15–32% protein and 4–8% lipid composition; Aranyakananda and Lawrence, 1993; Unnikrishnan and Paulraj, 2010). HSC diets were also optimized to ensure that macronutrients (i.e., protein and lipids) and micronutrients (e.g., Vitamins B, C, E, and K, copper) were available for proper hemocyanin production, as such any deficiencies may be exacerbated with hemolymph extraction. All trial diets, with the exception of Diet A (commercially manufactured aquafeed), contained natural probiotics. However, further studies are required to characterize such probiotics in the nutritional blend to understand whether and how each enhances the HSC immune response.

Study feeds were also developed to satisfy required daily protein levels before reaching maximum energy requirements (Joules). The energy maintenance requirement for adult HSCs ranged between 153 and 235 kJ per kg of body weight per day as determined by pre-trial feed studies, which was similar to that of juvenile HSCs (224 kJ) and Pacific White Shrimp (23 kJ for maintenance and up to 345 kJ for maximum growth) (Kuresh and Davis, 2000; Lupatsch et al., 2008; Tzafrir-Prag et al., 2010). For comparison, adult humans require 135–195 kJ/kg/day and fish, 17–75 kJ/kg/day (Wilson and Halver, 1986; National Research Council, 1993; United Nations University, and World Health Organization, 2004; National Research Council, 2011). The protein levels in the hemolymph did not drop below the protein reference interval (3.4–11.7 mg/mL) over the feeding trial (or to date) and, all of the animals remained healthy (100% survival),

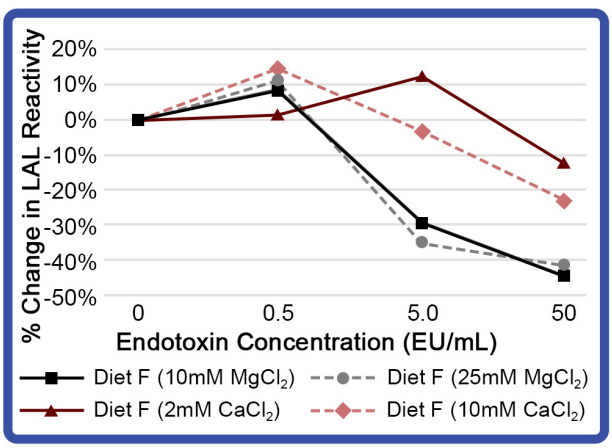

FIGURE 10 | Effect of different cations on reactivity of aquaculture LAL at different endotoxin concentrations. LAL extract (300 μg) derived from HSCs fed Diet F in Feed Study 3 analyzed to determine effect of $MgCl_2$ (10 and 25 mM) and $CaCl_2$ (2 and 10 mM). LAL was incubated with a range of endotoxin concentrations (0 – 50 EU/mL) for 1 h at 37°C. Background absorbance was subtracted from each at 0 EU/mL, as addition of $CaCl_2$ and $MgCl_2$ resulted in minor shifts in absorption. Percent change in LAL reactivity shown vs. Diet F LAL free of divalent cations.

suggesting that HSCs can be maintained in long-term captivity without risk of panhypoproteinemia using these diet formulations.

In the presence of high LPS concentrations (50 EU/mL, saturation levels), aquaculture LAL titrations displayed greater reactivity than the reference LAL (**Figures 2–5** and **Table 2**). LAL derived from HSCs fed Diets A and E in Feed Study 1 also had higher levels of clotting factors per μg than the reference LAL and revealed 2X greater clot formation at the same total protein concentrations after 1-h of incubation. Thus, the LAL coagulation cascade appeared to demonstrate a zero-order enzyme kinetic reaction in which rates were constant; such that, at a saturation substrate level, the reaction was directly proportional to the amount of enzyme present. This finding suggests that the aquaculture LAL had greater LPS-reactivity at higher endotoxin concentrations due to higher amounts of one or more of the clotting factors than in the reference.

Further, the lag in coagulin formation from LAL derived from the Diet A and B cohorts may have been caused by the slow disassociation of an enzyme-bound inhibitor upon dilution of the clotting enzymes in the assay. However, the lower reactivity in the reference LAL was not consistent with the presence of an inhibitor, as the reaction rate at lower LPS concentrations (e.g., 0.5 EU/mL) was similar to that of the aquaculture LAL (**Figure 2A**).

Notably, the HSC cohort fed Diet F showed the greatest level of LAL reactivity and concentration of clotting factors among all feed groups in Feed Study 3 (**Figures 4**, **6–9**). Results in **Figures 3**, **4** indicate lower levels of one or more of the clotting enzymes, Factor C, Factor B, or the proclotting enzymes in reference LAL compared to aquaculture LAL; whereby, clot formation (product) was weaker after a 1-h LPS saturation-level incubation (50 EU/mL). Western blot analysis also yielded lower clotting factors in the reference LAL than in the aquaculture LAL (Diet F).

In Feed Study 3, low LAL response also corresponded to low hemocyanin levels (**Figures 4A,D**), yet the highest LAL response in Feed Study 1 was observed in the HSC cohort fed Diet E with the lowest hemocyanin levels. Across all feed cohorts, amebocyte density remained consistent irrespective of changes in LAL reactivity. These results suggest that LAL reactivity may not be linked to hemocyanin levels or amebocyte density; rather, it appeared to be connected to diet, such that healthier amebocytes produced higher concentrations of immunologically essential protein factors. The variance between the 6 HSCs within the same tank was low; given that the amebocyte levels stayed consistent within each cohort between each feed trial, this health parameter served as a consistent internal control in the experimental design. Thus, variations in LAL between trials appear to be significant and did not arise from random environmental factors.

A gradual decline in LAL response (**Table 4**) was also observed in HSCs that were provided the same initial feed source across all three 8-week studies (**Table 1**; Cohort 1 – Diet A and Cohort 3 – Diet E). Whereas, the increase in diversity of natural sources between Feed Trials 1 through 3 (**Tables 1**, **2**; HSC Cohort 2) corresponded to a consistent increase in LAL reactivity [Diet B (2 sources): 1.1X < Diet C (6 sources): 1.4X < Diet D (12 sources): 1.6X] over the 6-month period. Without optimization, long term rearing on a fixed feed source may not deliver the complexity or essential nutrients needed to maintain animal vigor, as frequently observed in captive animals (Worthy, 2001).

While the natural feed diets and Diet F contained fewer calories (**Table 2**; gross energy) and higher moisture content than the manufactured commercial feed, they resulted in higher quality LAL (as measured by LPS-reactivity and protein concentration). While Diet E was higher in caloric levels than natural feeds (Diet B–D) and Diet F but had a similar moisture content, hemocyanin levels and LAL reactivity from that cohort declined over the course of multiple feed trials. These findings suggest that nutritional complexity, and the bioavailability of the feed ingredients, may be key drivers to maintaining and safeguarding HSC wellbeing, rather than total caloric density, at least until the nutritional requirements of the HSC have been fully elucidated.

Divalent cations play an essential role in stabilizing LPS dimerization via cross-links, which is thought to be required for LPS biological activity. In the presence of cation chelating agents *in vitro*, LPS dimers are liberated into monomers, which have been shown to be unresponsive to LAL (Tsuchiya, 2019). In this study, aquaculture LAL was prepared following standard SOPs; whereby, amebocytes were washed in 0.5 M NaCl prior to lysis in water. The presence of 0.15 M NaCl has been shown to reduce LAL reactivity; however, the addition of divalent cations ($CaCl_2$ or $MgCl_2$) can reverse this inhibition and increase its reactivity (Sullivan and Watson, 1974). In fact, the addition of divalent cations to aquaculture LAL preparations resulted in greater reactivity at low endotoxin levels; but remarkably, a slight decrease was seen with specific cations and concentrations as endotoxin levels increased above 5.0 EU/mL. As expected, the addition of divalent cations also increased the reactivity of the reference LAL preparations, as previously described (Sullivan and Watson, 1974). However, under optimal salt conditions for each lysate, aquaculture LAL nonetheless outperformed the reference LAL (**Figures 2**, **9** mM $CaCl_2$ and 25 mM $MgCl_2$, respectively). It appears that variables in amebocyte lysate preparations can affect the biochemical properties of LAL; therefore, the optimization of LAL extraction methods, buffer formulation, and lyophilization (for shelf-life stability) are planned for future studies to increase LAL sensitivity.

These observations of dietary effects on coagulation and HSC hemolymph clotting factors appear to share similarities with those of other organisms. For example, *Limulus* Factor C, Factor B, and proclotting enzyme suggest functional and evolutionary parallels with vertebrate complement factors and certain Vitamin-K-dependent coagulation factors (e.g., mammalian Factors VII, IXa, and X). The activity of human coagulation Factor VII (a homolog to *Limulus* Factor C) is regulated by macronutrients with respect to total lipid intake (Miller et al., 1998). Vitamin K is required for normal human blood coagulation and tyrosine kinase activation (and phosphorylation), which plays a central role in cell growth and metabolism (Saxena et al., 1997; Vermeer, 2012). In another vitamin-dependent metabolic process associated with coagulation, calcium, cholecalciferol (Vitamin D_3) and retinoic

acid (Vitamin A) control the protein expression of Kallikrein (a homolog to *Limulus* Factor B) in human epidermal keratinocytes (Morizane et al., 2010). Similarly, enhanced human coagulation Factor IXa (a homolog to *Limulus* proclotting enzyme) activity has been demonstrated in the presence of calcium and magnesium (Sekiya et al., 1996; Agah and Bajaj, 2009).

In this study, diet was specifically shown to influence the biochemical properties, concentration, and reactivity of LAL clotting factors. The relative abundance and ratios of Factor C, Factor B, and proclotting enzyme varied across diets and corresponded to protein expression of amebocyte clotting factors. Factor B was present at a higher concentration in the aquaculture LAL from all cohorts relative to reference LAL. However, the amount of Factor B did not directly correlate to LAL reactivity and may not be a rate-limiting factor in the HSC coagulation cascade. Whereas, higher Factor C and proclotting enzyme protein levels correlated to increased LAL reactivity. The LAL derived from HSCs fed Diet E demonstrated the least response and contained the lowest levels of Factor C and proclotting enzyme; thus, suggesting that these clotting factors may be rate-limiting component(s) of the LAL cascade and therefore determinants of the overall LAL reaction.

Diet also appeared to affect proclotting enzymes in the aquaculture HSC amebocytes. While a range of sizes (38, 54, 60, and >150 kDa) and amino acid compositions of the proclotting enzymes from North American (*L. polyphemus*) and Asian (*T. gigas, T. tridentatus,* and *C. rotundicauda*) HSCs have been described (Roth and Levin, 1992), variations of the proclotting factors were detected among the different feed groups. Neither glycosylation nor phosphorylation were responsible for the observed differences (**Figure 9**). However, an evolutionary basis for clotting enzyme size variations between different HSC species might explain why the beta-glucan-sensitive Factor G pathway is active in some HSC species and not others.

When Factor C is bound to the amebocyte membrane and activated by LPS *in vivo*, it can trigger a G-protein-coupled receptor (GPCR) signal transduction cascade, leading to the degranulation of the amebocyte and the release of defense molecules, including the zymogens of the coagulation cascade (Ariki et al., 2008). Most GPCR and receptor-mediated signaling pathways are regulated by phosphorylation (Lefkowitz and Whalen, 2004). For example, in HSCs the phosphorylation of both visual arrestin and its respective receptor (via a calcium/calmodulin-dependent protein kinase) plays a critical role in uncoupling membrane receptors from triggering GPCR-dependent cascades (Calman et al., 1996).

To date, a possible role of phosphorylation of Factor C-mediated amebocyte exocytosis has not been fully characterized, but this work has now shown that Factor C is a target of phosphorylation (**Figure 5B**). As illustrated in **Figure 6**, the L-chain of Factor C in the aquaculture LAL migrated at a higher molecular weight in SDS-Tris glycine gels than the Factor C (L-chain) in the reference LAL. The difference in migration was attributed to the phosphorylation of Factor C in aquaculture LAL (**Figure 6D**). In addition, AP treatment of the aquaculture-derived LAL did not affect the mobility of the B-chain subunit of Factor C, suggesting that the phosphorylation site(s) in the L-chain of Factor C is located in the A-chain subunit (**Figures 6**, **7**). Notably, LAL protein phosphorylation modification of Factor C was present in all aquaculture HSC cohorts regardless of feed inputs, but not in the reference LAL. Factor C in LAL sourced directly from the wild ("wild type") HSCs and collected within 1 h of habitat removal was also shown to be phosphorylated (data not shown), suggesting that this modification is not a consequence of indoor aquaculture or experimentally diets, *per se*. The observation that Factor C was not phosphorylated in the reference lysate would require additional information with respect to product preparation and possible additives to draw further conclusions. Future analysis is directed toward complete characterization of Factor C phosphorylation observed in the aquaculture LAL.

The role of glycosylation in protein folding, stability, reactivity, and macromolecular interactions has been well documented (Medzihradszky, 2005; Kosloski et al., 2009), including increased sensitivity to endotoxins with glycosylation of recombinant Factor C (Mizumura et al., 2017). As expected, Factor C in both reference and aquaculture LAL was glycosylated in the H-chain region (**Figures 5B**, **6C**). Since the activated Factor C B-chain was not found to be glycosylated in the reference LAL, Factor C L-chain glycosylation may occur in the A-chain subunit. The B-chain of intact Factor C has two predicted glycosylation sites (**Figure 5A**), but whether glycosylation modifications remain on the B-chain after Factor C activation has not been reported.

Remarkably, PNGase treatment of the Factor C L-chain caused an upward shift (not the expected downward shift) in mobility of this subunit derived from the reference LAL but not in the aquaculture LAL (**Figure 6C**). Post-translational modifications like phosphorylation or glycosylation of proteins typically change protein hydrophobicity and result in a slower migration. In some circumstances, highly glycosylated proteins that retain globularity (less frictional drag) have migrated faster; whereby, the glycosylation branches cannot be linearized after denaturation (Rath et al., 2009; Schwarz and Aebi, 2011). This may be the case for the Factor C L-chain in the reference LAL in this study (**Figure 6C**).

It further appears that Factor C can be modified by either glycosylation or phosphorylation and that it most likely occurs in the A-chain region of the L-chain, since these post-translational modifications were not detected in the B-chain subunit of activated Factor C (i.e., the A-chain was not detectable by Western blot analysis). Conversely, these modifications could instead reside in the B-chain and be removed after Factor C activation and L-chain conversion to the A- and B-chain forms (**Figures 5**, **6**). Future studies will be directed at describing the observed modifications of the Factor C protein.

The differences in the type of post-translational modification of Factor C (e.g., glycosylation and phosphorylation) in aquaculture versus reference LAL may also be directly related to those seen in clotting activity between the preparations (**Table 2**). As the experimental feeds are high in protein and lipids but low in carbohydrates (**Table 1**), it would follow that the quantity and quality of carbohydrates in the diets could lead to lower blood glucose levels. Whereas, diets low in carbohydrates have

been shown to reduce *in vivo* (non-enzymatic) protein glycation (Guilbaud et al., 2016). The significance of glycosylation and phosphorylation of Factor C in aquaculture-derived LAL and the relationship to the diet of the HSC nonetheless remains to be elucidated.

While Factor B was found to be a glycoprotein in both aquaculture and reference LAL, glycosylation was detected only in the double-chain form of Factor B in the H-chain and not in the intact single-chain form of Factor B (**Figures 5B**, **7**). Whether glycosylation of Factor B triggers the Factor C dependent nicking of the full-length single-chain form into the double-chain form of Factor B, or whether glycosylation is downstream of the nicking process that generates the double-chain form remains to be determined (**Figure 5B**). The presence of the double-chain form of Factor B typically found in standard LAL preparations is thought to be a result of trace contamination of Factor C generated during the purification process (Nakamura et al., 1986).

CONCLUSION

We consider this work to be important in the HSC global context and the potential role of aquaculture for the future of species viability. That is, given the valuable resources derived from these arthropods and the potential for long-term aquaculture solutions, a meticulous assessment of alternative feed strategies with respect to the quality of the resources for which they have been selected is both rational and necessary. This study demonstrated that protein-dense amebocytes, optimal hemocyanin levels, and high-quality LAL derived from HSCs in aquaculture can be produced with functional nutrient-rich feed. Aquaculture LAL contained higher amounts of clotting factors per μg of total protein compared to reference LAL preparations and could thus yield higher reactivity and therefore more tests per unit of harvested hemolymph. HSC sustentation and reproductive activities also continued during captivity, such that all aspects of natural behavior were observed without apparent stress or decline in wellbeing.

This study highlights the use of amebocyte protein components as novel biomarkers to objectively measure HSC nutritional status with respect to the intake of dietary constituents for usefulness in future studies. The data show that the expression levels and chemical modifications of such biomarkers are directly affected by feed inputs and therefore may provide a new tool to evaluate dietary patterns, especially with HSCs and possibly other species. We found that LAL reactivity to an endotoxin standard was similar in the indoor HSCs compared to LAL sourced directly from wild-type HSC collected within 1 h of habitat removal. However, LAL prepared from husbanded HSCs had higher reactivity compared to commercially available LAL preparations (reference) that are also sourced from wild type HSCs. Additional studies would be needed to identify and assess possible variations in reference LAL preparation compared to these methods that do not involve lyophilization. In addition, batch-to-batch and seasonal variations in commercial LAL prepared from wild harvested HSC populations have been shown to differ in their sensitivity to LPS (Lindberg et al., 1972), although these findings may not reflect differences in sensitivity to LPS *per se* but reactivity related to the potency of LAL. Controlled-environmental aquaculture using diverse feedstocks may alleviate these LAL variations and result in more reproducibility and batch-to-batch product consistencies while producing more robust LAL reactivity.

Ultimately, this work has shown that nutrition imparts an effect on the LAL derived from an aquaculture cohort. The research further suggests that highly reactive LAL can be reproducibly prepared from aquaculture HSCs on a year-round basis if a properly defined and economical dietary regimen is established. As such, aquaculture and an enriched feeding strategy for a finite cohort of HSCs representing a fraction of the current annual wild catch could satisfy the global demand for LAL biomedical testing over the long term. Thus, enduring HSC aquaculture and the resulting raw material quality represent a promising, sustainable new supply paradigm for the biomedical industry.

In turn, HSC husbandry also promises unparalleled conservation advantages for dwindling wild populations and dependent species, including myriad migrating shorebirds.

With respect to future research, the elemental HSC immune system based on a single circulating cell also provides an elegant model to investigate the effects of nutrition on immunity and vigor at an ingredient and cellular level. Such studies will center on further examination of the relationship between nutrition and production and modification of specific, bioactive LAL proteins. Notably, these strategies to ensure wellbeing in captivity could also be applied in other sectors, from agriculture stocks to companion animals and zoos, to human medicine, etc.

Following the submission of this manuscript and during the peer-review process, it was noteworthy that the United States Pharmacopeia (USP) ruled to retain LAL as the industry BET amid new demands for drug and vaccine development and manufacturing emerging from the novel coronavirus pandemic, suggesting that LAL will remain the standard for the foreseeable future (United States Pharmacopeia, 2020a,b).

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

RT-K, AD, TB, MG, and KD conceived the project. RT-K, AD, TB, and KD developed the research objectives and experimental plan. RT-K, KD, CK, and TB verified the materials and methods. RT-K, AD, LR, and KD conducted the research. KD and AD supervised the work. All the authors prepared analysis of the primary research and contributed to the final writing of the manuscript. JB and SA provided the research support for the nutritional effects on performance and physiology and alternative feedstuff development for sustainable production and safety. MG performed the editing of the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2020.541604/full#supplementary-material

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Extraction of People's Perception Toward Horseshoe Crab Existence in Northeast Coast of India

Siddhartha Pati[1,2], Salwa Shahimi[1,3], Hisham Atan Edinur[4]*, Bryan Raveen Nelson[1,2]*, Diptikanta Acharya[5] and Bisnu Prasad Dash[6]*

[1] Institute of Tropical Biodiversity and Sustainable Development, University Malaysia Terengganu, Kuala Nerus, Malaysia, [2] Research Division, Association for Biodiversity Conservation and Research (ABC), Balasore, India, [3] School of Marine and Environmental Sciences, Universiti Malaysia Terengganu, Kuala Nerus, Malaysia, [4] Forensic Science Programme, School of Health Sciences, Universiti Sains Malaysia, Kota Bharu, Malaysia, [5] Department of Biotechnology, GIET University, Gunupur, India, [6] Department of Biosciences and Biotechnology, Fakir Mohan University, Balasore, India

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***Correspondence:**
Bryan Raveen Nelson
bryan.nelson@umt.edu.my
Hisham Atan Edinur
edinur@usm.my
Siddhartha Pati
patisiddhartha@gmail.com

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Understanding local community attitudes toward wildlife and their environment is critical for making sensitive conservation planning and management decisions particularly for conservation-neglected species like *Tachypleus gigas*. A questionnaire-based interview was carried out on 388 local households from 12 different villages in Balasore, Odisha, between September 2018 and February 2019, which uses a theoretical mapping on attitudes toward horseshoe crabs. We found that 53% of interviewees accepted the presence of horseshoe crabs in their area, 27% have oppressive attitudes, and the remaining 20% were having mixed feelings. Most respondents (>60%) considered horseshoe crabs to bring tangible benefits such as esthetic, monetary, and cultural significance. However, a handful of respondents expressed oppressive attitudes because horseshoe crabs damage their fishing nets (<20%). Both principal component and stepwise analyses revealed that age, gender, and education were demographic components that closely relate local community perceptions toward the conservation-neglected horseshoe crabs that remain threatened by by-catch. We encourage socioeconomic monitoring particularly during rapid economic and infrastructure development to minimize knowledge erosion and to appreciate local ecological knowledge for better conservation management in India.

Keywords: community, threats, estuary, relationship, fisheries, management

INTRODUCTION

Expansions of human populations in coastal areas result in exploitation (Brash, 1987; Newmark et al., 1993; Walters, 2004) and the reduction of species (Gregory, 2005; Ndenecho, 2009) as precursors to the sixth extinction era (Ceballos et al., 2015; Corlett, 2015). Humans have overlooked the context of biological diversity. Biotic assemblages are present to serve a certain function in the ecosystem (Manfredo, 2008; Rands et al., 2010; Nelson et al., 2018, 2020; Jaffar et al., 2019). Yet humans portray themselves as stakeholders with commensalism attitudes toward resources and their interaction with wildlife in their area (Nepal, 2002; Braga and Schiavetti, 2013;

Fakhrul-Hatta et al., 2018; Khalib et al., 2018; Liao et al., 2019). While planning for development, authorities often proposed actions based on top-down decision that may disregard wildlife and communities who inhabit a certain area of interest (Sutherland et al., 2004; Segan et al., 2011). We propose an "otherwise" approach where the community governs and manage their habitat. In this sense, opinions of *Odia* community were bottom-up suggestions that call for transparent management decisions, particularly for resource exploitation and human–wildlife co-habitations.

India is a South Asian country with large land plains, and 68% of its boundaries are surrounded by seas that support some 1.38 billion humans that unleash anthropic stress to wildlife and surrounding environments, particularly the water bodies (Schipper et al., 2008; Sodhi et al., 2010). We perceived the local communities are engaging themselves in experiential learning from the environment because their livelihood and well-being relate with resource governance in the area. Hence, the gathering of opinions related to non-keystone species produces genuine opinions from the community particularly, when they are sharing resources with this animal. Therefore, we introduce conservation-neglected horseshoe crabs like *Tachypleus gigas* and *Carcinoscorpius rotundicauda* as our tool because they are available in India and also throughout Southeast Asia (John et al., 2018; Nong et al., 2020).

Horseshoe crab species currently under conservation purview are *Limulus polyphemus* and *Tachypleus tridentatus* because their respective statuses in the wild were recently changed from "data deficient" to "vulnerable" and "endangered" in International Union for Conservation of Nature (IUCN) Red List (Smith et al., 2018; Laurie et al., 2019). This means that *T. gigas* and *C. rotundicauda* statuses in the wild are "data deficient" and yet to receive recommendations for updating. Separately, horseshoe crabs spend their entire life cycle (spawning grounds, embryogenesis, nursery grounds, and feeding grounds) interacting with humans (Smith et al., 2009). Therefore, India has placed horseshoe crabs under Wildlife Protection Act 1972 and Fisheries Act 1897 protection. Further, fishery pressure is relieved during the peak spawning season of horseshoe crabs (between May and September), which falls on no-fishing season (April–July) in India (Pati et al., 2015; Pati, 2017; John et al., 2018). Moreover, the Association for Biodiversity Conservation and Research, a non-governmental organization, has implemented projects related to beach cleaning (Mohanty, 2017b; Kundu, 2018), culture and tradition (Mohanty, 2017a), awareness (Pati and Dash, 2016; Pati et al., 2017; Reuters,, 2020), horseshoe crab rescue (Senapati, 2018; Pati, 2019a,b; ENS,, 2020), celebrations (Mohanty, 2020), and art (Patsani, 2018).

However, all efforts are inadequate if knowledge raised from community engagements is not documented and made available to the public. Hence, with an objective to document community perceptions toward horseshoe crabs after a series of conservation-leadership programs, we design our instrument (questionnaire) using layman's terms so that community members can freely express their opinions without a scientific stigma. The *Odia* community opinions and views are respected by maintaining a non-disclosure approach while welcoming rural folks into horseshoe crab conservation-based programs in Balasore (Odisha), northeast India. Overall, this community perception survey enables us to understand community values and their attitudes toward horseshoe crabs in India.

METHODOLOGY

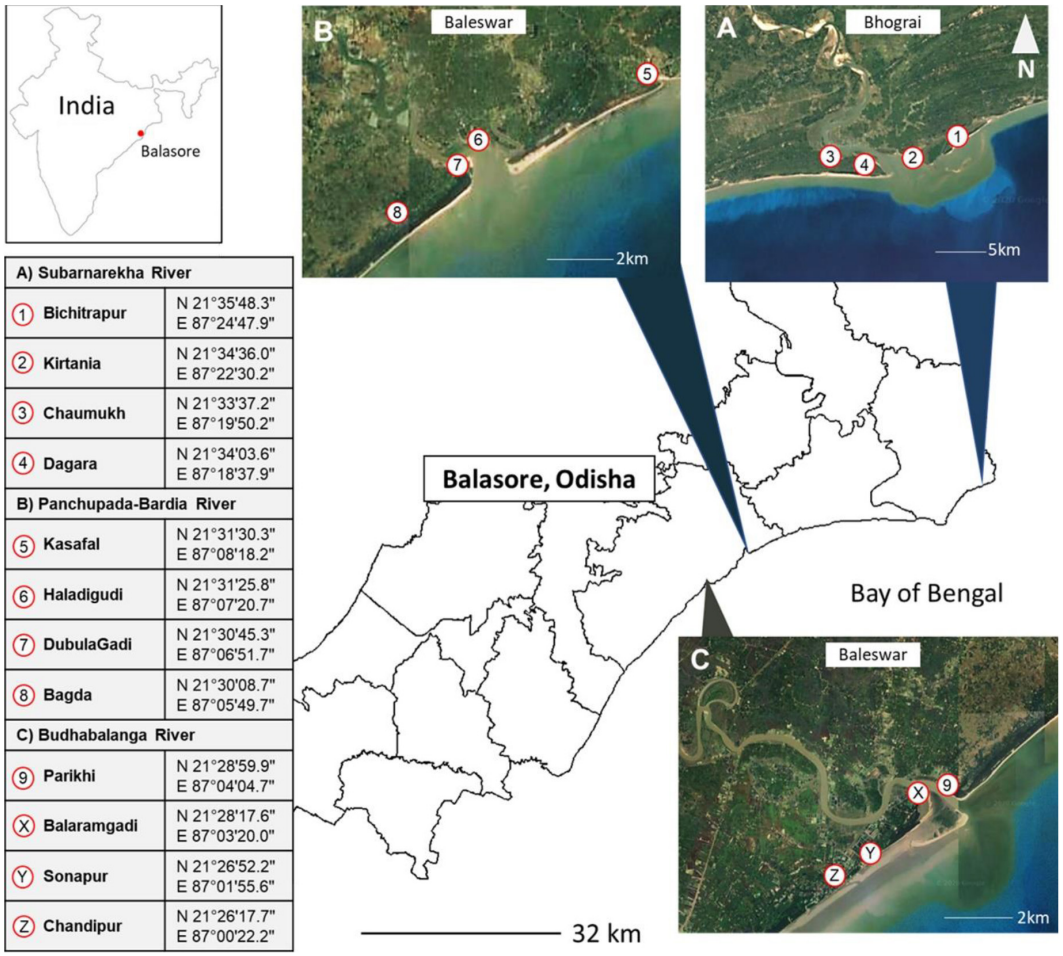
Experimental Design

The present study involved the participation of coastal communities in Balasore (Odisha, India) within an 88-km^2 (N 21°30′; E 86°56′) intersection of the Subarnarekha, Panchupada-Bardia, and Budhabalanga estuaries (**Figure 1**). Since both rivers extend into northeast Bay of Bengal (the most productive waters in northeast India), capture fisheries and horseshoe crab (*Tachypleus gigas*) by-catch are human–wildlife interactions of the *Odia* community. While coastal communities in Baleswar and Bhograi areas (Balasore, **Figure 1**) are risking their capture fishery efforts with unpredictable weather and stormy waters of the Indian Ocean, we also learned that horseshoe crabs cause fisher net damage and income loss. This is challenging because *T. gigas* is placed in Schedule IV of Wildlife Protection Act 1972, and deleterious acts toward the listed wildlife (in either schedules) are punishable with fines (between USD 16 and USD 254) that are unaffordable by these communities. However, out of the six schedules, Schedules I and II provide absolute protection, and offenses under these are prescribed with the highest penalties. The horseshoe crab *T. gigas* is listed in Schedule IV of Wildlife Protection Act 1972, but penalties for violations are minimal (from USD 16 to USD 50). For effective conservation to stabilize horseshoe crab population structure in the wild, absolute protection is needed, and it is imperative that the current Wildlife Protection Act be updated.

Here, we partake discussions with the community before designing our questionnaire. All questions are tested using 50 randomly selected members from the public before every set of questionnaires is validated using Cronbach α test (SPSS v.16, IBM, United States). We adopted the procedures of Creswell and Tashakkori (2007) to amend the questionnaire until Cronbach α test score >0.70 was achieved. A pre-interview (during 16 December 2018) is carried out on another 50 random members from the public to finalize the contents of our questionnaire (Cronbach α = 0.87). With this, our questionnaire covers sociodemographic information, local beliefs on the animal, local knowledge of horseshoe crab, and also their perceptions, attitudes, and opinions toward the horseshoe crab.

Data Collection

We identified a total of 500 households from the Subarnarekha (Bichitrapur, Kirtania, Dagra, and Chaumukha), Panchupada-Bardia (Kasafal, Haladigudi, Dubulagadi, and Bagda), and Budhabalanga (Parikhi, Balaramgadi, Sonapur, and Chandipur) river estuaries using random respondent selection and also "snowball sampling" for their interaction with aquatic species (cf. Newing et al., 2011; **Figure 1**). We further limited the respondents by their age (between ages 18 and 65), and only one representative is allowed per household (assures responses are independent).



A) Subarnarekha River	
① Bichitrapur	N 21°35'48.3" E 87°24'47.9"
② Kirtania	N 21°34'36.0" E 87°22'30.2"
③ Chaumukh	N 21°33'37.2" E 87°19'50.2"
④ Dagara	N 21°34'03.6" E 87°18'37.9"
B) Panchupada-Bardia River	
⑤ Kasafal	N 21°31'30.3" E 87°08'18.2"
⑥ Haladigudi	N 21°31'25.8" E 87°07'20.7"
⑦ DubulaGadi	N 21°30'45.3" E 87°06'51.7"
⑧ Bagda	N 21°30'08.7" E 87°05'49.7"
C) Budhabalanga River	
⑨ Parikhi	N 21°28'59.9" E 87°04'04.7"
Ⓧ Balaramgadi	N 21°28'17.6" E 87°03'20.0"
Ⓨ Sonapur	N 21°26'52.2" E 87°01'55.6"
Ⓩ Chandipur	N 21°26'17.7" E 87°00'22.2"

FIGURE 1 | Location of the study site, Balasore in Odisha (East India). This study site indicates the existence of horseshoe crab populations, regardless of *Tachypleus gigas* or *Carcinoscorpius rotundicauda* in the area.

Thus, our final sample size is 388 respondents who fulfilled all the desired criteria while also answering most of the questions (>80%). Every respondent needed approximately 1 h both for the face-to-face interview session (verbal consent, identity non-disclosure, and eligibility) in *Odia* language and to complete the questionnaire with Likert scale (5 = strongly agree, 4 = agree, 3 = neutral, 2 = disagree, and 1 = strongly disagree) and open-ended questions. Our data collection was supported by two volunteers from the Association of Biodiversity Conservation and Research because our intension is to understand coastal community perception on wildlife (marine animal) threats, actions to conserve them, and the willingness to participate in conservation programs (horseshoe crab volunteerism in annual *Raksha Bandhan*, art contests, participatory horseshoe crab research, beach cleanups, and horseshoe crab tourism) that were held annually in Balasore (Odisha, India).

Data Analysis

We registered all data into both SPSS v.16 (IBM, United States) and also Microsoft Excel as platforms for descriptive and statistical analyses. We develop principal components to analyze the community perceptions using key-word strings. The stepwise analysis that amalgamates Bray–Curtis similarity index and Spearman's correlation is used to organize data into statistical associations that give the highest value for the best relationship. Both the principal component and stepwise analyses were developed in Primer v.6.1 using protocols of Gorley and Clarke (2006).

RESULTS

Data on Local Knowledge

To explore opinions from 388 respondents, we classify them into age groups of 18–24 (Group 1), 25–34 (Group 2), 35–44 (Group 3), 45–54 (Group 4), and 55–64 (Group 5). The second-line arrangement involved education through the separation of respondents into upper primary (UP), middle elementary (ME), and high school/college (HC) groups (**Table 1**). However, we only considered opinions of 350 individuals (90.2% of men and

women) who possess ME (45.1%) or higher (32.0%) education because they correctly identified the shape and form of horseshoe crab (*Tachypleus gigas*) from a series of aquatic life pictures (ghost crab, freshwater and marine rays, horseshoe crab in mating pairs, porcelain crab, and hermit crab) presented to them. Only 252 (69.4%, $P = 0.921$, eigenvalue 5.7, cumulative variation 88.8%) respondents from whom a majority (167 persons) are between 35 and 54 years old (**Figure 2A**) and possess ME education (**Figure 2B**) knew about "*Nilarakta Kankda*" (*T. gigas*) as mentioned in folk stories from their culture. Comparatively, a majority of 173 individuals aged between 35 and 54 years from the 277 (75.4%, $P = 0.927$) respondents who knew *T. gigas* shape and form (particularly women; **Figure 2C**) associated this arthropod with aphrodisiac tonic, traditional medicine, and alchemy preparations. Only a handful ranging from 12 to 39 female respondents with a majority of them aging 25–54 years were uncertain about *T. gigas* relevance to their culture ($P = 0.898$) and tradition ($P = 0.726$; **Table 1**), although this arthropod coexist with other fishery resources in their area.

With 173 male and 92 female respondents acknowledging the year-long availability of *T. gigas* ($P = 0.924$), we learned that horseshoe crab emergence into shallow waters was not influenced by season (rain, summer, or winter, $P = 0.456$, eigenvalue 8.71; cumulative variation 79.9%; **Figure 3**) in this northeast territory unless the fishermen were fishing exclusively for horseshoe crabs. A total of 29 respondents suggest that environmental conditions *vis-à-vis T. gigas* populations are recovering ($P = 0.454$), whereas five male and four female respondents claimed that coastal conditions and horseshoe crab populations remained similar to their first contact with this arthropod ($P = 0.457$). A vast collection of 321 (91.7%, $P = 0.920$, **Figure 3A**) respondents expressed either uncertain or negative views on *T. gigas* population recovery because their opinions closely relate with wild capture fisheries as resource (**Table 1**). On the other hand, although 148 individuals (with ME and HC education; **Figure 3B**) were uncertain about *T. gigas* role in the environment, 77 (32.9%) male and 38 (32.8%) female respondents expressed views on "biological control" for bivalve and small crab populations after highlighting presence of empty bivalve shells and crab remnants after day-time net casting in the lowest tide (1–1.2 m) waters during the summer months (**Figure 3C**). These 115 respondents knew about their environment and were able to express their opinions with scientific logic, although these 35 (UP), 47 (ME), and 33 (HC) individuals were deprived of advanced education.

Tachypleus gigas and the *Odia* Community

We learned that 184 individuals aging between 25 and 54 years (from the total of 350 respondents) who correctly identified horseshoe crabs closely associate their livelihood to *T. gigas* ($P = 0.883$, eigenvalue 5.63; cumulative variation 84.0%; **Figure 4A**; **Table 1**). In their perception, although horseshoe crabs are not fished as alternative protein or exotic food, occasionally, there are middlemen who purchase both male (small) and female (large) *T. gigas*. However, these middlemen would only pick healthy-looking crabs (absence of encrusting/fouling organisms on the carapace or absence of morphological abnormalities), as these men inform (some respondents) of lucrative demands in the north and west territories of India. While come respondents routinely fish from the estuary, they would not purposely collect *T. gigas* and sell (USD 0.50 to USD 0.72) these arthropods to middlemen simply because arrival of middlemen are uncertain. The fishermen do not want to be seen with a large number of horseshoe crabs in their possession and fear heavy fines. Moreover, the crabs are often seen in inundated areas for amplexus, but artisanal fishermen make contact with horseshoe crabs during net casting. These individuals either sell or consume all resources (finfish and shellfish including horseshoe crabs) gathered from their routine activity. With this, a total of 212 respondents ($P = 0.794$) comprising 137 male and 75 female individuals from all age groups (and having UP or ME education; **Figure 4B**) referred to horseshoe crabs as by-catch. On the contrary, 155 male and 72 female respondents ($P = 0.879$) expressed dissatisfaction because entangled horseshoe crabs either damage or make their nets unusable (**Figure 4C** and **Table 1**). Although fishermen do not immediately discard their nets into the sea when horseshoe crab entangling is common, there are incidences where horseshoe crabs are forcefully removed, but only when *T. gigas*' limbs and tail cease to move (inactive or presume dead).

Capture fisheries are carried out by 226 respondents from age groups 3 and 4 (35–54 years old; 62.9–69.9%, $P = 0.941$, eigenvalue 8.49; cumulative variation 88.2%; **Figure 5A**) for their survival, livelihood, and income (**Table 1**). Therefore, horseshoe crabs are indirectly exploited (by-catch) because all respondents (regardless of their education; **Figure 5B**) stressed on nets becoming entangled by individual crabs rather than by *T. gigas* in pairs. The community fears that reduced horseshoe crab capture in pairs relates to reduced finfish and shellfish availability. In their perception, resource depletion may force their community into starvation. On the contrary, 223 respondents ($P = 0.958$) aging between 35 and 54 were a majority of the 388 respondents who collectively agreed (94.3–100% across all age groups; **Figure 5C**) that horseshoe crabs and capture fisheries are threatened by degraded habitat. Though not associated, but collectively, 228 men and 111 women made 75 observations of crows, and 188 witnessed accounts of dogs and 56 counts of pig predation on *T. gigas* ($P = 0.735$), which suggests that horseshoe crabs are at risk of terrestrial animal predation after becoming stranded or when discarded on the beach (**Table 1**).

DISCUSSION

The present study reached out to 117 respondents from Subarnarekha, 119 respondents from Panchupada-Bardia, and 152 respondents from Budhabalanga river estuaries in northeast India where 65% and 35% of the respondents were, respectively, men and women. A vast majority of respondents received ME education (175 persons; 45%) compared with HC (124 persons; 32%) and UP (89 persons; 23%), which means that education was reformed in India to include science, mathematics,

TABLE 1 | Processed data from the instrument (questionnaire) after receiving responses from Odia folks in Balasore.

Criteria	G1	G2	G3	G4	G5	M	F	UP	ME	HC
Demography										
Education	19	75	118	136	40	254	134	89	175	124
+ ID	18	68	106	123	35	234	116	80	155	115
−ID	1	7	12	13	5	20	18	9	20	9
Beliefs										
Cultural significance (yes)	(15) 14	(42) 36	(75) 73	(92) 92	(28) 28	(157) 156	(95) 87	(57) 55	(111) 106	(84) 82
Traditional significance (yes)	(11) 11	(56) 56	(91) 81	(97) 92	(22) 21	(178) 182	(99) 82	(64) 59	(124) 112	(93) 89
Tradition (not sure)	2	1	4	5	2	0	12	3	6	3
Local ecological knowledge										
Availability (throughout the year)	14	55	76	99	27	173	92	67	105	93
Hsc. recovering (yes)	2	6	6	11	4	19	10	10	7	12
HSc. recovering (same)	0	3	5	1	0	5	4	4	1	4
Ecological role (yes)	9	22	37	33	14	77	38	35	47	33
Ecological role (not sure)	7	28	42	57	14	102	46	29	64	55
Relationship										
Economic importance (yes)	6	42	58	84	22	137	75	43	98	71
Causes net damage (yes)	12	41	69	80	25	155	72	57	103	67
Threats										
Hsc. habitat degraded (yes)	18	65	101	122	33	228	111	76	153	110
Predation (crow)	2	16	20	28	9	54	21	19	31	25
Predation (dog)	12	27	58	72	19	121	67	40	81	67
Predation (pig)	2	14	18	16	6	38	18	15	26	15
Hsc. as by-catch (yes)	9	46	63	86	22	147	79	50	98	78

Table headings are abbreviated as G = group, M = male, F = female, UP = upper primary, ME = middle elementary, and HC = high school/college. Abbreviated components listed in criteria include + ID = number of respondents who successfully identified horseshoe crabs, −ID = number of respondents who were not able to identify horseshoe crabs, and Hsc. = horseshoe crab(s). Values in (brackets) for "beliefs" section refer to total number of respondents. Values in other sections refer to number of respondents who successfully identified horseshoe crab from pictures presented to them.

and geography (Suchkov, 1959). Education is relevant to their scientific logic when reasoning on biology–ecology opinions (Gafoor and Narayan, 2012). For instance, respondents from Subarnarekha, Panchupada-Bardia, and Budhabalanga estuaries used the term "biological control" for horseshoe crabs against bivalves and small crabs. It shows that these folks understand prey–predator interactions from their basic science education, and also experience working with predators provides them with local ecological knowledge.

We also learned that horseshoe crabs compete with fisher folk for shellfish, but we did not expect that the *Odia* community ignores the crab's presence even though this arthropod threatens their livelihood. Instead, the by-catch (horseshoe crab) is removed from nets with caution and returned back to the sea. But when opportunity arises, they are sold to the middlemen for extra income. In addition, most of the respondents were within ages 35–54 (254; 65%), which means that these rural folks may possess traditional and cultural knowledge that was passed from one to the next generation. Perhaps, this passed knowledge allowed them to correctly identify mating horseshoe crab pairs (amplexus), size disparity between male (small) and female (large) crabs, and also their species ("*Ram Lakhan*," rounded tail, *Carcinoscorpius rotundicauda*; and "*Nilarakta Kankda*," triangular tail, *Tachypleus gigas*). Rural communities are known to possess traditional knowledge of forest and aquatic resources (Demunshi and Chugh, 2010), and they pioneer on resource bioprospecting as well as shared-benefit practices (Moran, 2000; Torri, 2011; Pati et al., 2020a). We have learned the benefits of horseshoe crab blood lysate for endotoxin recovery through research, but it was commercialization that lures communities into horseshoe crab exploitation (Bolden et al., 2016). Also, confidence in biomedical bioprospecting concerning blood proteins (tachyplesin-*a*, *b*, *1*, and *2*) gained urban community support for horseshoe crab-derived antibiotics (Dash et al., 2017; Mans, 2017), the use of embryonic peri-vitelline fluid to trigger stem cell differentiation into cardiomyocyte (Ghaskadbi et al., 2008; Alam et al., 2015b) and dendritic cells (Chinnari et al., 2015), and horseshoe crab chitosan use for food preserves and wound healing (Alam et al., 2015a; Pati et al., 2018, 2020b).

We learned about perceived (38 persons) and acquired knowledge (350 persons) through this study because relationships concerning *T. gigas* and community (culture), its use for traditional applications (tradition), and local ecological knowledge (role, availability, and recovery of *T. gigas*) were unexplored prior to this study. Tribal traditional knowledge considers arthropods as a dietary and medicinal resource (Jayashankar et al., 2016), whereas coastal communities in India are experts on culture and capture fisheries (Nair et al., 1990; Kumari and Singh, 2020). Unfortunately, past and present research did not focus on *T. gigas* and *C. rotundicauda*. Local ecological knowledge that entails sociology and indigenous practices remain top-down and targeted toward community

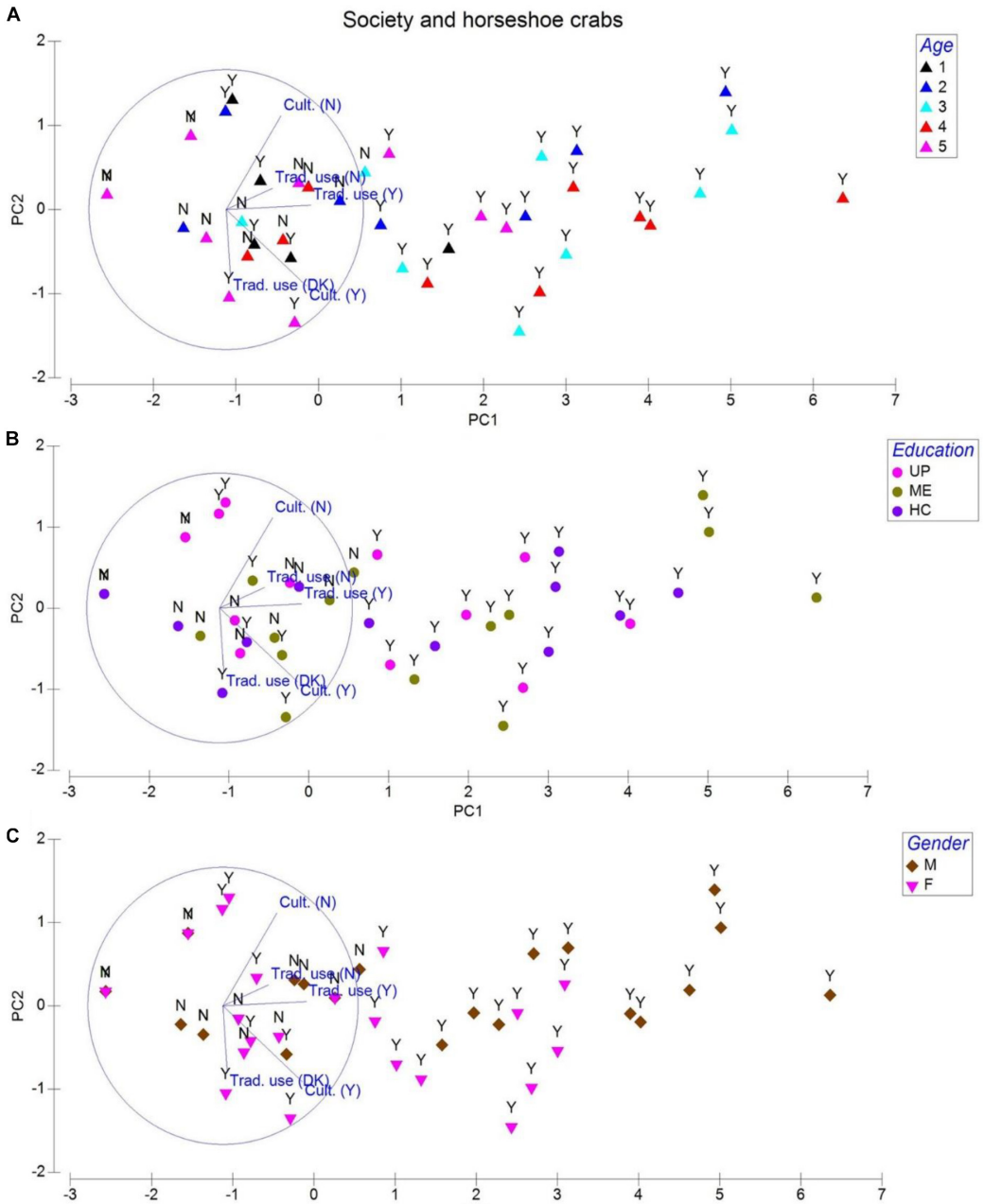


FIGURE 2 | Principal component analysis for society interaction (beliefs) and the horseshoe crab *Tachypleus gigas*. Abbreviations cult. = culture, Trad. use = traditional use, Y = yes, and N = no are components of the questionnaire that are assigned with values like 1 = yes and 0 = no to develop this analysis. Legends represented by age are 1 = 18–24(Group 1), 2 = 25–34 (Group 2), 3 = 35–44 (Group 3), 4 = 45–54 (Group 4), and 5 = 55–64 (Group 5). Education is denoted with UP = upper primary, ME = middle elementary, and HC = high school. Gender is represented by M = male and F = female respondents.

development in India (Davis and Wagner, 2003; Aswani et al., 2018). This means that biodiversity well-being continues to remain neglected. In our approach, community opinions (local economy support and impacts to the community) vis-à-vis expressions on existing threats (resource pool reductions from habitat loss) are relevant with livelihood and ecosystem interactions (cf. Joa et al., 2018) such as social-shared perceptions, values, norms, and also local experiences.

We translate the questionnaire into a conservative mindset for resource pool governance, which also means that there is concern for resource reduction and that the community is attentive toward yield of wildlife. Therefore, our action to replace keystone species like mangroves (*Avicennia* sp. and *Sonneratia* sp.) and mud crab (*Scylla* sp.) with a neglected species like *T. gigas* allowed us to harness the *Odia* community's knowledge about their environment as well as the biology of different

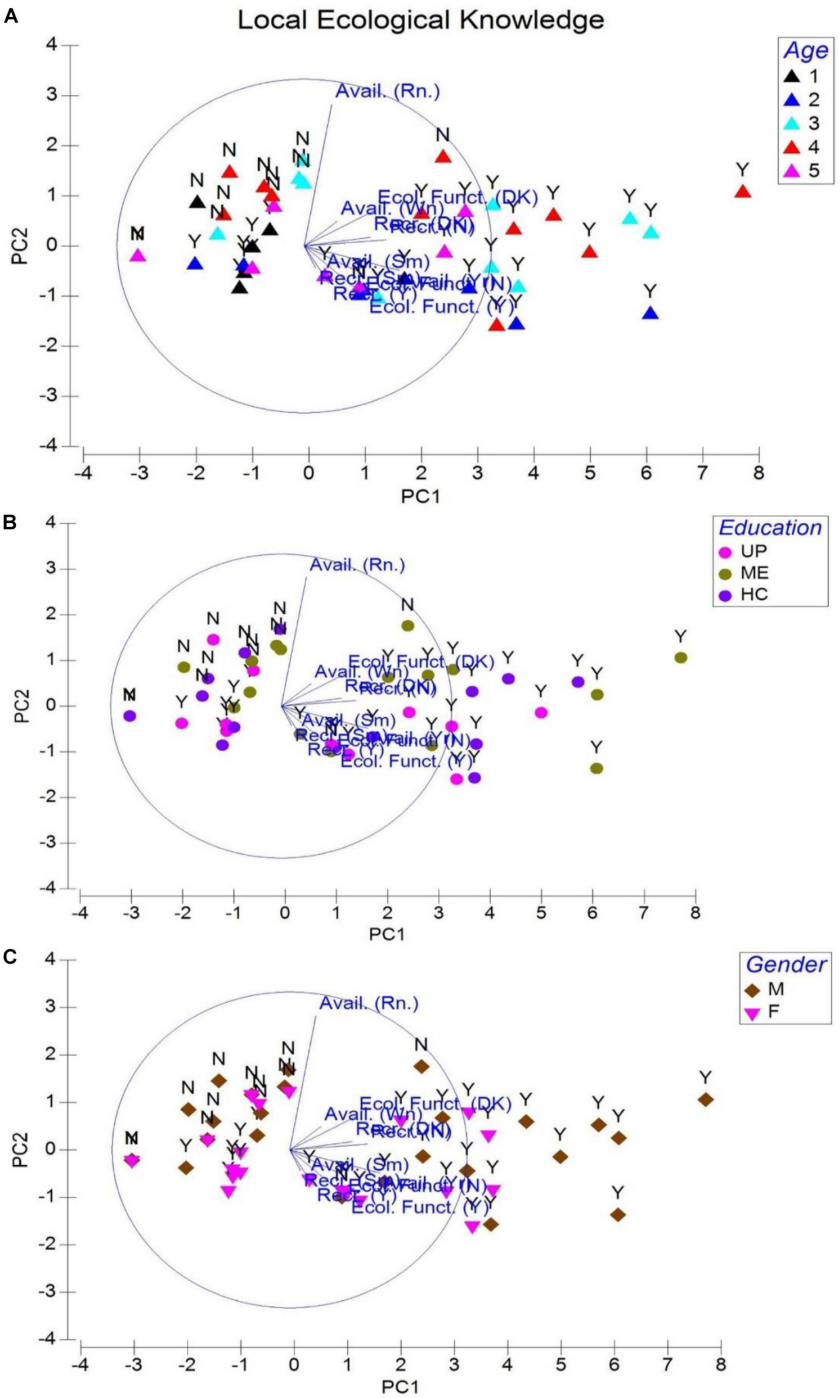


FIGURE 3 | Principal component analysis for local ecological knowledge on *Tachypleus gigas.* Abbreviations are avail. = availability, Ecol. funct. = ecological function, Recr. = recovery, DK = don't know, Y = yes and N = no, Rn. = rainy season, Wn. = winter season, and Sm. = summer season are components of the questionnaire that are assigned with values like 1 = yes and 0 = no to develop this analysis. Legends represented by age are 1 = 18–24 (Group 1), 2 = 25–34 (Group 2), 3 = 35–44 (Group 3), 4 = 45–54 (Group 4), and 5 = 55–64 (Group 5). Education is denoted with UP = upper primary, ME = middle elementary, and HC = high school. Gender is represented by M = male and F = female respondents.

types of aquatic species. For instance, we learned from *Odia* folks that horseshoe crabs are available throughout the year in Subarnarekha, Panchupada-Bardia, and Budhabalanga estuaries. But researchers elsewhere wrongly assumed that horseshoe crabs have seasonal emergence (cf. Moore and Perrin, 2007; John et al., 2012). Compared with researchers, communities

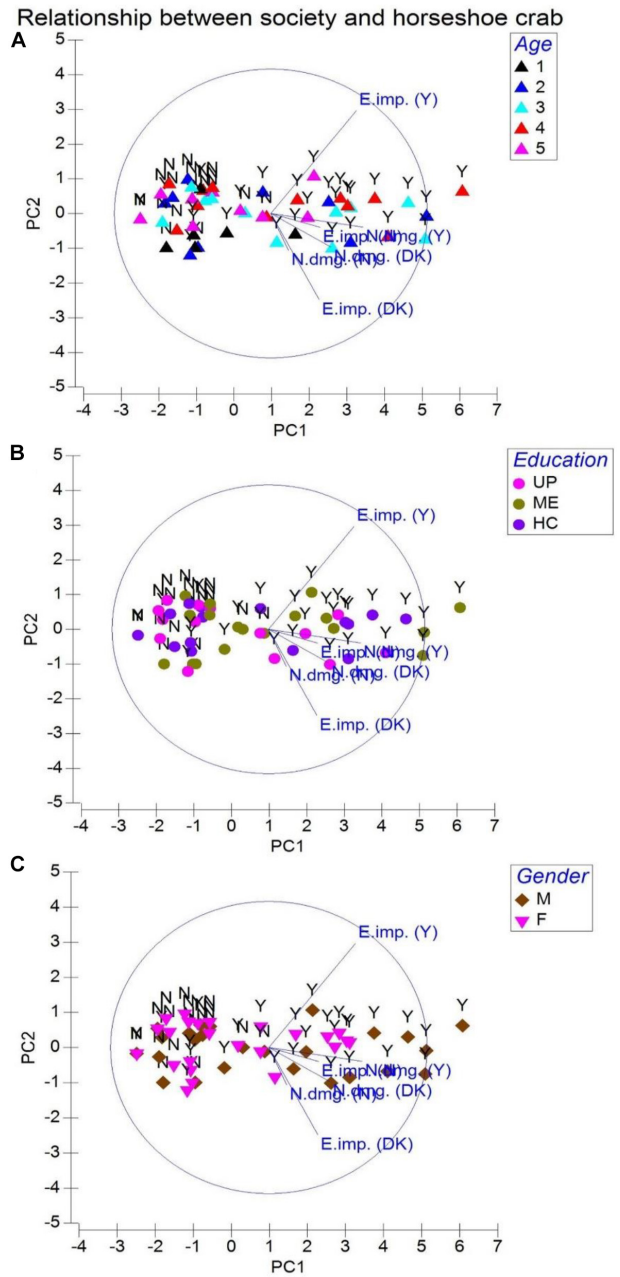


FIGURE 4 | Principal component analysis for society relationships with the horseshoe crab *Tachypleus gigas*. Abbreviations are E. imp. = ecological importance, N. dmg. = net damage, DK = don't know, Y = yes, and N = no. Legends represented by age are 1 = 18–24 (Group 1), 2 = 25–34 (Group 2), 3 = 35–44 (Group 3), 4 = 45–54 (Group 4), and 5 = 55–64 (Group 5). Education is denoted with UP = upper primary, ME = middle elementary, and HC = high school. Gender is represented by M = male and F = female respondents.

reside and constantly utilize their environments. Only after two simultaneous long-term studies were carried out in Malaysia (Nelson et al., 2015, 2016a) and throughout Southeast Asia (John et al., 2018) that it is widely accepted that *T. gigas* are available throughout the year. Similarly, we learned about the impacts of stranding toward horseshoe crab predation (Fraser et al., 2010; Pati and Dash, 2016), but the *Odia* community already witnessed this form of opportunistic predation on *T. gigas* by many other animals like crow, pigs, and dogs; all of these would have remained undocumented if not for this study.

Horseshoe crabs like *T. gigas* and *C. rotundicauda* do not receive conservation attention (ca. Smith, 1993; Sekhar, 2003; Tsetan and Ramanibai, 2011; Behera et al., 2014; Gupta et al., 2014) when compared with the Bengal tiger (*Panthera tigris tigris*), South Asian river dolphin (*Platanista gangetica*), Indian flapshell turtle (*Lissemys punctata*), or golden mahseer (*Tor putitora*), although the horseshoe crabs are listed in Wildlife Protection Act 1972 in India (John et al., 2018). These arthropods are less received for conservation projects in Indonesia. A similar (to the present) type of study was carried out on 277 respondents, but differently, the findings show that *T. gigas* is impacted by fishing gear (gill nets and bottom trawl), its habitat is impoverished by rubbish dumping, and the crab is consumed by locals in 62 districts of Java, Sumatra, Kalimantan, and Sulawesi Island (Meilana and Fang, 2020). With this, the Indian horseshoe crabs remain excluded from IUCN Red List updating simply because information on their population size and spatial occupancy by country and by region remains incompletely explored. For instance, studies in Thailand, Cambodia, and Vietnam relate horseshoe crabs to tetrodotoxin poisoning, but these studies do not map their availability and population size (John et al., 2018).

Although the coastal community of Balasore exploits finfish and shellfish through capture fisheries, they are governed by rules and regulations of Fisheries Act 1897. A seasonal fishing ban regulated by Fisheries Act 1897 was imposed for 45 days since 1998 in fishing zones (0–12 nautical miles) of maritime states (east and west coasts). This ban was revised to 60 days (15 April to 14 June onward year 2015) for resource recovery (Narayanakumar et al., 2017). Unfortunately, some respondents assert that horseshoe crab populations are not reducing since the last decade. Perhaps, these folks organize deep sea fishing where chances to entrap *T. gigas* in their nets are low. Comparatively, vast majority of *Odia* folks from Subarnarekha, Panchupada-Bardia, and Budhabalanga estuaries (Balasore) are near shore fishers. They witness unusual reductions in mating horseshoe crab pairs becoming entangled in their casted nets particularly when these folks compare the yields during their early fishing experiences. Researchers would rely on sampling time and project money to monitor horseshoe crab populations (Pati et al., 2015, 2017), and the outcome would be similar to ecological knowledge extractions from the *Odia* community.

While horseshoe crab spawning grounds are situated in shallow surf protected areas (estuaries or shallow seafront), these grounds are easily accessible, attractive for human encroachments (Berkson et al., 2009; Faurby et al., 2010; Lee and Morton, 2016; Nelson et al., 2019; Zauki et al., 2019a), and therefore, would provide substantial evidence on the declining population size of Asian horseshoe crabs (Nelson et al., 2016b; Pati et al., 2017; Fairuz-Fozi et al., 2018; John et al., 2018;

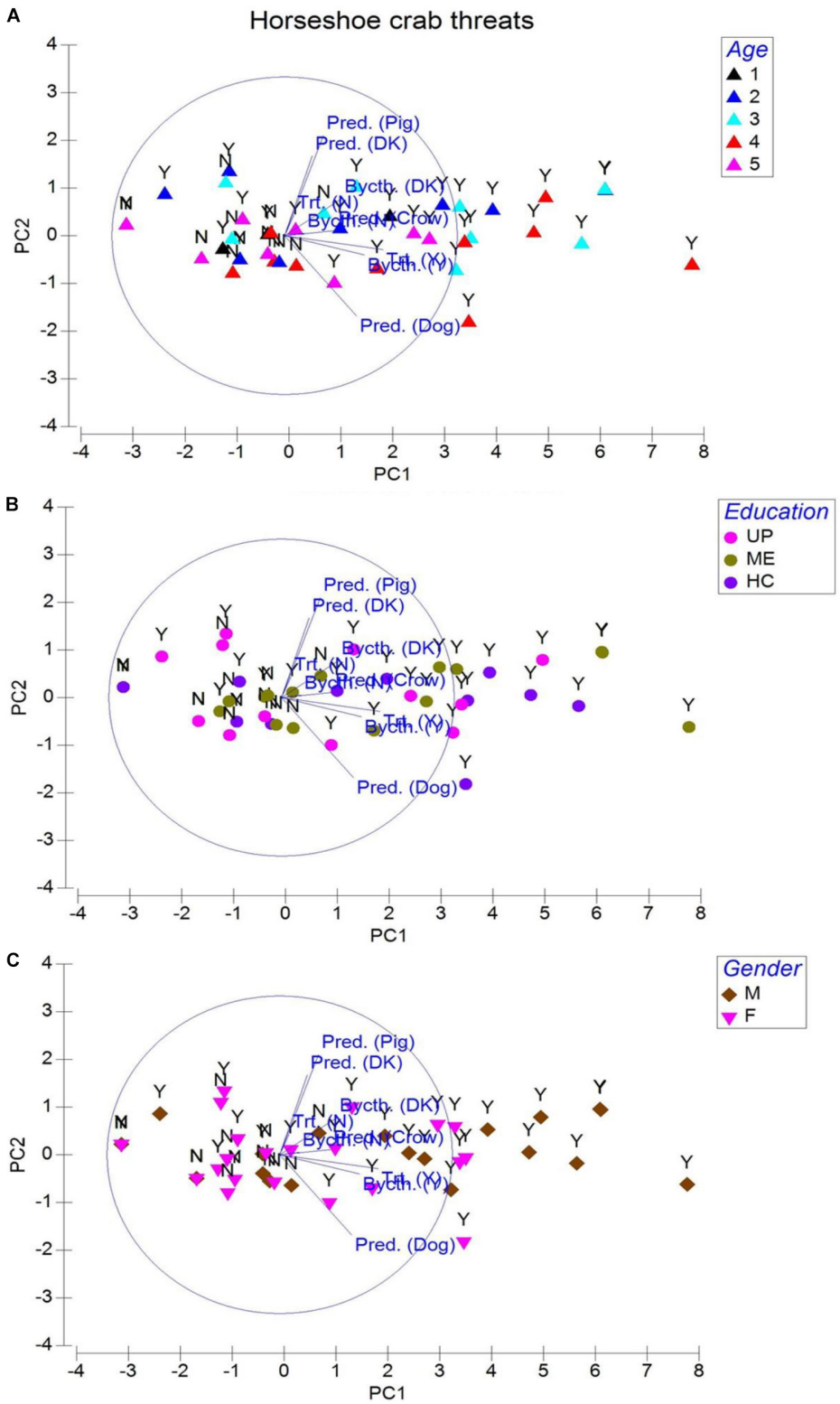


FIGURE 5 | Principal component analysis for society perception of threats to the horseshoe crab, *Tachypleus gigas.* Abbreviations are pred. = predation, bycth = by-catch, Trt. = threats, DK = don't know, Y = yes, and N = no. Legends represented by age are 1 = 18–24 (Group 1), 2 = 25–34 (Group 2), 3 = 35–44 (Group 3), 4 = 45–54 (Group 4), and 5 = 55–64 (Group 5). Education is denoted with UP = upper primary, ME = middle elementary, and HC = high school; gender is represented by M = male and F = female respondents.

Zauki et al., 2019b). Fortunately, the *Odia* community in Balasore relates "*Nilarakta Kankda*" with memory, grace, and love, whereas "*Ram Lakhan*" regards the crab as a goddess (grace and completion). Therefore, our 5-year continuous monitoring through citizen science conservation projects shows that *Odia* folks do not harm horseshoe crabs, although the crabs become entangled in the nets. This is strong evidence that local ecological knowledge has an outline of conservation awareness, which is constantly practiced by rural communities, although in the presence of local economy threats (net damage and resource competition).

CONCLUSION AND RECOMMENDATIONS

Horseshoe crab entry into the sixth extinction era is possible. Likewise, local ecological knowledge baselines are shifting by each passing human generation. This study shows that we wrongly practice conservation because our efforts are top-down due to community absence in policymaking and land-use decisions. In fact, by acknowledging people's perceptions, we are reducing expenditure on monitoring programs and, instead, use these funds for awareness projects and compassion instilment among folks in vulnerable areas. This questionnaire communicates science at the community level of understanding, and it appreciates community experiences and their interaction with horseshoe crabs. We highly recommend reliance on artisanal fishers who already bridged gaps between acquired and perceived knowledge in their area through their experiences. This study also creates awareness on fishing gears regardless of their application because these instruments cause horseshoe crab removal and unintentional mortalities in capture fisheries. In short, we recommend conservation projects that educate and empower communities with ecological roles whereby their interaction with communities and leaders enhance monitoring and surveillance. This bottom-up approach allows communities to convey opinions that then are easily translated by researchers into practical applications for policies and resource good-governance, particularly conservation-neglected aquatic species like the Indian horseshoe crabs.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors.

ETHICS STATEMENT

Ethical review and approval was not required for the study on human participants, in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

SP and BRN conceived and designed and performed the survey, analyzed the data, wrote the manuscript, prepared figures and tables, and reviewed drafts of the manuscript. DA, BRN, BPD, and HAE reviewed drafts of the manuscript and gave their support during fieldwork. SS and HAE analyzed the data and reviewed drafts of the manuscript. SP, SS, HAE, BRN, DA, and BPD approved the final manuscript. All authors contributed to the article and approved the submitted version.

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Substantial Gaps in the Current Fisheries Data Landscape

Gordon D. Blasco[1], Danielle M. Ferraro[1,2,3,4], Richard S. Cottrell[1], Benjamin S. Halpern[1,2] and Halley E. Froehlich[5,6]*

[1] National Center for Ecological Analysis and Synthesis, University of California, Santa Barbara, Santa Barbara, CA, United States, [2] Bren School of Environmental Science and Management, University of California, Santa Barbara, Santa Barbara, CA, United States, [3] Environmental Market Solutions Lab, University of California, Santa Barbara, Santa Barbara, CA, United States, [4] Marine Science Institute, University of California, Santa Barbara, Santa Barbara, CA, United States, [5] Ecology, Evolution, and Marine Biology, University of California, Santa Barbara, Santa Barbara, CA, United States, [6] Environmental Studies, University of California, Santa Barbara, Santa Barbara, CA, United States

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***Correspondence:**
Gordon D. Blasco
blasco@nceas.ucsb.edu

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Effective management of aquatic resources, wild and farmed, has implications for the livelihoods of dependent communities, food security, and ecosystem health. Good management requires information on the status of harvested species, yet many gaps remain in our understanding of these species and systems, in particular the lack of taxonomic resolution of harvested species. To assess these gaps we compared the occurrence of landed species (freshwater and marine) from the United Nations Food and Agriculture Organization (FAO) global fisheries production database to those in the International Union for Conservation of Nature (IUCN) Red List and the RAM Legacy Stock Assessment Database, some of the largest and most comprehensive global datasets of consumed aquatic species. We also quantified the level of resolution and trends in taxonomic reporting for all landed taxa in the FAO database. Of the 1,695 consumed aquatic species or groups in the FAO database considered in this analysis, a large portion (35%) are missing from both of the other two global datasets, either IUCN or RAM, used to monitor, manage, and protect aquatic resources. Only a small number of all fished taxa reported in FAO data (150 out of 1,695; 9%) have both a stock assessment in RAM and a conservation assessment in IUCN. Furthermore, 40% of wild caught landings are not reported to the species level, limiting our ability to effectively account for the environmental impacts of wild harvest. Landings of invertebrates (44%) and landings in Asia (>75%) accounted for the majority of harvest without species specific information in 2018. Assessing the overlap of species which are both farmed and fished to broadly map possible interactions – which can help or hinder wild populations - we found 296 species, accounting for 12% of total wild landings globally, and 103 countries and territories that have overlap in the species caught in the wild and produced through aquaculture. In all, our work highlights that while fisheries management is improving in many areas there remain key gaps in data resolution that are critical for fisheries assessments and conservation of aquatic systems into the future.

Keywords: wild capture, overexploited, seafood, data uncertainty, global oceans, knowledge gaps

INTRODUCTION

Fisheries are critical for livelihoods, nutrition, and food security worldwide (Hicks et al., 2019). Marine and freshwater capture fisheries produced ca. 97 million tons (live weight) of fish and invertebrates for human consumption and indirect uses, and employed over 40 million people in 2018 (FAO, 2020a). Yet in many regions, seafood supply from wild sources is still threatened by mismanagement and overexploitation of wild populations and ecosystems (Pauly et al., 2005; Maxwell et al., 2016; Link and Watson, 2019). While the abundance of many assessed fish stocks has been increasing in the last decade, largely due to the application of effective management strategies (Hilborn et al., 2020), over a third remain overfished (FAO, 2020a). Overfishing not only influences target species but can have wider implications for ecosystem state and function through bycatch of other species, habitat destruction, and the subsequent effects on food web interactions (Davies et al., 2009; Smith et al., 2011; Mumby et al., 2012; Link and Watson, 2019). Of considerable concern is that the vast majority of stocks are not formally assessed and data on many stocks are limited, constraining our understanding of the status of wild populations harvested for food and the ecosystems that support them. As human pressures on aquatic ecosystems increase in response to rising demands for food and other resources (Halpern et al., 2019), these information gaps pose considerable blind-spots for meeting sustainability targets around food security and protecting life below water (United Nations, 2015; Roberson et al., 2020).

Fish and seafood consumption has increased faster than any other animal-sourced food over the last 60 years, and global demand is expected to increase by 18% between 2018 and 2030 as countries urbanize and consumer affluence grows (FAO, 2020a). While aquaculture will be key to meeting this demand, unassessed artisanal fisheries are still the dominant form of fish production and coastal livelihoods in many regions (e.g., West and East Africa, Southeast Asia, Pacific Islands; FAO, 2020a). Without careful accounting of the species harvested for food, overcapacity and mismanagement of aquatic resources could severely threaten food and nutrition security and exacerbate biodiversity loss in the areas most fisheries-dependent (Hicks et al., 2019; Link and Watson, 2019). Moreover, growth in the aquaculture sector highlights a need for a greater understanding of the status of wild populations. Poor planning and management practices in fish farming can lead to negative impacts on fisheries through deoxygenation, genetic introgression, disease transmission, or shifts in local food webs (Alleway et al., 2019; Barrett et al., 2019; Clavelle et al., 2019; Gentry et al., 2019; Theuerkauf et al., 2019). But aquaculture also has the capacity to augment fisheries through stock enhancement, restoration of biogenic habitat, and the replacement of wild capture seafood in some contexts (Asche et al., 2001; Froehlich et al., 2017; Alleway et al., 2019; Gentry et al., 2019; Theuerkauf et al., 2019). Understanding the complex trade-offs among sectors requires greater knowledge of their overlap and interactions (Barrett et al., 2019); a key step in that process is careful accounting of which species are both farmed and fished, and where.

The current data landscape concerning aquatic food species, their extraction, distribution, and status relies on just a handful of key datasets, each with their own strengths and limitations. The United Nations' Food and Agricultural Organization (FAO) compiles the most complete global account of the quantity of aquatic species harvested for food by synthesizing production statistics from over 200 countries and territories, and is an invaluable resource for understanding trends in global fisheries (Garibaldi, 2012). Others have extended these data to estimate small-scale or illegal, unreported, and unregulated (IUU) fishing and have filled gaps in the FAO data where possible (Pauly and Zeller, 2016; Watson, 2017), however the FAO remains a key dataset used by a variety of stakeholders and across food sectors (Ye et al., 2017).

Data on fisheries landings, as with FAO's data, however, are a limited proxy for population status due to uncertainty around discards, non-commercial reporting gaps, changes to fishing effort, and/or management implementation (Mutsert et al., 2008; Pauly et al., 2013). Stock assessments go further in providing biomass estimates and management reference points for exploited aquatic populations by combining catch data with indices of stock status including, but not limited to, independent surveys, fishery-dependent catch per unit effort, and age structures (Ricard et al., 2012). But such high resolution data is inherently expensive to acquire and is thus limited to stocks harvested by industrial or commercial fisheries, such as those cataloged in the RAM Legacy Stock Assessment database (Ricard et al., 2012). And while various data-limited approaches to stock assessment methods have been developed, they tend to produce imprecise and biased estimates of stock status relative to data-intensive methods (Free et al., 2020). As a result, the population status of species targeted purely by artisanal, recreational, and subsistence fisheries, which represent more than half of global fishing effort (Rousseau et al., 2019), are not included in stock assessments. Many of these harvested species may not individually represent a large portion of global aquatic food, but prioritizing good management based on fishery yields overlooks the critical role that other species play in food and nutrition security (Hicks et al., 2019).

For unassessed aquatic species harvested for food, the International Union for Conservation of Nature (IUCN) Red List database provides an important resource. The IUCN Red List does not produce limit reference points for fisheries like a stock assessment from RAM; instead the IUCN is primarily concerned with a risk-of-extinction metric deemed the "conservation status." Conservation status classification requires detailed assessments for various threats to each species such as vulnerabilities to specific fishing gear types, aquaculture development, and habitat loss along with trends in population size (Mace et al., 2008). While the IUCN Red List does not provide an as comprehensive analysis of stock health as a stock assessment from RAM, this dataset provides an important suite of information to help guide decisions in the absence of formal assessments (Gullestad et al., 2017). Moreover, this resource is a valuable supplement to stock assessments where robust estimates of population status are restricted to the spatial contexts of an assessment region. In reality, a species can have a wide geographic

range, and while populations in a given region may be well managed, the threat from fisheries exploitation may extend over large, unassessed areas.

Given the differences in species coverage and conservation metrics between these datasets, identifying gaps within and among these data, and across different regions, presents an important opportunity for prioritizing research and policy aimed at ensuring sustainable management of aquatic resources into the future. To identify key gaps in our knowledge and assessment of aquatic (freshwater, brackish, and marine) species used for food, we compare available global data for harvested species across the FAO, RAM, and IUCN Red List databases. While the fact that many fisheries remain unassessed is well known, we illustrate the current state and trajectory of the fisheries data landscape, and define taxonomic and spatial gaps in our knowledge of aquatic food species. In doing so, we hope to help prioritize future research efforts that improve our knowledge, and ultimately support better management of aquatic ecosystems.

MATERIALS AND METHODS

Selection of Data Types and Target Species

We aimed to harmonize and broadly characterize the data landscape of harvested aquatic species using three of the principle global datasets which use separate criteria important for conservation and management: production, conservation status, and scientific stock assessments. We compared species representation across these datasets to quantify current data gaps, as well as highlight species and taxa with the most comprehensive coverage (those represented across the datasets). The FAO global production database is the backbone from which we define a list of unique harvested species, hereafter "target species," which are those species that have any reporting of wild catch in any year in the FAO database. We then extracted the available data on these species from the RAM Legacy Database and the IUCN Red List. We excluded mammals and reptiles because a majority of those species are not reported in terms of biomass in the FAO, but instead as the number of individuals harvested, thus complicating analysis of their contribution to global production. Additionally, we are focused on species that contribute to nutrition in this analysis, so we also exclude species listed as ornamental species by the FAO, which includes corals, shells of molluscs, cuttle-bone, and ornamental fish and plants for display purposes.

Our analysis largely focuses on gaps in terms of the number of harvested species rather than weighting a species by its contribution to global production because of differences in the local importance of a given species for ecosystem and human health (Hicks et al., 2019). Firstly, some fish and invertebrates represent important keystones in aquatic food webs that disproportionately influence the integrity of their surrounding ecosystem and that is not reflected in catch data Anderson et al., 2011). And second, the global scale of a fishery does not reflect its nutritional value, for instance, many small-scale tropical fisheries are richer in essential micronutrients such as zinc, calcium, and iron than those of a large global production scale (Hicks et al., 2019). This focus shifts when assessing 'Not elsewhere included' classifications, where we analyze the biomass of these groupings rather than the number NEI classifications. See section "Analysis of Not Elsewhere Included Landings."

Fisheries Production

Fisheries production data were sourced from the FAO global production database, which contains time series of reported freshwater and marine fishery landings and aquaculture production from 247 countries and territories for 2,416 species or taxonomic groups since 1950 (FAO, 2020b). The FAO database is heavily cited in the scientific literature and stands as the principal dataset of global seafood production (Garibaldi, 2012). The data collection relies on voluntary submission from national correspondents asking for the "best scientific estimate" of their annual landings. While the FAO has a thorough data validation process that includes following up with the reporting correspondents for clarification and rejecting and flagging questionable estimates, the FAO has always recognized that their database does not include all fish removals and there are still uncertainties in the reported numbers (Ye et al., 2017). Not all landings are reported to the species level and are instead designated as "Not elsewhere included" (NEI) observations with various levels of specificity, ranging from order, family, genus, or mixed species. NEI groups represent landings that are not taxonomically resolved and they introduce another level of uncertainty to the data, which we highlight here.

For this analysis, we extracted uniquely reported landed species or taxonomic groups from the 2,416 listed in the FAO, aside from those we excluded *a priori* (mammals, reptiles, and species harvested for ornamental purposes), to generate the list of target species (n = 2,077) that we collected conservation status and stock assessment data on. These target species include NEI groupings as well as individual species.

In order to approximate the extent to which captured species overlap with farmed species at a global level, we evaluated the presence of aquaculture for each target species. Each target species was classified as being produced by aquaculture or not by assessing if it had at least 1 year of aquaculture production recorded in the FAO database, regardless of producing country. For each country, we identified the number of species with any amount of both capture and aquaculture production reported in 2018. Additionally, we evaluated the number of countries per year since 1950 that reported at least one species both produced by aquaculture and captured in the wild.

Conservation Status

To represent the conservation status of target species, we used the IUCN Red List (IUCN, 2020). The Red List provides detailed reports on the conservation status of more than 120,000 species and assigns a global extinction threat score on a seven-point scale from "least concern" to "extinct" using a robust and consistent framework (Mace et al., 2008). The IUCN assesses species against a set of criteria based on the size and decline rate of the population and home range in order to determine its conservation status. Once a species is assessed on a global level, the IUCN then accepts assessments for subspecies and geographically distinct

populations, allowing the database to be leveraged for both global trends and local scale analysis, which in turn can be used to inform nuanced fisheries management plans (Gullestad et al., 2017). Although there have been comments on how to improve the assessment methodology (Hayward et al., 2015), the Red List remains a powerful tool for reporting trends in biodiversity, including those that are harvested. For this analysis we extracted the conservation status for the marine (n = 1,571) and freshwater (n = 293) species designated as target species from the FAO landings database.

Stock Assessment

The last dataset we included was the RAM Legacy Stock Assessment Database, the largest global collection of scientific stock assessments (Ricard et al., 2012; RAM Legacy Stock Assessment Database, 2018). These assessments are conducted on a specific geographic and/or genetically distinct population of a species designated as (or part of) a "stock." A stock assessment is a data intensive method for determining the status of a fishery or fisheries. At minimum, these assessments require data describing an index of abundance, such as catch-per-unit-effort (Hilborn and Ovando, 2014). These outputs allow a comparison to the current stock size (i.e., biomass) or fishing effort in relation to various reference points (e.g., maximum sustainable yield, spawning stock biomass), used for setting management reference points for fisheries managers to work toward and ideally sustain.

The RAM database compiles data from stock assessments of 882 stocks across 360 unique species and has been widely leveraged to evaluate global fisheries' status and stock improvements over time and space (e.g., Worm et al., 2009; Costello et al., 2012; Hilborn and Ovando, 2014). As noted above, stock assessments are an important tool in fisheries management but can be resource intensive, and as such are typically biased toward high-value, highly landed stocks in developed regions (Neubauer et al., 2018). Despite these drawbacks, there continue to be improvements in spatial and species coverage (Hilborn et al., 2020). For this analysis we considered a species represented in the RAM database if it was listed in RAM's metadata table, regardless of the year of assessment, assessment type, or number of stocks assessed. We take this conservative approach in order to highlight species that are fully excluded from the database. Of note, 14 multispecies stocks did not have explicit species listed (e.g., *Penaeus* spp.) and were not included in this analysis.

Taxonomic Alignment

We joined the databases at the species level in order to quantify species coverage and relative taxonomic distribution across the datasets. Discrepancies in the scientific name for a given species may result from differences in data entry across databases, as well as revisions to species classifications that emerge as the field of taxonomy continues to evolve. In order to best match species names among the datasets FishBase (Froese and Pauly, 2000), SeaLifeBase (Palomares and Pauly, 2020), and the World Register of Marine Species (WoRMS Editorial Board, 2020) were used to update synonyms and deprecated names across the three datasets. Because the fishery datasets are built for different purposes, they operate on different spatial resolutions. Landings in the FAO database are attributed to the country reporting the catch of a specific species in large marine regions (i.e., FAO regions), while stock assessments in the RAM database are attributed to area-specific stocks. Importantly, not all stocks of a given species will be assessed. However, if at least one stock of a given species has a stock assessment, we identified the species as represented in the RAM database. In contrast, the conservation status reports of species in the IUCN Red List are generated primarily at the global scale but also less frequently at the population scale. We used the presence of a global assessment, excluding 151 species categorized as "data deficient," as the criterion for considering a species covered in the IUCN Red List.

Analysis of Database Representation

In order to reconcile these databases in the interest of species coverage, we did not consider the spatial occurrence of a species within each dataset but rather relied on the binary evaluation of presence or absence of any data describing a species in a dataset. In other words, a species is considered better understood if any information is available in the datasets, but does not mean all stocks of that species are accounted for. Therefore, in cases where only a subset of a species fisheries have been assessed in RAM we will be overestimating the assessed biomass coverage of the dataset, this is not so for the IUCN where assessments used in this analysis are based on the status of the global population. We applied this conservative approach to coverage in order to highlight species with zero or incomplete coverage in the databases.

We compared the number of target species from each major taxonomic group (pisces, aquatic invertebrates, molluscs, crustaceans, and aquatic plants) represented by each dataset to highlight gaps in coverage across taxa. While the target list of species was the primary baseline for comparisons of species coverage across databases, we also identified species with stock assessments in RAM that were not listed as captured species in the FAO database. These discrepancies, though few, represent how differences in taxonomic reporting between the databases can contribute to the data landscape gaps described here. We also report the subset of target species that are the most represented, i.e., have both a stock assessment in the RAM database and an entry in the IUCN Red List. In order to better understand the role these species play in food systems, we calculated the proportion of total global landings these species represent. We took a conservative approach where if a species was classified as included in a dataset, based on the criteria laid out above, then we attributed all landed tonnage recorded in 2018, the most recent year published in the FAO, of that species to the datasets that it appears in. Finally, we report the number of target species also produced by aquaculture.

Analysis of Not Elsewhere Included Landings

In order to determine the taxonomic resolution of each of the 2,077 species codes in the FAO fisheries production database

considered in this analysis, we first classified each code as either a species level observation or an NEI. This was done by testing if the reported scientific name followed classic binomial nomenclature rules (genus species). Species codes that only reported a higher level taxonomic group (e.g., family or class), or a genus followed by "spp." were classified as NEI. Additionally, species codes that were composed of more than one explicit species (e.g., Auxis thazard/Auxis rochei for the bullet tunas) were designated as "mixed species" NEI. Next we determined the taxonomic resolution of each NEI observation. To do this we searched for defined text patterns in the reported name of each observation in order to classify the reported name as a genus, family, class, order, or mixed species, e.g., any name ending in "dae" was classified as a family, and any name ending with "spp." was classified as a genus. The classifications were verified with the Aquatic Sciences and Fisheries Information System List of Species for Fisheries Statistics Purposes (FAO, 2020b).

After defining the classifications of NEIs and the respective taxonomic resolution of those observations, we looked at the trends over time and space for freshwater and marine systems, as identified by FAO. In order to determine the trends in the proportion of total landings categorized as NEI globally and the median of all countries' proportions over time, we fit a linear model (proportion $\sim$ year) for the proportion of NEI landings by year. To test if NEIs in one sector were indicative of the quality of reporting in another, we also assessed the correlation between freshwater and marine NEIs and wild capture and aquaculture NEIs per country using Pearson's correlation coefficient (r). Finally, we compared the NEI reporting across taxonomic groups for the total percentage of reported NEI biomass per major group, as well as the taxonomic specificity of each of the NEIs.

We assess the biomass represented in NEI classifications rather than the number of NEI classifications for two reasons. Firstly, because NEI data are often at very different taxonomic resolutions (as narrow as mixed species and as broad as subphlya), each NEI group can hold a very different level of information on species richness, with highly uncertain implications for biodiversity or food security. And secondly, the number of NEI classifications can be misleading when assessed on temporal scales. For instance, the taxonomic resolution of nei classifications for any given country could plausibly degrade from, for example, the genus level with a handful of closely related species being lumped into one group, to data resolved to order or subphylum that could contain hundreds more species and considerably more biomass, while being represented as a single NEI group. For these reasons we assess the amount of biomass categorized as NEI then assess the level of taxonomic specificity of those reports.

For all data synthesis and analysis, we used R version 3.6.3 (R Core Team, 2020) with the tidyverse collection of packages (Wickham et al., 2019). The RAM database version 4.44 was accessed via the ramlegacy R package (RAM Legacy Stock Assessment Database, 2018), and the IUCN Red List version 2020-2 was accessed via the rredlist R package (Chamberlain, 2020). The taxize and rfishbase R packages were used for taxonomic validation (Boettiger et al., 2012; Chamberlain and Szocs, 2013).

RESULTS

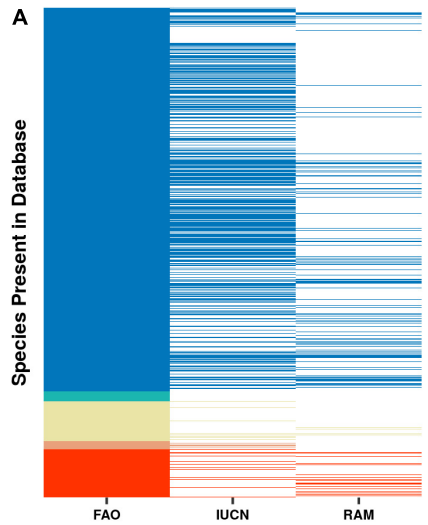
Species Representation Across Datasets

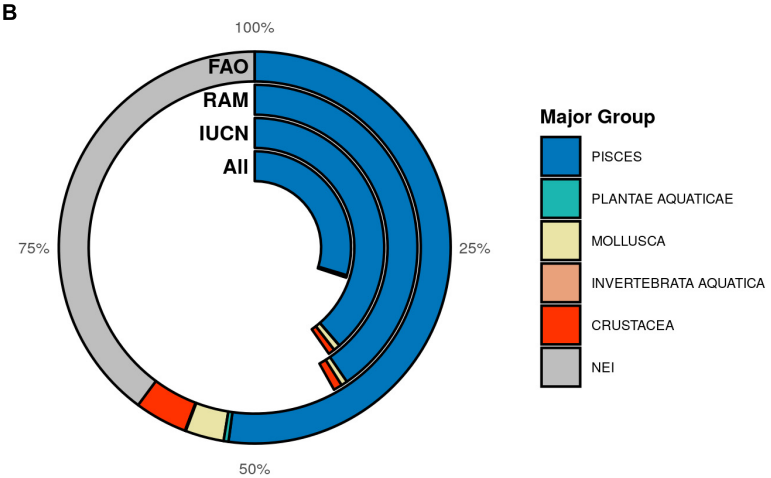
The quantity and types of species covered by FAO, RAM, and IUCN differ substantially (**Figure 1**). FAO reports 1,695 explicit species (i.e., non-NEI groupings), which account for 60% of wild capture landings in 2018 (57 million tonnes). The IUCN Red List covers 955 of the target species, which account for 40% of 2018 landings (39 million tonnes). The RAM database covers 289 of the target species considered in this analysis, which account for 42% of 2018 landings (41 million tonnes). However, because 40% of the tonnage in the FAO database is not resolved to the species level (**Figure 1B**), there is uncertainty around what percentage of landed biomass can truly be attributed to each species. The Red List covers more fished species in each of the major groups than RAM does, although some of these are taxonomic groups that are not covered by RAM in general, such as aquatic plants.

When looking across data sets for highly represented species, we found 150 target species that have a conservation status record in the IUCN Red List and at least one stock assessment in the RAM database (**Figure 1C** and **Supplementary Table 1**). These species only account for 9% of the explicit species in the target list, yet make up 30% of total capture production in 2018 (29 million tonnes). The majority of these species are finfish ($n = 139$), with sharks, rays, and chimeras being the most represented of the fish group ($n = 32$). The remaining non-fish species are lobsters ($n = 6$) and squids and octopuses ($n = 5$). All other exploited species have either only a scientific assessment in RAM ($n = 136$), only a conservation status on the IUCN Red List ($n = 805$), or neither ($n = 601$) (**Figure 1A**). The 601 species that appear neither in the IUCN Red List nor the RAM Legacy database make up 35% of unique species harvested from wild systems recorded by the FAO, and made up 8% of landed tonnage in 2018. However, this percentage changes considerably when looking within different taxonomic groups. Most target aquatic plant species (96%) have no representation in either RAM or IUCN, neither do mollusc species (76%) or crustacea (67%), while only 26% of fish species considered in this analysis are unassessed by either database.

Stock assessments are typically conducted on caught species of high volume or value; however, 49 explicit species with stock assessments recorded in the RAM database were notably absent from the list of target species, as well as six species which were reported in the FAO database as produced by aquaculture only, despite having stock assessments which by definition categorize them as caught species (**Supplementary Table 2**). These species not having any landings records in the FAO could be for two reasons; either the species names used by RAM and FAO were not recognized as valid synonyms of each other in either Fishbase, Sealifebase, or the World Register of Marine Organisms, or landings of these species were never recorded to the species level inside of FAO and instead were lumped into an NEI grouping.

Of the target species, 296 were recorded as produced via aquaculture at some point over the time series. Capture of these





C

Major group	Num. total taxa	Num. NEI	Num. resolved to species	Included in IUCN	Included in RAM	Included in Both	Included in Neither	Produced Via Aquaculture
PISCES	1627	274	1353	892	246	139	354	203
PLANTAE AQUATICAE	41	15	26	1	0	0	25	9
MOLLUSCA	171	40	131	22	15	5	99	46
INVERTEBRATA AQUATICA	34	14	20	7	0	0	13	4
CRUSTACEA	204	39	165	33	28	6	110	34
Total	2077	382	1695	955	289	150	601	296

FIGURE 1 | (A) Composition of the FAO target species across major taxonomic groups and the presence or absence of those species in the IUCN Red List and RAM Legacy Stock Assessment databases. **(B)** Comparison of the landed biomass per major taxonomic group accounted for by the species or groups included in each database. **(C)** The number of total species or groups, taxa not elsewhere included (NEI), and fully resolved species in the list of FAO target species per major group, as well as the number included in the IUCN Red List and RAM databases, and the number produced by aquaculture.

species accounted for 12% (12 million tons) of the total wild landings in 2018, while that same year farming of these species accounted for 56% (64 million tons) of the total aquaculture production. Of the species farmed, 55% (n = 164) have their wild counterparts represented in the IUCN Red List, most being fish (n = 150), followed by crustaceans (n = 8). Only 16% (n = 47) of these species' wild counterparts are represented in RAM, the majority being fish (n = 36), followed by molluscs (n = 4). In order to track possible interactions between wild and farmed species, we report the number of countries that are fishing and farming the same species over time. In 1950 only 27 countries were farming at least one of the species they reported as also captured in the wild, and by 2018 that number rose to 103 countries (**Figure 2A**). In 2018, Taiwan reported tonnage for 32 species that were produced both via aquaculture and from wild capture methods, the most of any other country (**Figure 2B**). Other countries or regions with relatively high numbers of both captured and farmed species include Southeast Asia, Europe, Russia, and the United States; the majority of countries in Africa are notable for their lack of any species with both farmed and caught production. However, because 32% of aquaculture production was reported as an NEI group in 2018, the true number of species that are *de facto* farmed is unknown with the current level of data resolution recorded in the FAO database.

Not Elsewhere Included

NEI catch accounts for over a third (35%) of the cumulative fished tonnage (4.8 billion tons) of target species since the earliest reported year (c. 1950) in the FAO production database, and has been present to some degree in every year of the time series. In 2018, 40% of total landed tonnage globally was categorized as NEI (**Figure 3A**). There has been an increase of approximately 10% (SD = 0.04) in the percentage of total annual landings reported as NEI over the last 70 years [$F_{(1, 67)}$ = 125, p < 2.2e-16, R^2adj = 0.65]. While the percentage of NEI landings are increasing at a global scale, the median percentage of national catch classified as NEI for all reporting countries has decreased by more than half from 93% of annual landings in 1950 to 40% in 2018 at a rate of −0.8% (SD = 17.3) per year [$F_{(1, 67)}$ = 1,342, p < 2.2e-16, R^2adj = 0.95; **Figure 3B**]. Across the entire time series, countries in Asia account for more than 50% of all annual global NEI landings, and upwards of 75% starting in 2000. Countries in Africa, the Americas, Europe, and Oceania collectively remained below 25% after 2000 (**Figure 3C**).

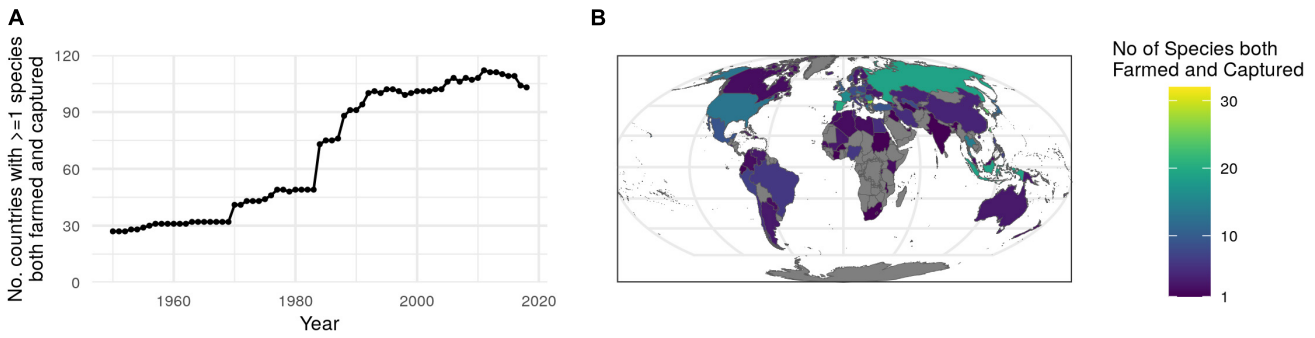


FIGURE 2 | Countries with overlap in aquatic species harvested through wild capture and aquaculture. **(A)** The number of countries that farm the same species they are catching in the wild over time. **(B)** Number of species both farmed and captured in the wild in 2018.

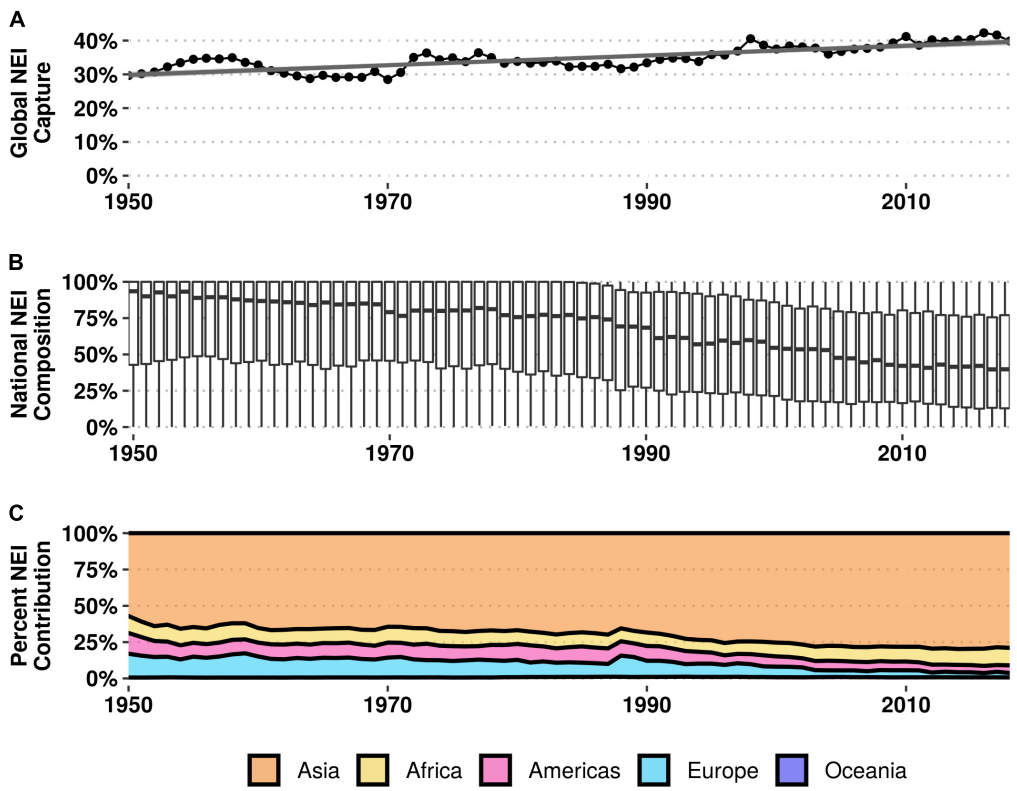


FIGURE 3 | (A) Percentage of global capture landings categorized as not elsewhere included (NEI) from 1950 to 2018; line represents linear regression. **(B)** Percentage of NEI landings at the national level for 247 countries and territories from 1950 to 2018. **(C)** Percentage of global NEI landings contributed by geographic region; Oceania contributed < 1% of global NEI landings throughout the entire time series.

Countries with high NEI tonnage in one sector (i.e., marine, freshwater, aquaculture) trend toward reporting higher NEI in other sectors as well (**Figure 4**). Countries with high levels of marine NEI landings in 2018 tend to also have a high level of freshwater NEI landings (r = 0.70, 95% CI = 0.60–0.78, p < 2.2e-16). And while globally the percentage of aquaculture production reported as NEI (35%) is lower than that of capture landings (40%), countries that have high levels of capture NEI production in 2018 also have high levels of aquaculture NEI production (r = 0.88, CI = 0.83–0.91, p < 2.2e-16).

Of all NEI tonnage landed in 2018, 47% was reported at the major group level, the least taxonomically resolved, while 2% was reported at the mixed species level, the most resolved (**Figure 5**). The majority of the remaining NEI production was reported at the genus (24%), family (18%), and order (9%) levels. However, there are differences in the resolution of the reporting between the major taxonomic groups. Notably, invertebrates (excluding molluscs and crustaceans) are the group with the largest amount of reported NEI production, with 88% of landings in 2018 not reported at the species level. Within the NEI production of each

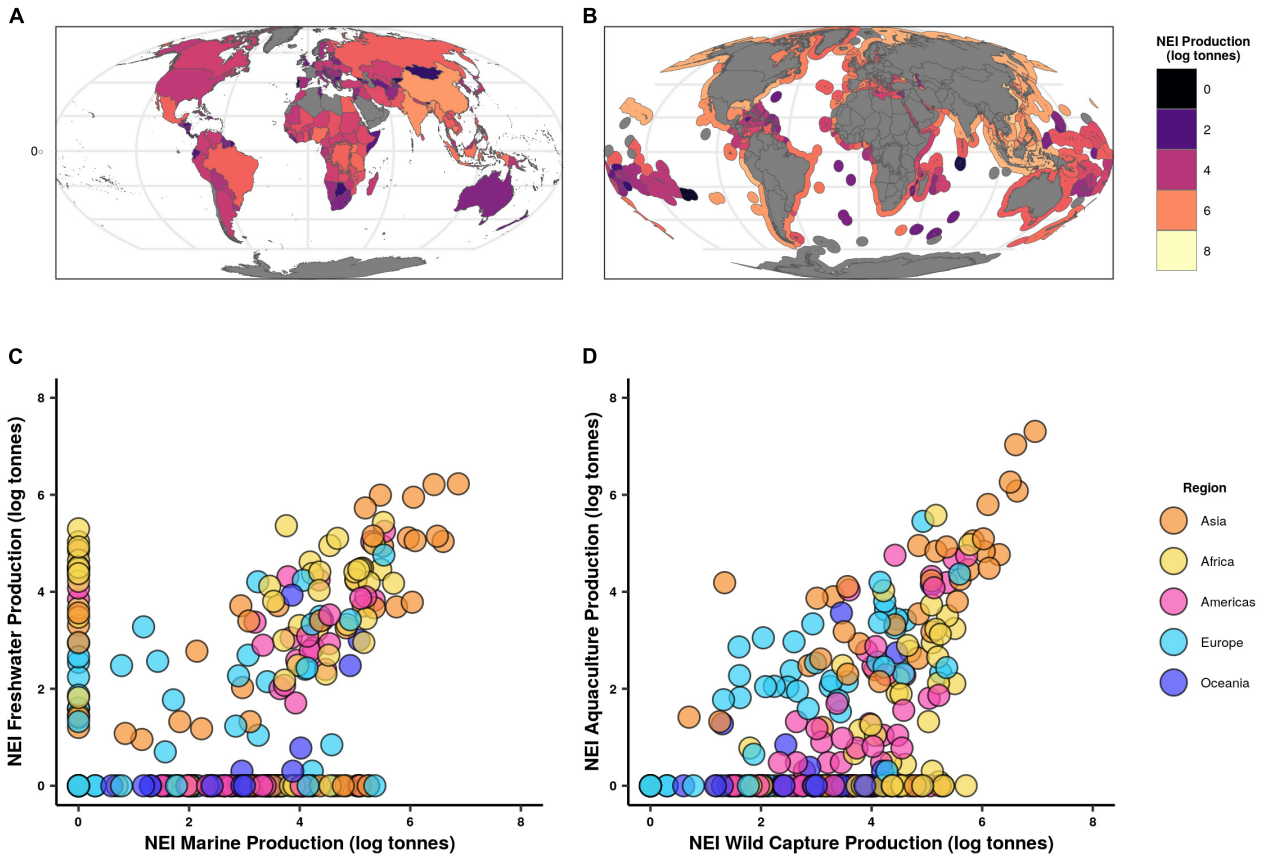


FIGURE 4 | Production designated as not elsewhere included (NEI) by country and sector **(A)** Total national wild capture NEI freshwater production. **(B)** Total national wild capture NEI marine production. **(C)** National freshwater NEI production against marine NEI production. **(D)** National aquaculture NEI production against wild capture NEI production. Production in all plots is $\log_{10}$ scaled and represents 2018 values.

major taxonomic group, the resolution of the NEI landings can differ greatly; the majority of fish NEI landings are resolved to major group, the least taxonomically resolved level (52%), while the majority of non-mollusc and non-crustacean invertebrates (included in the major group of invertebrata aquatica) NEI landings are resolved to the finer scale genus level (60%).

DISCUSSION

A substantial proportion of aquatic species we harvest for food worldwide is not evaluated in terms of their conservation status or through formal stock assessments. Of the 1,695 explicitly identified species reported to the FAO as wild-caught, 601 (35%) of them are not represented by either RAM or IUCN. Furthermore, 40% of wild landings reported to the FAO are not identified to the species level but are aggregated to groups of various taxonomic resolutions ranging from mixed species up to categories as broad as "Pisces." While species absent from the RAM and IUCN databases comprise a smaller proportion of reported fisheries tonnage, due to regional differences in reporting and regulatory practices, discards, and illegal fishing activity, these figures are unlikely to be representative of the fishing pressure exerted on associated populations. Moreover, proportional representation within fisheries production data does little to fully capture a species' sensitivity to fishing, particularly where production is reported in groups that may conceal important interspecific differences. Without greater understanding of which species are specifically influenced by fishing, these gaps in data coverage hinder our understanding of the broader impacts of aquatic food production.

Coverage differs among taxonomic groups but invertebrates constitute the largest foundational knowledge gap in fisheries data. Of the non-fish species, 44% are not identified to the species level even when landed. Molluscs are particularly poorly represented; over 40% of global landings are resolved only to family level or broader, and only 12% of the species explicitly identified in landings have data assessments in either RAM and IUCN databases. Given the growing proportion of global landings invertebrates represent, and their importance for human livelihoods, and ecosystem function, these are concerning voids in our knowledge of aquatic foods (Berkes et al., 2006; Anderson et al., 2011; Eddy et al., 2015; Miller et al., 2017). Historic overharvesting of oysters in Chesapeake Bay contributed to increased eutrophication and hypoxia (i.e., low dissolved oxygen), leading to declines in other local fisheries and wildlife

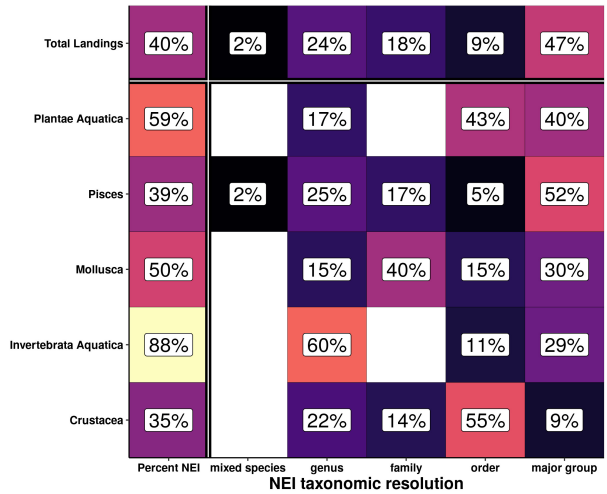


FIGURE 5 | Percentage of not elsewhere included (NEI) capture fisheries landings and the highest level of resolution for the different major groups. Column 1 shows the percentages of all landings and landings per major group that are categorized as NEI. Row 1 shows the percentages of all NEI landings identified to each taxonomic level. The remainder of the boxes show the percentage of NEI landings of the associated major group that are identified to each taxonomic level.

(e.g., rays, sharks, river otters) (Jackson, 2001; Randall, 2003), providing a stark example of how overexploitation of important invertebrates can influence the wider ecosystem function and diversity. Looking forward, this knowledge gap is of considerable concern given the trajectory of increasing invertebrate fisheries combined with a greater vulnerability of many calcareous species to ocean acidification occurring under climate change (Marshall et al., 2017; Miller et al., 2017).

Although commercially exploited finfish species are a more widely harvested and well-studied group, there also remain considerable data gaps in our knowledge of these species and their population status. Three-quarters (74%) of finfish species reported in landings to the FAO are missing from either the RAM or IUCN datasets, 26% are missing from both, leaving these species partially or completely unassessed in two of the principle datasets used to manage and monitor our aquatic resources. Notably, one fifth of global finfish landings reported to the FAO are resolved only to broad groupings (e.g., "marine fishes nei," "freshwater fishes nei," "Pelagic fishes nei"), which makes it impossible to understand their representation among other data sets and thus the sustainability of their harvest. Although NEI classification is a decreasing proportion of domestic landings for most countries, the global increase in NEI resolved production, driven by landings in Asia and to a lesser extent Africa, is somewhat troubling. Diversification of catch into lesser known species can be an indication of expanding fishing pressure down, up, or across food webs in response to fully exploited or even dwindling resources in more traditionally- or economically preferred stocks (Shen and Heino, 2014; Branch, 2015). With population and fish-dependency set to grow considerably in many tropical Asian and African regions (FAO, 2020a), finding ways to address or account for these resource data gaps is necessary to better manage and support the associated fisheries and livelihoods.

Addressing gaps in harvest and conservation-relevant data is a significant challenge and thus requires a suite of broad and bold tactics at multiple governance scales. Increased support for maintaining and building capacity of existing organizations, such as the FAO, their networks and infrastructure is of critical importance, yet often overlooked and underfunded (Halpern et al., 2019). In doing so, greater harmonization across existing resources becomes of greater possibility. For instance, our analysis identified 55 species with stock assessments represented within the RAM database which are absent from FAO landings (**Supplementary Table 2**). While these species may potentially be accounted for in FAO landings classified as NEI or otherwise not resolved to the species level, such mismatches in taxonomic resolution challenge the synthesis of these datasets. Further, addressing the growing proportions of NEI tonnage in key regions such as Asia, requires greater transparency across interregional ocean and freshwater commons, and equitable sharing to transition toward better ecosystem-based management and protection of aquatic environments (Visbeck, 2018; Rudolph et al., 2020). For example, with China as a major presence in the South China Sea and being the largest contributor to catch globally (Gephart and Pace, 2015; Crona et al., 2020), it will need to play a pivotal role in the adoption of cooperative practices in the region. Prioritizing additional conservation-relevant data collection must also balance trade-offs in social-ecological impact. Targeting species that comprise a significant proportion of global or regional landings prioritizes a food production perspective, but focusing research and data collection efforts only on groups of species that play the largest roles in the aquatic food system can undermine the ability for managers to make comprehensive policy decisions (Halpern et al., 2019). Ultimately, shifts toward more stable sociopolitical structures and governance strategies, with science-based policy decisions are at the heart of addressing these and many other sustainability targets.

Technological innovation will likely continue to be an important part to improving confidence in fisheries reporting and associated data. For instance, video surveillance technologies on fishing vessels can offer accurate, objective, and cheaper additions or even alternatives to on-board observers for tracking quota, species, and size compliance (Ames et al., 2007; Hold et al., 2015). DNA barcoding is showing promise as a tool that can be used to increase the taxonomic resolution of landings, enforce catch restrictions around threatened species, and reduce seafood fraud in the consumer facing marketplace (Rasmussen and Morrissey, 2008; Ardura et al., 2013). DNA barcoding also can play a role in deterring IUU fishing by empowering ports and landing sites with quick testing for illegal catch of protected species (Ogden, 2008; Ardura et al., 2013). Other efforts to combat IUU fishing have shown promise, like Global Fishing Watch which has been employed to identify suspect or illegal activities via satellite imagery, typically linked to overexploitation, such as the "dark fishing fleets" (Park et al., 2020). Furthermore, data technologies such as blockchains can help traceability of a fished

product along the supply chain, and provide a decentralized system for logging catch statistics that does not rely on different national institutions with varying capacity and incentives for accurate reporting (Probst, 2020). It may also encourage fishers to more accurately report species caught if quotas can be traded among vessels and trade information is available from different ports to determine where the best prices for catch may be obtained (Branch, 2009; Probst, 2020). It's important to highlight that while technological advances such as these have potential to "nudge" fisheries actors toward greater compliance or efficiency, they should be viewed as supplements to, rather than replacements of, robust fisheries management.

Improvements in management and data become increasingly important under the context of an aquaculture sector that continues to expand and cohabit aquatic environments that also produce wild seafood. Better understanding the status of wild fish and invertebrate populations cannot only minimize negative interactions among fisheries and aquaculture sectors, but create opportunities for aquaculture to be used as a tool in fisheries management (Froehlich et al., 2017). For example, culture of extractive species (e.g., bivalves, seaweeds, corals) can provide similar habitat complexity for native species as the natural benthos, and improve water quality with equivalent efficacy as established biogenic reefs (Dumbauld et al., 2009; Humphries et al., 2016), functioning as powerful tools in degraded or overfished environments. Greater knowledge of which species are fished, and where, can also inform whether a farmed equivalent can provide options for stock enhancement of struggling wild populations or whether risks from genetic pollution may be too great from candidate aquaculture species (Froehlich et al., 2017; Clavelle et al., 2019). Nonetheless, given that countries with poorly resolved fisheries data typically experience the same issues with aquaculture reporting, more widespread cooperation and improved coordination across management and reporting agencies is needed if these mutualisms are to be realized.

Currently the knowledge needed to prioritize future conservation and policy interventions is incomplete, with significant gaps surrounding important exploited taxa. Fisheries management for a number of major commercial fisheries stocks and populations has improved through time. However, the lack of conservation-relevant data surrounding a substantial number of species, as well as increasing proportions of taxonomically unresolved landings in some areas, highlights the challenge of correcting aquatic ecosystem degradation and protecting life below water as we aim to feed a growing human population. Our work takes steps toward outlining the scale of the data gaps among three principal datasets relevant to the conservation and resource management, which we hope will help target what and where to focus resources and efforts. And while technology could help lower costs to track and improve accounting for some wild harvested species, there is no substitute for good governance because people are at the heart of better data and management. Filling these data gaps is a considerable challenge for resource managers and the scientific community alike, but addressing these voids in our knowledge is a fundamental aim of the UN Decade for Ocean Science, and critical for meeting sustainability targets by 2030.

DATA AVAILABILITY STATEMENT

The primary datasets used in this study are available publicly. The FAO global production is available at (http://www.fao.org/fishery/statistics/global-production/en), the RAM legacy database at (https://www.ramlegacy.org/database/), and the IUCN Red List at (https://www.iucnredlist.org). All code used in this analysis is available in a public repository at (https://github.com/GordonBlasco/data_landscape).

AUTHOR CONTRIBUTIONS

HF conceived the idea. GB collected, analyzed the data, and wrote the first draft. All authors contributed to writing and editing of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2020.612831/full#supplementary-material

Supplementary Table 1 | Target species from the FAO Global Production database that also have at least one assessment recorded in the RAM Legacy Stock Assessment database and IUCN Red List.

Supplementary Table 2 | Species with stock assessments in the RAM Legacy Database that had no landings reported in the FAO Global Capture Production Database.

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Conflict of Interest: HF sits on the Technical Advisory Board of the Aquaculture Stewardship Council.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Marine Science

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The Long-Term Effect of Bleeding for Limulus Amebocyte Lysate on Annual Survival and Recapture of Tagged Horseshoe Crabs

David R. Smith[1], Joshua J. Newhard[2], Conor P. McGowan[3] and C. Alyssa Butler[4]

[1] U.S. Geological Survey, Leetown Science Center, Kearneysville, WV, United States, [2] U.S. Fish and Wildlife Service, Maryland Fish and Wildlife Conservation Office, Annapolis, MD, United States, [3] U.S. Geological Survey, Alabama Cooperative Fish and Wildlife Research Unit, Auburn University, Auburn, AL, United States, [4] Alabama Cooperative Fish and Wildlife Research Unit, School of Forestry and Wildlife Sciences, Auburn University, Auburn, AL, United States

In the U.S., 525,000 horseshoe crabs (*Limulus polyphemus*) per year have been captured during 2013–2017, brought to biomedical facilities, and bled to produce Limulus amebocyte lysate (LAL), then mostly released to the area of capture. The Atlantic States Marine Fisheries Commission estimates short-term bleeding-induced mortality to be 15% (4% to 30%), resulting in mortality of approximately 78,750 horseshoe crabs annually in recent years comprising a minor portion (<13%) of the up to one million annual coastwide landings dominated by harvest for bait. However, the long-term effect of bleeding for LAL on annual survival and spawning behavior is unknown; thus, results from short-term studies alone might underestimate bleeding effects at the population level. To address this knowledge gap, we analyzed data from the U.S. Fish and Wildlife horseshoe crab tagging database to estimate the differences in survival and recapture rates of bled and not bled horseshoe crabs tagged in the same years and geographic area. Contrary to expectation, survival was not lower for bled crabs compared to unbled crabs. Differences varied, but survival estimates tended to be higher for bled crabs than for unbled crabs. However, biomedical culling and selection for younger or healthier animals could have resulted in biomedically tagged individuals representing a healthier subset of the overall population with subsequent higher survival. Furthermore, the tagging analysis revealed a post-bleeding reduction in capture probability, which could indicate decreased spawning activity, evident in males more than females. Continued tagging of bled and unbled crabs in the same geographic area while recording age class and sex will contribute to the further resolution of LAL production's effect on horseshoe crab populations.

Keywords: *Limulus polyphemus*, Limulus amebocyte lysate, LAL, Delaware Bay, tagging analysis, survival, spawning activity, horseshoe crab

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***Correspondence:**
David R. Smith
drsmith@usgs.gov

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INTRODUCTION

Horseshoe crabs are the sole remaining representatives of the order of Xiphosura, with the four extant species of horseshoe crabs closely resembling their fossilized relatives from hundreds of million years ago, indicative of extraordinary evolutionary success (Rudkin and Young, 2009; BłaŻejowski, 2015). However, modern stressors associated with expanding harvest and habitat

loss threaten horseshoe crabs' current status (Berkson and Shuster, 1999; Anderson and Shuster, 2003; Smith et al., 2016; John et al., 2018). Commercial harvest of horseshoe crabs is for bait in the U.S., human consumption in Asia, and biomedical use in the U.S. and Asia (Atlantic States Marine Fisheries Commission [ASMFC], 2019; Laurie et al., 2019). The International Union for Conservation of Nature (IUCN) Red List ranks are Vulnerable for the American horseshoe crab (*Limulus polyphemus*), Endangered for the Asian species *Tachypleus tridentatus*, and Data Deficient for the two remaining Asian species (*T. gigas* and *Carcinoscorpius rotundicauda*) (Smith et al., 2016; Laurie et al., 2019).

Biomedical companies produce an assay from the hemolymph (blood) of adult *L. polyphemus* in the U.S. and *T. tridentatus* in Asia (Levin et al., 2003). The generic source of the hemolymph names the assay – LAL for Limulus amebocyte lysate and TAL for Tachypleus amebocyte lysate (Krisfalusi-Gannon et al., 2018). The lysate test detects minute quantities of bacterial endotoxin in injectable medicine or implantable devices (Levin et al., 2003). In the U.S., over 525,000 horseshoe crabs per year on average between 2013–2017 have been captured and brought to biomedical facilities and bled to produce LAL (Schmidtke et al., 2018). Although most bled horseshoe crabs are released, mortality and potential behavioral effects from hemolymph harvest are sources of uncertainty in stock assessments (Atlantic States Marine Fisheries Commission [ASMFC], 2019). The importance of LAL to human health and the need to understand LAL-harvest effects on horseshoe crabs are heightened by the potential demand to test coronavirus vaccines for bacterial contamination (Smithsonian Magazine, 8 June 2020, https://www.smithsonianmag.com/smart-news/race-coronavirus-vaccine-runs-horseshoe-crab-blood-180975048/; National Geographic, 2 July 2020, https://www.nationalgeographic.com/animals/2020/07/covid-vaccine-needs-horseshoe-crab-blood/#close).

To minimize bleeding-induced mortality due to hemolymph harvest, the Atlantic States Marine Fisheries Commission (ASMFC), which governs interstate fisheries along the Atlantic coast of the U.S., recommended best management practices (BMPs) for the biomedical bleeding process to mitigate for post-bleeding effects (Atlantic States Marine Fisheries Commission [ASMFC], 2011). The BMPs considered all steps of the process, including capturing and collecting, transport, holding, bleeding, and returning to the sea. The 41 recommended practices comprise trawl-tow times during capture, environmental conditions during transport and holding, bleeding procedures, return to the sea, monitoring, and reporting. The practices are not mandated by ASMFC, but states where the LAL process occurs can stipulate that BMPs are conditions for collection permit issuance. For example, in Maryland a chain of custody is required from harvest until release with records for number of animals, mortalities, and temperatures during transport and holding; animals must be returned to sea within 48 h (Steve Doctor, Maryland Department of Natural Resources).

Multiple studies have assessed the short-term effects of biomedical bleeding (cf Rudloe, 1983; Hurton and Berkson, 2006; Leschen and Correia, 2010; John et al., 2011; Owings et al., 2019), but long-term effects remain understudied. The short-term studies, which examine effects over weeks or months, found that post-bleeding mortality depended on the percent volume of hemolymph removed and animal handling before, during, and after the bleeding procedure. A meta-analysis of 47 bleeding-mortality studies estimated an acute post-bleeding mortality rate of 15% with a 95% confidence interval of 4% to 30% (Atlantic States Marine Fisheries Commission [ASMFC], 2019). The objective of most such studies focused on survival, but some observed post-bleeding effects on behavior as well (Kurz and James-Pirri, 2002; Anderson et al., 2013; Owings et al., 2019). Anderson et al. (2013) found that bleeding caused reduced activity and physiological changes potentially altering immune function. Owings et al. (2019) tracked 28 telemetry-tagged horseshoe crabs, 14 unbled (control), and 14 bled, and found that bled animals had less frequent spawning bouts than control animals and remained in deeper waters than control animals, but significant impacts lasted less than two weeks post bleeding.

In contrast to the numerous studies of short-term bleeding effects, studies of bleeding effects on long-term survival are lacking. A paucity of data for tracking post-bleeding fate beyond weeks or months has been an overriding reason for the lack of attention to long-term survival effects. The inability to maintain adult horseshoe crabs in captivity for long periods contributes to the absence of long-term studies of post-bleeding survival (Carmichael and Brush, 2012). The exception was a tagging data analysis conducted by Butler (2012), who found evidence of a positive effect of biomedical bleeding on annual survival, but the tag releases were not geographically constrained to control for regional differences in survival.

To address this fundamental knowledge gap and expand on the work of Butler (2012), we used capture-recapture models to analyze data from the U.S. Fish and Wildlife (USFWS) horseshoe crab tagging database to compare annual survival rates of adult horseshoe crabs based on bleeding status within the same geographic area and years. Since 1999, the USFWS has established standardized tagging protocols, facilitated tag reporting, and managed a coastwide tagging database. We focused on analyzing the USFWS database on the Delaware Bay population within the estuary and adjacent coastal waters, where there has been consistent tagging of crabs that had been bled or not bled. Our working hypothesis was that – all else equal – bled horseshoe crabs would have lower annual survival and spawning rates than not-bled (unbled) crabs due to the stress of capture and bleeding. Because most tag recaptures occur during spawning when the animal is accessible to observation, we hypothesized that lower spawning rates of bled crabs would appear as lower tag-recapture rates or capture probability estimates.

MATERIALS AND METHODS

The USFWS provides tags to qualified tagging program coordinators and manages a centralized tagging database of tag releases and recaptures. The tagging program must demonstrate consistent tagging and recapture effort over multiple years,

adhere to a standardized protocol, and promptly submit tagging records. Two biomedical companies, Lonza Walkersville, Inc., (hereafter Lonaz) and Wako Chemical (hereafter Wako), have tagged crabs after bleeding them for LAL; this represents the only consistent tagging effort of bled crabs in the USFWS database. Both companies captured crabs in coastal Delaware to Virginia, which extends from the mouth of Delaware Bay south to Virginia Beach (**Figure 1**).

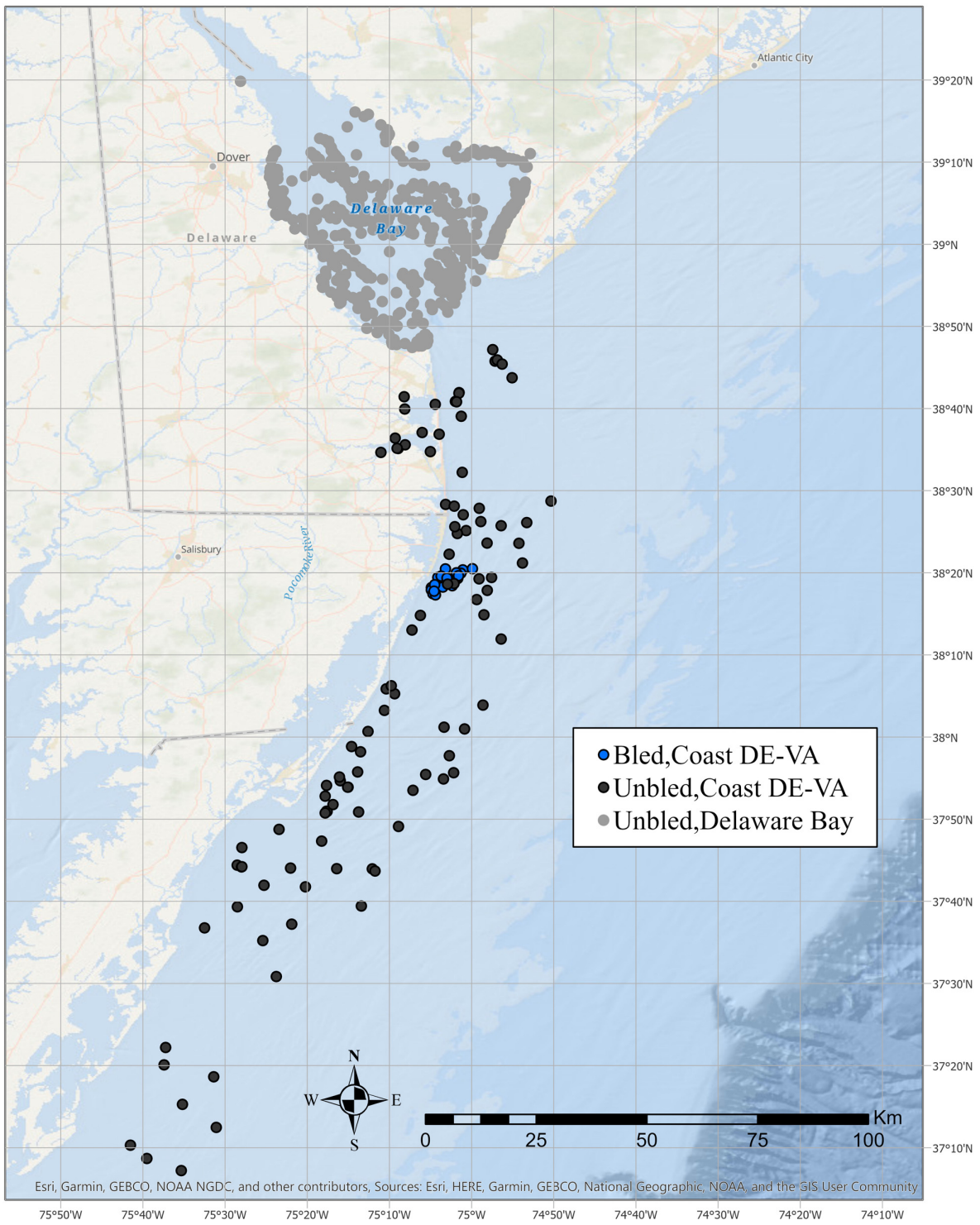

FIGURE 1 | Tag release locations in coastal Delaware to Virginia and Delaware Bay for adult horseshoe crabs that were bled for LAL or not bled.

The two biomedical companies have followed standard protocol but tagged at different levels of effort (personal communication: Sasha Charleron, Salisbury Site Manager, Lonza Walkersville, Inc., and Christina Lecker, Plant Manager, Wako Chemical). The majority (96%) of tagged and bled crabs came from Lonza Walkersville, Inc., (Walkersville, MD, United States) with the remaining fraction tagged by Wako Chemicals. Lonza tagged approximately 3,000 per year starting in 1999 and tagged every day that bleeding operation occurs from June to November. Wako started tagging in 2008, tagged approximately 75 per year before 2012, and have tagged approximately 300 per year since 2012. No intentional sorting of animals occurred before selection for tagging, although only mature animals, as evidenced by amplexus scars in females or claspers in males, were tagged. Tag placement followed the USFWS tagging protocol. Tagged animals were treated the same as other bled animals during holding and transport.

We could not determine with certainty the bleeding status of crabs tagged by an entity other than a biomedical company. However, evidence suggests that a tagged crab is unlikely to have been bled unless it was tagged by a biomedical company. The number of crabs bled coastwide in Massachusetts, the Delaware Bay states, and South Carolina is approximately 2% of the adult population abundance only in the Delaware Bay area (0.5 million divided by 30 million in 2017; Atlantic States Marine Fisheries Commission [ASMFC], 2019), suggesting that the crabs not tagged by a biomedical company, by chance alone, were not bled. The more relevant number of crabs bled within the Delaware Bay region is much lower than the coastwide number but is considered confidential business information and not publicly available (Schmidtke et al., 2018).

A consistent minimum tagging effort was an important consideration in the selection of data for the analyses. Although >1,000 bled horseshoe crabs have been tagged in most years since 1999 within coastal Delaware to Virginia, tagging of unbled crabs within the coastal area had remained below 1,000 until 2008 (**Table 1**). In contrast, the number tagged within the Delaware Bay exceeded 5,000 several years before 2008 (**Figure 1**, **Table 1**). ASMFC defines the Delaware Bay population to include adults that spawn at least once in the Delaware Bay estuary (Atlantic States Marine Fisheries Commission [ASMFC], 2019). Because there is considerable movement between the Atlantic Ocean off the mouth of Delaware Bay, the coastal embayments in Delaware, Maryland, and Virginia, and Delaware Bay estuary (Swan, 2005; King et al., 2015; McGowan, 2018), these areas are considered to be part of the Delaware Bay population (Atlantic States Marine Fisheries Commission [ASMFC], 2019).

We proceeded with two analyses – one geographically expansive, which included tags released in Delaware Bay or coastal Delaware to Virginia from 1999 to 2017, and one geographically restrictive, which included tags released only in coastal Delaware to Virginia from 2008 to 2017. The geographically extensive analysis allowed for the most years in the comparison. The geographically restrictive analysis, which includes the regional coastline except for Delaware Bay proper, had the tightest focus on the area where animals were harvested for LAL production. Expanding the analysis to include Delaware Bay and coastal Delaware to Virginia areas from 1999 to 2017 provided a sample of 174,936 animals tagged with sex and bleeding status: 33,883 unbled females, 32,310 bled females, 72,216 unbled males, 36,098 bled males, and 429 with unknown sex. The disposition of recaptures included alive (75%), dead (16%), and unknown (9%) status. Among the bled animals, 1,283 (4%) females and 2,217 (6%) males were recaptured at least once; among the unbled animals, 2,525 (7%) females and 7,217 (10%) males were recaptured at least once. Restricting the analysis to coastal Delaware to Virginia from 2008 to 2017 provided a sample of 63,903 animals tagged with sex and bleeding status: 7,194 unbled females, 18,454, bled females, 17,311 unbled males, 20,846 bled males, and 98 with unknown sex. Among the bled animals, 752 (4%) females and 1,186 (6%) males were recaptured at least once; among the unbled animals, 287 (4%) females and 1,144 (7%) males were recaptured at least once. We used both mark-recapture modeling and relative risk estimation to infer bleeding effects on survival and recapture.

TABLE 1 | Tag releases within coastal Delaware to Virginia and Delaware Bay from 1999 to 2017.

Year	Coastal Delaware to Virginia		Delaware Bay
	Unbled	Bled	Unbled
1999	975	2500	0
2000	1	2500	0
2001	350	2500	4434
2002	175	2499	5139
2003	307	0	19432
2004	579	3122	10308
2005	192	5496	9170
2006	101	5000	143
2007	301	5596	525
2008	7728	5571	51
2009	543	4178	546
2010	378	5035	1976
2011	1810	5034	3625
2012	5570	4303	2277
2013	2098	4715	1314
2014	1401	2837	4222
2015	1662	1913	4232
2016	1476	2723	5840
2017	1896	3032	5605

*Tags placed on horseshoe crabs after bleeding for LAL production occurred only in coastal Delaware to Virginia. For geographic reference, see **Figure 1**.*

A description of the sampling and modeling process for capture-recapture studies can help illustrate how the parameters, i.e., probabilities of survival and capture, are related to the observed tag releases and recaptures. Sampling begins when an animal is caught, tagged, released, and available for recapture in future encounters. The tagged animal confronts alternative fates (**Figure 2**, **Table 2**). The animal could survive to the next year, die, or emigrate permanently from the study area. If the animal survives, then it could be captured or not captured. Survival is the biological parameter of interest.

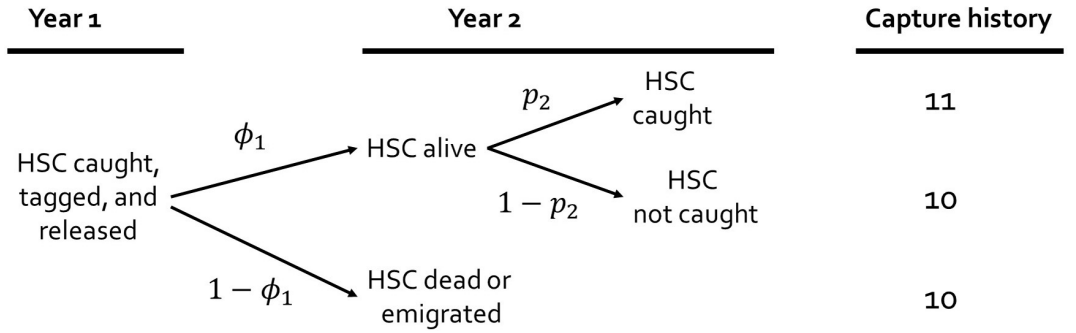

FIGURE 2 | Diagram illustrating the capture-recapture sampling process over two years. The parameters represent the apparent survival (φ) and capture probabilities (p). The capture histories represent capture by '1' and not capture by '0.'

Capture probability is a function of sampling design and effort, catchability of the species, and environmental conditions at the time of sampling. For example, most horseshoe crab tags are recaptured on the spawning beaches; thus, decreased spawning activity reduces capture probability. To estimate survival, the probability of capture must also be estimated because otherwise these parameters are confounded. That is why raw frequencies of recapture cannot be used to infer survival. Despite accounting for capture probability (p), there is an additional complication; mortality remains confounded with permanent emigration from the study area. Thus, we qualify the parameter for survival by referring to it as 'apparent survival' (φ). Because permanent emigration from the Delaware Bay population is infrequent (Swan, 2005), apparent survival closely approximates actual survival for the geographically expansive analysis.

The statistical theory and methods underlying inference from capture-recapture studies are well-developed (Pollock et al., 1990; Nichols, 1992; White and Burnham, 1999). The capture-recapture data form a capture history, where '1' indicates the animal was captured and '0' indicates the animal was not caught. Expectations for the possible capture histories are functions of the underlying probabilities for apparent survival and capture probabilities (**Table 2**). Capture probability and survival are not mechanistically correlated. Thus, low capture probability can be coincident with high survival or high capture probability with low survival because different processes determine capture as opposed to survival. The statistical approach to estimation is analogous to fitting a regression model by comparing model expectations to observed data to solve for the model that best fits the data. In the capture-recapture case, the observed frequencies of the capture histories are compared to the expected capture probabilities (**Table 2**), and the model fitting optimization estimates survival and capture using maximum likelihood methods (Pollock et al., 1990).

TABLE 2 | Example capture histories and their expected probabilities as a function of apparent survival (ϕ_i) and capture probability (p_i) for a 3-year tagging study.

Capture history	Probability
111	$\phi_1 p_2 \phi_2 p_3$
110	$\phi_1 p_2 (1 - \phi_2 p_3)$
101	$\phi_1 (1 - p_2) \phi_2 p_3$
100	$(1 - \phi_1) + \phi_1 (1 - p_2) (1 - \phi_2 p_3)$

In the capture history, each 1 or 0 represents a year when the crab was captured or not, respectively.

We fit Cormack-Jolly-Seber (CJS) models using program MARK (Pollock et al., 1990; White and Burnham, 1999) to capture histories from horseshoe crabs tagged and released in the Delaware Bay population. Models were fit using Jeff Laake's RMark v2.2.6 (R version 3.4.0, R Core Team, 2017) to manage input for and output from program MARK (White and Burnham, 1999). The CJS models estimated apparent survival and capture probability, including sex, bleeding status, and time as possible covariates using a logit link (Pollock, 2002). Different relationships between the covariates and the parameters (survival and capture probabilities) defined alternative models. For example, in one model, bleeding status affected survival, and in an alternative model, survival was unaffected by bleeding status. The covariates' significance was inferred by ranking the candidate models based on the Akaike Information Criteria (AIC) and using the minimum AIC to identify the best model (Burnham and Anderson, 1998). The AIC implemented in program MARK, denoted by AICc, is adjusted for small samples.

The models allowed for year-specific survival and capture probabilities. We found that year-specific survival was not estimable for many years based on zero or uninformative standard errors. Thus, we binned the years into periods defined by 2, 3, or 4 consecutive years and added those models to the candidate set, thereby estimating average annual survival within the binned periods. We used model selection (AICc) to determine which binning period best fit the data. The inference from all models relate to annual survival.

While the focus was on annual survival, we were also interested in capture probability because of the potential for an indirect impact on spawning behavior. Either beachcombers or spawning surveyors reported most (69%) recaptures in the USFWS database. But only spawning adults are found on the beach, and a decline in spawning activity would reduce capture probability. Mature males can migrate to

the beach exhibiting spawning behavior as individuals or in amplexus with a mature female (Brockmann et al., 2015). Thus, a change in capture probability is a reasonable proxy for a change in spawning behavior. The capture probability for unbled crabs can be considered the baseline and the ratio of capture probabilities for unbled to bled crabs can be used to indicate a potential change in spawning activity. We calculated relative risk to infer differences in capture probability between bled and unbled individuals (Agresti, 1990; Van Sickle et al., 2006). Relative risk (RR) was calculated as $RR = {p_u}/{p_b}$, where capture probability for unbled crabs (p_u) was divided by capture probability for bled crabs (p_b) controlling for same year and sex. The variance of relative risk was $var\,(RR) = var\,(p_u) + var\,(p_b) - 2var\,(p_u)\,var\,(p_b)\,corr\,(p_u, p_b)$, and approximate 95% confidence interval (CI) was $RR \pm 2\sqrt{var\,(RR)}$. A CI for RR entirely above 1 implies capture probability was significantly lower for bled than unbled; a CI entirely below 1 implies capture probability was significantly higher for bled than unbled; and a CI that includes 1 implies capture probability was not different for bled and unbled animals.

Our analytical approach using tag and relative risk analyses controlled for sex, time, and geographic area while comparing survival and capture probability between bled and unbled crabs. Age would have been a logical covariate because adult survival declines with age; for example, the probability of stranding increases by age class (Smith et al., 2010), and stranding is the primary source of natural mortality among adults (Botton and Loveland, 1989). However, information on age was not available consistently enough to be included as a covariate. Age cannot be determined directly for horseshoe crabs, but age can be classified based on shell condition (Shuster, 2009). Thus, we compared age-class distribution across bleeding status for the subset of years when tagging programs recorded age class.

RESULTS

For the geographic-expansive analysis covering the Delaware Bay population from 1999 to 2017, the best models included covariate effects on survival due to sex, bleeding, and time (**Table 3**). The top model with the most empirical support (i.e., with the minimum AICc) included interactive effects on apparent survival from sex, bleeding status, and time binned into 3-year periods. The ΔAICc for the 2nd best model was 40.20, indicating the top model fits the best among the models considered.

Survival differed by bleeding status (bled vs. unbled) in some years, but not consistently (**Table 4**, **Figure 3**). The point estimates for survival among males tended to be higher for bled crabs than for unbled crabs, but the pattern was mixed among females. The average survival was 0.69 and 0.71 for unbled males and females, respectively, and 0.72 and 0.68 for bled males and females.

For the analysis restricted to the coastal area from 2008 to 2017, the best models included covariates effects on survival due to bleeding and time, but not sex (**Table 5**). The top model with the most empirical support (i.e., with the minimum AICc) included bleeding status and time binned into 2-year periods. The

TABLE 3 | Statistics for the top 6 candidate models fit to the capture histories for horseshoe crabs tagged in the Delaware Bay population (Delaware Bay and coastal Delaware to Virginia, cf **Figure 1**) from 1999 to 2017.

Model	Number of parameters	AICc	ΔAICc	AIC weight
Phi(∼sex * bled * timebin3) *p*(∼sex * bled * time)	144	136288.55	0.00	1.00
Phi(∼sex * timebin3) *p*(∼sex * bled * time)	126	136328.75	40.20	0.00
Phi(∼bled * timebin3) *p*(∼sex * bled * time)	120	136333.80	45.25	0.00
Phi(∼sex + bled + timebin3) *p*(∼sex * bled * time)	117	136375.46	86.90	0.00
Phi(∼bled + timebin3) *p*(∼sex * bled * time)	115	136380.77	92.22	0.00
Phi(∼sex * bled * timebin2) *p*(∼sex * bled * time)	162	136392.11	103.55	0.00

Phi (ϕ) denotes apparent survival, and p denotes capture probability. The covariates are listed parenthetically with the parameter that they modify. An asterisk indicates that the covariates' effects are interactive, and a plus means the effects are additive. Covariates are sex (male, female), bled (yes, no), time (year-specific), timebin2 (2 -year periods), and timebin3 (3-year periods). AICc is the small-sample adjusted version of Akaike Information Criteria. ΔAICc is the model AICc minus the minimum AICc from the candidate model set. The AIC weight is $e^{-0.5*\Delta AICc}$ for the candidate model in question divided by the sum of that quantity for all candidate models.

ΔAICc for the 2nd best model was 6.86 indicating the top model fit best of the models considered. Estimates of annual survival were higher for bled than unbled crabs, although confidence intervals overlapped in most years (**Table 6**; **Figure 4**). Survival declined steeply in the last period (2014 to 2016) for both bled and unbled crabs (**Figure 4**). The average survival was 0.63 and 0.75 for unbled and bled animals excluding the last period, which could be an anomaly, and 0.51 and 0.68 for unbled and bled animals including the last period.

Although age is a potentially significant covariate of survival, the tagging database did not consistently include age class. Lonza recorded age class for 12,496 bled crabs released from 1999 to 2002 and in 2004. Age class was recorded sporadically for 41,562 unbled crabs: 36,138 released in Delaware Bay between 2003 and 2005, 4,811 released in coastal Delaware to Virginia between 2013 and 2017, and 613 released over years and areas. Nevertheless, a composite view of age distributions of tagged crabs indicates selection for young adults among bled crabs (**Figure 5**).

Capture probability tended to be higher for unbled than bled crabs, but the difference depended on sex (**Tables 7**, 8). For females in the Delaware Bay population, the relative risk was significantly above 1 in about half (56%) of the years (**Table 7**). For males in the Delaware Bay population, relative risk exceeded 1 in all years. For females in coastal Delaware to Virginia, relative risk trended from being below 1 in 2009–2011 and not different from 1 in 2012 to remaining above 1 in 2013–2016 (**Table 8**). For males in coastal Delaware to Virginia, the relative risk was significantly >1 except for one year (2010) early in the time series. Capture probability was high for unbled crabs in 2016 and 2017, particularly in coastal Delaware to Virginia (**Table 8**), corresponding to the years with low survival (**Figure 4**).

TABLE 4 | Estimates of apparent survival, standard error (SE), and 95% confidence limits (LCL, UCL) for the top model for tagged horseshoe crabs released in Delaware Bay population (Delaware Bay and coastal Delaware to Virginia regions, cf **Table 3** and **Figure 1**) from 1999 to 2017.

Sex	Middle year of period	Unbled				Bled			
		Apparent survival	SE	LCL	UCL	Apparent survival	SE	LCL	UCL
F	2000	0.99	7.30E-06	0.004	1.000	0.85	0.054	0.713	0.929
	2003	0.68	0.032	0.611	0.738	0.68	0.043	0.591	0.759
	2006	0.68	0.049	0.574	0.765	0.57	0.022	0.531	0.616
	2009	0.71	0.045	0.615	0.791	0.79	0.036	0.714	0.853
	2012	0.65	0.042	0.565	0.729	0.80	0.066	0.643	0.903
	2015	0.53	0.114	0.3163	0.736	0.36	0.109	0.184	0.589
M	2000	1.00	0.000	0.000	1.000	0.89	0.030	0.813	0.934
	2003	0.69	0.013	0.660	0.710	0.74	0.031	0.675	0.797
	2006	0.70	0.017	0.661	0.729	0.70	0.022	0.660	0.746
	2009	0.65	0.016	0.617	0.679	0.73	0.024	0.680	0.773
	2012	0.73	0.020	0.690	0.769	0.83	0.043	0.729	0.901
	2015	0.37	0.034	0.306	0.437	0.45	0.093	0.285	0.634

The interaction among covariates indicated that survival was related to bleeding status, sex, and year. In the top model, time was binned into 3-year periods.

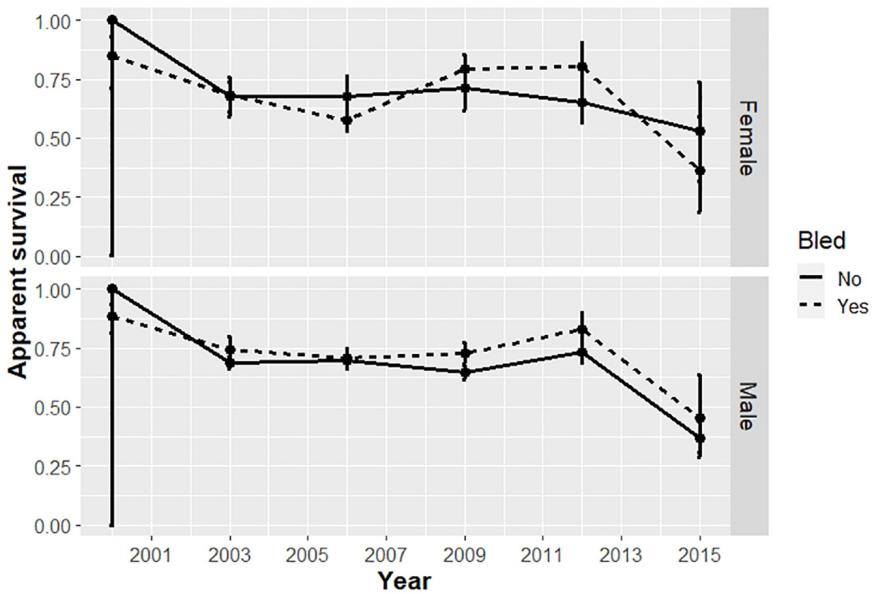

FIGURE 3 | Apparent survival of horseshoe crabs tagged and released in the Delaware Bay population, including Delaware Bay and coastal Delaware to Virginia areas (cf **Figure 1**) from 1999 to 2017. Estimates with 95% confidence intervals from the candidate model with minimum AICc, which binned time into 3-year periods, are plotted for adult males and females.

DISCUSSION

Short-term mortality and behavioral effects due to bleeding for LAL production have been well-established (Rudloe, 1983; Kurz and James-Pirri, 2002; Hurton and Berkson, 2006; Leschen and Correia, 2010; Anderson et al., 2013; Owings et al., 2019). With limited exception (Butler, 2012), long-term bleeding effects have not been studied. Until recently, tagging programs lacked enough recaptures of bled and unbled animals required to overcome the low and variable recapture rates (<10%) for tagged horseshoe crabs. The USFWS horseshoe crab tagging database, started in 1999, provides an opportunity to examine bleeding effects over years rather than weeks or months.

In our analysis of the USFWS tagging data, the working hypotheses were that – all else equal – bled crabs survive at a lower annual rate and exhibit lower capture probabilities than unbled crabs. Our results showed mixed support for these hypotheses. There was no evidence that bleeding reduced survival based on multiple years of tagging contrary to our expectations. The geographically expansive analysis revealed a mixed pattern for female survival and a slightly higher survival for bled male crabs (**Figure 3**). The analysis restricted to coastal areas revealed consistently higher survival for bled than unbled crabs

TABLE 5 | Statistics for the top 6 candidate models fit to the capture histories for horseshoe crabs tagged in coastal Delaware to Virginia (cf **Figure 1**) from 2008 to 2017.

Model	Number of parameters	AICc	ΔAICc	AIC weight
Phi(∼bled * timebin2) *p*(∼sex * bled * time)	62	35604.03	0.00	0.97
Phi(∼bled * timebin3) *p*(∼sex * bled * time)	60	35610.89	6.86	0.03
Phi(∼sex * bled * timebin2) *p*(∼sex * bled * time)	78	35626.19	22.16	0.00
Phi(∼sex * bled * timebin3) *p*(∼sex * bled * time)	72	35628.45	24.42	0.00
Phi(∼bled + timebin2) *p*(∼sex * bled * time)	59	35635.98	31.95	0.00
Phi(∼sex + bled + timebin2) *p*(∼sex * bled * time)	61	35637.32	33.29	0.00

Phi (ϕ) denotes apparent survival, and p denotes capture probability. The covariates are listed parenthetically with the parameter that they modify. An asterisk indicates that the covariates' effects are interactive, and a plus means the effects are additive. Covariates are sex (male, female), bled (yes, no), time (year-specific), timebin2 (2 -year periods), and timebin3 (3-year periods). AICc is the small-sample adjusted version of Akaike Information Criteria. ΔAICc is the model AICc minus the minimum AICc from the candidate model set. The AIC weight is $e^{-0.5*\Delta AICc}$ *for the candidate model in question divided by the sum of that quantity for all candidate models.*

(**Figure 4**). However, capture probability was lower for bled crabs especially for males based on a higher relative risk of capture for unbled crabs.

Our comparison of survival rates between unbled and bled crabs assumed that all other relevant factors were equal. We accounted for geography at the regional scale and controlled for time and sex. However, differences in geography at a subregional scale and demographic characteristics of bled versus unbled crabs could have confounded differences in survival. Releases for bled crabs were concentrated around Ocean City, Maryland, near the LAL bleeding facilities, while unbled crabs came from the broad region encompassing the Delaware Bay population (**Figure 1**). Horseshoe crabs were collected for LAL production from multiple locations mostly by trawl and released near the facility after bleeding. But tagged animals were given a year to mix with the population before recaptures were noted. Horseshoe crabs in the Delaware Bay population are mobile and highly migratory (Swan, 2005: McGowan, 2018), and behavioral effects due to bleeding occur over weeks (Owings et al., 2019), so we expect that the bled crabs mixed readily with the general population during the first year at large after release.

Demographic differences in age distribution could confound survival comparisons between bled and unbled crabs. Biomedical companies cull injured crabs and crabs with a well-worn carapace and release them without bleeding or tagging, causing the demographics of bled crabs to differ from unbled (Atlantic States Marine Fisheries Commission [ASMFC], 2011). Available data revealed that biomedical companies select for younger animals for bleeding (**Figure 5**). Using carapace wear indicative of age class (Shuster, 2009), Brockmann et al. (2015) reviewed the evidence that young males have been found to be more vigorous than older males as shown by higher activity, quicker amplexus with mature females, longer duration of amplexus, and higher sperm concentration. Stranding mortality during spawning, which is a primary source of natural mortality (Botton and Loveland, 1989), increases with age (Penn and Brockmann, 1995; Smith et al., 2010). Overall, we expect young animals to survive at a higher rate than older animals. Continued tagging of bled and unbled in the same geographic area with consistent recording of age class and sex will help further resolve how age interacts with post-bleeding mortality. Shuster (2009) outlined a protocol for determining age class, which could be used for standardized monitoring.

The numerous tag recaptures on beaches supports a change in capture probability being indicative of a change in spawning activity, and our tagging analysis affirmed a post-bleeding effect on capture probability and thus potentially on spawning activity. The effect was more apparent in males than in females. Capture probability for unbled male crabs was roughly twice that for bled male crabs (median relative risk was 2.03 and 1.60 for Delaware Bay and coastal Delaware to Virginia, respectively). The difference in capture probability between unbled and bled female crabs was smaller compared to males (median relative risk was 1.11 and 1.20 for Delaware Bay and coastal Delaware to Virginia, respectively). It was important to control for sex in the comparison between bled and unbled because males spend more time on spawning beaches, and thus have higher capture probability, than females (Smith et al., 2010). Also note that although bled crabs are released from trawls while unbled crabs are released from both trawls and beaches, the first recorded recapture is the year after release, which allows time for the tagged animals to mix and distribute with the population. Future studies may consider incorporating a temporal component to this

TABLE 6 | Estimates of apparent survival, standard error (SE), and 95% confidence limits (LCL, UCL) for the top model for tagged horseshoe crabs released in coastal Delaware to Virginia from 2008 to 2017.

	Unbled				Bled			
First year of period	Apparent survival	SE	LCL	UCL	Apparent survival	SE	LCL	UCL
2008	0.61	0.023	0.566	0.655	0.64	0.019	0.606	0.680
2011	0.68	0.046	0.585	0.764	0.83	0.038	0.747	0.896
2013	0.61	0.046	0.519	0.699	0.77	0.058	0.635	0.861
2015	0.13	0.007	0.115	0.143	0.47	0.084	0.309	0.628

The covariates indicated that survival was related to bleeding status and year. In the top model years were binned into 2-year periods with the first period covered three years.

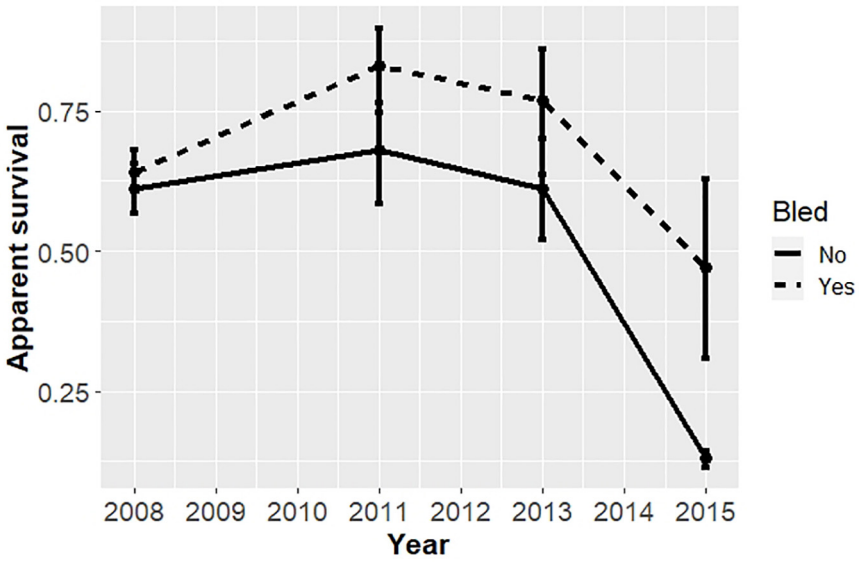

FIGURE 4 | Apparent survival of horseshoe crabs tagged and released in the coastal Delaware to Virginia areas (cf **Figure 1**) from 2008 to 2017. Estimates with 95% confidence intervals from the candidate model with minimum AICc, which binned time into 2-year periods, are plotted.

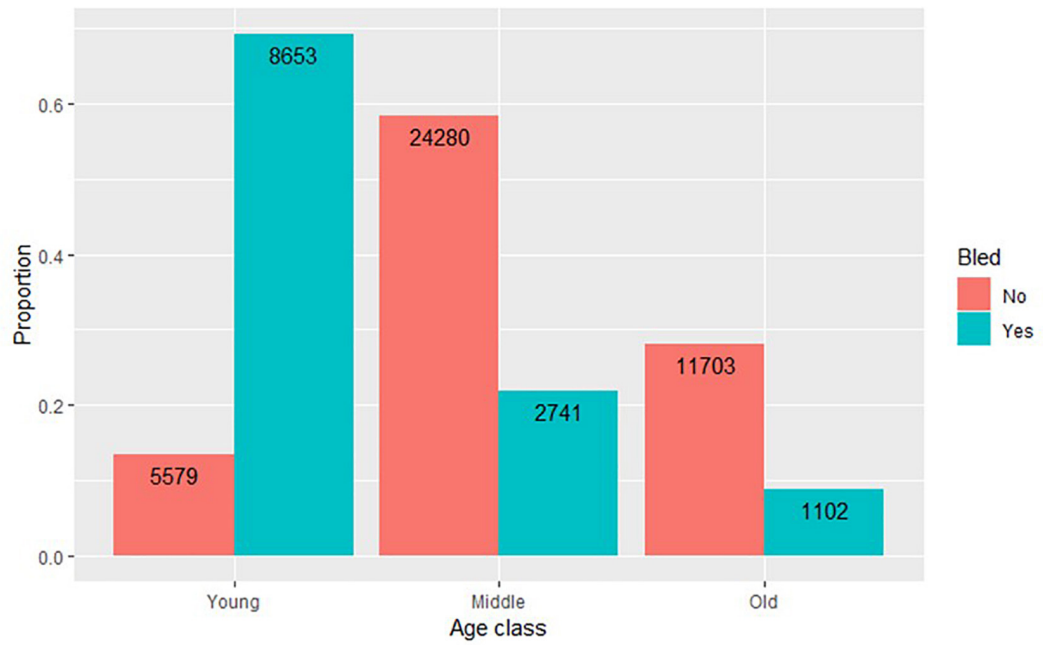

FIGURE 5 | Age distribution for tagged horseshoe crabs. Age was recorded sporadically. Bled crabs were aged only from 1999 to 2002 and in 2004. Most unbled crabs were aged in Delaware Bay from 2003 to 2005 and in coastal areas from 2013 to 2017.

assessment under the hypothesis that the effects of bleeding on spawning behavior may dissipate over time.

Although our research focused on the contrast between bled and unbled crabs, the analysis revealed a reduction in survival during recent years, which has potential management implications. The drop in survival appears for both bled and unbled crabs so the exploration of causes is outside the scope of this paper. However, potential causes include temporal changes in mortality or emigration. Alternatively, the drop could be an artifact of estimates for the last years of the study being based on less data. In part to resolve the nature and source of the apparent drop in survival in recent years, we are conducting an analysis using multistate models (Bopp et al., 2019) using data up to 2019 in relation to harvest regulations.

TABLE 7 | Capture probability (*p*) for the top model for tagged horseshoe crabs released in the Delaware Bay population (Delaware Bay and coastal Delaware to Virginia regions) from 1999 to 2017.

Sex	Year	Unbled				Bled				rho	Risk ratio (RR)	LCL RR	UCL RR
		p	SE	LCL	UCL	*p*	SE	LCL	UCL				
F	2000	0.01	0.007	0.006	0.037	0.01	0.003	0.003	0.018	0.0005	1.95	1.94	1.97
F	2001	0.00	0.004	0.001	0.025	0.00	0.002	0.002	0.011	−0.0002	0.73	0.72	0.73
F	2002	0.01	0.002	0.007	0.016	0.01	0.003	0.008	0.019	0.0002	0.82	0.81	0.83
F	2003	0.01	0.001	0.011	0.017	0.01	0.002	0.006	0.015	0.0000	1.51	1.50	1.51
F	2004	0.17	0.009	0.157	0.194	0.02	0.003	0.010	0.023	0.0003	11.54	11.52	11.56
F	2005	0.01	0.002	0.011	0.018	0.01	0.002	0.008	0.018	0.0001	1.21	1.20	1.22
F	2006	0.02	0.003	0.013	0.024	0.02	0.003	0.011	0.022	0.0001	1.14	1.13	1.14
F	2007	0.01	0.002	0.009	0.017	0.02	0.002	0.012	0.020	0.0000	0.80	0.80	0.81
F	2008	0.02	0.004	0.017	0.032	0.03	0.003	0.024	0.036	0.0001	0.80	0.79	0.81
F	2009	0.03	0.004	0.021	0.038	0.05	0.005	0.045	0.065	0.0002	0.52	0.51	0.54
F	2010	0.02	0.003	0.011	0.021	0.03	0.003	0.028	0.040	0.0001	0.45	0.44	0.46
F	2011	0.02	0.003	0.011	0.023	0.02	0.003	0.020	0.030	0.0000	0.63	0.63	0.64
F	2012	0.02	0.004	0.014	0.028	0.02	0.002	0.014	0.024	−0.0001	1.08	1.07	1.09
F	2013	0.02	0.003	0.018	0.031	0.01	0.002	0.010	0.016	0.0000	1.93	1.92	1.94
F	2014	0.02	0.004	0.017	0.032	0.01	0.002	0.009	0.017	0.0000	1.90	1.89	1.91
F	2015	0.02	0.004	0.015	0.029	0.01	0.002	0.007	0.016	0.0000	2.07	2.06	2.07
F	2016	0.03	0.007	0.019	0.047	0.02	0.005	0.011	0.034	−0.0003	1.54	1.53	1.56
F	2017	0.04	0.012	0.018	0.067	0.05	0.023	0.019	0.118	0.0004	0.72	0.67	0.77
M	2000	0.02	0.006	0.013	0.036	0.01	0.002	0.004	0.013	−0.0002	2.99	2.98	3.01
M	2001	0.02	0.005	0.009	0.031	0.01	0.002	0.007	0.015	0.0005	1.64	1.63	1.66
M	2002	0.02	0.002	0.016	0.024	0.01	0.002	0.006	0.013	0.0001	2.19	2.19	2.20
M	2003	0.02	0.002	0.015	0.023	0.01	0.002	0.009	0.017	0.0000	1.47	1.46	1.48
M	2004	0.16	0.004	0.152	0.170	0.03	0.004	0.028	0.043	0.0000	4.67	4.66	4.69
M	2005	0.05	0.002	0.041	0.050	0.02	0.003	0.015	0.025	−0.0001	2.34	2.33	2.35
M	2006	0.05	0.003	0.043	0.052	0.02	0.002	0.017	0.026	0.0001	2.29	2.28	2.29
M	2007	0.03	0.002	0.028	0.036	0.02	0.002	0.017	0.026	0.0000	1.51	1.50	1.52
M	2008	0.06	0.003	0.052	0.065	0.04	0.003	0.032	0.045	0.0000	1.53	1.52	1.54
M	2009	0.06	0.004	0.056	0.071	0.04	0.003	0.034	0.047	0.0000	1.57	1.56	1.58
M	2010	0.05	0.003	0.040	0.052	0.03	0.003	0.030	0.041	0.0000	1.31	1.30	1.31
M	2011	0.08	0.005	0.071	0.089	0.04	0.003	0.033	0.046	0.0000	2.03	2.02	2.04
M	2012	0.06	0.004	0.053	0.068	0.03	0.003	0.025	0.036	0.0000	2.03	2.02	2.03
M	2013	0.06	0.003	0.051	0.063	0.03	0.003	0.024	0.034	0.0000	1.98	1.98	1.99
M	2014	0.04	0.003	0.039	0.052	0.02	0.003	0.019	0.030	0.0000	1.88	1.87	1.89
M	2015	0.04	0.003	0.033	0.045	0.02	0.003	0.014	0.024	0.0000	2.09	2.08	2.10
M	2016	0.07	0.007	0.057	0.084	0.03	0.006	0.022	0.048	−0.0001	2.12	2.10	2.14
M	2017	0.14	0.019	0.109	0.184	0.06	0.019	0.029	0.106	−0.0002	2.54	2.49	2.59

Standard error (SE) and 95% confidence intervals (LCL, UCL) for annual estimates of p are shown for unbled and bled adults by sex (females, F, and males, M). The correlation coefficients (rho) between the unbled and bled capture probabilities are shown. Risk ratio (RR) is the ratio of capture probabilities for unbled to bled animals; 95% confidence interval limits for RR are denoted 'LCL RR' and 'UCL RR.'

The recent ASMFC stock assessment concluded that the short-term bleeding effect resulting in mortality of approximately 78,750 annually comprising a minor portion (<13%) of the up to one million annual coastwide landings dominated by harvest for bait and <1% of adult abundance in Delaware Bay (Atlantic States Marine Fisheries Commission [ASMFC], 2019). The male to female operational sex ratio (i.e., the ratio on the spawning beach) in 2017 was 5.2 in the Delaware Bay population and has increased in recent years (Atlantic States Marine Fisheries Commission [ASMFC], 2019) despite a male dominated bait harvest that greatly exceeds biomedical harvest. The ASMFC concluded that biomedical harvest has not significantly affected the Delaware Bay population's fishing mortality or dynamics.

Two effects of bleeding have management implications: (1) the effects on individual behavior and mortality and (2) the effect on population status. Effects on individual behavior and mortality are well-established but mitigated through best practices, to some extent. However, results from the tagging-data analysis and the recent stock assessment do not indicate an effect of LAL production on the Delaware Bay population's status. A population impact would require a significant effect on enough individuals relative to the population abundance. The ASMFC

TABLE 8 | Capture probability (*p*) for the top model for tagged horseshoe crabs released in coastal Delaware to Virginia from 2008 to 2017.

Sex	Year	Unbled				Bled				rho	Risk ratio (RR)	LCL RR	UCL RR
		p	SE	LCL	UCL	*p*	SE	LCL	UCL				
F	2009	0.04	0.005	0.032	0.053	0.08	0.007	0.045	0.065	0.001179	0.53	0.51	0.54
F	2010	0.02	0.004	0.010	0.026	0.05	0.005	0.028	0.040	0.000010	0.35	0.34	0.36
F	2011	0.02	0.005	0.010	0.032	0.03	0.003	0.020	0.030	0.000004	0.59	0.58	0.61
F	2012	0.02	0.005	0.013	0.032	0.02	0.002	0.014	0.024	−0.000005	0.99	0.98	1.00
F	2013	0.03	0.005	0.023	0.041	0.01	0.002	0.010	0.016	0.000000	2.59	2.58	2.60
F	2014	0.03	0.004	0.019	0.036	0.01	0.002	0.009	0.017	0.000002	2.15	2.14	2.16
F	2015	0.01	0.003	0.008	0.021	0.01	0.002	0.007	0.016	−0.000002	1.20	1.19	1.21
F	2016	0.08	0.020	0.053	0.132	0.02	0.003	0.011	0.034	−0.000007	5.53	5.49	5.57
F	2017	0.36	0.074	0.231	0.513	0.03	0.010	0.019	0.118	−0.000001	10.81	10.66	10.96
M	2009	0.09	0.006	0.082	0.107	0.06	0.006	0.034	0.047	−0.001907	1.47	1.46	1.49
M	2010	0.02	0.003	0.015	0.028	0.05	0.005	0.030	0.041	0.000000	0.44	0.43	0.45
M	2011	0.05	0.007	0.035	0.064	0.05	0.004	0.033	0.046	0.000000	1.03	1.01	1.05
M	2012	0.03	0.005	0.025	0.044	0.03	0.003	0.025	0.036	−0.000003	1.15	1.14	1.16
M	2013	0.06	0.007	0.052	0.079	0.03	0.003	0.024	0.034	0.000001	2.38	2.36	2.39
M	2014	0.04	0.005	0.031	0.048	0.02	0.003	0.019	0.030	0.000002	1.61	1.60	1.62
M	2015	0.04	0.007	0.030	0.057	0.02	0.003	0.014	0.024	−0.000007	1.96	1.94	1.97
M	2016	0.18	0.022	0.136	0.223	0.03	0.006	0.022	0.048	−0.000002	5.34	5.29	5.38
M	2017	1.00	0.000	1.000	1.000	0.06	0.017	0.029	0.106	0.000001	17.11	17.07	17.14

Standard error (SE) and 95% confidence interval limits (LCL, UCL) for annual estimates of p are shown for unbled and bled adults by sex (females, F, and males, M). The correlation coefficients (rho) between the unbled and bled capture probabilities are shown. Risk ratio (RR) is the ratio of capture probabilities for unbled to bled animals; 95% confidence interval limits for RR are denoted 'LCL RR' and 'UCL RR.'

stock assessment found that Limulus's biomedical harvest had not limited population growth or abundance in the Delaware Bay (Atlantic States Marine Fisheries Commission [ASMFC], 2019). Adult horseshoe crab abundance in the Delaware Bay area in 2017 was 30,852,000 comprising 8,665,000 females and 22,187,000 males (Hata and Hallerman, 2018). By comparison, bait landings from the Delaware Bay population were 339,976 (10,136 females and 329,840 males), which is only 1.1% (0.1% females and 1.5% males) of population abundance (Atlantic States Marine Fisheries Commission [ASMFC], 2019). The coastwide mortality from biomedical harvest from Massachusetts, the mid-Atlantic states, and South Carolina in 2017 was 78,750 (<13% of coastwide bait landings), and biomedical mortality from Delaware Bay would be fewer (Atlantic States Marine Fisheries Commission [ASMFC], 2019). Thus, ASMFC's findings are not surprising given that the total harvest was less than 1.4% of abundance and biomedical mortality is a minor fraction of total harvest.

Our analysis detected a reduced relative risk for bled crabs to be recaptured, which could indicate reduced spawning activity and, in turn, reduced population productivity. An effect at the population level would require sufficient numbers relative to abundance to reduce their fecundity or spawning activity. Changes in population productivity for horseshoe crabs in the Delaware Bay area have not been detected but productivity is very difficult to measure in this species (Atlantic States Marine Fisheries Commission [ASMFC], 2019). Furthermore, current evidence reveals that the effects at the individual level are short-lived (Owings et al., 2019) or, in the long term, modest (based on this tagging analysis), and the number affected is small relative to the population size (Atlantic States Marine Fisheries Commission [ASMFC], 2019). Thus far, the evidence does not support that LAL-production has impacted the Delaware Bay population status. However, continued monitoring is warranted as the demand for LAL might increase.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

All co-authors approved the publication and contributed to the concept and design of the analysis and the writing. DS and JN conducted the analyses. DS was the lead writer. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2020.607668/full#supplementary-material

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The *hsp40* Gene Family in Japanese Flounder: Identification, Phylogenetic Relationships, Molecular Evolution Analysis, and Expression Patterns

*Weijie Yan[1], Yingjie Qiao[1], Jiangbo Qu[1], Xiumei Liu[2], Quanqi Zhang[1] and Xubo Wang[1]**

[1] *Key Laboratory of Marine Genetics and Breeding, Ministry of Education, Ocean University of China, Qingdao, China,*
[2] *College of Life Sciences, Yantai University, Yantai, China*

Heat shock proteins (hsps) are cellular chaperones that are involved in developmental stages and stress responses. Hsp40 is the major subfamily of hsps, but has not been fully characterized in Japanese flounder (*Paralichthys olivaceus*), especially their roles in immune response. In this study, a comprehensive identification and analysis of hsp40 in flounder is presented, including gene structures, evolutionary relationships, conserved domains, molecular evolution analysis, and expression patterns. Sequence features and phylogenetic analysis revealed that *hsp40* genes could be grouped into 40 distinct subfamilies and most of them (96%) in Japanese flounder possessed no less than two introns. Molecular evolution analysis indicated that the *hsp40* genes were conservative during evolution and were functional-constrained. Meanwhile, *hsp40* genes were found to express in different embryonic and larval stages and might play the role of sentinel in healthy organisms. Furthermore, *hsp40* genes' expression profiles after *Edwardsiella tarda* injection were determined in Japanese flounder without precedent, and 88% (44/50) of *hsp40* genes showed differential expression patterns after bacterial challenge. Our findings provide basic and useful resources for understanding the immune responsibilities of *hsp40* genes in flatfish.

Keywords: heat shock protein, Hsp40, Japanese flounder, *Edwardsiella tarda*, immune response

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****Correspondence:***
Xubo Wang
wangxubo@ouc.edu.cn

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INTRODUCTION

In fisheries, fish are often affected by a variety of stresses during growth and development, including abiotic and biotic stresses such as poor water quality, thermal stress, environmental pollution, osmotic pressure, as well as bacterial and viral infections, which may influence the balance between fish and the environment and cause the stress responses of fish (Chen et al., 2010; Roberts et al., 2010; Zhang et al., 2011; Eissa, 2014; Eissa et al., 2018; Lee et al., 2018; Wen et al., 2019; Xu Z. N. et al., 2019). Over the course of long-term evolution, fish proceed changes in reaction to a variety of stressors, which are named the "general adaptation syndrome" (GAS) (Pickering, 1998). "Cellular stress response" is one feature of GAS that has been little studied.

Heat shock proteins (hsps), first reported in *Drosophila* under heat stress, are a superfamily of stress proteins expressed ubiquitously in most species from bacteria to human beings (Ritossa, 1962; Whitley et al., 1999). Although first discovered in response to thermal stress, hsps were also

found to participate in a great number of stress conditions including radiation (UV), heavy metals, pesticides, hypoxia, oxygen radicals, anti-inflammatory drugs, malignant transformation, bacterial, and viral infection (Fuller et al., 1994; Sørensen et al., 2003; Gehrmann et al., 2004; Multhoff, 2006; Akira et al., 2008). Based on the molecular weight, sequence homology, and domain structures, the hsp superfamily can be classified into several subfamilies, including Hsp90, Hsp70/Hsp110, Hsp10/Hsp60, Hsp40, and Hsp20 (sHsp) families (Gething, 1997).

Hsp40 proteins (also referred to DnaJ proteins) constitute one of the largest subfamilies among the hsp superfamily. Each member of hsp40 proteins contains the J domain (JD), a 70-amino-acid domain with similarity to the initial 73 amino acids of the *Escherichia coli* hsp40 (Georgopoulos et al., 1980; Zylicz et al., 1985). The conserved JD is necessary for hsp40 to bind to hsp70 and regulate the ATPase activity of hsp70 proteins (Ohtsuka and Hata, 2000; Qiu et al., 2006; Li et al., 2009). Except for JD, members of *E. coli* hsp40 proteins typically have three other distinct regions: glycine/phenylalanine-rich region (G/F domain), cysteine-rich region (CRR domain), and variable C-terminal domain (CTD) (Bork et al., 1992; Hennessy et al., 2005; Qiu et al., 2006). Based on the homology of hsp40 proteins of *E. coli*, DnaJ proteins are divided into three categories: Type I DnaJ proteins (DnaJA) possess all four regions of DnaJ protein in *E. coli*; Type II hsp40 proteins (DnaJB) lack the CRR domain; and Type III hsp40 proteins (DnaJC) only possess the JD, which is not necessarily located at N-terminus of the protein (Cheetham and Caplan, 1998; Kampinga et al., 2009). The concept of type IV DnaJ protein family was raised, which owns a "J-like" domain (Walsh et al., 2004; Botha et al., 2007; Morahan et al., 2011) containing a wide range of mutations in a highly conserved histidine, proline, and aspartic acid–HPD motif located between helices II and III in the DnaJ domain (Tsai and Douglas, 1996; Mayer et al., 1999; Hennessy et al., 2000). Cooperating with hsp70, another molecular chaperone that couples the cycles of ATP binding, hydrolysis, and ADP release, the hsp40 proteins are involved in numerous cellular functions, including regulation of protein folding, translocation, and assembly (Cheetham and Caplan, 1998; Ohtsuka and Hata, 2000).

Except for the traditional functions, previous studies suggested that hsps may actually play important roles in immune reactions (Srivastava, 2002; Roberts et al., 2010). Hsps took part in humoral and cellular responses in innate immunity (Sung and MacRae, 2011) and also played a role as the danger signal *in vitro* to communicate innate immune responses by activating various cells (Chen et al., 1999; Kol et al., 2000; Singh-Jasuja et al., 2000). Besides, hsps can induce a variety of cytokines including interleukin-12, nitric oxide, tumor necrosis-a, interleukin-1β, and several chemokines (Basu et al., 2000; Lehner et al., 2000; Moré et al., 2001; Panjwani et al., 2002). Additionally, in adaptive immunity, hsps can also function as powerful danger signals and antigen carriers. For example, Hsp60, Hsp70, and Hsp90 act as ligands for a number of clusters of differentiation and cell-surface receptors (Ohashi et al., 2000; Basu et al., 2001; Vabulas et al., 2001; Habich et al., 2002). Furthermore, the increase of *hsp* gene expressions induced by stressors influences the immune resistance of aquatic animals (Wilhelm et al., 2005; Sung and MacRae, 2011). However, little is known about the participation of *hsp40* genes in the immune reactions of flatfish except the differential expression of three *hsp40* genes in the embryonic cells of Japanese flounder after virus infection (Dong et al., 2006).

Because of various advantages such as rapid growth rate and delicious taste, Japanese flounder is a high-value flatfish in Asian countries including China, Japan, and Korea (Fuji et al., 2006). Nevertheless, the development of industrial farming has caused the Japanese flounder's susceptibility to various pathogens like bacteria, parasites, and viruses, resulting in numerous diseases that are infectious and severe losses in aquaculture recently (Isshiki et al., 2001; Moustafa et al., 2010). Edwardsiellosis is a serious illness caused by *Edwardsiella tarda*, which has led to sizable economic losses all over the world in aquaculture (Hoshina, 1962; Meyer and Bullock, 1973; Yasunaga, 1982; Bang et al., 1992; Nougayrede et al., 1994). Japanese flounder that suffers from Edwardsiellosis often shows various symptoms including a swollen abdomen, pigmentation loss, dermal damages, and spiral movement; as a result, Edwardsiellosis has affected the flounder breeding industry tempestuously (Bang et al., 1992; Moon et al., 2014). Recently, immune responses in different organs of Japanese flounder after *E. tarda* affection have been reported (Takano et al., 2006; Taechavasonyoo et al., 2013; Li et al., 2014; Liu et al., 2017; Thanasaksiri et al., 2017).

Up to now, the systematic efforts to reveal the roles of *hsps* in economically important fish' immune reactions have not been completed. In order to avoid commercial losses caused by *E. tarda* in aquaculture, more in-depth comprehension about the involvement of *hsps* in the resistance to *E. tarda* is of great importance. Since the first discovery of *hsp40* in bacteria, thousands of *hsp40* genes have been identified in prokaryotes and eukaryotes (Yochem et al., 1978; Song et al., 2014; Chen T. et al., 2018; Huang et al., 2018; Xu Y. et al., 2019). Previous researches illustrated that the expression of several *hsp* subfamilies from Japanese flounder could be affected after the infection of several pathogens, e.g., *Streptococcus parauberis* (Dong et al., 2006; Chen et al., 2010; Sung and MacRae, 2011; Cha et al., 2013; Wei et al., 2013). Nevertheless, with respect to the roles of *hsp40* genes in the disease resistance in Japanese flounder, extensive understanding is lacking. In this study, we conducted the genome-wide characterization of 50 *hsp40* genes, including sequence information, selective pressures, and phylogenies. We also determined their expression profiles in embryonic and larval stages in *E. tarda*-infected and healthy tissues, to give early reference about the characteristics of *hsp40* genes in the immune reaction in Japanese flounder.

MATERIALS AND METHODS

Identification of *hsp40* Family Members in Flounder Genome

To identify the *hsp40* genes, the whole genome database of Japanese flounder (NCBI accession number MPLB00000000.1) was searched using available *hsp40* sequences from teleosts [zebrafish (*Danio rerio*), medaka (*Oryzias latipes*), tilapia

(*Oreochromis niloticus*), channel catfish (*Ictalurus punetaus*), yellow catfish (*Tachysurus fulvidraco*), spotted gar (*Lepisosteus oculatus*), and fugu (*Takifugu rubripes*)] from the Heat Shock Protein Database Information Resource (Sinha et al., 2012). The *E*-value was set at 1e-10 to acquire as many candidate *hsp40* genes as possible. Redundant sequences were removed. Afterward, Pfam (El-Gebali et al., 2018) and SMART (Letunic and Bork, 2017) databases were applied to confirm the conserved *hsp40* domain, namely, DnaJ_CXXCXGXG (PF00684), DnaJ_C (PF01556), DnaJ (PF00226), and Pam16 (PF03656), and sequences without any *hsp40* domain were excluded. Newly identified *hsp40* genes of flounder were named following Zebrafish Nomenclature Guidelines and the Guidelines for the nomenclature of the human hsps (Kampinga et al., 2009), and the genes were named after the zebrafish orthologs whenever possible (**Table 1**).

Phylogenetic Tree Conduction of *hsp40* Gene Families

The amino acid sequences of hsps of seven teleosts above and Japanese flounder were used for phylogenetic analysis. The MEGA7 (Kumar et al., 2016) software was used to construct a phylogenetic tree, using parameters of the WAG model and the maximum-likelihood method. Further, we applied the Evolview (Zhang et al., 2012) to visualize the tree.

Sequence Structure and Motif Prediction

We utilized the MEME program (Bailey et al., 2009) to evaluate the motifs in the hsp40 sequences; the parameters were as follows: any number of repetitions, maximum of eight motifs, and an optimum motif width of 6–200 amino acid residues. Then, we used the Gene Structure Display Server (GSDS[1]) to identify exon–intron organizations of the *hsp40* genes from Japanese flounder. Then, TBtools software was applied to construct a diagrammatic sketch (Chen C. et al., 2018). Finally, the ProtParam Tool (Walker, 2005) predicted the biophysical properties of each hsp40 protein.

Molecular Evolution Analysis

To investigate the selective pressure of *hsp40* genes, the relative rates of non-synonymous substitutions (dN) and synonymous substitutions (dS) were used to represent the natural selective pressure of eight different teleosts above (Kryazhimskiy and Plotkin, 2008). According to previous research, a dN/dS ratio of greater than 1 forecasts positive selection and less than 1 forecasts negative selective pressure, whereas a ratio equal to 1 hints neutral selection (Nei and Gojobori, 1986). We applied the ClustalW for codon-based alignment and removed the terminator. Then, the single likelihood ancestor counting (SLAC) method (Kosakovsky Pond and Frost, 2005) in Datamonkey (Delport et al., 2010) was utilized to predict selective pressure on individual codons (sites) within the CDS of the *hsp40* genes. SLAC is good at exploring non-neutral evolution in over 50 sequences, using likelihood-based branch lengths, nucleotide and codon substitution parameters, and ancestral sequence reconstructions (Pond and Frost, 2005).

Subcellular Localization and Secondary Structure Analysis of hsp40 Proteins

The secondary structure of *hsp40* genes in Japanese flounder was predicted by SOPMA (Geourjon and Deleage, 1995) with the following parameters: output width, 70; number of conformational states, four (helix, sheet, turn, and coil); similarity threshold, 8; and window width, 17. The subcellular localization was performed using the amino acid sequences of hsp40 proteins by PSORT (protein subcellular localization prediction tool) (Nakai, 1999).

Expression Profiles in Developmental Stages, Challenged and Unchallenged Tissues

We analyzed the expression profiles of *hsp40* genes in unchallenged Japanese flounder tissues and *E. tarda*-infected Japanese flounder, namely, 11 tissues (heart, spleen, liver, kidney, intestines, muscle, brain, gill, stomach, testis, and ovary) and six embryonic and larval stages (stages 1–6) (NCBI accession number: SRX500343, PRJNA319595).

To analyze the expression levels of *hsp40* mRNA in Japanese flounder blood, gill, and kidney samples during *E. tarda* infection, we applied the RNA-seq data from previous research of our lab (Liu et al., 2017; Li et al., 2018a,b). The individuals for infection provided by the Yellow Sea Aquatic Product Co. Ltd. in China were approximately 1 year old with an average body length of 16.3 ± 1.5 cm (mean ± SD) and an average weight of 70.5 ± 7.9 g (mean ± SD). They were acclimatized in aerated seawater at 19°C for 7 days before injection. The *E. tarda* strain EIB202 was acquired from the Key Laboratory of Microbial Oceanography, Ocean University of China. It was a chloramphenicol, tetracycline, rifampicin, and streptomycin strain isolated from an outbreak in farmed turbot in Shandong province of China (Xiao et al., 2008; Wang et al., 2009). We did not detect pathogenic *E. tarda* from the Japanese flounder before the experiment utilizing primers based on the specific esaV gene of pathogenic pathogen (Tan et al., 2005). We incubated the *E. tarda* strain in Luria–Bertani (LB) medium to mid-logarithmic stage at 28°C and then harvested it by centrifugation and re-suspended it to a final concentration of 2×10^7 colony-forming units (CFU) ml^{-1} in Ringer's solution.

Before the formal injection, we performed two pre-experiments to confirm that the injections did make healthy individuals sick and even die. After we validated that *E. tarda* infections were virulent, the formal injections were performed. The Japanese flounder was randomly spilt into three groups: 60 individuals as bacteria-challenge experiment group (BCEG), 60 in the Ringer's solution control group (RSCG), and 10 as the blank control group (BCG). Japanese flounder in BCEG was injected intraperitoneally with 1 ml of the abovementioned pathogen suspension. Additionally, RSCG individuals were injected with the same dosage of Ringer's solution, and individuals in BCG have not been injected. Then, their kidney,

[1]http://gsds.cbi.pku.edu.cn/

TABLE 1 | Comparison of copy numbers of *hsp40* genes among selected teleosts genomes.

Species	*Ictalurus punetaus*	*Tachysurus fulvidraco*	*Danio rerio*	*Oryzias latipes*	*Takifugu rubripes*	*Oreochromis niloticus*	*Paralichthys olivaceus*	*Lepisosteus oculatus*	Total
dnaja1	3	2	1	2	1	1	2	2	14
dnaja2	2	2	2	2	2	2	1	1	14
dnaja3	2	1	3	2	2	2	1	1	14
dnajb1	3	3	2	3	3	3	2	2	21
dnajb3	1	1	1	1	1	1	1	1	8
dnajb4	1	1	1	1	1	1	1	0	7
dnajb5	2	2	2	2	3	2	2	1	16
dnajb6	2	2	1	2	2	2	2	1	14
dnajb9	2	1	2	2	2	3	2	2	16
dnajb11	2	1	1	0	1	1	1	0	7
dnajb12	2	2	2	2	2	2	2	1	15
dnajb14	1	1	1	1	1	1	0	1	7
dnajc1	1	1	1	1	1	1	0	0	6
dnajc2	1	1	1	1	1	1	1	0	7
dnajc3	2	2	2	2	2	2	2	1	15
dnajc4	1	1	1	1	1	1	1	0	7
dnajc5	4	4	4	4	5	4	2	2	29
dnajc6	1	1	2	1	1	1	1	1	9
dnajc7	0	1	1	2	2	2	2	0	10
dnajc8	0	1	1	1	1	1	1	1	7
dnajc9	1	1	1	1	1	1	1	1	8
dnajc10	1	1	1	1	1	1	1	1	8
dnajc11	0	2	2	2	2	2	2	1	13
dnajc12	1	1	1	1	1	1	1	1	8
dnajc14	0	1	1	1	1	1	1	0	6
dnajc15	1	1	2	1	0	1	1	1	8
dnajc16	2	2	2	2	2	2	2	0	14
dnajc17	0	1	1	1	1	1	1	1	7
dnajc18	1	1	1	1	1	2	1	1	9
dnajc19	2	2	1	1	1	1	1	1	10
dnajc21	1	1	1	1	1	1	1	0	7
dnajc22	1	1	1	1	1	1	1	1	8
dnajc24	1	1	1	1	1	3	1	1	10
dnajc25	1	1	1	1	1	1	1	1	8
dnajc27	1	0	1	1	1	1	1	0	6
dnajc30	1	1	2	2	2	2	2	0	12
dnajgak	0	1	2	1	1	1	1	1	8
dnajpam16	0	1	1	2	1	2	1	1	9
dnajsec63	1	1	1	1	1	1	1	1	8
hscb	1	1	1	1	1	1	1	1	8
Total	50	53	57	57	57	61	50	33	418

gill, and blood samples were extracted and stored in liquid nitrogen until RNA extraction. Three time points (0, 8, and 48 h) were chosen to sample collection at each group: BCG for 0 h (Bl-BC), BCEG for 8 h (Bl-8hE), RSCG for 8 h (Bl-8hC), BCEG for 48 h (Bl-48hE), and RSCG for 48 h (Bl-48hC). We have set the necessary biological duplication, and equal molar ratios of two individuals' RNA were pooled as one replicate for further study. Sequencing libraries were constructed using NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (#E7530L, NEB, United States) following the manufacturer's recommendations, and index codes were added to attribute sequences to each sample. Raw reads were cleaned by removing adaptor sequences, low-quality sequences (Sanger base quality < 20), and reads with unknown nucleotides larger than 10%. The TopHat–Cufflinks–Cuffmerge–Cuffdiff pipeline was used to analyze the clean data by using default parameters, and then the parameter fragments per kilobase of transcript per million mapped reads (FPKM) was used to quantify the abundance of assembled transcripts (Trapnell et al., 2012; Ghosh and Chan, 2016; Li et al., 2018b). Thirty sequencing libraries from four groups were

constructed totally. The raw sequencing reads were submitted to Sequence Read Archive (SRA) in NCBI with accession numbers PRJNA359626, PRJNA359627, SRR5713071, SRR5713072, SRR5713073, SRR5713074, SRR5713075, SRR5713076, SRR5713077, SRR5713078, SRR5713079, and SRR5713080. Last, the expression levels in unchallenged (RSCG) and challenged groups (BCEG) were examined to find out further information about *hsp40* genes' differential expression in reaction to *E. tarda* injection (Liu et al., 2017; Li et al., 2018a,b). Then, we applied R package pheatmap to visualize the profiles in different tissues (Kolde, 2018).

RESULTS

Sequence Extraction From Japanese Flounder Genome

We applied 57 zebrafish *hsp40* sequences extracted from the Heat Shock Protein Database Information Resource as a query to search against the Japanese flounder genome. Afterward, SMART and Pfam databases were applied to validate the putative *hsp40* genes by the standard of hsp40 domains (PF00226, PF00684, PF01556, and PF03656). Sequences that were repetitive or lacked any hsp40 domains were deleted. As a result, 50 *hsp40* sequences (*dnaja1*, *dnaja1a*, *dnaja2*, *dnaja3*, *dnajb1*, *dnajb1a*, *dnajb3*, *dnajb4*, *dnajb5*, *dnajb5a*, *dnajb6*, *dnajb6a*, *dnajb9*, *dnajb9a*, *dnajb11*, *dnajb12*, *dnajb12a*, *dnajc2*, *dnajc3*, *dnajc3a*, *dnajc4*, *dnajc5aa*, *dnajc5ga*, *dnajc6*, *dnajc7*, *dnajc7a*, *dnajc8*, *dnajc9*, *dnajc10*, *dnajc11*, *dnajc11a*, *dnajc12*, *dnajc14*, *dnajc15*, *dnajc16*, *dnajc16a*, *dnajc17*, *dnajc18*, *dnajc19*, *dnajc21*, *dnajc22*, *dnajc24*, *dnajc25*, *dnajc27*, *dnajc30*, *dnajc30a*, *dnajgak*, *dnajpam16*, *dnajsec63*, and *hscb*) identified from the Japanese flounder genome were named based on the Guidelines for the nomenclature of the human hsps as well as the Zebrafish Nomenclature Guidelines (**Table 1**).

Phylogenetic Analysis

To evaluate the evolutionary history of the *hsp40* genes collected from eight teleosts above, a phylogenetic tree (maximum-likelihood method, WAG model) was constructed by MEGA7 (**Figure 1**). The 418 *hsp40* genes fell into 40 specific subfamilies: 14 *dnaja1*, 14 *dnaja2*, 14 *dnaja3*, 21 *dnajb1*, eight *dnajb3*, seven *dnajb4*, 16 *dnajb5*, 14 *dnajb6*, 16 *dnajb9*, seven *dnajb11*, 15 *dnajb12*, seven *dnajb14*, six *dnajc1*, seven *dnajc2*, 15 *dnajc3*, seven *dnajc4*, 29 *dnajc5*, nine *dnajc6*, 10 *dnajc7*, seven *dnajc8*, eight *dnajc9*, eight *dnajc10*, 13 *dnajc11*, eight *dnajc12*, six *dnajc14*, eight *dnajc15*, 14 *dnajc16*, seven *dnajc17*, nine *dnajc18*, 10 *dnajc19*, seven *dnajc21*, eight *dnajc22*, 10 *dnajc24*, eight *dnajc25*, six *dnajc27*, 12 *dnajc30*, eight *dnajgak*, nine *dnajpam16*, eight *dnajsec63*, and eight *hscb*. Each of the homologous *hsp40*

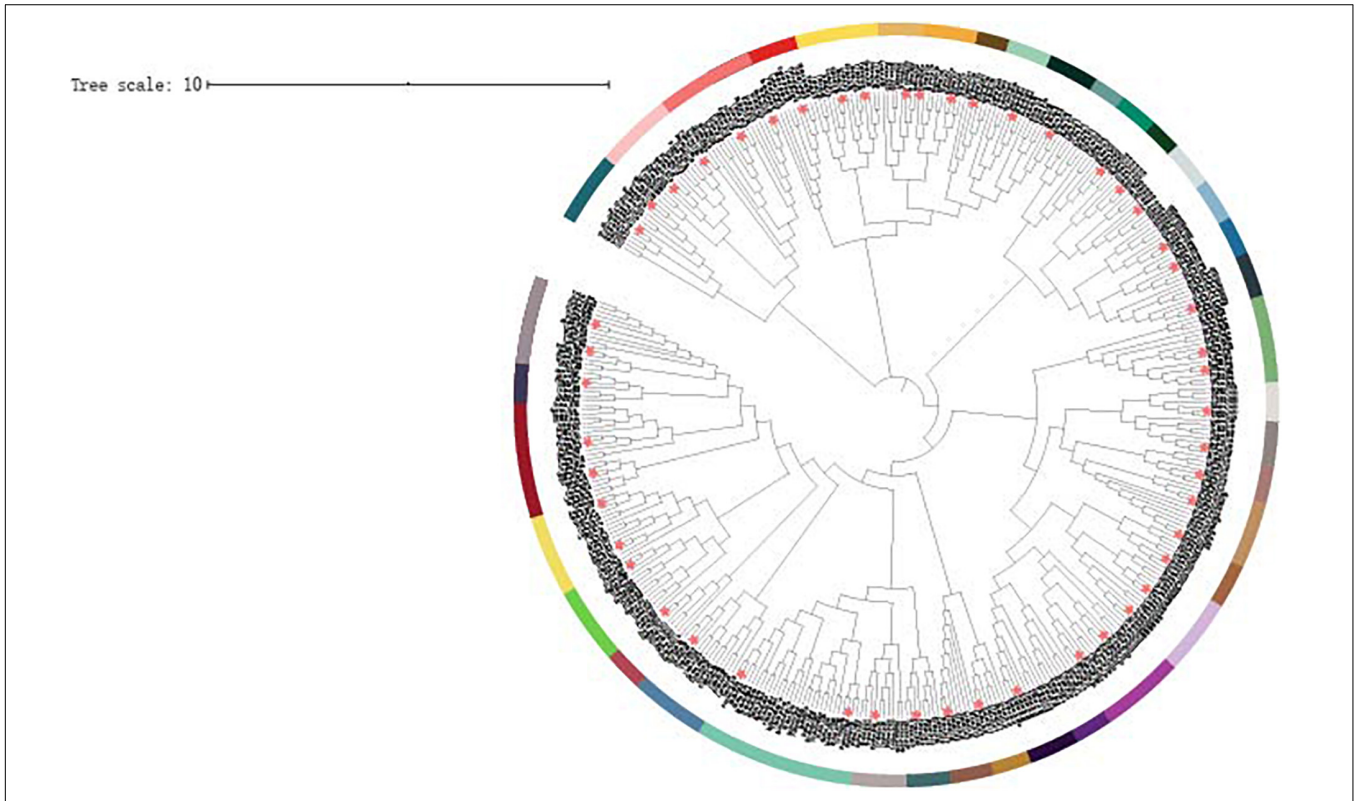

FIGURE 1 | Phylogenetic analysis of 418 *hsp40* genes constructed using ML method and WAG models. Different subfamilies were distinguished in different colors, and the Japanese flounder *hsp40* genes were asterisk labeled. Abbreviations: On, *Oreochromis niloticus*; Tr, *Takifugu rubripes*; Dr, *Danio rerio* Ol, *Oryzias latipes*; Ip, *Ictalurus punetaus*; Tf, *Tachysurus fulvidraco*; Lo, *Lepisosteus oculatus*.

genes from eight teleosts was divided into the same cluster. Furthermore, *hsp40* genes whose protein shows high similarity in structure share a close range in the phylogenetic tree.

Exon–Intron Organizations and Motif Patterns

Gene structure and motif patterns of *hsp40* genes were analyzed to illustrate their similarities and differences and provide deeper comprehension about their evolutionary relationship (**Figure 2**). As for the 50 *hsp40* genes of Japanese flounder, two (*dnajc30* and *dnajc30a)* had no introns, belonging to the "no intron" group, whereas other genes had no less than two introns and were divided into the "multiple introns" group. We identified eight evolutionary-conserved motifs from flounder *hsp40* genes. Results indicated that *hsp40* genes had diverse motif patterns, and genes with close phylogenetic

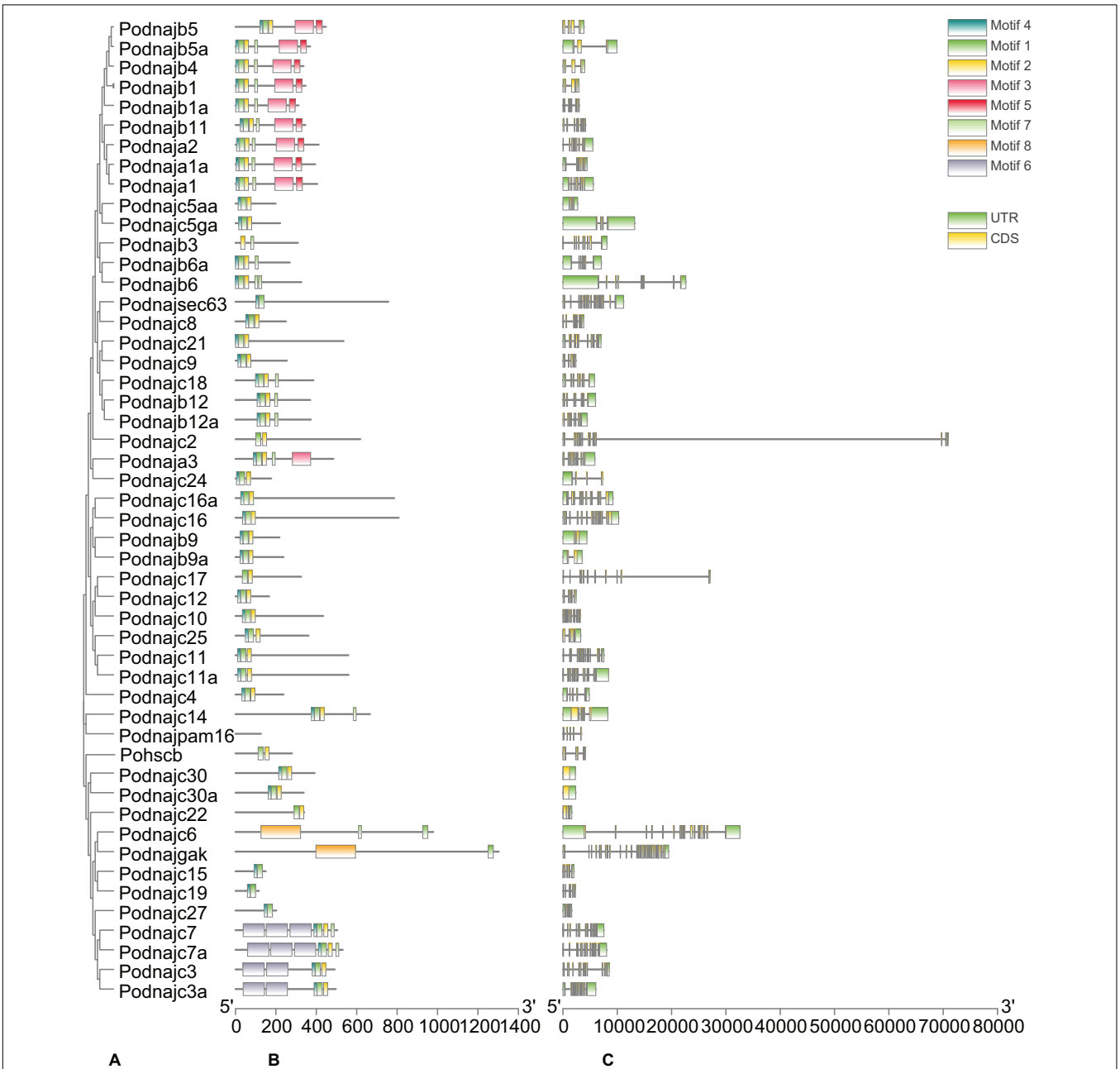

FIGURE 2 | Phylogenetic analysis, exon/intron organization, and motif analysis of *hsp40* genes. **(A)** Phylogenetic tree of 50 hsp40 proteins. The maximum-likelihood phylogenetic tree was constructed using amino acid sequences of 50 hsp40 proteins with MEGA7 software. **(B)** Distributions of conserved motifs in *hsp40* genes. Eight motifs were distinguished in different colored boxes. **(C)** Gene structure of *hsp40* genes. Yellow lines represent CDSs and green boxes with the same length represent UTRs. The length of exons can be inferred by the scale.

relationship had similar motif patterns. Additionally, the biophysical properties are listed in **Table 2**. The amino acid numbers of hsp40 proteins were with the scope of 116 (dnajc19) to 1303 (dnajgak); the molecular masses were between 12.48259 kDa (dnajc19) and 143.23479 kDa (dnajgak), and the pI values are confined from 4.89 (dnajc24) to 10.42 (dnajc30).

Molecular Evolution Analysis of the *hsp40* Genes

In order to get more information about the evolutionary history of *hsp40* gene interspecies, we used the synonymous rate to infer whether fixation of non-synonymous mutations is strengthened or weakened by selection pressure, and the coding sequences of the 50 *hsp40* genes were applied for calculating the dN/dS ratio (**Table 3**). In this study, the dN/dS ratio of all the 50 *hsp40* genes was less than 1, indicating pronounced negative selective pressure. We cannot find any positive selection sites of all the *hsp40* genes from Japanese flounder.

Subcellular Localization and Secondary Structure Analysis of hsp40 Proteins

Subcellular localization analysis showed that the *hsp40* genes of Japanese flounder were mainly expressed in the cytoplasm, nucleus, mitochondria, and endoplasmic reticulum (ER) (**Table 4**), and less expressed in peroxisome, cytoskeleton, and Golgi apparatus. In detail, most *hsp40* genes were expressed mainly in the nucleus, nine genes (*dnaja1a*, *dnaja2*, *dnajb1*, *dnajb1a*, *dnajb4*, *dnajb6*, *dnajc7*, *dnajc11*, and *dnajc27*) were mainly expressed in the cytoplasm, six genes (*dnaja3*, *dnajb9a*, *dnajc10*, *dnajc14*, *dnajc19*, and *dnajc25*) were expressed highly in mitochondria, and four genes (*dnajb11*, *dnajc16*, *dnajc16a*, and *dnajc22*) were mainly expressed in ER. The secondary structure of hsp40 proteins consisted of alpha helix, extended strand, beta turn, and random coil (**Table 4**). Among the 50 hsp40 proteins in Japanese flounder, danjb12 was alpha helix = random coil > extended strand > beta turn, dnajc9 was alpha helix > random coil > beta turn > extended strand, 19 proteins (dnajc2, dnajc3, dnajc3a, dnajc4, dnajc7, dnajc7a, dnajc8, dnajc10, dnajc15, dnajc17, dnajc19, dnajc21, dnajc22, dnajc24, dnajc25, dnajc27, dnajsec63, hscb, and dnajpam16) were alpha helix > random coil > extended strand > beta turn, and the other 29 proteins were random coil > alpha helix > extended strand > beta turn. In summary, the alpha helix and random coil were the main components of the hsp40 secondary structure.

Expression Profiles Analysis

The expression profiles of *hsp40* genes at embryo and larval developmental stages and tissues extracted from healthy individuals and after *E. tarda* injection were illustrated using our previous data. In order to visualize the expression profiles in detail, heat maps with the phylogenetic tree were constructed (**Figure 3**). Results showed that most *hsp40* genes participated in six embryonic and larval stages of Japanese flounder, while three of them (*dnajb1a*, *dnajb5*, and *dnajb12a*) were

TABLE 2 | Features of hsp40 proteins in Japanese flounder identified.

ID	NCBI accession number	Size (aa)	pI	Mw (kDa)
Podnaja2	XP_019934545.1	412	5.91	45.18112
Podnaja1a	XP_019935083.1	395	5.9	44.77599
Podnaja1	XP_019955997.1	405	8.33	45.92839
Podnajb4	XP_019963110.1	337	8.81	37.77469
Podnajb5	XP_019937162.1	448	9.48	50.09499
Podnajb1	XP_019940040.1	347	8.96	38.23021
Podnajb11	XP_019960126.1	346	5.16	39.48552
Podnajb5a	XP_019956370.1	370	8.91	41.8922
Podnajb1a	XP_019941143.1	312	8.67	35.15
Podnaja3	XP_019965729.1	485	9.32	52.23182
Podnajc5aa	XP_019962986.1	199	5.21	22.2869
Podnajb6a	XP_019967712.1	269	7.24	29.63975
Podnajb12	XP_019951913.1	370	8.93	42.16221
Podnajc18	XP_019941514.1	386	8.43	44.52194
Podnajc16a	XP_019944601.1	786	7.13	90.59462
Podnajc10	XP_019968568.1	434	5.98	49.65526
Podnajb12a	XP_019938503.1	373	9.1	42.75287
Podnajc5ga	XP_019965682.1	221	4.91	24.40564
Podnajb6	XP_019943374.1	326	8.35	36.09796
Podnajb9	XP_019934058.1	218	9.08	25.5084
Podnajc16	XP_019942216.1	808	7.21	93.06036
Podnajb9a	XP_019947865.1	238	7.4	27.77144
Podnajc21	XP_019938005.1	535	5.07	62.21628
Podnajc14	XP_019942158.1	666	6.74	74.7478
Podnajc11	XP_019963719.1	559	8.09	63.42023
Podnajc7	XP_019949855.1	504	6.28	57.59444
Podnajc7a	XP_019938771.1	531	6.72	60.31686
Podnajc11a	XP_019941868.1	560	6.98	63.12494
Podnajc9	XP_019952654.1	255	5.34	29.36504
Podnajc3	XP_019945309.1	491	6.64	56.04434
Podnajc3a	XP_019951274.1	497	5.58	56.891
Podnajc24	XP_019934316.1	177	4.89	20.54465
Podnajc4	XP_019965877.1	238	8.96	27.99151
Podnajc25	XP_019963320.1	362	8.76	43.16232
Podnajb3	XP_019945294.1	309	5.28	34.48404
Podnajc30	XP_019964323.1	392	10.42	44.95075
Podnajc27	XP_019967980.1	202	5.73	22.72074
Podnajc30a	XP_019940424.1	338	9	37.44297
Podnajc17	XP_019940273.1	325	8.94	36.936
Podnajsec63	XP_019947337.1	757	5.18	86.6702
Podnajc6	XP_019954137.1	979	7.33	104.25921
Podnajgak	XP_019954890.1	1303	5.69	143.23479
Podnajc12	XP_019945743.1	167	5.4	19.38179
Podnajc15	XP_019966627.1	150	9.8	16.22657
Podnajc19	XP_019959696.1	116	10.12	12.48259
Podnajc22	XP_019944261.1	340	8.79	38.01626
Pohscb	XP_019954647.1	280	5.87	31.79024
Podnajc8	XP_019958083.1	250	9.14	29.70666
Podnajpam16	XP_019963217.1	126	9.52	13.8867
Podnajc2	XP_019955430.1	618	6.8	71.15799

TABLE 3 | Selection pressure of *hsp40* genes (dN/dS) in teleosts.

Gene	dN/dS	No. of positive sites	No. of negative sites
dnaja1	0.135	0	131
dnaja1a	0.11	0	71
dnaja2	0.0551	0	201
dnaja3	0.148	0	179
dnajb1	0.103	0	142
dnajb1a	0.117	0	140
dnajb3	0.259	0	82
dnajb4	0.0557	0	122
dnajb5	0.0911	0	109
dnajb5a	0.139	0	70
dnajb6	0.159	0	103
dnajb6a	0.142	0	51
dnajb9	0.135	0	77
dnajb9a	0.182	0	51
dnajb11	0.0798	0	86
dnajb12	0.14	0	123
dnajb12a	0.168	0	79
dnajc2	0.1	0	174
dnajc3	0.129	0	152
dnajc3a	0.102	0	114
dnajc4	0.0506	0	194
dnajc5aa	0.0864	0	29
dnajc5ga	0.118	0	16
dnajc6	0.127	0	272
dnajc7	0.0622	0	105
dnajc7a	0.109	0	26
dnajc8	0.0809	0	89
dnajc9	0.171	0	76
dnajc10	0.144	0	270
dnajc11	0.0624	0	224
dnajc11a	0.0642	0	136
dnajc12	0.227	0	40
dnajc14	0.21	0	97
dnajc15	0.137	0	30
dnajc16	0.141	0	213
dnajc16a	0.152	0	192
dnajc17	0.182	0	85
dnajc18	0.123	0	128
dnajc19	0.0812	0	44
dnajc21	0.198	0	105
dnajc22	0.212	0	52
dnajc24	0.251	0	35
dnajc25	0.128	0	99
dnajc27	0.0185	0	82
dnajc30	0.375	0	34
dnajc30a	0.379	0	18
Dnajgak	0.117	0	349
dnajpam16	0.0991	0	45
dnajsec63	0.065	0	280
Hscb	0.329	0	60

expressed only at a certain part of developmental stages. Furthermore, there were six genes (*dnaja2*, *dnajb3*, *dnajc3a*, *dnajc4*, *dnajc5aa*, and *dnajc19*) that showed high expression levels at all stages and 12 genes (*dnaja1*, *dnaja1a*, *dnaja3*, *dnajb6a*, *dnajb9*, *dnajb11*, *dnajb12*, *dnajc8*, *dnajc9*, *dnajc10*, *dnaj16*, and *dnajc21*) had relatively high expression levels at one or several developmental stages. Under normal conditions, all *hsp40* genes were expressed to maintain homeostasis, as a reserve in case of sudden pathogen invasion. The expression of *hsp40* genes in different tissues had various patterns, and some genes showed preferential expression in certain tissues. For instance, several *hsp40* genes were highly expressed in one specific tissue, but had low expression levels or not expressed in other tissues, namely, *dnajc22* in the heart, *dnajc3a* in the gill, *dnaja3* and *dnajc21* in the muscle, *dnajb11* in the stomach, *dnajb6* and *dnajc7* in the intestines, and *dnajb5*, *dnajb12a*, *dnajc5aa*, *dnajc6*, and *dnajc11a* in the brain. Additionally, we also found that *dnajb1a* had weak expression levels in 11 tissues, which required deeper studies.

To investigate the role of *hsp40* family members in response to *E. tarda*, we analyzed the expression levels of the 50 *hsp40* genes from three tissues and three time points after *E. tarda* infection from the RNA-seq data of previous research (**Figure 4**); the summary of the comparison of Ringer's solution and bacterial-challenge group, namely, log2(fold_change) and *p*-value, is shown in **Supplementary Table 1**. In total, 41 *hsp40* genes except *dnajb1a*, *dnajb4*, *dnajb5*, *dnajb6a*, *dnajb12a*, *dnajc5aa*, *dnajc6*, *dnajc22*, and *dnajc30* were significantly influenced by Ringer's solution and the differential expression showed a tissue- and gene-specific pattern. After the injection with Ringer's solution in the blood, the expression of eight genes (*dnaja1*, *dnajb5a*, *dnajc3*, *dnajc16a*, *dnajc18*, *dnajc24*, *hscb*, and *dnajsec63*) was significantly influenced at 8 h and the amount of 10 genes' transcripts (*dnaja3*, *dnajb9*, *dnajb9a*, *dnajc4*, *dnajc7*, *dnajc11a*, *dnajc12*, *dnajc15*, *dnajc18*, and *dnajc25*) was not influenced until 48 h, whereas that of eight genes (*dnaja1a*, *dnajb1*, *dnajb3*, *dnajb12*, *dnajc5ga*, *dnajc9*, *dnajc10*, and *dnajgak*) was influenced from 8 to 48 h. After Ringer's injection in the gill, gene expression of 11 genes (*dnaja2*, *dnajb9a*, *dnajc10*, *dnajc11*, *dnajc14*, *dnajc15*, *dnajc16*, *dnajc17*, *dnajc18*, *dnajc21*, and *dnajc30a*) was prominently influenced at 8 h whereas the expression of eight genes (*dnaja1a*, *dnajb1*, *dnajb9*, *dnajc2*, *dnajc12*, *dnajc19*, *dnajgak*, and *hscb*) was influenced at 48 h. In addition, gene expression of 13 genes (*dnaja3*, *dnajb3*, *dnajb6*, *dnajb11*, *dnajc3*, *dnajc3a*, *dnajc5ga*, *dnajc7a*, *dnajc8*, *dnajc25*, *dnajc27*, *dnajsec63*, and *dnajpam16*) was influenced by Ringer's solution throughout all the time points investigated. After the injection with Ringer's solution in the kidney, the expression of 11 genes (*dnaja2*, *dnajb9a*, *dnajc10*, *dnajc11*, *dnajc14*, *dnajc15*, *dnajc16*, *dnajc17*, *dnajc18*, *dnajc21*, and *dnajc30a*) was up- or downregulated at 8 h, whereas that of eight genes (*dnaja1a*, *dnajb1*, *dnajb9*, *dnajc2*, *dnajc12*, *dnajc19*, *dnajgak*, and *hscb*) was influenced at 48 h. The expression of 13 genes (*dnaja3*, *dnajb3*, *dnajb6*, *dnajb11*, *dnajc3*, *dnajc3a*, *dnajc5ga*, *dnajc7a*, *dnajc8*, *dnajc25*, *dnajc27*, *dnajsec63*, and *dnajpam16*) was influenced from 8 to 48 h.

TABLE 4 | Subcellular localization and secondary structure of hsp40 proteins.

Gene name	Alpha helix	Extended strand	Beta turn	Random coil	Subcellular localization
Podnaja2	106 (25.73%)	67 (16.26%)	40 (9.71%)	199 (48.3%)	Cytoplasmic
Podnaja1a	99 (25.06%)	74 (18.73%)	33 (8.35%)	189 (47.85%)	Cytoplasmic
Podnaja1	108 (26.67%)	74 (18.27%)	37 (9.14%)	186 (45.93%)	Nuclear
Podnajb4	79 (23.44%)	63 (18.69%)	27 (8.01%)	168 (49.85%)	Cytoplasmic
Podnajb5	110 (24.55%)	91 (20.31%)	30 (6.7%)	217 (48.44%)	Nuclear
Podnajb1	78 (22.48%)	65 (18.73%)	23 (6.63%)	181 (52.16%)	Cytoplasmic
Podnajb11	109 (31.5%)	64 (18.5%)	31 (8.96%)	142 (41.04%)	Endoplasmic reticulum
Podnajb5a	76 (20.54%)	65 (17.57%)	24 (6.49%)	205 (55.41%)	Nuclear
Podnajb1a	82 (26.28%)	59 (18.91%)	22 (7.05%)	149 (47.76%)	Cytoplasmic
Podnaja3	113 (23.3%)	87 (17.94%)	35 (7.22%)	250 (51.55%)	Mitochondrial
Podnajc5aa	63 (31.66%)	30 (15.08%)	10 (5.03%)	96 (48.24%)	Nuclear
Podnajb6a	59 (21.93%)	51 (18.96%)	31 (11.52%)	128 (47.58%)	Nuclear
Podnajb12	167 (45.14%)	29 (7.84%)	7 (1.89%)	167 (45.14%)	Nuclear
Podnajc18	174 (45.08%)	27 (6.99%)	9 (2.33%)	176 (45.6%)	Nuclear
Podnajc16a	306 (38.93%)	123 (15.65%)	26 (3.31%)	331 (42.11%)	Endoplasmic reticulum
Podnajc10	173 (39.86%)	86 (19.82%)	16 (3.69%)	159 (36.64%)	Mitochondrial
Podnajb12a	163 (43.7%)	28 (7.51%)	8 (2.14%)	174 (46.65%)	Nuclear
Podnajc5ga	72 (32.58%)	17 (7.69%)	7 (3.17%)	125 (56.56%)	Nuclear
Podnajb6	87 (26.69%)	55 (16.87%)	38 (11.66%)	146 (44.79%)	Cytoplasmic
Podnajb9	87 (39.91%)	25 (11.47%)	6 (2.75%)	100 (45.87%)	Nuclear
Podnajc16	304 (37.62%)	120 (14.85%)	23 (2.85%)	361 (44.68%)	Endoplasmic reticulum
Podnajb9a	98 (41.18%)	23 (9.66%)	6 (2.52%)	111 (46.64%)	Mitochondrial
Podnajc21	288 (53.83%)	25 (4.67%)	17 (3.18%)	205 (38.32%)	Nuclear
Podnajc14	276 (41.44%)	56 (8.41%)	42 (6.31%)	292 (43.84%)	Mitochondrial
Podnajc11	180 (32.2%)	131 (23.43%)	29 (5.19%)	219 (39.18%)	Cytoplasmic
Podnajc7	319 (63.29%)	29 (5.75%)	22 (4.37%)	134 (26.59%)	Cytoplasmic
Podnajc7a	322 (60.64%)	27 (5.08%)	24 (4.52%)	158 (29.76%)	Nuclear
Podnajc11a	177 (31.61%)	132 (23.57%)	27 (4.82%)	224 (40%)	Nuclear
Podnajc9	166 (65.1%)	9 (3.53%)	16 (6.27%)	64 (25.1%)	Nuclear
Podnajc3	327 (66.6%)	28 (5.7%)	21 (4.28%)	115 (23.42%)	Nuclear
Podnajc3a	344 (69.22%)	31 (6.24%)	21 (4.23%)	101 (20.32%)	Nuclear
Podnajc24	68 (38.42%)	36 (20.34%)	9 (5.08%)	64 (36.16%)	Nuclear
Podnajc4	130 (54.62%)	15 (6.3%)	2 (0.84%)	91 (38.24%)	Nuclear
Podnajc25	227 (62.71%)	32 (8.84%)	11 (3.04%)	92 (25.41%)	Mitochondrial
Podnajb3	109 (35.28%)	52 (16.83%)	23 (7.44%)	125 (40.45%)	Nuclear
Podnajc30	162 (41.33%)	49 (12.5%)	13 (3.32%)	168 (42.86%)	Nuclear
Podnajc27	98 (48.51%)	24 (11.88%)	11 (5.45%)	69 (34.16%)	Cytoplasmic
Podnajc30a	123 (36.39%)	32 (9.47%)	16 (4.73%)	167 (49.41%)	Nuclear
Podnajc17	199 (61.23%)	31 (9.54%)	11 (3.38%)	84 (25.85%)	Nuclear
Podnajsec63	327 (43.2%)	106 (14%)	23 (3.04%)	301 (39.76%)	Nuclear
Podnajc6	212 (21.65%)	140 (14.3%)	56 (5.72%)	571 (58.32%)	Nuclear
Podnajgak	394 (30.24%)	160 (12.28%)	65 (4.99%)	684 (52.49%)	Nuclear
Podnajc12	74 (44.31%)	11 (6.59%)	5 (2.99%)	77 (46.11%)	Nuclear
Podnajc15	75 (50%)	18 (12%)	11 (7.33%)	46 (30.67%)	Nuclear
Podnajc19	71 (61.21%)	10 (8.62%)	7 (6.03%)	28 (24.14%)	Mitochondrial
Podnajc22	171 (50.29%)	42 (12.35%)	17 (5%)	110 (32.35%)	Endoplasmic reticulum
Pohscb	135 (48.21%)	33 (11.79%)	6 (2.14%)	106 (37.86%)	Nuclear
Podnajc8	164 (65.6%)	16 (6.4%)	9 (3.6%)	61 (24.4%)	Nuclear
Podnajpam16	78 (61.9%)	14 (11.11%)	1 (0.79%)	33 (26.19%)	Nuclear
Podnajc2	392 (63.43%)	38 (6.15%)	14 (2.27%)	174 (28.16%)	Nuclear

DISCUSSION

The industrial farming of Japanese flounder was affected by *E. tarda* infection seriously (Egusa, 1976; Nakatsugawa, 1983; Miyazaki and Kaige, 1985; Isshiki et al., 2001; Moustafa et al., 2010; Park et al., 2012). Previous studies indicated that hsps participated in disease defense and resistance of teleosts, e.g., channel catfish (Xie et al., 2015; Song et al., 2016).

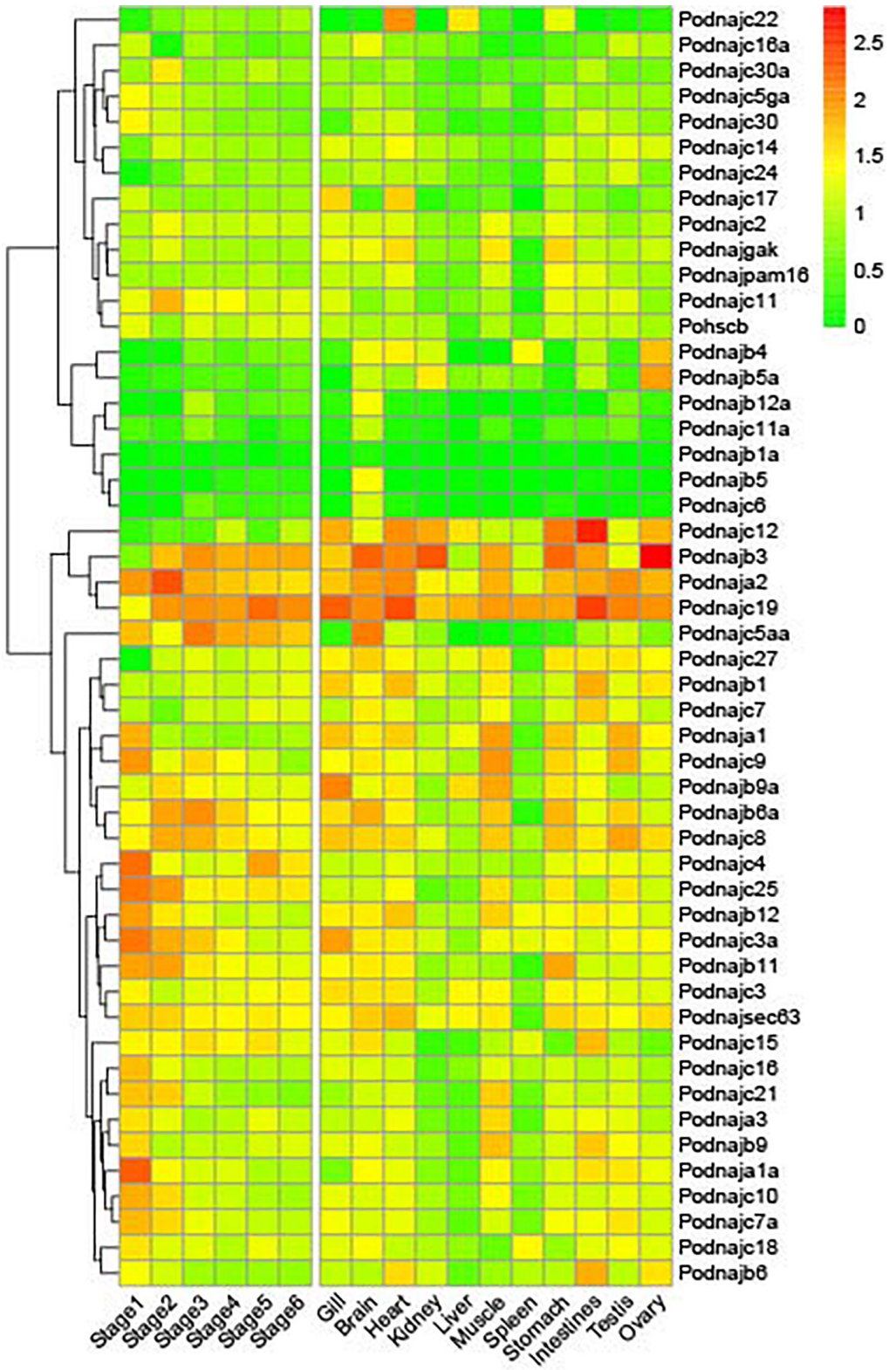

FIGURE 3 | Expression patterns of *hsp40* genes in 11 tissues and six embryonic and larval stages in healthy individuals. Each cell in the heat map corresponds to an expression level and the numbers in cell are FPKM values. Six stages of embryonic and larval development: stage 1 (from two cells to morula), stage 2 (from early gastrula to late somites), stage 3 (from hatching stage to 2 days after hatching), stage 4 (before metamorphosis), stage 5 (metamorphosis stages 1–2), and stage 6 (metamorphosis stages 3–5).

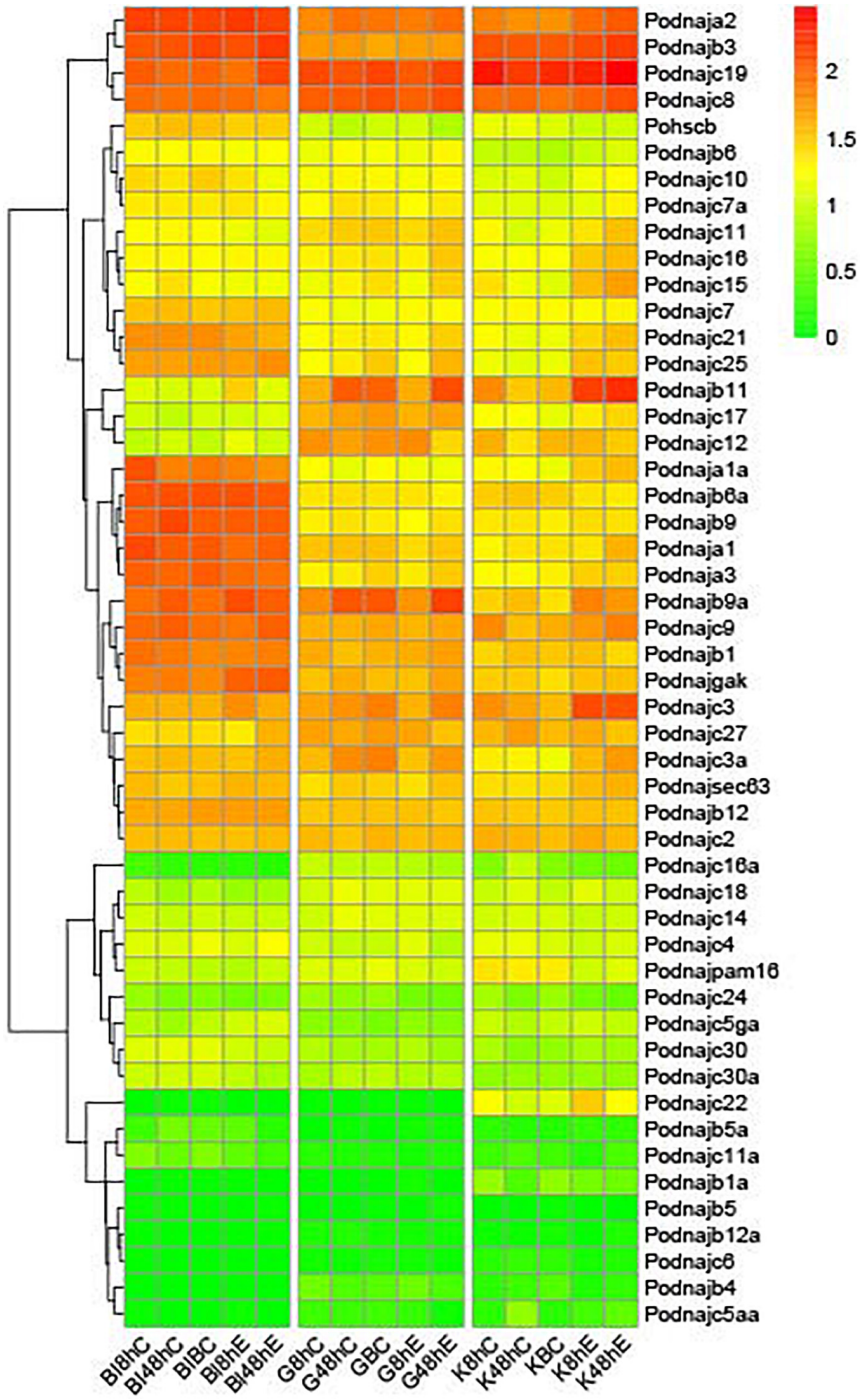

FIGURE 4 | Expression profiles of *hsp40* genes after *E. tarda* injection. Each row represents a gene, and each column represents a time point. The relative expression level is indicated by the color bar on the top right. G represents gill, K represents kidney, and Bl represents blood. 0 h represents the blank control group at the beginning of the experiment, C 8 h and C 48 h indicate Ringer's solution control group, whereas E 8 h and E 48 h indicate *E. tarda*-challenged experimental group, and BC represents blank control.

However, despite these observations, a deeper understanding of the molecular mechanism of Japanese flounder immune responses is required. In this study, we identified 61 *hsp40* genes from tilapia, 57 from medaka, 57 from fugu, 57 from zebrafish, 53 from yellow catfish, 50 from channel catfish, 50 from Japanese flounder, and 33 from spotted gar, indicating a relatively similar *hsp* gene number. In order to elucidate the evolution history of hsp40 proteins, we conducted a maximum-likelihood

phylogenetic gene tree of eight teleosts above. As shown in **Figure 1**, each of the *hsp40* genes was divided into 40 subfamilies (*dnaja1*, *dnaja2*, *dnaja3*, *dnajb1*, *dnajb3*, *dnajb4*, *dnajb5*, *dnajb6*, *dnajb9*, *dnajb11*, *dnajb12*, *dnajb14*, *dnajc1*, *dnajc2*, *dnajc3*, *dnajc4*, *dnajc5*, *dnajc6*, *dnajc7*, *dnajc8*, *dnajc9*, *dnajc10*, *dnajc11*, *dnajc12*, *dnajc14*, *dnajc15*, *dnajc16*, *dnajc17*, *dnajc18*, *dnajc19*, *dnajc21*, *dnajc22*, *dnajc24*, *dnajc25*, *dnajc27*, *dnajc30*, *dnajgak*, *dnajpam16*, *dnajsec63*, and *hscb*) with no obvious extension between species, and Japanese flounder harbored most genes, indicating a high evolutionary conservativeness. Although we conducted complete searches with all Japanese flounder genomic resources available, the *dnajb14* and *dnajc1* have not been found in the Japanese flounder genome. Together with selective pressure analysis, dN/dS analysis of all *hsp40* genes experienced pronounced negative selection, indicating that there were no non-synonymous nucleotide changes at that codon. There existed three genes (*dnajc30*, *dnajc30a*, and *hscb*) whose dN/dS ratio was more than 0.3, which is greater than that of others, hinting that they may experience a higher evolutionary dynamic. Previous studies have found that the evolution of new genes was usually accompanied by changes that occurred in both their sequence and structure, while mutation is the original condition in the evolution of genes. Furthermore, positive Darwinian selection may be another important power forcing new genes' evolution. In addition, natural selection pressure might affect particular sites but not the whole gene, namely, site-specific selection (Koester et al., 2012). These results revealed that the *hsp40* genes between teleosts above were evolutionary-constrained and they were function-conserved; therefore, negative selection might account for the loss of genes in the Japanese flounder genome. In addition, according to the subcellular localization analysis, we assume that most *hsp40* genes function in the nucleus of cells because of their expression preference. As for the structural characteristics of hsp40 proteins, the results above could predict their potential roles and ultimately provide their sequence–structure–function relationships by binding and acting with other proteins.

During embryogenesis, there exist vigorous cell proliferation and differentiation as well as gene expression and protein synthesis, the intra- and extracellular environments experience steady changes, and cells are extremely sensitive to external stimuli (Haanen and Vermes, 1996). Thus, the change and function of *hsps* may be more vital (Walsh et al., 1997; Neuer et al., 1999). *Hsp* genes are regarded as chaperones in morphologic development of cells and organisms and are believed to be related to normal and abnormal development of embryo (Neuer et al., 1999; Brown et al., 2007). Nevertheless, among the *hsp* superfamily, compared to the abundant researches about *hsp20* and *hsp10/60* genes with respect to embryonic and larval development, there is a lack of systemic studies about the role of *hsp40* genes in normal development of embryo and larva (Mao and Shelden, 2006; Elicker and Hutson, 2007; Xu et al., 2011; Middleton and Shelden, 2013; Wang et al., 2017). As for the model species, *hsp40* gene was reported to participate in the embryonic development of mouse forelimbs (Zhu et al., 2010). With regard to marine animals, we also found reports about the roles of *hsp40* genes in sea urchin cilia regeneration during embryogenesis (Casano et al., 2003). Furthermore, previous research also indicated that *hsp40* genes were upregulated after pathogen injection in Japanese flounder embryonic cells (FECs) (Dong et al., 2006). Herein, results indicated that *hsp40* genes had different expression profiles during different embryonic and larval stages and six of them (*dnaja2*, *dnajb3*, *dnajc3a*, *dnajc4*, *dnajc5aa*, and *dnajc19*) had high expression levels at all developmental stages, which hints that *hsp40* genes may be involved in the development of Japanese flounder embryo and larva.

Previous studies have shown the roles of *hsp40* genes in the reactions of a large number of stressors of aquatic animals; however, they emphasized on the abiotic stresses such as thermal, acidity/alkalinity, and salinity challenges (Chen T. et al., 2018; Huang et al., 2018; Xu Y. et al., 2019). As for the immune response, *hsp40* genes were found to be significantly regulated after *Edwardsiella ictaluri* and *Flavobacterium columnare* challenges in channel catfish (Song et al., 2014). Interestingly, in this study, we also found that a large percentage of *hsp40* family members, with a number of 44 genes (88%), were significantly influenced after pathogen injection. Though the mechanisms behind are ambiguous, *hsp40* genes were dramatically regulated after *E. tarda* challenge. These results indicate that *hsp40* genes participated in pathogen reactions and disease resistance against pathogens. In detail, the expression patterns showed a tissue-dependent feature after *E. tarda* infection: 35 genes were up- or downregulated in kidney, whereas 34 genes were regulated in blood while 31 were regulated in gill. In summary, 44 out of 50 *hsp40* genes participated in *E. tarda* defense reactions. After *E. tarda* infection in the blood, expression of three genes (*dnaja1a*, *dnajc11a*, and *dnajc21*) decreased from 8 to 48 h, whereas that of seven genes (*dnaja2*, *dnajb3*, *dnajb12*, *dnajc3*, *dnajc5ga*, *dnajsec63*, and *dnajgak*) was upregulated significantly. Gene expression of *dnajc19* and *dnajpam16* decreased at 8 h and then rose at 48 h, and gene expression of *dnajb5a* and *dnajc7a* was upregulated at 8 h but downregulated at 48 h. Gene expression of *dnajb9a*, *dnajb11*, and *dnajc11* was upregulated at 8 h but returned to the original level at 48 h, whereas that of nine genes (*dnaja1*, *dnaja3*, *dnajb1*, *dnajb6*, *dnajc9*, *dnajc10*, *dnajc16a*, *dnajc18*, and *dnajc24*) was downregulated at 8 h and kept in a normal standard at 48 h. In addition, of the 50 *hsp40* genes, nine did not show significant differential regulation until 48 h, among them, five (*dnajc3a*, *dnajc4*, *dnajc17*, *dnajc25*, and *dnajc27*) were upregulated at 48 h, whereas three genes (*dnajc15*, *dnajc30*, and *dnajc30a*) were downregulated at 48 h. After *E. tarda* injection in the gill, expression of *dnaja2* was dramatically increased from 8 to 48 h, whereas that of *dnajc24* decreased significantly after injection. The expression of *dnajc14* increased at 8 h and followed by a drop at 48 h. Gene expression of *dnajc19* dropped at 8 h and rose to a high level at 48 h. Besides, the expression of 19 *hsp40* genes (*dnaja1a*, *dnajb1a*, *dnajb3*, *dnajb4*, *dnajb5*, *dnajb5a*, *dnajb12a*, *dnajc4*, *dnajc5aa*, *dnajc5ga*, *dnajc6*, *dnajc9*, *dnajc10*, *dnajc11a*, *dnajc17*, *dnajc22*, *dnajc30*, *dnajc30a*, and *hscb*) was stable throughout all time period. Interestingly, *dnajb12* and *dnajc18* showed a different response pattern, which was upregulated at 8 h but returned

to the original level at 48 h, while that of *dnaja1* and *dnajb9* was downregulated at 8 h but restored to a normal level at 48 h. Additionally, gene expression of 16 genes (*dnaja3*, *dnajb1*, *dnajb6*, *dnajb9a*, *dnajb11*, *dnajc2*, *dnajc3*, *dnajc7*, *dnajc8*, *dnajc11*, *dnajc15*, *dnajc16*, *dnajc21*, *dnajc25*, *dnajsec63*, and *dnajgak*) was kept in a normal standard and showed a significant increase at 48 h, and the gene expression of seven genes (*dnajb6a*, *dnajc3a*, *dnajc7a*, *dnajc12*, *dnajc16a*, *dnajc27*, and *dnajpam16*) was kept normal and decreased prominently at 48 h. Besides, after *E. tarda* injection in the kidney, the gene expression of 20 genes (*dnaja1*, *dnaja1a*, *dnaja2*, *dnaja3*, *dnajb3*, *dnajb9a*, *dnajb11*, *dnajc3*, *dnajc3a*, *dnajc5ga*, *dnajc8*, *dnajc10*, *dnajc11*, *dnajc15*, *dnajc16*, *dnajc21*, *dnajc22*, *dnajc25*, *dnajcsec63*, and *dnajgak*) was prominently elevated after administration, and the expression of *dnajb6a*, *dnajpam16*, and *hscb* was downregulated from 8 to 48 h. The expression of *dnajb1* and *dnajc27* was elevated at 8 h and followed by a decrease at 48 h. Gene expression of six genes (*dnajb6*, *dnajc12*, *dnajc17*, *dnajc19*, and *dnajc30*) was kept in a normal standard and showed a significant increase at 48 h, whereas that of *dnajc16a* was kept normal but decreased at 48 h. Moreover, the expression of *dnajb9*, *dnajc7*, and *dnajc18* was upregulated at 8 h, whereas the original level was restored at 48 h, and gene expression of *dnajc24* was downregulated but restored to the normal standard at 48 h. Notably, 15 genes (*dnajb1a*, *dnajb4*, *dnajb5*, *dnajb5a*, *dnajb12*, *dnajb12a*, *dnajc2*, *dnajc4*, *dnajc5aa*, *dnajc6*, *dnajc7a*, *dnajc9*, *dnajc11a*, *dnajc14*, and *dnajc30a*) did not exhibit up- or downregulation from 8 to 48 h. In a word, results showed that a large percentage of *hsp40* gene in gill, blood, and kidney samples were likely to be involved in reaction to *E. tarda* injection, with the exception of *dnajb1a*, *dnajb4*, *dnajb5*, *dnajb12a*, *dnajc5aa*, and *dnajc6*. Interestingly, there existed a tissue-specific response pattern in these 44 regulated *hsp40* genes; namely, most *hsp40* genes had different reaction profiles in three tissues examined except *dnaja2*, which was upregulated in gill, kidney, and blood tissues from 8 to 48 h. In addition, there were still 14 *hsp40* genes (*dnaja1*, *dnajb3*, *dnajb6*, *dnajc3*, *dnajc5ga*, *dnajc16a*, *dnajc17*, *dnajc18*, *dnajc19*, *dnajc24*, *dnajc25*, *dnajc27*, *dnajgak*, and *dnajsec63*) that showed a similar response pattern in two of three particular tissues, among them, *dnaja1*, *dnajc19*, and *dnajc25* had the same patterns in blood and gill, while *dnajb3*, *dnajc3*, *dnajc5ga*, *dnajc17*, *dnajsec63*, and *dnajgak* had the same profiles in blood and kidney, whereas the remaining five genes had the same expression patterns in gill and kidney. Furthermore, the 44 regulated *hsp40* genes had different response efficiency: *dnajc2*, *dnajc4*, *dnajc17*, *dnajc30*, and *dnajc30a* did not show an up- or downregulation until 48 h after *E. tarda* injection, while the other 39 genes respond rapidly at 8 h after injection. Results above hint that five genes (*dnajc2*, *dnajc4*, *dnajc17*, *dnajc30*, and *dnajc30a*) showed a lower speed in regulating *E. tarda* challenge in flounder.

In total, 44 out of 50 *hsp40* genes seem to show a response in reaction to *E. tarda* administrations. The regulatory mechanism behind has not yet been fully validated nevertheless. Therefore, more in-depth research is needed to elucidate the mechanisms of differential expression and to verify the characteristics of *hsp40* genes in immune defenses. In summary, a full set of 50 *hsp40* genes derived from the Japanese flounder genome were identified and characterized in this study. As the largest subfamily of *hsp* superfamily that is involved in many vital physiological processes, *hsp40* genes were divided into 40 subfamilies in phylogenetic analysis, and selective pressure analysis indicated that *hsp40* genes experienced pronounced purifying selection. Additionally, we investigated the expression levels of the *hsp40* genes in *E. tarda*-injected and unchallenged organisms. In healthy individuals, *hsp40* genes played the sentinel role. In challenged individuals, 44 *hsp40* genes were up- or downregulated after *E. tarda* injection, hinting that they might play a role as a portion of the disease response while some of differential expression genes may participate in disease defense against *E. tarda*. These findings give elementary reference for in-depth validation of the characteristics of *hsp40* genes in the process of immune reactions and molecular evolutionary history in Japanese flounder.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/ **Supplementary Material**.

ETHICS STATEMENT

The animal study was reviewed and approved by the Institutional Animal Care and Use Committee of the Ocean University of China and the China Government Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research, and Training.

AUTHOR CONTRIBUTIONS

WY contributed to conceptualization, methodology, software, writing—original draft, writing—review and editing, and visualization. YQ contributed to software and resources. JQ contributed to software. XL contributed to formal analysis. QZ contributed to funding acquisition. XW contributed to conceptualization, methodology, project administration, and funding acquisition. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2020.596534/full#supplementary-material

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REVIEW
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Exploring the Diversity of the Marine Environment for New Anti-cancer Compounds

*Divya L. Dayanidhi[1], Beatrice C. Thomas[1], Joshua S. Osterberg[2], Mallissa Vuong[1], Giselle Vargas[1], Sarah K. Kwartler[1], Emma Schmaltz[1], Meagan M. Dunphy-Daly[2], Thomas F. Schultz[2], Daniel Rittschof[2], William C. Eward[3,4], Christopher Roy[5] and Jason A. Somarelli[1,3]**

[1] Department of Medicine, Duke University Medical Center, Durham, NC, United States, [2] Division of Marine Conservation, Nicholas School of the Environment, Duke University, Durham, NC, United States, [3] Duke Cancer Institute, Duke University Medical Center, Durham, NC, United States, [4] Department of Orthopaedics, Duke University Medical Center, Durham, NC, United States, [5] Department of Chemistry, Duke University, Durham, NC, United States

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Rob Keyzers, Victoria University of Wellington, New Zealand

***Correspondence:**
Jason A. Somarelli
jason.somarelli@duke.edu

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Marine ecosystems contain over 80% of the world's biodiversity, and many of these organisms have evolved unique adaptations enabling survival in diverse and challenging environments. The biodiversity within the world's oceans is a virtually untapped resource for the isolation and development of novel compounds, treatments, and solutions to combat human disease. In particular, while over half of our anti-cancer drugs are derived from natural sources, almost all of these are from terrestrial ecosystems. Yet, even from the limited analyses to date, a number of marine-derived anti-cancer compounds have been approved for clinical use, and several others are currently in clinical trials. Here, we review the current suite of marine-derived anti-cancer drugs, with a focus on how these compounds act upon the hallmarks of cancer. We highlight potential marine environments and species that could yield compounds with unique mechanisms. Continued exploration of marine environments, along with the characterization and screening of their inhabitants for unique bioactive chemicals, could prove fruitful in the hunt for novel anti-cancer therapies.

Keywords: cancer hallmarks, natural products, marine biodiversity, cancer therapy, marine medicine

INTRODUCTION

Cancer is a disease found in nearly all multicellular organisms (Aktipis et al., 2015). For most organisms, cancer is strongly associated with aging (Aunan et al., 2017). As individuals age, mutations accumulate within somatic cells of the body, giving rise to dysfunctional cells that forego their roles as members of a multicellular organism and activate functions, such as unregulated growth and invasion, which work against the health of the organism. In addition to uncontrolled growth, cancer displays several known "hallmarks" – phenotypic characteristics that are nearly universal across cancers. These hallmarks, described in two landmark papers by Hanahan and Weinberg, are genomic instability and mutation, sustained proliferative signaling, evading growth suppressors, enabling replicative immortality, resisting cell death, inducing angiogenesis, reprogramming of energy metabolism, tumor-promoting inflammation, avoiding immune destruction, and activating invasion and metastasis (Hanahan and Weinberg, 2000, 2011).

Decades-long research efforts into the fundamentals of cancer and its hallmarks have led to substantial improvements in clinical outcomes for patients with several cancer types through the development of new diagnostics and therapeutics. However, many of these new technologies also come at a tremendous cost. For example, in the U.S., the national expenditure on cancer totaled more than $147.3 billion in 2017, with some patients spending as much as $10,000 a month on their cancer treatments. Moreover, for many patients, even the best and most costly therapies extend survival by only a few months. Continued efforts are urgently needed to identify promising new therapies to treat cancer.

Cancer therapies can be broadly segregated into two general classes: (1) naturally derived compounds and (2) synthetic compounds. Within each of these classes are two subcategories: small molecules and biologics. Small molecules are low molecular weight compounds that can readily enter cells to elicit a biological effect. Biologics, on the other hand, are larger biomolecules, such as monoclonal antibodies or small RNAs, which often cannot penetrate cells and/or require a delivery system to enter the cell membrane and exert their effects (Mocsai et al., 2014). Over three-fourths of all anti-cancer compounds are derived from natural sources (Newman and Cragg, 2020). For example, paclitaxel and docetaxel, two of the primary chemotherapy agents used to treat breast, prostate, and other cancers, were discovered and purified from naturally occurring plant taxanes (Verweij et al., 1994). Further chemical diversification of these natural plant taxanes has led to potent derivatives with unique properties, such as cabazitaxel.

Interestingly, while the majority of approved therapeutics are derived from natural sources, almost all of these are from terrestrial ecosystems. Hundreds of thousands of natural compounds have been discovered for a variety of uses, with over 100,000 from plants alone. In contrast, in the past three decades, over 2,000 marine-derived compounds have shown a wide range of application (Hu et al., 2015).

Despite the lower number of compounds identified from the marine environment, the enormous volume of the ocean comprises 99% of the total living space on earth and is home to 80% of the world's species (Malve, 2016). In addition to its species richness, the ocean is also comprised of extraordinary environmental diversity, with temperatures that span from −2°C to over 400°C (Mullineaux et al., 2018), pressures from 1 to over 1000 atm (Trenberth et al., 1987), salinities from 0 to over 36 ppt (Srokosz and Banks, 2019), from anoxic to normoxic, bright light to no light, and chemicals including H_2S, CH_4 (Lyman and Fleming, 1940), heavy metals, allelopathic defenses, and anthropogenic pollutants. Based on this environmental diversity, the earth's marine-dwelling inhabitants contain a potential treasure trove of anti-cancer therapeutics.

Although extraordinary potential still lies unexplored within the oceans, many marine-derived compounds that have been discovered have been discovered possess extremely potent anti-cancer properties (Montaser and Luesch, 2011). These compounds are derived from diverse organisms, act through multiple molecular mechanisms, and collectively target numerous cancer hallmarks. In this review, we discuss natural products or compounds directly derived from those natural products from marine macroorganisms with known activity against the hallmarks of cancer. While a number of reviews have exhaustively cataloged the suite of marine natural products with anti-cancer activity, we focus here on the cancer hallmarks as a framework to understand the spectrum of anti-cancer activity from marine-derived sources. We highlight key features of these potential therapies and their sources, and pinpoint strategies to maximize future efforts to identify novel compounds from the marine environment.

MARINE-DERIVED CANCER THERAPIES SHOW ACTIVITY SPANNING THE HALLMARKS OF CANCER

Over 40 years of active research in marine natural products has identified a host of bioactive compounds. We focused on literature that makes explicit reference to one or more of the cancer hallmarks. Analysis of these marine-derived anti-cancer compounds across the cancer hallmarks reveals that most marine-derived therapies act on the specific hallmark of resisting cell death. Out of the 42 compounds reviewed here, including those that act against more than one hallmark, 31 act on the resisting cell death hallmark (**Figure 1A**). The majority of drugs we categorized under this hallmark affect microtubule dynamics and induce apoptosis through cell cycle arrest. The next largest category of 11 compounds represents drugs that inhibit angiogenesis (**Figure 1A**). Most of these drugs act by suppressing vascular endothelial growth factor (VEGF) signaling. Interestingly, nine compounds target multiple hallmarks (**Figures 1B,C**). All other hallmarks are targeted by at least one marine-derived compound, with the exception of the genomic instability and mutation hallmark.

This variation in coverage across the cancer hallmarks is likely due to several factors. For one, bias in the methods used to evaluate anti-cancer properties is very likely, with the vast majority of research directed toward rapid assays for biological activity, such as proliferation, apoptosis, and cytotoxicity. Studies designed to interrogate hallmarks related to immune function, metabolism, and invasion/metastasis often require more complex, costly, and time-consuming experiments. Integrating existing statistical frameworks to predict mechanism of action (Huang et al., 2006) with a panel of simple assays to test newly isolated compounds for activity across the spectrum of cancer hallmarks would aid in identifying compounds with unique activities. In addition to the potential bias in assays used, it is also likely that many marine-derived compounds evolved to be toxins (Haefner, 2003), which may explain the increased representation of agents that target cell death pathways.

Among the compounds reviewed in this paper (see **Figure 2**), 19 have been tested in clinical trials, with six United States Food and Drug Administration (US FDA) approvals (eribulin, trabectedin, brentuximab vedotin, polatuzumab vedotin, cytarabine, and aplidin) and one approval in Australia (aplidin). All of these tested compounds target the resisting cell death cancer hallmark. Below we summarize specific marine-derived

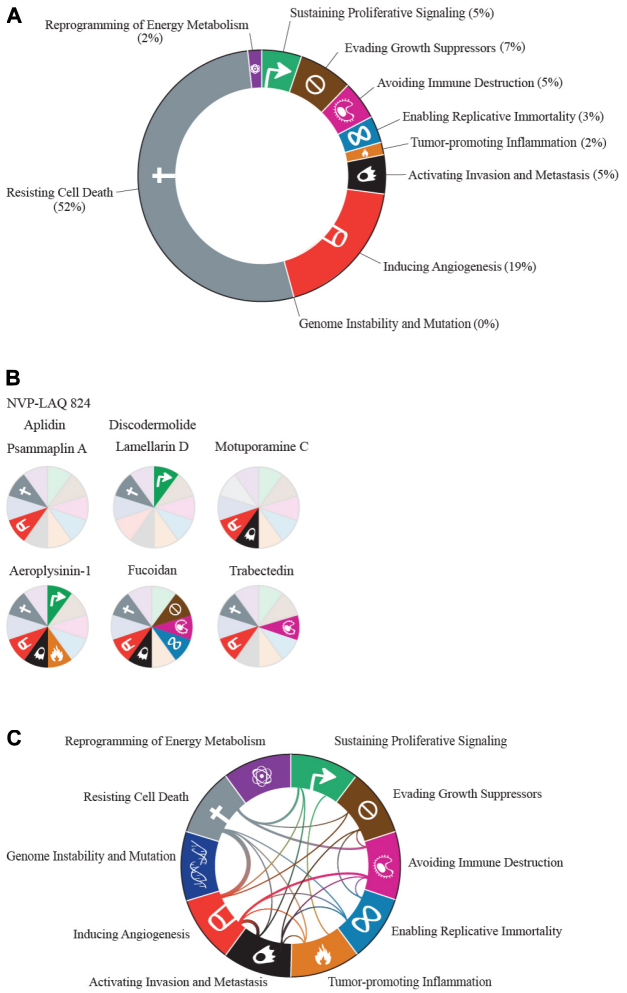

FIGURE 1 | The hallmarks of cancer, adapted from Hanahan and Weinberg (2011), visualized according to marine drug representation. **(A)** All proportions were calculated based on the number of drugs classified under each hallmark. Drugs that were categorized under more than one hallmark were counted multiple times. **(B)** Representation of compounds that are classified under multiple hallmarks. Hallmarks that the compound acted on are represented in brighter colors with hallmark symbols. Compounds that have the same classifications are represented with the same wheel. **(C)** Connections between hallmarks that share at least one compound. Thicker lines represent more compounds shared between the hallmarks.

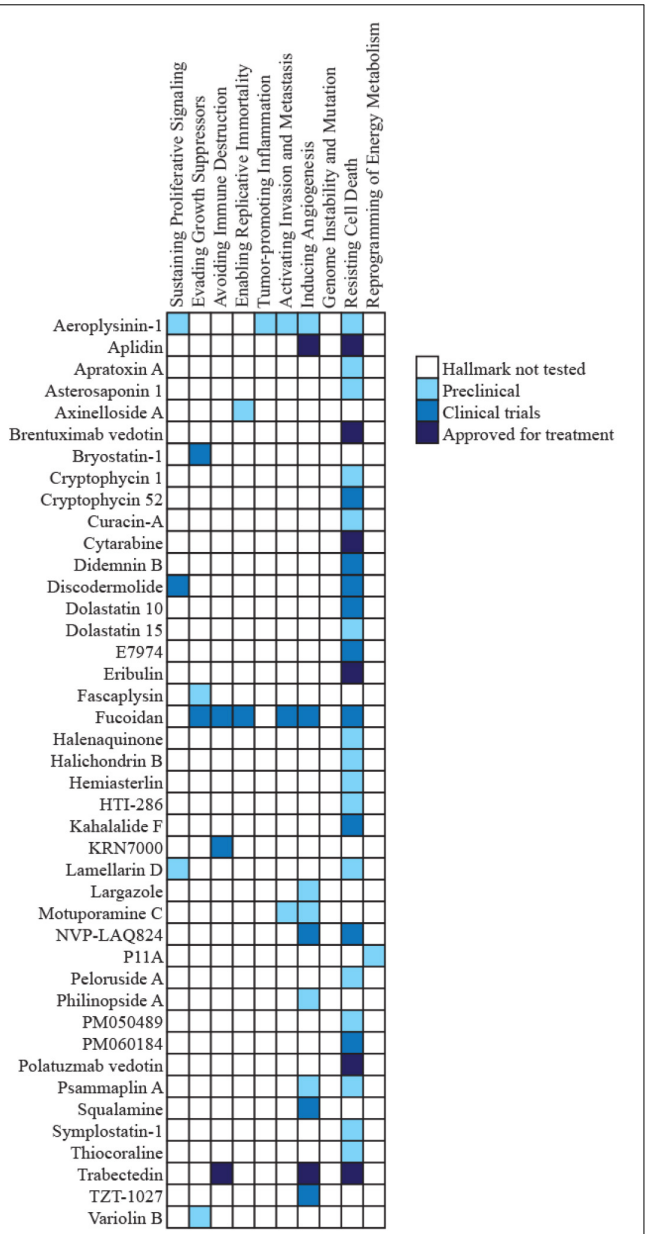

FIGURE 2 | Clinical trial status of marine-derived compounds. The heat map depicts the clinical trial status of each of the compounds discussed in this review, categorized by hallmark. Compounds that were categorized under more than one hallmark have the same color for clinical trial status across those hallmarks. Hallmarks that were not tested for the compound are colored white.

compounds and their known mechanisms of action across the cancer hallmarks.

Sustaining Proliferative Signaling

Healthy tissues carefully regulate cell division by controlling the release of growth-promoting signals that help determine precise timings in cycles of cell division (Jones and Kazlauskas, 2001). Cell cycle regulators only allow cell growth and division when all cellular requirements are met (Schafer, 1998). Unlike healthy cells, cancer cells co-opt growth signals to generate autocrine signals to divide or have dysfunctional receptors that are hypersensitive to growth signals (Hanahan and Weinberg, 2011). Imbued with these dysfunctional growth signaling properties, the cancer cells ultimately grow and divide more frequently than their neighboring normal cells, overtaking the space and resources available.

Inhibiting Epidermal Growth Factor Receptor (EGFR)

Some marine-derived drugs combat unchecked proliferation by preventing cell division through targeting of receptor-mediated growth signaling. For example, aeroplysinin-1 is a brominated

tyrosine metabolite first isolated from the marine sponge *Verongia aerophoba* originally collected from Capo Caccia (Fattorusso et al., 1972; Ciminiello et al., 1997). Aeroplysinin-1 is a cytostatic agent, which inhibits the growth of cells without killing them (Kreuter et al., 1989, 1990). Aeroplysinin-1 leads to growth arrest by inhibiting epidermal growth factor receptor (EGFR) phosphorylation and preventing downstream EGFR-mediated signaling (Kreuter et al., 1990).

Inducing Senescence

A group of growth-arresting marine compounds is the lamellarin family of alkaloids first isolated from the mollusk *Lamellaria* sp. found in Koror, Palau (Andersen et al., 1985). Lamellarins induce cancer cell growth arrest and subsequent cell death. The most notable of the lamellarins is lamellarin D, which has antiproliferative activity in cancer cells through a variety of mechanisms, including induction of senescence (Bailly, 2015). Ballot et al. (2014) reported that lamellarin D induces senescence in P388 mouse leukemia and HBL cutaneous melanoma cells by arresting them in the G2 phase of the cell cycle. Like lamellarin D, another compound that induces cell cycle arrest in G2 is discodermolide. Discodermolide is a polyketide first isolated from the sponge *Discodermia dissoluta* found at Lucaya, Grand Bahama Island (Gunasekera et al., 1990). Discodermolide stabilizes microtubules, which leads to cell cycle arrest and the induction of senescence (ter Haar et al., 1996; Klein et al., 2005).

Evading Growth Suppressors

In addition to growth promoting signals, normal cells use cell cycle checkpoints to limit cell division. One class of cell cycle checkpoint molecules is the cyclin-dependent kinases (CDKs). CDKs act as gatekeepers at key points in the cell cycle. In healthy cells, CDKs regulate whether a cell can progress to the next stage based on environmental and intracellular conditions (Canavese et al., 2012). A number of CDK inhibitors are currently approved for treatment of breast cancer (Schettini et al., 2018), and clinical trials of CDK inhibitors are completed or ongoing for liposarcoma (Dickson et al., 2013, 2016), non-small cell lung cancer (Gopalan et al., 2014), gastric and esophageal cancer (Karasic et al., 2020), ovarian cancer (Konecny et al., 2016), and prostate cancer[1] (NCT02905318).

Inhibiting Cyclins and Cyclin-Dependent Kinases

One marine-derived compound with known anti-CDK activity is fucoidan. Fucoidan is a sulfated polysaccharide that is a cell wall constituent first isolated in many types of brown seaweed, including *Laminaria* spp. (Bird and Haas, 1931). Fucoidan causes G1 arrest in 5637 and T-24 human bladder cancer cells as well as MCF-7 human breast cancer cells, which is mediated by downregulation of CDK2 and CDK4 (Banafa et al., 2013; Cho et al., 2014). Like fucoidan, fascaplysin also directly affects CDKs. It is a bis-indole alkaloid first isolated from the sponge *Fascaplysinopsis* Bergquist sp. in Ndravuni, Fiji (Roll et al., 1988; Hamilton, 2014). Fascaplysin inhibits CDK4 and induces G1 arrest. Fascaplysin also induces the build-up of reactive oxygen species and induces apoptosis (Soni et al., 2000; Hamilton, 2014). Another marine-derived CDK inhibitor, variolin B, is a pyridopyrrolopyrimidine alkaloid first extracted from the sponge *Kirkpatrickia varialosa* found in Cape Armitage in McMurdo Sound, Antarctica (Perry et al., 1994). Variolin B inhibits the activity of CDK1-cyclin B, CDK2-cyclin A, and CDK2/cyclin E complexes, causing HCT-116 human colon adenocarcinoma and A2780 human ovarian carcinoma cells to arrest in G2 and undergo apoptosis (Simone et al., 2005). One compound that inhibits cyclins is bryostatin-1, a PKC agonist. It is a macrocyclic lactone first isolated from *Bugula neritina* in the Gulf of Mexico (Pettit et al., 1982). Bryostatin-1 activates GSK3β, which induces the degradation of cyclin D1. This results in the induction of cell cycle arrest in the G2 phase (Wang et al., 2019). Bryostatin-1 has been tested in phase II clinical trials, but did not progress due to minimal activity (Raghuvanshi and Bharate, 2020). However, it is an effective anti-cancer agent when administered in combination with other drugs (Raghuvanshi and Bharate, 2020), such as dolastatin 10 or paclitaxel (Kortmansky and Schwartz, 2003; Kollar et al., 2014).

Resisting Cell Death

Apoptosis is a type of programmed cell death that is tightly regulated by multiple signaling cascades. The process can be initiated by either extrinsic or intrinsic pathways. In the extrinsic pathway, an extracellular ligand activates a death receptor that induces a signaling cascade to trigger cell death (Galluzzi et al., 2018). In the intrinsic pathway, mitochondrial instability induces mitochondrial outer membrane permeabilization through the B-cell lymphoma 2 (BCL2) apoptosis regulatory protein family (Galluzzi et al., 2018). Apoptosis occurs naturally within populations of normal cells to regulate cell number and control tissue and organ patterning, such as during embryonic development (Elmore, 2007). Tumor cells evade apoptosis in multiple ways, including loss of function in tumor suppressor genes and increased expression of anti-apoptotic regulators (Fernald and Kurokawa, 2013). By preventing apoptosis, cancer cells are able to survive for longer periods of time, which could lead to the acquisition of additional genetic and epigenetic lesions that contribute to the development of therapy resistance and metastasis.

Inducing Apoptosis Through Cell Cycle Arrest

Apoptosis is intimately linked to cell cycle regulation. Many genes involved in cell cycle progression are also involved in initiating apoptosis, and disrupting the cell cycle can trigger apoptosis (Evan et al., 1995). Drugs developed from marine sources combat the anti-apoptotic effects of cancer cells by inducing cell death in a variety of ways. Several of these compounds act by targeting cell cycle arrest, which subsequently induces apoptosis. One example of a class of compounds that inhibits cell cycle function is dolastatins. Dolastatins were first extracted from the sea hare, *Dolabella auricularia*, collected from the eastern coast of Mauritius (Pettit et al., 1993). Fifteen structurally unique dolastatins were identified (Pettit et al., 1989, 1993), of which the most active were dolastatins 10 and 15. Although dolastatin 10 is a pentapeptide and dolastatin 15 is a depsipeptide, both are

[1] clinicaltrials.gov

thought to bind to the vinca domain of tubulin and interfere with tubulin polymerization, disrupting mitotic cell division (Bai et al., 1990, 1992). Interfering with the formation and function of microtubules triggers the intrinsic mitochondrial apoptosis pathway (Bai et al., 1992; Bhalla, 2003). Like dolastatin 10, a variety of other marine-derived compounds exhibit similar ability to induce apoptosis (**Table 1**). Dolastatin 10 reached Phase II clinical trials in multiple cancers (Vaishampayan et al., 2000; Margolin et al., 2001; Saad et al., 2002; Hoffman et al., 2003; Kindler et al., 2005; Perez et al., 2005), but despite its favorable toxicity profile, it failed to show efficacy in any of these solid tumors.

Subsequent studies have developed antibody-drug conjugates using dolastatin 10 derivatives. In brentuximab vedotin, the dolastatin 10 analog monomethyl auristatin E (MMAE) is covalently bound to an antibody against CD30, an activator of B and T cells (Muta and Podack, 2013), to make an antibody-drug conjugate (Younes et al., 2010). While antibodies against CD30 were not significantly effective against lymphomas by themselves, the addition of MMAE greatly increased its efficacy (Younes et al., 2010). In March 2018, brentuximab vedotin was approved by the FDA for the treatment of Hodgkin's lymphoma[2]. Another similar antibody-drug conjugate is polatuzumab vedotin. This conjugate is comprised of MMAE covalently bound to an antibody against CD79b, a component of B cell receptors (Deeks, 2019). The antibody helps guide the drug to its target cells, where the drug is internalized and induces apoptosis (Deeks, 2019). This form of targeting and drug delivery greatly reduces the toxicity to normal cells, so long as the antibody target is highly specific to a target on tumor cells. Polatuzumab vedotin was approved for treatment of relapsed/refractory diffuse large B-cell lymphoma in June 2019 (Deeks, 2019).

Other marine-derived compounds induce cell-cycle arrest and apoptosis through mechanisms that are independent of the disruption of microtubule dynamics. For example, the disulfide bromotyrosine derivative, psammaplin A, first isolated from the sponge *Psammaplin aplysilla* in the Kingdom of Tonga (Quinoa and Crews, 1987), is a histone deacetylase inhibitor. In Ishikawa endometrial cancer cells, psammaplin A induces cell cycle arrest and apoptosis through the downregulation of cyclins and CDKs in a p53-independent manner (Ahn et al., 2008). The cinnamic hydroxamic acid NVP-LAQ824 is a synthetic derivative of psammaplin A and is also a histone deacetylase inhibitor (Atadja et al., 2004). It activates the p21 promoter, selectively causes cell cycle arrest at the G2/M phase, and induces apoptosis in H1299, HCT116, A549, DU145, PC3, and MDA435 cancer cells (Atadja et al., 2004). NVP-LAQ824 successfully passed Phase I clinical trials for advanced solid tumors (de Bono et al., 2008). Aplidin, which was first isolated from ascidian *Aplidium albicans* in the Mediterranean Sea (Erba et al., 2002), is a cyclic depsipeptide that causes cell cycle arrest in G1/G2 through an unknown mechanism (Erba et al., 2002). In addition, the cyclodepsipeptide apratoxin A first extracted from the cyanobacterium *Lyngbya majuscula* found in Finger's Reef, Guam induces apoptosis through cell cycle arrest (Luesch et al., 2001, 2006). Paatero et al. (2016) showed that apratoxin A specifically prevents protein translocation by inhibiting Sec61α, and thus blocks biogenesis.

[2]https://www.fda.gov/

TABLE 1 | Compounds classified under the resisting cell death hallmark that have a mechanism involving microtubules and cell cycle arrest to induce apoptosis.

Compound	Class	Species	Location	References
Asterosaponin 1	Sulfated steroidal glycoside	*Culcita novaeguineae*	Sanya Bay, South China Sea	Cheng et al., 2006; Tang et al., 2006
Cryptophycin 1	Cyclic depsipeptide	*Nostoc* sp. GSV 224	Of aquatic origin but grown in labs	Golakoti et al., 1995; Kerksiek et al., 1995
Curacin-A	Thiazole lipid	*Lyngbya majuscula*	Curaçao	Gerwick et al., 1994; Chang et al., 2004
Discodermolide	Polyhydroxylated lactone	*Discodermia dissoluta*	Lucaya, Grand Bahama Island	Gunasekera et al., 1990; Balachandran et al., 1998
E7974	Tripeptide	Synthetic analog of Hemiasterlin	–	Kuznetsov et al., 2009
Eribulin	Macrocyclic ketone	Synthetic derivative of Halichondrin B	–	Kuznetsov et al., 2004; Huyck et al., 2011
Halichondrin B	Polyether macrolide	*Halichondria okadai* Kadota	Aburatsubo, Japan	Hirata and Uemura, 1986; Bai et al., 1991
Hemiasterlin	Peptide	*Hemiasterella minor*	Sodwana Bay, South Africa	Talpir et al., 1994; Bai et al., 1999
HTI-286	Linear peptide	Synthetic analog of Hemiasterlin	–	Loganzo et al., 2003
Peloruside A	Polyketide	*Mycale hentscheli* (Carmia)	Pelorus Sound, New Zealand	West et al., 2000; Hood et al., 2002; Huzil et al., 2008; Chan et al., 2017
PM050489	Polyketide	*Lithoplocamia lithistoides*	Madagascar	Martin et al., 2013
PM060184	Polyketide	*Lithoplocamia lithistoides*	Madagascar	Martin et al., 2013
Symplostatin-1	Pentapeptide	*Symploca hydnoides*	Guam	Harrigan et al., 1998a; Khalifa et al., 2019; Mooberry et al., 2003
Thiocoraline	Depsipeptide	*Micromonospora*	Mozambique	Romero et al., 1997; Erba et al., 1999

Among these compounds, discodermolide (Mita et al., 2004) and E7974 (Rocha-Lima et al., 2012) reached Phase I clinical trials. PM060184 is in Phase II (see footnote 1, NCT03427268). Eribulin is FDA-approved for the treatment of metastatic breast cancer and liposarcoma, and aplidin is FDA-approved to treat multiple myeloma.

Inducing Apoptosis Through Replication, Translation, or Signaling Inhibition

While some marine-derived compounds act by inducing apoptosis through cell cycle arrest, other compounds induce apoptosis by targeting molecules that either activate apoptosis directly or are essential for cell survival. For example, didemnins are a class of depsipeptides that were first extracted from the Caribbean tunicate, *Tridemnum solidum* (Rinehart et al., 1981a). Among these peptides, didemnin B was the most potent (Rinehart et al., 1981a). Didemnin B inhibits protein synthesis by preventing eukaryotic elongation factor 2 (EEF2)-dependent translation elongation (Li et al., 1984; SirDeshpande and Toogood, 1995). Didemnin B reached Phase II clinical trials for the treatment of small cell lung cancer, but failed to show efficacy (Shin et al., 1994).

Another mechanism of inducing apoptosis is by disrupting pro-survival signaling cascades. The pentacyclic polyketide halenaquinone was first extracted from the sponge *Xestospongia exigua* found in Palau (Roll et al., 1983). Halenaquinone inhibits phosphoinositide 3-kinase (PI3K) and induces apoptosis in PC12 adrenal phaeochromocytoma cells (Fujiwara et al., 2001). Aeroplysinin-1, in addition to its effects on multiple hallmarks (**Figure 1B**), induces cytotoxic effects by inhibiting β-catenin signaling (Park et al., 2016).

Other compounds induce apoptosis by interacting with DNA in unique ways, such as trabectedin and cytarabine. Trabectedin is a tetrahydroisoquinoline alkaloid first isolated from the Caribbean tunicate *Ecteinascidia turbinata* (Rinehart et al., 1981b, 1991; D'Incalci and Galmarini, 2010). By binding to the minor groove of DNA, it causes DNA to bend at the major groove. The binding of trabectedin interferes with other factors that interact with DNA, such as transcription factors and DNA repair machinery, and ultimately results in induction of apoptosis (D'Incalci and Galmarini, 2010). Moreover, trabectedin can cause DNA strands to break by interacting with transcription-coupled nucleotide excision repair machinery (Takebayashi et al., 2001). Cytarabine, a synthetic analog of naturally occurring spongothymidine, also interferes with DNA replication (Bergmann and Feeney, 1951; Jimenez et al., 2018). This compound inserts into newly replicating DNA in place of nucleotides, which prevents elongation of the new daughter strand, and induces apoptosis (Iacobini et al., 2001; Jimenez et al., 2018). Cytarabine is FDA-approved for the treatment of leukemia and lymphoma.

Inducing Apoptosis Through Mitochondrial Interactions

In healthy cells, apoptosis can be initiated through mitochondrial instability. Outer mitochondrial membrane permeabilization releases cytochrome c into the cytoplasm where it activates apoptosis-inducing caspases (Lopez and Tait, 2015). Lamellarin D induces mitochondrial permeability by opening pores in the inner mitochondrial membrane, which leads to caspase activation and subsequent apoptosis (Kluza et al., 2006). Aplidin also disturbs mitochondrial permeability in addition to its ability to induce cell cycle arrest. It activates the Fas/CD95 pathway, which leads to mitochondria-mediated apoptosis in AML HL-60, T-lymphoid Jurkat, and HEL cells (Gajate et al., 2003).

Cancer cells overcome apoptotic signaling by upregulating anti-apoptotic proteins, such as BCL-2, to inhibit mitochondrial permeabilization (Lopez and Tait, 2015), or by inhibiting caspase-mediated downstream targets (Lopez and Tait, 2015). Compounds directed toward overcoming these resistance mechanisms by targeting multiple pathways or apoptosis mechanisms could represent promising candidates to treat apoptosis-resistant cancers. For example, some cryptophycins, first extracted from cyanobacteria *Nostoc* sp. GSV 224 (Golakoti et al., 1995), have anti-cancer properties. Treatment of human H460 non-small cell lung carcinoma cells with the depsipeptide cryptophycin 52 induces phosphorylation of BCL-2 and causes apoptosis in lung carcinoma cells (Lu et al., 2001). Cryptophycin 52 reached Phase II clinical trials. Similarly, fucoidan, in addition to targeting other hallmarks (**Figure 1B**), induces apoptosis by targeting parallel apoptotic-inducing pathways, including release of cytochrome c from mitochondria (Xue et al., 2012), and activation of caspases 3, 7, 8, and 9 (Kim et al., 2010; Xue et al., 2017).

Inducing Cell Death by Necrosis

While apoptosis is a well-studied mechanism of cell death, numerous mechanisms and pathways of cell death exist (Galluzzi et al., 2018). One of these mechanisms, necrosis, involves vacuolization and autolysis of cells in response to extreme stress. Necrosis can occur either as a result of ATP depletion or through specific signaling pathways (Edinger and Thompson, 2004). One drug that can induce cancer cell necrosis is kahalalide F, a peptide first isolated from the sea slug, *Elysia rufescens,* in Black Point, O'ahu, Hawaii (Hamann et al., 1996; Becerro et al., 2001). Experiments with HeLa cells suggested that kahalalide F targets lysosomes, which results in extreme vacuolization and swelling (Garcia-Rocha et al., 1996). Moreover, PC3, DU1445, LNCaP, SKBR-3, BT474, MCF7, MDA-MB-231, and LoVo cells treated with kahalalide F die from a combination of swelling, karyolysis, and necrosis (Suarez et al., 2003; Molinski et al., 2009). Kahalalide F reached Phase II clinical trials for melanoma, but showed no clinical benefit (Martin-Algarra et al., 2009).

Enabling Replicative Immortality

Normal cells have a limited number of cell divisions before they either die or enter senescence (Hayflick, 1992). This limit is driven by telomere shortening at each cell division (Allsopp et al., 1995), ultimately leading to crisis and death (Muraki et al., 2012). Cancer cells, however, enable replicative immortality, often through alterations in telomere maintenance, such as re-activation of telomerase or the alternative lengthening of telomeres pathway (Muraki et al., 2012).

Drugs That Inhibit Telomerase

Axinelloside A, a sulfated lipopolysaccharide first isolated from the sponge *Axinella infundibula* near Shikine-jima Island (Warabi et al., 2005), inhibits telomerase activity with an IC_{50} of 400 nM (Warabi et al., 2005). Given the potency of axinelloside A and its mechanism of telomerase inhibition, subsequent efforts have established a scalable system to develop compounds similar to the structure of axinelloside A (Guang et al., 2017). Like axinelloside A, fucoidan, in addition to its other mechanisms of cancer cell inhibition (**Figure 1B**), also inhibits telomerase activity by reducing human telomerase reverse transcriptase (hTERT) expression (Han et al., 2017).

Inducing Angiogenesis

Tissues require sufficient blood supply to transport oxygen, nutrients, and waste products (Potente et al., 2011). Under normal circumstances, the growth of new blood vessels, known as angiogenesis, only occurs in certain conditions, such as wound healing, menstruation, and embryonic development (Tonini et al., 2003). Cancer cells induce angiogenesis, which provides nutrient supplies to sustain tumor growth. One of the main activators of angiogenesis is VEGF (Carmeliet, 2005; Goel and Mercurio, 2013). VEGF signals to its receptor, VEGFR, which induces signaling and gene expression pathways related to cell survival, actin reorganization, cytoskeletal rearrangement, migration, and vascular cell permeability.

Inhibiting Vascular Endothelial Growth Factor (VEGF)

A number of compounds affect tumor vasculature through VEGF inhibition. The cyclodepsipeptide TZT-1027, a synthetic derivative of dolastatin 10, causes necrosis by damaging tumor vasculature (Otani et al., 2000). TZT-1027 enhances vascular permeability, resulting in the accumulation of red blood cells and damage to tumor vasculature (Watanabe et al., 2007). Damage to the vasculature causes the depletion of oxygen and nutrients to the tumor, resulting in necrosis-mediated cell death (Natsume et al., 2003). However, a Phase II clinical trial of TZT-1027 in non-small cell lung cancer was inconclusive (Riely et al., 2007). Similarly, largazole, first extracted from cyanobacteria of *Symploca* sp. in Key Largo, Florida Keys (Taori et al., 2008), is a cyclodepsipeptide that inhibits VEGF signaling. Largazole rapidly metabolizes into a thiol in the cell and inhibits histone deacetylases (Ying et al., 2008). It also inhibits VEGF and its receptor, thus inhibiting angiogenesis and inducing apoptosis (Liu et al., 2013). Like largazole, Motuparamine C also affects VEGF signaling (Roskelley et al., 2001). It is an alkaloid first extracted from the sponge *Xestospongia exigua* in Motupore Island, Papua New Guinea (Williams et al., 1998).

Interestingly, several compounds that affect VEGF signaling also affect the hallmark of resisting cell death, including aplidin, trabectedin, NVP-LAQ824, and fucoidan. A schematic representation of all drugs that inhibit multiple hallmarks is provided in **Figure 1B**. Aplidin prevents angiogenesis by inhibiting VEGF signaling. It also blocks VEGF secretion and inhibits the formation of capillary-like structures (Broggini et al., 2003; Taraboletti et al., 2004). Trabectedin inhibits VEGF mRNA expression, inhibits the activity of cytokines, and induces the activity of anti-angiogenic cytokines, such as tissue inhibitor metalloproteinase 1 (TIMP-1) and Serpin E1 (Atmaca and Uzunoglu, 2014). NVP-LAQ824 downregulates the expression of VEGF *in vivo* in mouse models (Qian et al., 2004). In a similar manner, philinopside A affects VEGF signaling through the VEGF receptor. Philinopside A is a sulfated triterpene glycoside first extracted from the sea cucumber, *Pentacta quadrangulari*, found in the South China Sea (Tong et al., 2005; Aminin et al., 2015). Fucoidan, which acts across multiple hallmarks (**Figure 1B**), including resisting cell death, evading growth suppressors, and enabling replicative immortality, also prevents angiogenesis by inhibiting VEGF and suppressing neovascularization (Xue et al., 2012; Senthilkumar et al., 2013).

Inhibiting Angiogenesis Through Other Mechanisms

In addition to direct inhibition or downregulation of VEGF signaling/expression, a number of compounds suppress angiogenesis through alternative mechanisms. For example, squalamine is an aminosterol first isolated in a dogfish shark, *Squalus acanthias*, from off the New England Coast (Moore et al., 1993). Squalamine was originally identified as an antibiotic and fungicide, but was later found to inhibit neovascularization by suppressing mitogen-induced proliferation of endothelial cells that form blood vessels (Sills et al., 1998). It was successful in a Phase I/II trial for non-small cell lung cancer (Herbst et al., 2003). By contrast, psammaplin A, in addition to its role in suppressing cell death resistance, also inhibits aminopeptidase N, a galectin 3 binding partner that induces endothelial cell migration (Yang et al., 2007).

While squalamine and psammaplin A have defined mechanisms of action, other compounds, such as aeroplysinin-1, have unknown mechanisms of anti-angiogenic activity. Aeroplysinin-1 caused severe disorganization in existing blood vessels and inhibition of new blood vessel growth in the areas where the drug was applied (Rodriguez-Nieto et al., 2002). Moreover, blood vessels outside the area of application grew around the site (Rodriguez-Nieto et al., 2002). Aeroplysinin-1 also affects the sustaining proliferative signaling and resisting cell death hallmarks (**Figure 1B**).

Activating Invasion and Metastasis

The cause of death for nearly all patients with solid tumors is by metastatic spread of their cancer to a new site in the body. Metastasis is often a multi-step process involving detachment from neighboring tumor cells, invasion, intravasation, dissemination, extravasation, seeding, and colonization. Drugs that target one or more steps in this process have potential to prolong the lives of patients with advanced, metastatic disease.

One marine-derived compound with potential anti-metastatic capability is motuparamine C. Motuparamine C inhibits the migratory ability of cancer cells (Roskelley et al., 2001) by inducing actin filament disassembly, ultimately resulting in a decrease in cancer cell invasion (Roskelley et al., 2001). Aeroplysinin-1 downregulates integrin β1, preventing cell

migration and adhesion (Bechmann et al., 2018). Another potential anti-metastatic agent is fucoidan, which targets multiple cancer hallmarks (**Figure 1B**). Fucoidan inhibits cancer cell invasion and metastasis through numerous mechanisms, including preventing interactions between tumor cells and fibronectin within the extracellular matrix, interfering with tumor cell-platelet interactions, and inhibiting invasion through suppression of matrix metallopeptidase 2 (MMP-2) (Liu et al., 2005; Cumashi et al., 2007; Lee et al., 2012; Atashrazm et al., 2015). Fucoidan also prevents epithelial-mesenchymal transition (Senthilkumar et al., 2013), a key process in metastasis of many solid tumors in which epithelial-like cancer cells undergo a reversible phenotypic transition to a more invasive, mesenchymal-like state (Jolly et al., 2017, 2019). Fucoidan is currently in Phase II clinical trials for metastatic colorectal cancer (see footnote 1, NCT04066660).

Genome Instability and Mutation

For normal cells to become cancerous, they must acquire mutations that enable the traits of the cancer hallmarks. These new mutations can lead to tumor formation if they favor long-term proliferation and allow cells to dominate their environment (Hanahan and Weinberg, 2011). Mutations in DNA repair machinery or telomeres are particularly harmful to healthy cells and can increase the rate of mutations, and thus the likelihood of developing cancer (Kinzler and Vogelstein, 1997). Unfortunately, no marine-derived compounds have been identified to date that can target cells with dysfunctional DNA repair machinery.

Reprogramming of Energy Metabolism

Metabolic reprogramming by cancer cells has been recognized as a key hallmark for almost a century, with landmark discoveries by Otto Warburg in the 1920s showing that cancer cells can utilize aerobic glycolysis to produce ATP even in oxygen-rich conditions (Warburg et al., 1927; Liberti and Locasale, 2016). Renewed focus on this cancer hallmark has also revealed numerous other metabolic alterations and has pinpointed how these alterations affect both gene expression and interactions between cancer cells and the tumor microenvironment (Pavlova and Thompson, 2016).

The compound P11A is a streptodepsipeptide that was first isolated from the actinobacterium, *Streptomyces* S11-23B in the East China Sea (Ye et al., 2017). It counteracts alterations in metabolism by downregulating genes involved in glycolysis and glutaminolysis in glioma cells, including HK2, PFKFB3, PKM2, GLS, and FASN (Ye et al., 2017).

Tumor-Promoting Inflammation

The relationship between inflammation and cancer is complex. The inflammatory response can be either a cancer-protective or cancer-enabling feature. For example, inflammation at the sites of solid tumors has been shown to promote tumorigenesis and aid cancer cells in the process of gaining the characteristics of other hallmarks (Qian and Pollard, 2010). Moreover, factors secreted by immune cells can increase mutation rates in cancer cells, thus enabling cancer cells to take on characteristics of other hallmarks (Grivennikov et al., 2010). Aeroplysinin-1 has the potential to inhibit inflammation by decreasing the levels of total reactive oxygen species (Garcia-Vilas et al., 2018), which may make this compound a promising agent to reduce the tumor-promoting effects of inflammation.

Evading Immune Destruction

The immune system is critically important for surveilling and eliminating precancerous and cancerous cells. Cancer cells evade immune surveillance by (1) escaping immune recognition and (2) inducing immune suppression through the secretion of immunosuppressive ligands (Gonzalez et al., 2018). The burgeoning area of immuno-oncology seeks to activate a patient's immune system by targeting tumor-immune interactions to eradicate cancer cells. Interestingly, several marine-derived compounds act on the immune system to inhibit cancer, making these compounds promising agents for use as single agents or in combination with existing immunotherapies.

For example, KRN7000 is a synthetic analog of agelasphin-9b, which was extracted from the sponge *Agelas mauritianus* (Haefner, 2003). KRN7000 is a potent immunostimulant that induces lymphocyte proliferation (Morita et al., 1995), enhances invariant natural killer cell activity (Tashiro, 2012), and inhibits both tumor growth (Morita et al., 1995) and metastasis in mouse models (Yamaguchi et al., 1996). Natural killer cell activity is stimulated by interaction of KRN7000 with the CD1d receptor (Nicol et al., 2000). In a Phase I-II study of KRN7000-pulsed peripheral blood mononuclear cells in non-small cell lung cancer, KRN7000 stimulation was associated with increased production of interferon gamma and prolonged median survival times in a subset of patients (Motohashi et al., 2009). Since the discovery of KRN7000, a number of analogs and modifications have been reported that have increased immunostimulatory effects (Shiozaki et al., 2013; Tashiro et al., 2013).

Like KRN7000, fucoidan also improves the activity of the immune system against cancer cells. As mentioned above, fucoidan has demonstrated activity across multiple cancer hallmarks (**Figure 1B**). In addition to its roles across these hallmarks, fucoidan also works broadly as an immune stimulant across multiple immune subsets, including decreasing neutrophil apoptosis, activation of splenic dendritic and T-cells (Zhang et al., 2015), and activation of natural killer cells (Ale et al., 2011).

Trabectedin, which is approved for the treatment of soft tissue sarcoma, has been shown to have anti-tumor efficacy by inducing cell death of monocytes. The selective induction of apoptosis in monocytes is due to the differential expression of signaling tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) receptors on monocytes and decoy receptor expression in other immune subsets, such as neutrophils and lymphocytes (Germano et al., 2013). Trabectedin increased the number of infiltrating T-lymphocytes in a spontaneous model of osteosarcoma metastasis, many of which expressed programmed cell death-1 (PD-1) (Ratti et al., 2017). Combination with an anti-PD-1 therapeutic antibody led to significantly increased control of osteosarcoma growth (Ratti et al., 2017), highlighting the importance of testing biologically informed immunotherapy combinations with these marine-derived agents.

Compound Classification by Common Mechanisms

The compounds discussed in this review were broadly classified by mechanism of action in **Figure 3**. Of these, the largest group of compounds with a common mechanism inhibits the activity of microtubules (**Figure 3A**). These compounds predominantly belong to the peptide and polyketide classes. There were a similar number of compounds that inhibit the growth of blood vessels and interfere with the cell cycle (**Figures 3B,C**). Compounds that inhibit new blood vessel growth are more diverse in their structures (**Figure 3B**) while those that interfere with the cell cycle are mostly alkaloids and peptides (**Figure 3C**).

BIOGEOGRAPHY OF MARINE SPECIES THAT ARE SOURCES OF ANTI-CANCER COMPOUNDS

The marine organisms that harbor anti-cancer chemicals span many phyla, but share some phenotypic characteristics. Many of these organisms lack significant structural defenses and have evolved the ability to create or harness secondary metabolites to reduce predation by being unpalatable or toxic. Many are benthic and either sessile, like sponges (phylum Porifera), bryozoans (phylum Bryozoa), and tunicates (phylum Chordata) or slow moving, like sea hares and sea slugs (phylum Mollusca) and sea cucumbers (phylum Echinodermata) (see **Table 2** for examples). Sponges and cnidarians have long been used as sources of marine chemicals and are the two most common marine macrofaunal taxa from which bioactive compounds have been isolated (Mehbub et al., 2014). Indeed, soft, sessile, and slow marine species of any taxa are good candidates for the presence of novel metabolites to test, since they often lack other means of avoiding predation or foraging. The annual reviews of these compounds by Carroll et al. (2019, 2020) provide a comprehensive overview of compounds, their origins, and biological activities.

Not all novel compounds are necessarily created by the macrofauna from which they were isolated. Many come from symbiotic bacteria, like *Candidatus Endobugula sertula*, the symbiont in *Bugula neritina* that produces bryostatin-1 (Davidson et al., 2001). Others come from the microorganisms an animal consumes. For example, Luesch et al. (2001) proposed

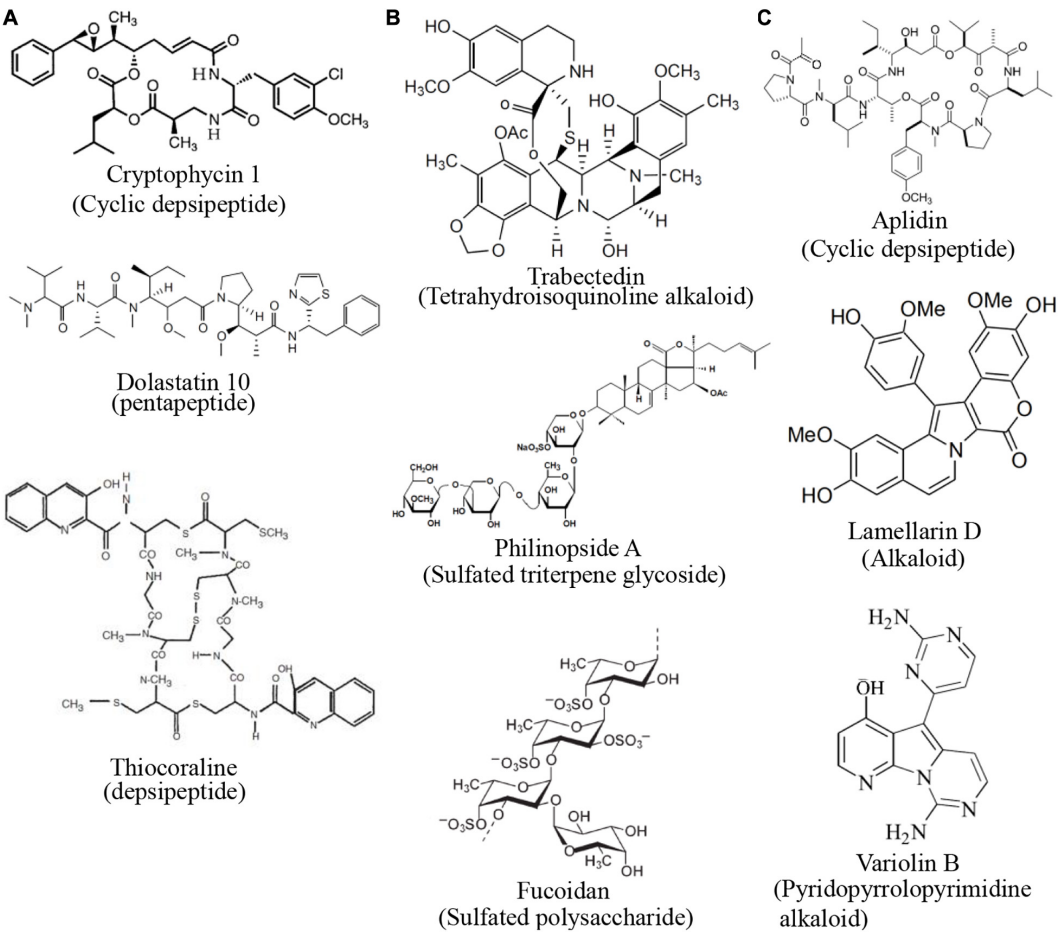

FIGURE 3 | Structures of compounds with common mechanisms. **(A)** Some compounds that inhibit the activity of microtubules **(B)** Some compounds that inhibit blood vessel growth **(C)** Some compounds that interfere with the cell cycle. Structure images were taken from cited journals.

TABLE 2 | Compound use in marine species.

Compound	Species	Compound use	Symbionts	References
Aeroplysinin-1	*Verongia aerophoba* (sponge)	Chemical defense	–	Garcia-Vilas et al., 2015
Apratoxin A	*Lyngbya majuscula* (cyanobacteria)	Secondary metabolite formed during photosynthesis	–	Paerl et al., 2016
Asterosaponin 1	*Culcita novaeguineae* (starfish)	Chemical defense	–	Tang et al., 2006
Bryostatin-1	*Bugula neritina*	Antipredator chemical defense role	Similar to metabolites produced by bacterial symbiont *Candidatus Endobugula sertula*	Davidson et al., 2001
Dolastatin 10	*Dolabella auricularia* (sea hare /shell-less mollusk)	Dietary origin	Also found in Paulauan Cyanobacteria	Luesch et al., 2001
Dolastatin 15	*Dolabella auricularia* (sea hare/shell-less mollusk)	–	Found in *Lyngbya majuscula/Schizothrix calcicola* (cyanobacteria)	Harrigan et al., 1998b
Halichondrin B	*Halichondria okadai Kadota* (sponge)	Collect micro-organism by filteration of sea-water	Produced by symbiont bacteria	Hirata and Uemura, 1986; Lichota and Gwozdzinski, 2018
Kahalalide F	*Elysia rufescens* (sea slug)	Chemical defense	Possible bacterial origin	Davis et al., 2013
Lamellarin D	*Lamellaria* sp. (prosobranch mollusc)	Used by host as a predatory mechanism	–	Bailly, 2015
Peloruside A	*Mycale hentscheli (Carmia)* (sponge)	Seasonal and spatial variation in metabolite production	Multiproducer consortium	Page et al., 2005; Rust et al., 2020; Storey et al., 2020
Psammaplin A	*Psammaplin aplysilla* (sponge)	–	Also found in marine microalgae, cyanobacteria, and symbiotic, heterotrophic bacteria	Lichota and Gwozdzinski, 2018
Trabectedin	*Ecteinascidia turbinata* (tunicate)	–	Produced by bacterial symbiont, *Candidatus Endoecteinascidia frumentensis*.	Le et al., 2015; Lichota and Gwozdzinski, 2018
Variolin B	*Kirkpatrickia varialosa* (sponge)	Aposematic coloration against predation	–	Perry et al., 1994

that dolastatin 10 may not be produced by the sea hare *Dolabella auricularia*, but rather by the cyanobacteria that it consumes. Likewise, the sea slug *Elysia rufescens* accumulates high concentrations of the anti-cancer compound Kahalalide F by consuming algae and their associated bacteria (Zan et al., 2019).

However, the interaction between the sea hare and the cyanobacterium is important, since the sea hare is responsible for accumulating the compound at higher concentrations in its tissues, facilitating identification and isolation. Thus, marine bacteria, fungi, algae, and the species that host them or eat them represent worthwhile candidates for further investigation.

Like the diversity of organisms that contain anti-cancer compounds, the habitats from which marine anti-cancer chemicals have been isolated are also diverse. These habitats span the globe, with the majority located near coastal areas or small islands in the tropical Pacific Ocean (**Figure 4A**). This, like most historical marine research, is likely due to ease of access to species at those locations. Estuarine and coastal environments are often home to vibrant communities of the taxa outlined above. Tropical coral reefs and shallow fouling communities are amongst the most studied and can have high abundance, biodiversity, and species richness with plentiful predators. These biological factors likely contribute to evolution of defensive chemicals in soft bodied organisms (Bakus, 1981).

Very few chemical isolations have been reported from the open ocean or in extreme environments, which is likely the result of substantial barriers associated with exploring these environments. While the barriers to exploration are often substantial, extreme environments can provide the biological, chemical, and/or physical challenges that might lead to the evolution of compounds with anti-cancer properties, and may be prime locations in which to search. One such extreme environment is Antarctica with extremes in day length and ocean temperatures normally below 0°C and a variety of known physiological adaptations. In addition, Antarctica is also home to abundant benthic communities composed of soft-bodied species likely because of a lack of large crustacean predators (Aronson and Blake, 2001). Interesting examples of compounds isolated from the cold waters of Antarctica include Variolin B, which was isolated from the sponge *Kirkpatrickia variolosa*, and displays pro-apoptotic activity and inhibits CDK activity (Simone et al., 2005), and Palmerlolide A and other macrolides isolated from the tunicate *Synoicum adareanum*, with cytotoxicity against melanoma cells by inhibiting vacuolar ATPase (Diyabalanage et al., 2006; Noguez et al., 2011).

Another extreme environment that is a promising location in which to search for anti-cancer compounds is the deep sea. The deep sea is the largest environment on Earth. With its

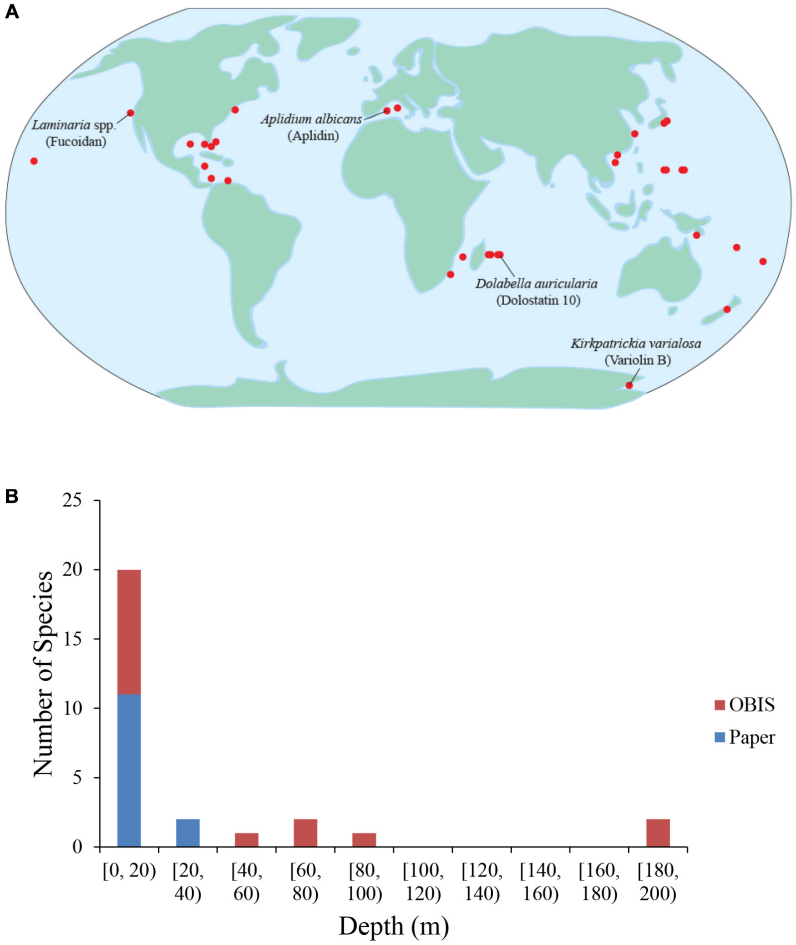

FIGURE 4 | Geographic and depth distribution of known marine drug sources. **(A)** Each dot represents one marine source for one drug. No synthetic compounds were included. Some species and their compounds are labeled. **(B)** Depths of the marine drug source species at times of collection. The red color depicts species whose depth was determined by data from the Ocean Biogeographic Information System (OBIS), while the blue color depicts species whose depth was reported in the original papers describing the characterization/extraction/etc. of the compound (indicated as "Paper").

decreasing sunlight and available organic carbon and increasing hydrostatic pressure with depth, the deep sea pelagic and benthic realms are full of novel biological adaptations. An analysis of the sampling depths across identified species and compounds reviewed herein reveals that nearly all reported isolations thus far have taken place between the surface and approximately 200 m (**Figure 4B**). The lack of isolations at depths deeper than 200 m is likely due to the difficulties associated with sampling at greater depths and/or the inability to recollect promising organisms. The deep-sea floor is commonly home to sea cucumbers, and rocky outcrops are often the sites of deep-sea coral reefs, many of them made of soft corals. In the deep-sea, other even more extreme environments exist that add toxic chemicals to the physical challenges. Deep-sea hydrothermal vents and cold seeps host abundant endemic communities dependent on organic carbon from hydrogen sulfide- or methane-based chemosynthesis (Van Dover, 2000). These environments are so toxic that often only endemic species can survive. Often, these species either utilize the abundant sulfide available or employ detoxification strategies to remove it from their systems (Zierenberg et al., 2000). Some species from these deep-sea environments that are particularly promising with respect to anti-cancer compounds are those that produce chemical deterrents to render them unpalatable as food (Kicklighter et al., 2004). Interestingly, all the species in the study that employed chemical defenses contained chemoautotrophic bacteria, which emphasizes the importance of symbiotic relationships with bacteria and the role their hosts play in accumulating these chemicals.

The sea also contains many hypoxic areas that support organisms with adaptations to low-oxygen conditions, which may promote the adaptation of compounds with unique properties. Hypoxic zones are home to microbes, invertebrates, and fish with adaptations for survival under chronic hypoxic stress (Childress and Seibel, 1998). Adaptations to these oxygen-poor zones include smaller and thinner bodies, more efficient respiratory surface area, and symbiosis with

sulfide-oxidizing organisms (Levin, 2003). Deep bodies of water, like the Black Sea and many high-latitude fjords, with limited deep circulation often become hypoxic or anoxic at depth. The hypoxic marine ecosystems that are easiest to access are muddy, coastal benthic areas. The interstitial water within the sediments is decreasingly oxygenated with depth, yet can be full of organisms across the spectrum of taxa. Bivalved molluscs (e.g., clams and mussels) often close their valves for hours at a time and transition to anaerobic metabolism for the duration (McMahon, 1988). The adaptations observed throughout marine hypoxic environments are often unique to these locations, which increases the probability that species living in such environments utilize unique compounds.

CONCLUSION

With their diverse and highly complex habitats and lifestyles, marine species represent a vast, largely unexplored source of potential anti-cancer agents. Despite the current successes and enormous potential of exploring the marine chemical landscape, several barriers must be overcome before additional, substantial progress can be made. First, improvements in synthesis, isolation chemistry, and production of synthetic derivatives are needed to address the high complexity of many of these compounds, which often have multiple chiral centers. Indeed, with thousands of these bioactive compounds having been isolated and tested over the years, the few compounds that have entered clinical trials were among the rare minority that were abundant enough or simple enough to isolate for testing. Second, there is an urgent need to conserve and preserve marine natural habitats, both for its inherent and anthropocentric value: Innovations in synthesis and isolation must be combined with sustainable efforts to increase sampling across more diverse and extreme marine environments. Third, the development of a platform to analyze newly isolated compounds for activity across the hallmarks of cancer would provide a robust and rapid system to identify new anti-cancer therapies with enhanced specificity to target multiple hallmarks and hallmarks of advanced disease, such as metastasis and immune evasion. Encouragingly, among the compounds already discovered, they collectively target nearly all of the hallmarks of cancer, mirroring the substantial diversity inherent within the marine ecosystem. With many marine species yet to be discovered, countless novel compounds may exist with the potential to enhance and complement the existing arsenal of anti-cancer therapeutics.

AUTHOR CONTRIBUTIONS

DD, JS, SK, ES, and CR conceived and designed the study. DD, BT, SK, and ES performed the literature review. DD, BT, JO, SK, ES, MD-D, TS, DR, WE, CR, and JS wrote the manuscript. All the authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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Enhanced Growth Performance Physiological and Biochemical Indexes of *Trachinotus ovatus* Fed With Marine Microalgae *Aurantiochytrium* sp. Rich in *n*-3 Polyunsaturated Fatty Acids

Shuangfei Li[1,2,3], Boyu Wang[1,2,3], Liangxu Liu[1,2,3], Yingjie Song[1,2,3], Chuhan Lv[1,2,3], Xingyu Zhu[1,2,3], Yanqing Luo[1,2,3], Christopher H. K. Cheng[4], Huapu Chen[5], Xuewei Yang[1,2,3] and Tao Li[6]*

[1] Guangdong Key Laboratory of Plant Epigenetics, Guangdong Technology Research Center for Marine Algal Bioengineering, College of Life Sciences and Oceanography, Shenzhen University, Shenzhen, China, [2] Shenzhen Key Laboratory of Marine Biological Resources and Ecology Environment, Shenzhen Key Laboratory of Microbial Genetic Engineering, College of Life Sciences and Oceanography, Shenzhen University, Shenzhen, China, [3] Longhua Innovation Institute for Biotechnology, Shenzhen University, Shenzhen, China, [4] School of Biomedical Sciences, The Chinese University of Hong Kong, Hong Kong, China, [5] Guangdong Research Center on Reproductive Control and Breeding Technology of Indigenous Valuable Fish Species, Fisheries College, Guangdong Ocean University, Zhanjiang, China, [6] Shenzhen Base of South China Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences, Shenzhen, China

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Anthony Lee Dellinger,
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***Correspondence:**
Xuewei Yang
yangxw@szu.edu.cn

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Aurantiochytrium sp. is a major source of *n*-3 polyunsaturated fatty acids (*n*-3 PUFAs), which are essential nutrients for marine fish. *n*-3 PUFAs have drawn increasing attention because of their great potential for improving the biological functions of fish. The growth performance and immune response of *Trachinotus ovatus* were studied by applying diets with various microalgae content from 1.00 to 11.00% for 8 weeks. The results showed that, with the addition of *Aurantiochytrium* sp., the survival rate, weight gain rate, and specific growth rate of fish increased by a maximum of 1.02, 1.16, and 1.08 times, respectively, indicating that the intake of marine microalgae *Aurantiochytrium* sp. was beneficial for fish growth. As the microalgae content increased, the feed utilization efficiency index feed conversion ratio decreased (maximum 15.00%) and feed efficiency increased (maximum 1.17 times), showing that the addition of *Aurantiochytrium* sp. contributed to the assimilation of fish feed. Furthermore, our results showed that as the addition of *Aurantiochytrium* sp. increased (from 1.00 to 11.00%), the glucose content increased in the blood (from 9.04 to 27.80%). The content of fatty acids ARA, ALA, DHA, and EPA in fish was significantly increased after adding *Aurantiochytrium* sp. in diets. In liver, ARA content increased from 1.17 to 1.63%, ALA increased from 0.56 to 0.85%, DHA increased from 14.44 to 20.61%, and EPA increased from 1.86 to 4.40%. In muscle, ARA content increased from 0.97 to 1.24%, ALA increased from 0.59 to 0.81%, DHA increased from 14.63 to 14.82%, and EPA increased from 4.58 to 5.19%. Positive changes were observed in the blood cell count of

immune related cells (white blood cells, lymphocytes, monocytes, neutrophils, and red blood cells). These results indicated that microalgae rich in *n*-3 PUFAs could increase the number of immune cells, thus helping to improve fish immunity and disease resistance.

Keywords: *n*-3 polyunsaturated fatty acids, docosahexaenoic acid, polyunsaturated fatty acids, *Aurantiochytrium* sp., *Trachinotus ovatus*

INTRODUCTION

The demand for fish feed is rapidly increasing globally. Improving the functionality of feed through the proper addition of nutrients could not only reduce the overuse of antibiotics but also promote the immunity and growth of aquatic products (Wang et al., 2020). Unsaturated fatty acids (FAs), which are essential nutrients for marine fish, show a wide range of important biological functions (Jin et al., 2019). *n*-3 polyunsaturated FAs (*n*-3 PUFAs) are an important category of unsaturated FAs, whose first unsaturated double bond appears between the third and fourth carbon atoms from the methyl end (ω terminus) of the FA. N-6 PUFAs arachidonic acid (20:4 ω6, ARA) and the several principal species of *n*-3 PUFAs eicosapentaenoic acid (20:5 ω3, EPA), linolenic acid (18:3 ω3, ALA), and docosahexaenoic acid (22:6 ω3, DHA), play an essential role in promoting the growth or boosting the immunity of fish (Leshno et al., 2018). Previous research has shown that *n*-3 PUFAs can increase leukocyte phagocytosis, T cell proliferation, and enhance cellular immunity in fish (Thompson et al., 1996; Wu et al., 2003). In addition, fish can also increase lectin activity and improve non-specific immune function by ingesting *n*-3 PUFAs (Pilarczyk, 1995). A previous study found that EPA and DHA help fish reduce inflammatory factors and reduce inflammation (Zhao et al., 2007). Moreover, as essential nutrients for the development of marine fish (Tocher, 2010), EPA and DHA can enrich the components of the mitochondrial phospholipid membrane and promote the growth of fish (Duda et al., 2009).

However, as a traditional source of unsaturated FAs, the extraction of *n*-3 PUFAs from fish oil is costly and inefficient. Therefore, finding novel, inexpensive, and edible sources of *n*-3 PUFAs is important (Tocher, 2015). In recent years, marine microalgae has been reported as a vital source of unsaturated FAs (Yadav et al., 2020) and have been increasingly used as a fish feed additive (Yin, 2014). *Aurantiochytrium* sp., a marine protist with confirmed food security benefits as determined by the Food and Drug Administration (FDA), has drawn increasing attention due to its rich production of *n*-3 PUFAs (Ren et al., 2020). *Aurantiochytrium* sp. can be mass-produced at low cost through industrial fermentation (Scott et al., 2011), and is a stable source of *n*-3 PUFAs (Li et al., 2013). The total FA content of *Aurantiochytrium* sp. reached 389 mg/g (Chang et al., 2015) with 68–74% PUFA and 42–44% essential PUFAs (EPA, ARA, and DHA) (Chang et al., 2011). *Aurantiochytrium* sp. has been used as a fish feed additive, indicating that the addition of *Aurantiochytrium* sp. can increase the content of *n*-3 PUFAs, especially DHA (Miller et al., 2007) in marine fish (Yamasaki et al., 2007). Kissinger et al. (2016) found that *Aurantiochytrium* sp. can be a suitable substitute for establishing protein in fish feed. Kousoulaki et al. (2016) reported that, with the increase in *Aurantiochytrium* sp. content in feed, the fat level in the fish liver decreased, and the FA accumulation and meat quality of fish continued to increase (García-Ortega et al., 2016). Some studies have shown that adding *Aurantiochytrium* sp. to fish feed can reduce energy and FA apparent digestibility (Tibbetts et al., 2020), and improve the growth performance of marine fish (Xie et al., 2019). *Trachinotus ovatus* is an carnivorous hard-bony marine fish whose meat is highly regarded by consumers, and it is a valuable edible fish in the southeast coastal areas of China (Liao, 2017). Nevertheless, the effects of *Aurantiochytrium* sp. as a feed supplement on the development and immunity of *T. ovatus* have barely been reported (Li Y. et al., 2019).

The purpose of the present study was to evaluate the effects of dietary *Aurantiochytrium* sp. on the growth performance and immune response of *T. ovatus*. Different proportions of *Aurantiochytrium* sp. powder were added to the feed, and the growth index, whole body FA content, blood composition, and other indicators of *T. ovatus* were measured.

MATERIALS AND METHODS

Fish Feed Preparation

Aurantiochytrium sp. SZU445 was isolated from mangroves (22°31′13.044″ N, 113°56′58.560″ E) in the coastal waters of southern China. *Aurantiochytrium* sp. SZU 445 (used name as SW7-7) was used for powder preparation and was screened by our laboratory. Various percentages of *Aurantiochytrium* sp. powder (Diet 1: 0.00%, Diet 2: 1.00%, Diet 3: 3.00%, Diet 4: 5.00%, Diet 5: 7.00%, Diet 6: 9.00%, and Diet 7: 11.00%) were added to the fish feeds. Fish feeds were prepared according to the method presented by Ma et al. (2009). All the feed materials were crushed through an 80-mesh steel sieve. After mixing, the feed was formed into hard pellets with a diameter of 3 min. Fish feed was dried at 30°C for 72 h. Feed was stored in a refrigerator at −20°C for later use. The composition of the fish feed is shown in **Tables 1**, **2**. The nutrient composition of the feed is shown in **Table 3**.

Animal Rearing

The *T. ovatus* fry used in this experiment had an initial weight of 8.00 ± 1.00 g and were purchased from Shenzhen Taifeng Oriental Marine Biotechnology Co., Ltd. Prior to the start of the experiment, *T. ovatus* were bred for 14 days. At the beginning of feeding, the fish were starved for 24 h, weighed, and then fish of similar size (initial body weight 8.00 ± 1.00 g) were randomly allocated to 14 sea cages (0.7 m × 0.5 m × 1.0 m; two cages

per diet treatment); each cage was stocked with 50 fish. The total weight of 50 fish in each repetition was similar to ensure that there were no significant differences.

The cages had an overflow system, and the water speed was ∼19 L/rain. An aeration system was set in each cage, and the amount of dissolved oxygen in the water was maintained above 5.0 mg/L. During the test, the water temperature was controlled at 28 ± 1℃, and the salinity of the breeding water body was 29–31 g/L. The water quality was regularly monitored to ensure that it met breeding standards throughout the whole experiment.

Feeding occurred twice daily at 10:00 a.m. and 18:00 p.m., and lasted for 8 weeks. Each feeding weight was the percentage of the total weight. The body length and body weight were recorded for each fish every week to observe the weight gain of the fish. The number of fish deaths per day was recorded, and whether the fish exhibited pathological symptoms, such as *n*-3 PUFA deficiency, was observed.

Sample Collection

At the end of the feeding trial, fish were starved for 24 h. The fish were anesthetized with anesthetics (MS222, 60 mg/L) before sample collection. The number of fish in each cage and the length and weight of the fish were recorded. To collect whole fish, three fish were randomly selected from each cage, and the

TABLE 1 | Ingredients and proximate composition of experimental diets.

Ingredients (%)	Groups						
	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6	Diet 7
Fish meal	30.00	29.00	27.00	25.00	23.00	21.00	19.00
Algal flour	0.00	1.00	3.00	5.00	7.00	9.00	11.00
Peeled soybean meal	20.00	20.00	20.00	20.00	20.00	20.00	20.00
Peanut meal	12.00	12.00	12.00	12.00	12.00	12.00	12.00
Flour	20.42	20.42	20.42	20.42	20.42	20.42	20.42
Shrimp head powder	2.00	2.00	2.00	2.00	2.00	2.00	2.00
Fish oil	7.00	7.00	7.00	7.00	7.00	7.00	7.00
Choline (50%)	0.50	0.50	0.50	0.50	0.50	0.50	0.50
VC Phosphate	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Soybean phosphate	2.00	2.00	2.00	2.00	2.00	2.00	2.00
Multi-vitamins	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Compound mineral salt	1.00	1.00	1.00	1.00	1.00	1.00	1.00
$Ca(H_2PO_4)_2$	2.00	2.00	2.00	2.00	2.00	2.00	2.00
Methionine	0.15	0.15	0.15	0.15	0.15	0.15	0.15
Lysine	0.14	0.14	0.14	0.14	0.14	0.14	0.14
Threonine	0.29	0.29	0.29	0.29	0.29	0.29	0.29
Sodium alginate	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Total	100.00	100.00	100.00	100.00	100.00	100.00	100.00

TABLE 2 | Main polyunsaturated fatty acid content of feed.

	Groups						
Percentage of PUFAs	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6	Diet 7
ARA (%)	0.23	0.25	0.26	0.26	0.28	0.27	0.28
EPA (%)	0.45	3.89	3.93	4.05	4.04	4.06	4.34
DHA (%)	0.91	4.39	4.91	5.74	5.97	6.41	6.66
Total (%)	1.59	8.53	9.10	10.05	10.29	10.74	11.28

ARA, arachidonic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid.

TABLE 3 | The nutrients composition of the feed.

	Groups						
Feed composition	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6	Diet 7
Crude protein g/100g	39.40	38.90	38.30	37.70	37.10	36.50	36.00
Crude fat g/100 g	12.50	14.00	14.60	15.10	15.50	16.40	17.60
Energy kJ/100 g	1580.00	1617.00	1635.00	1645.00	1651.00	1656.00	1658.00

body surface was dried. Samples were quick-frozen with liquid nitrogen and stored at −20°C for later analysis. To collect plasma, we used the tail arterial blood sampling method. Three test fish were randomly selected from each cage. The side of the test fish was placed on an anatomic table. The vacuum blood collection needle was inserted into the posterior part of the gluteal fin and moved the blood to the ventral side of the vertebral body where to was collected. Blood samples were centrifuged at 900 × *g* (4°C) for 10 min to obtain plasma samples. Samples were stored at −20°C for later analysis. To collect liver samples, three fish were randomly selected from each cage. The liver of the fish was frozen immediately with liquid nitrogen and then placed in a sealed bag. Samples were stored at −20°C for later analysis. To collected back muscle samples, three fish were randomly selected from each cage. The back muscles of the fish were frozen immediately with liquid nitrogen and put into a sealed bag. Samples were stored at −20°C for later analysis.

Growth Performance Analysis

The following variables were calculated:

Survival rate (SR,%) = 100 × (final number of fish)/(initial number of fish);

Weight gain rate (WGR,%) = 100 × (final body weight – initial body weight)/initial body weight;

Specific growth rate (SGR,% day – 1) = 100 × (Ln final individual weight – Ln initial individual weight)/number of days;

Hepatosomatic index (HSI,%) = 100 × (liver weight, g)/(whole body weight, g);

Feed conversion ratio (FCR,%) = dry diet fed/wet weight gain;

Feed efficiency (FE,%) = wet weight gain/dry diet fed;

Condition factor (CF, g/cm^3) = 100 × (body weight, g)/(body length, cm^3).

Determination of Plasma Biochemical Indicators

The health status of fish was determined by analyzing plasma biochemical indicators. The content of protein, glucose, triglyceride (TG), and glutamic oxaloacetate (GOT) in fish plasma were determined using a kit provided by Nanjing Jiancheng Biotechnology Research Institute.

Fatty Acid Composition Analysis

The water content in the tissue samples was measured using the drying weight loss method. The protein content of tissue was determined using a quantitative protein kit after the tissue was ground and broken by ultrasound. FAs were extracted from tissue samples by Soxhlet extraction, and FA composition and content were determined by GC/MS. The detailed method was as follows: before the experiment, filter paper bags (Hanjiang Road, Shuncheng District, Fushun City, Liaoning Province, China) were pretreated with a solvent mixture [hloroform:methanol = 2:1 (v/v)] for 48 h and dried at 50°C. Five hundred milligrams of dried cells were placed in a pretreated filter paper bag as a filter paper package and extracted in a Soxhlet extractor at 70°C for 48 h (solvent as described above). Then, the filter paper package was dried and weighed. The weight of FAs was determined by subtracting the weight of FAs extracted from the weight before extraction. The remaining liquid was evaporated to dryness at 70°C using a rotary evaporator. The FAs were completely rinsed with 5 mL of n-hexane and placed in a 10-ml glass tube. Three biological replicates were examined for each sample.

Immune Related Cell Count in Fish

The number of red (RBC) and white (WBC) blood cells can be used as a reference indicator of immunity (Köllner and Kotterba, 2002). Fish blood cells were counted using an HB7510 five classification hemocytometer (Jiangsu China SINNOWA Medical Technology Co., Ltd.).

All data are presented as the mean ± standard deviation (SD) and were subjected to one-way ANOVA, independent-sample *f*-test, and Duncan multiple comparisons to test the effects of experimental diets using SPSS (v.16.0, United States). Statistical significance was considered at $P < 0.05$ unless otherwise noted.

RESULTS

Trachinotus ovatus Growth Performance

Growth performance, feed utilization, and biometric parameters of *T. ovatus* fed different dietary levels are shown in **Table 4**. The high SR of fish in this experiment (SR was the lowest in Diets 3 and 7 at 86%, and the highest in Diet 2 at 92%) indicated that the test subjects barely suffered from the adverse stress caused by the feeding operation. In fish feeding experiments, the WGR and SGR of fish can reflect their growth performance. The results showed that adding *Aurantiochytrium* sp. rich in *n*-3 PUFAs to feed had a significant effect on the WGR and SGR of *T. ovatus* ($P < 0.05$). Furthermore, with increased addition of *Aurantiochytrium* sp. (from 1.00 to 11.00%), the WGR of fish also increased (from 264.95 to 306.65%). The SGR increased from 2.31 to 2.50% with increasing addition of *Aurantiochytrium* sp. This indicated that the addition of *n*-3 PUFAs had a positive effect on the growth of *T. ovatus*.

FCR and FE are often used to measure the ability of animals to absorb feed. A higher FE indicates that fish can more effectively absorb energy from feed. In contrast, a higher FE indicates that fish need more feed to accumulate the same weight. The present results indicated that *T. ovatus* fed diets supplemented with *Aurantiochytrium* sp. showed higher FE (from 73.63 to 79.28%) and lower FCR (from 126.14 to 148.39%) than the control ($P < 0.05$). With increasing *n*-3 PUFAs in the feed, FE increased (Diet 7 was 1.18 times that of the control), whereas FCR decreased [that in Diet 7 (126.14%) was lower than that in Diet 2 (135.96%)]. This indicated that the addition of *n*-3 PUFAs improved feed utilization in fish.

The CF is an indicator of fish weight and growth, and the higher the CF, the higher the nutritional level of fish. When *Aurantiochytrium* sp. was added to the feed, the CF of *T. ovatus* was significantly higher (from 290.76 to 302.32%) ($P < 0.05$). This indicates that *n*-3 PUFAs improved the nutritional level of fish.

Based on the above indices and dynamic characteristics, the growth and development of fish and their feed utilization capacity

TABLE 4 | Effect of different diets on growth performance of *Trachinotus ovatus*.

	Groups						
Growth performance	**Diet 1**	**Diet 2**	**Diet 3**	**Diet 4**	**Diet 5**	**Diet 6**	**Diet 7**
SR (%)	90.00	92.00	86.00	82.00	90.00	78.00	86.00
WGR (%)	264.95 ± 0.77a	276.06 ± 5.8ab	286.69 ± 2.07bc	286.97 ± 3.58bc	294.34 ± 3.02c	296.44 ± 3.26cd	306.65 ± 15.48d
SGR (%/day)	2.31 ± 0.00a	2.37 ± 0.03b	2.42 ± 0.01bc	2.42 ± 0.02bc	2.45 ± 0.01cd	2.46 ± 0.01cd	2.50 ± 0.07d
HSI (%)	0.95 ± 0.06b	0.78 ± 0.16ab	0.92 ± 0.08b	0.81 ± 0.02ab	0.83 ± 0.08ab	0.70 ± 0.15a	0.88 ± 0.02ab
FCR (%)	148.39 ± 10.89a	135.86 ± 3.01b	133.35 ± 4.65bc	128.81 ± 2.15bc	126.17 ± 0.95bc	127.45 ± 1.73c	126.14 ± 0.69c
FE (%)	67.63 ± 4.85a	73.63 ± 1.63b	75.05 ± 2.61bc	77.65 ± 1.29bc	79.26 ± 0.60c	78.47 ± 1.07c	79.28 ± 0.43c
CF (g/cm^3)	276.88 ± 13.82	290.76 ± 22.32	294.41 ± 13.25	297.44 ± 11.59	299.02 ± 12.05	297.93 ± 7.18	302.32 ± 7.43
Final body weight (g)	30.17	33.83	33.00	35.47	36.93	37.13	37.37

SR, survival rate; WGR, weight gain rate; SGR, specific growth rate; HSI, hepatosomatic index; FCR, feed conversion ratio; FE, feed efficiency; CF, condition factor; Diets 1–7 with 0.00, 1.00, 3.00, 5.00, 7.00, 9.00, and 11.00% Aurantiochytrium sp. powder, respectively. Values are mean l' SEM of three replicates, and values in the same row with different letters are significantly different (P < 0.05).

were summarized. With the addition of *Aurantiochytrium* sp. to the feed, the growth performance and feed utilization ability of *T. ovatus* significantly increased. *Aurantiochytrium* sp. as a feed additive has a positive effect on the growth and development of *T. ovatus* and can replace traditional fish oil to become a benign source of essential FAs.

Trachinotus ovatus Plasma Biochemical Index

The plasma biochemical index of *T. ovatus* fed different levels of dietary *Aurantiochytrium* sp. are shown in **Figure 1**. Compared with the control group, *T. ovatus* fed with *n*-3 PUFAs as a supplement showed a significant increase in blood glucose level ($P < 0.05$). The results showed that, as the addition of *Aurantiochytrium* sp. increased (from 1.00 to 11.00%), the glucose content in the blood increased (from 9.04 to 27.80%). Notably, the blood glucose of the fish fed Diet 6 (468.15 mg/L) increased the most among the experimental groups (by 28.47%). Adding 9.00% of *Aurantiochytrium* sp. significantly stimulated glucose absorption in the blood.

When *n*-3 PUFAs were added to the *T. ovatus* feed, the TG content in the blood of the experimental group significantly decreased (5.70–3.29%) compared with that in the control group ($P < 0.05$). Among them, the TG content in the blood of the fish fed Diet 2 decreased by 42.28%, whereas the Diet 4 fish had the lowest TG content (32.80%). *n*-3PUFAs regulate blood lipids and are often used to treat cardiovascular diseases. The results showed that *n*-3 PUFAs could decrease TG content in blood, regulate blood lipid composition, and enhance the health of *T. ovatus*.

Adding *n*-3 PUFAs to the feed had a significant effect on the protein content of blood ($P < 0.05$). With increasing *n*-3 PUFAs in the feed, the protein content in fish blood significantly increased from 31.50 to 158.75 g/L ($P < 0.05$). Compared with the control group, when 3.00% *Aurantiochytrium* sp. was added to the feed (Diet 3), the blood protein content increased by almost two times (190.06%). Furthermore, when 11.18% *n*-3 PUFAs was added (Diet 7), the blood protein content increased by nearly four times (403.96%). The results showed that the amount of *Aurantiochytrium* sp. in the feed significantly influenced the blood protein content, with higher *n*-3 PUFA content significantly increasing blood protein content.

The addition of dietary *Aurantiochytrium* sp. had no significant effect on blood GOT content of *T. ovatus* ($P > 0.05$). This indicates that using *Aurantiochytrium* sp. to supplement *T. ovatus* diets will maintain liver function without causing pathological damage.

Trachinotus ovatus Fatty Acid Composition

The FA composition of the back muscles and liver of *T. ovatus* fed with the different diets is shown in **Figure 2** and **Table 5**. The changes in FA content in the liver and muscles can directly reflect the effect of *n*-3PUFAs on the accumulation of FAs in *T. ovatus*. The content of ALA in liver FAs of each experimental group significantly increased (from 0.56 to 0.85%) after adding dietary *Aurantiochytrium* sp. ($P < 0.05$). Diet 2 exhibited the highest increase (by 51.78%), whereas Diet 3 exhibited the lowest increase (by 37.50%). The ARA content in the liver of all experimental groups decreased with increasing dietary *Aurantiochytrium* sp. The results showed that the content of ARA was 0.46% when the amount of *Aurantiochytrium* sp. was 1.00%, and 0.03% when the amount of *Aurantiochytrium* sp. was 3.00%.

EPA and DHA are essential FAs for the growth of marine fish. After adding *Aurantiochytrium* sp. to the diet, the content of EPA in each experimental group was significantly higher than that in the control. Compared with the group without *Aurantiochytrium* sp., the group with the lowest increase (Diet 2) increased by 46.23%, whereas the group with the highest increase (Diet 5) increased by 136.56%. Similar to EPA, the content of DHA in the liver of each group also increased initially followed by a decrease. The lowest DHA content was observed in Diet 1 (14.44%), and the highest was observed in Diet 5 (20.61%). The results showed that the content of the four main FAs (ALA, ARA, EPA, and DHA) in the liver significantly increased with the addition of *n*-3 PUFAs. Moreover, with the increase in *Aurantiochytrium* sp., the four FAs showed a general trend of first increasing and then decreasing, which indicated that *Aurantiochytrium* sp. intake

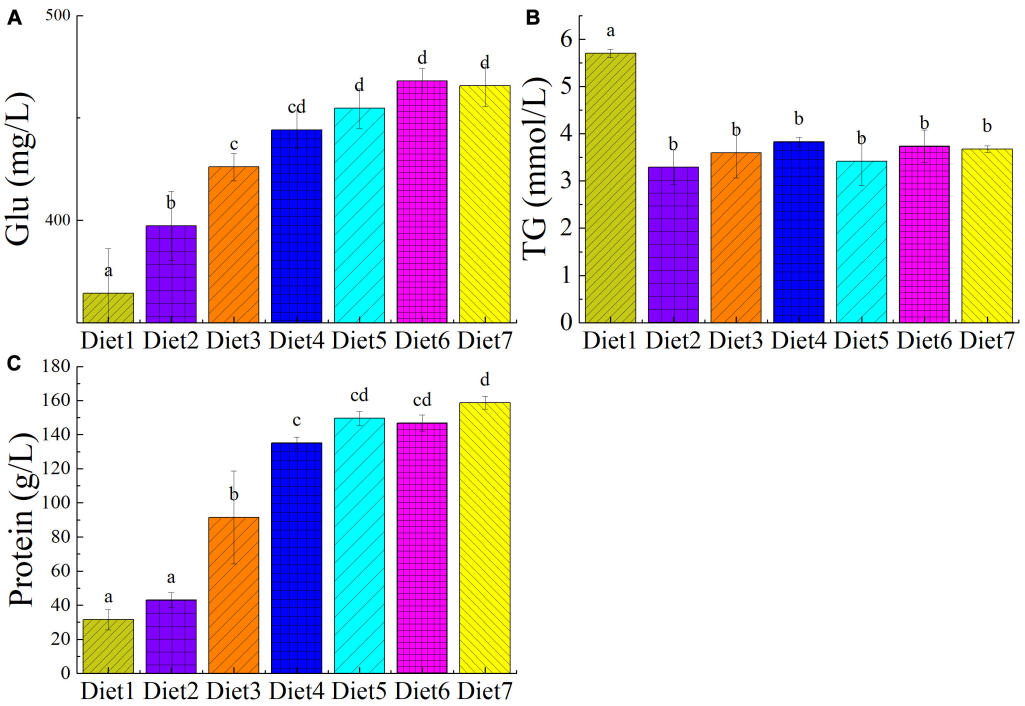

FIGURE 1 | The plasma biochemical indicators of *T. ovatus* sp. The plasma biochemical index of *T. ovatus* sp. fed with diets in different levels of *n*-3 PUFAs Diet1∼7 for Fish feed with different PUFA contents in 7 groups. Glucose **(A)**, triglyceride **(B)**, and protein **(C)** content in blood. The amount of *Aurantiochytrium* sp. powder in feed is 0.00%, 1.00%, 3.00%, 5.00%, 7.00%, 9.00%, and 11.00% respectively.

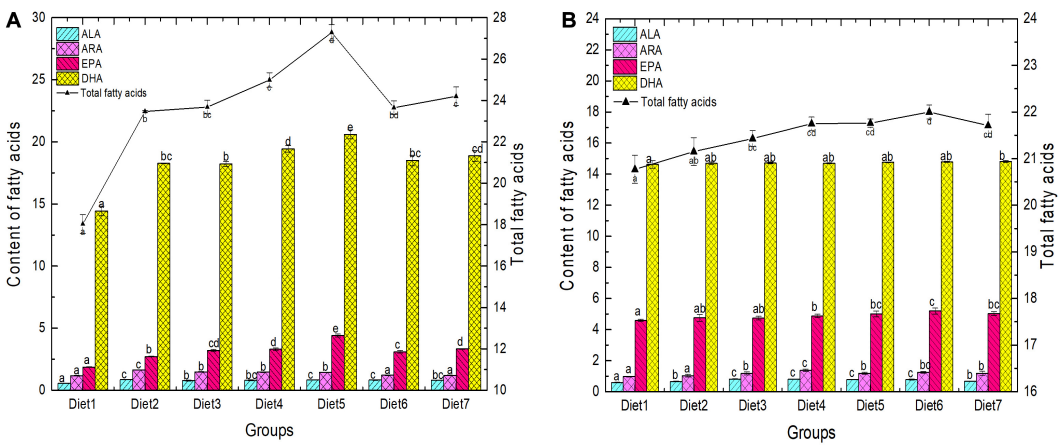

FIGURE 2 | Fatty acid composition in liver **(A)** and muscle **(B)** of *T. ovatus* sp. Diet1∼7 for Fish feed with different *n*-3 PUFAs contents in 7 groups. The amount of Aurantiochytrium sp. powder in feed is 0.00%, 1.00%, 3.00%, 5.00%, 7.00%, 9.00%, and 11.00%, respectively.

could increase the accumulation of FAs in the liver, but excessive intake of *Aurantiochytrium* sp. inhibited the accumulation of FAs.

The effect of dietary *Aurantiochytrium* sp. on FA content in muscles was non-significant, but the proportion of the main *n*-3 PUFAs still increased. Among the groups with added *Aurantiochytrium* sp., the highest proportion of ALA was observed in Diets 3 and 4 (0.81%), and the lowest was observed in Diet 2 (0.66%). The highest ratio of ARA was observed in Diet 4 (1.37%), and the lowest was observed in Diet 1 (0.97%). With the increase in FA content in feed, the proportion of EPA in muscles increased until peaking at 5.19% (Diet 6), and then decreasing to a certain extent (Diet 7, 5.03%). With increased addition of the *Aurantiochytrium* sp. (from 0.00 to 11.00%), the DHA content in muscles increased (from 14.63 to 14.82%), but there was no significant difference among the groups.

The results showed that *T. ovatus* fed a diet supplemented with *Aurantiochytrium* sp. showed more *n*-3 PUFA accumulation than those in the control. However, the changes in *n*-3 PUFA composition in muscle tissue were more subtle. Moreover, with the increase in algae powder content, ALA, ARA, and EPA

TABLE 5 | The percentage content of fatty acids in liver of *Trachinotus ovatus*.

	Diets						
Fatty acid	**Diet 1**	**Diet 2**	**Diet 3**	**Diet 4**	**Diet 5**	**Diet 6**	**Diet 7**
C14:0	2.73 ± 0.02b	2.71 ± 0.04b	2.72 ± 0.11b	2.68 ± 0.07a	2.77 ± 0.12c	2.73 ± 0.05b	2.69 ± 0.00a
C15:0	0.47 ± 0.01a	0.47 ± 0.03a	0.52 ± 0.14c	0.50 ± 0.09b	0.48 ± 0.04a	0.46 ± 0.00a	0.50 ± 0.11b
C16:0	26.69 ± 0.15d	26.13 ± 0.25c	25.58 ± 0.14b	25.97 ± 0.08bc	25.32 ± 0.35b	24.78 ± 1.47a	25.37 ± 0.41b
C17:0	0.48 ± 0.03	0.48 ± 0.00	0.47 ± 0.21	0.48 ± 0.15	0.47 ± 0.08	0.47 ± 0.03	0.48 ± 0.03
C18:0	9.48 ± 0.13ab	9.87 ± 0.24b	9.75 ± 0.09b	9.22 ± 0.24a	9.81 ± 35b	9.88 ± 1.23b	9.44 ± 0.88c
C24:0	0.36 ± 0.07bc	0.31 ± 0.12ab	0.28 ± 0.05a	0.34 ± 0.09b	0.38 ± 0.07c	0.34 ± 0.03b	0.29 ± 0.11a
SFa	39.71 ± 0.35c	39.11 ± 0.80ab	39.25 ± 0.41b	39.10 ± 1.12ab	39.00 ± 0.74b	38.87 ± 0.63a	38.87 ± 1.32a
C16:1	4.71 ± 0.24c	4.20 ± 0.09b	4.13 ± 0.18ab	4.19 ± 0.02b	3.93 ± 0.77a	4.20 ± 0.14b	4.03 ± 0.40a
C18:1	21.57 ± 0.40	19.84 ± 0.35	19.77 ± 0.09	19.50 ± 0.17	19.24 ± 0.47	19.37 ± 0.41	19.47 ± 0.27
C20:1	2.64 ± 0.10c	2.01 ± 0.21a	2.12 ± 0.08b	2.14 ± 0.21b	2.10 ± 0.17b	2.10 ± 0.48b	2.15 ± 0.92b
C22:1	1.01 ± 0.07b	1.05 ± 0.12c	1.01 ± 0.20b	0.99 ± 0.28b	0.90 ± 0.09a	1.00 ± 0.00b	1.01 ± 0.04b
C24:1	0.79 ± 0.31b	0.74 ± 0.08a	0.77 ± 0.25ab	0.79 ± 0.10b	0.76 ± 0.44ab	0.78 ± 0.22b	0.80 ± 0.14b
MUFa	30.71 ± 0.77d	27.83 ± 0.40c	27.85 ± 1.44c	27.51 ± 0.28b	27.15 ± 0.74a	27.42 ± 0.41b	27.44 ± 0.70b
C18:2 *n*–6	10.80 ± 0.45	8.72 ± 0.31	8.80 ± 0.54	7.92 ± 0.55	6.84 ± 0.20	9.04 ± 0.02	8.70 ± 0.17
C20:2	0.84 ± 0.28	0.76 ± 0.30	0.79 ± 0.11	0.88 ± 0.09	1.00 ± 0.09	0.80 ± 0.22	0.91 ± 0.34
C18:3 *n*-3	0.56 ± 0.02a	0.85 ± 0.00c	0.77 ± 0.04b	0.80 ± 0.04bc	0.83 ± 0.01c	0.83 ± 0.03c	0.80 ± 0.01bc
C20:4 *n*–6	1.17 ± 0.02a	1.63 ± 0.02c	1.48 ± 0.03b	1.45 ± 0.02b	1.45 ± 0.02b	1.21 ± 0.03a	1.20 ± 0.02a
C20:5 *n*-3	1.86 ± 0.03a	2.72 ± 0.02b	3.20 ± 0.11cd	3.30 ± 0.11d	4.40 ± 0.12*e*	3.10 ± 0.11c	3.33 ± 0.03d
C22:6 *n*-3	14.44 ± 0.39a	18.27 ± 0.03bc	18.23 ± 0.20b	19.44 ± 0.27d	20.61 ± 0.36*e*	18.51 ± 0.43bc	18.87 ± 0.44cd
PUFa	29.77 ± 0.68a	32.87 ± 0.47b	33.27 ± 0.21b	33.77 ± 0.30b	35.15 ± 0.58c	33.49 ± 0.09b	33.81 ± 0.24b

Values are mean l' SEM of three replicates, and values in the same row with different letters are significantly different ($P < 0.05$).

content increased initially, and then decreased gradually, but EPA and DHA showed an increasing trend. The results showed that a particular *Aurantiochytrium* sp. intake can promote the accumulation of ALA and ARA in muscles, while DHA accumulation in the fish required higher *Aurantiochytrium* sp. content in the feed.

Immune Related Cell Count in Fish

The numbers of RBCs, WBCs, lymphocytes, and granulocytes in the plasma of *T. ovatus* fed different levels of dietary *Aurantiochytrium* sp. are shown in **Figure 3**. When the amount of dietary *Aurantiochytrium* sp. reached 1.00% (Diet 2), the number of WBCs in *T. ovatus* blood began to significantly increase ($P < 0.05$). The number of WBCs in the blood of the fish fed Diet 3 increased by 8.64% and those fed Diet 7 increased by 30.27% compared with those in the control. In addition, the two main types of WBCs, lymphocytes and granulocytes, exhibited the same trend as the total number of WBCs in the blood. The number of lymphocytes in the fish fed Diet 4 was 1.13 times that of those in the control, and those fed Diet 7 had 1.30 times that of the fish in the control. When the fish were fed with 5.00% dietary *Aurantiochytrium* sp. (Diet 4), the number of granulocytes in *T. ovatus* blood reached 1.15 times that in the blood of those in the control. When the *Aurantiochytrium* sp. content was increased to 11.00% (Diet 7), the number of granulocytes was 1.37 times that of the control. The ratio of the number of lymphocytes and granulocytes to the total number of WBCs in the blood of each group fed with different feeds was normal, and there was no significant difference ($P > 0.05$). This result showed that, after adding *Aurantiochytrium* sp. to the feed, the number of WBCs increased, and the ratio of WBCs did not change outside of the normal range. Experimental results showed that adding *Aurantiochytrium* sp. to feed increased the number of immune cells and improved fish immunity and disease resistance.

With the increase in *Aurantiochytrium* sp. content in the feed, RBCs in the blood of *T. ovatus* significantly increased ($P < 0.05$). The RBC content of the fish fed Diet 3 was 1.53 times that of those in the control group. When the content of *Aurantiochytrium* sp. in the feed reached 11.00% (Diet 7), the number of RBCs peaked (2.64×10^{12}/L, 2 times that of the control), and it no longer significantly changed with increasing *Aurantiochytrium* sp. content. This showed that the addition of *Aurantiochytrium* sp. increased the number of RBCs in *T. ovatus*. In addition to the function of transporting oxygen, teleost fish RBCs also have immune functions, such as enhanced phagocytosis, immune adhesion, and defense against infection. The increase in the number of RBCs indicated that after adding *Aurantiochytrium* sp., *T. ovatus* aerobic respiration capacity and RBC immunity-related functions increased.

DISCUSSION

Growth Performance of *Trachinotus ovatus*

n-3 PUFAs are essential FAs that play important roles in fish growth and metabolism. However, the minimum requirements for PUFAs differ for different types of fish (Chen et al., 2018). Fish lacking PUFAs exhibit growth retardation and lack of vitality, and will suffer from seriously reduced feed-conversion efficiency

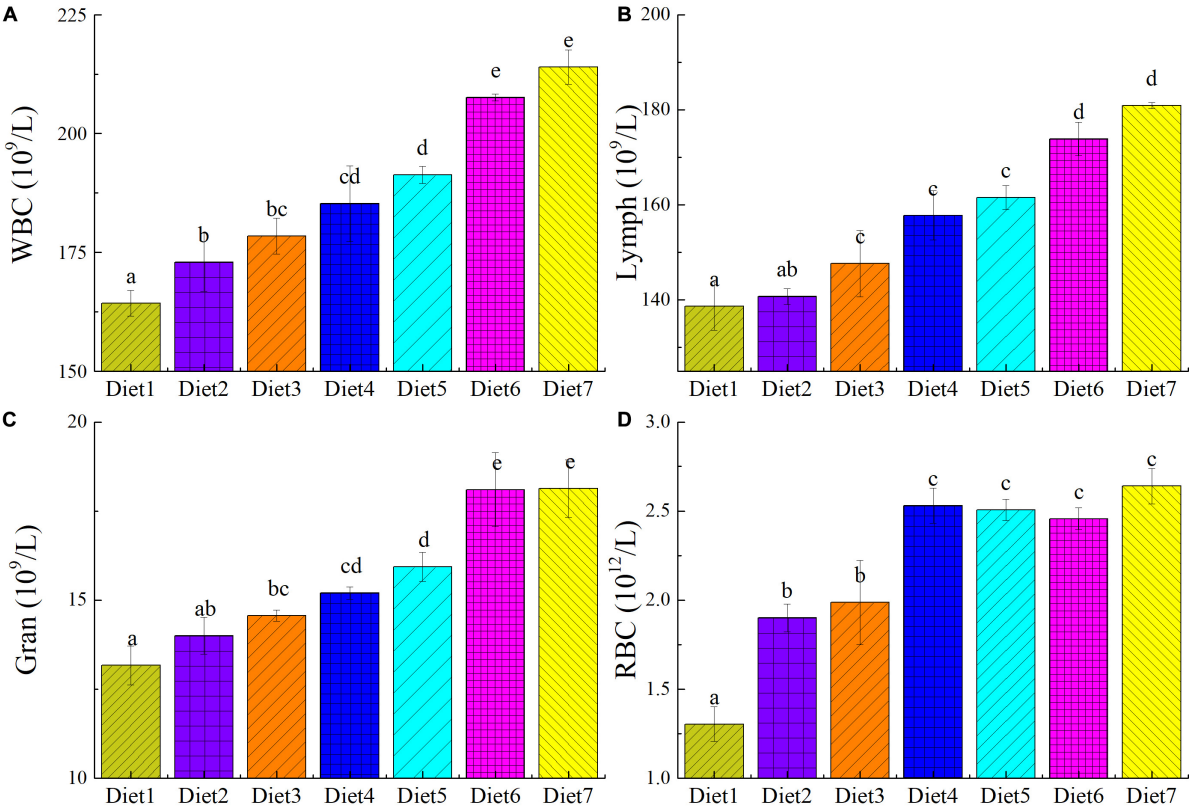

FIGURE 3 | The blood cell count of *T. ovatus* sp. fed diets with different levels of *n*-3 PUFAs Diet1∼7 for Fish feed with different *n*-3 PUFAs contents in 7 groups. The number of white blood cells **(A)**, lymphocyte **(B)**, granulocyte **(C)**, and red blood cells **(D)** in blood. The amount of *Aurantiochytrium* sp. powder in feed is 0.00%, 1.00%, 3.00%, 5.00%, 7.00%, 9.00%, and 11.00%, respectively.

(Xu et al., 2010). Growth performance parameters, such as WGR and SGR, are the most direct reflection of fish growth and nutritional status. The SR and FE can also reflect health status and energy-use efficiency.

Dietary *Aurantiochytrium* sp. supplementation significantly improved the WGR and SGR of *T. ovatus* compared with the supplemented group. The FE and other results related to energy-conversion efficiency also showed that the ability of *T. ovatus* to absorb energy from their diet improved after supplementation with dietary *Aurantiochytrium* sp. As seen from the results, *Aurantiochytrium* sp. provides sufficient DHA and other FAs necessary for the growth of *T. ovatus*. In addition, the algal-derived DHA can also increase the FE rate (Li et al., 2009) and stimulate the growth performance of aquatic animals (Glencross and Smith, 2001; Glencross and Rutherford, 2011). These results indicate that *Aurantiochytrium* sp. addition promotes the growth of *T. ovatus* by improving its energy-conversion efficiency.

Trachinotus ovatus Plasma Biochemical Index

Fish plasma is closely involved in fish metabolism, nutrition, and health status. Blood biochemical indicators are widely used to evaluate fish health, nutrition, and environmental adaptation (Abdel-Tawwab et al., 2010). Plasma protein concentration can be affected by dietary protein intake and can be used as an indicator of individual nutritional status to a certain extent. The serum total protein content can reflect protein metabolism in animals (Baldi et al., 1999). The higher the total protein content in fish serum, the higher the fish's absorption and metabolism levels, and the higher the efficiency of protein synthesis and nitrogen deposition (Sun et al., 2014). In the present study, as dietary *Aurantiochytrium* sp. increased from 0.00 to 11.00%, the protein content in the blood also significantly increased and was maintained at a high level. When dietary *Aurantiochytrium* sp. was at 11.00% the weight of the feed, the total amount of protein in the blood was 4 times that in the control. Previous research has shown that adding *Aurantiochytrium* sp. to feed can increase the protein content in juvenile black sea bream blood (Ma et al., 2009). These results indicate that adding *Aurantiochytrium* sp. to feed can improve protein efficiency and increase the nutritional status of *T. ovatus*.

Blood glucose and triglyceride levels reflect energy and fat metabolism in fish. Generally, when the blood glucose level is high, the fish feed has a higher energy level and greater vitality (Imsland et al., 2001), but excess glucose is more likely to be converted into fat for storage in the body (Guo et al., 2006). TG content is an important indicator of blood fat levels. When the body fat level increases, the blood TG content also increases. *n*-3

PUFAs can affect insulin sensitivity and regulate blood glucose levels (Luo et al., 1998), and, as the main type of *n*-3 PUFAs, ARA can reduce the TG level in the blood (Kris-Etherton et al., 2003). The results of the present study showed that, as the *n*-3 PUFA content in the feed increased, the glucose content in the blood increased from 9.00 to 27.00%. In addition, after adding *Aurantiochytrium* sp. powder to the feed, compared with the control, the blood TG content in each experimental group significantly decreased. These results indicate that *n*-3 PUFAs can reduce the energy expenditure index and improve the energy absorption efficiency of *T. ovatus* (Tibbetts et al., 2020).

GOT mainly exists in the cytoplasm of hepatocytes, and is an indispensable enzyme for hepatocytes to synthesize proteins (Zhong et al., 2020). This enzyme is released into the blood when hepatocytes degenerate, and necrosis or cell membrane permeability increases, so blood GOT levels can well reflect the functional status of liver cells (Li B. et al., 2019). Results showed that adding *Aurantiochytrium* sp. to the diet well-maintained *T. ovatus* liver function.

Fatty Acid Composition of *Trachinotus ovatus*

The liver is an organ rich in FAs and is more sensitive to changes in FA content in animals (Castaño-Moreno et al., 2020). Therefore, the variety of FA components in the liver was more significant with the increase in *n*-3 PUFA intake. This result could be due to ALA being the precursor of EPA/DHA synthesis in the process of FA metabolism (Deng et al., 2017). Compared with fish oil, marine microalgae as a source of fatty acids can inhibit the desaturation pathway in the liver and increase the accumulation of fatty acids (Carvalho et al., 2020a). The present study indicated that oils from microalgae, when engineered to contain high levels of EPA and DHA can replace fish oil in feeds for sea bream juveniles and allowed complete replacement of fish oil in combination with more cost-effective lipid sources, such as poultry and rapeseed oils (Carvalho et al., 2020b). Furthermore, more EPA-DHA synthesis will lead to a decrease in ALA content. We speculated that with the increase in DHA content, the conversion process of ALA to DHA may be inhibited by feedback. In addition, the direct absorption and deposition of DHA may be inhibited by increased DHA conversion. However, the increase in FA content can protect the fish liver (Zirnheld et al., 2019) and increase the economic value of *T. ovatus*.

Immune Related Cell Count in Fish

The blood composition of fish is affected by a variety of endogenous factors, including nutrition status, individual size, and exogenous factors, including light, temperature, and dissolved oxygen. Some blood indicators (RBCs and WBCs) are used to assess the physiological and pathological conditions of fish (Harikrishnan et al., 2011). In addition, RBC, WBC, and lymphocytes are also important components of the cellular and humoral immunity of fish (Köllner and Kotterba, 2002).

As an important component of the RBC membrane, PUFAs can maintain a stable number of RBCs in the blood through the ingestion of dietary *n*-3 PUFAs (Ma et al., 2004). In the present study, compared with the control, the RBCs in the feed groups was significantly increased, which indicates that *Aurantiochytrium* sp. can indeed enhance the RBC immunity of *T. ovatus*. Leukocytes mainly function in defense. On the basis of traditional immunology, granulocytes are mainly responsible for host defense, immune regulation, and tissue damage (Kruger et al., 2015). Recent studies have shown that granulocytes exhibit a defense response to tumor cells (Fridlender and Albelda, 2012). Lymphocytes are a large group of immune cells that mainly participate in the specific immune response of the body. In the present study, the number of WBCs, granulocytes, and lymphocytes in the blood of the experimental group significantly increased compared with the control group, and the overall trend in growth remained stable. Generally, the number of WBCs in fish will increase due to environmental stress (Zuo et al., 2019), but there was no significant difference in the proportion of lymphocytes and granulocytes in the total number of WBCs in each experimental group. This shows that the increase in leukocyte number was not caused by external environmental stress, but rather by improvements in immunity (Köllner and Kotterba, 2002). Through a comprehensive analysis of the number of immune cells, it can be seen that the supplementation of dietary *n*-3 PUFAs can increase immunity in *T. ovatus*.

n-3 PUFAs have been widely used as health products owing to their ability to regulate blood lipids. The mechanism of PUFAs, as components of cell membranes, involves changing the composition of cell membranes (Gawrisch et al., 2008), but also changing the process of intracellular messenger transmission and cytokines. All phospholipids and some secondary messengers, such as triglycerides and ceramide, contain FA chains, so their functions can be changed by altering FA composition (Issazadeh-Navikas et al., 2012). In the field experiment, *n*-3 PUFAs can enhance natural killer cell activity, $CD8^+$ T cell (a cytotoxic T lymphocyte) activation, and interleukin-γ and tumor necrosis factor-α production in tumor-bearing animals (Robinson et al., 2001). Previous studies have shown that appropriate intake of *n*-3 PUFAs can stimulate lymphocyte proliferation and enhance immune function (Hedelin et al., 2007; Gawrisch et al., 2008). This is consistent with the increase in the number of leukocytes and lymphocytes in *T. ovatus* blood after the addition of dietary *Aurantiochytrium* sp. in the present study. These results indicate that the immune function of *T. ovatus* was indeed increased by adding *Aurantiochytrium* sp. to its feed.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The animal study was reviewed and approved by Experimental Animal Ethics Committee of Medical Department of Shenzhen University.

AUTHOR CONTRIBUTIONS

LL, BW, XZ, YL, and CL analyzed the data and wrote the manuscript. XY and SL designed the experimental concept. CC, HC, and YS checked and revised the details of the manuscript. XY was responsible for critical reading and finalization of the manuscript. All authors have read and approved the final manuscript.

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frontiers in Marine Science

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A New Marine Biomaterial: The Shell of Mangrove Horseshoe Crabs, *Carcinoscorpius rotundicauda* (Latreille, 1802) Emphasizing Its Physico-Chemical Characteristics

Yusli Wardiatno[1,2,3], Bambang Riyanto[4], Nopa Aris Iskandar[4], Sonja Kleinertz[5,6], Peter Funch[7] and Fery Kurniawan[1,3]*

[1] Department of Aquatic Resources Management, Faculty of Fisheries and Marine Sciences, IPB University, Bogor, Indonesia, [2] Environmental Research Center, IPB University, Bogor, Indonesia, [3] Center for Coastal and Marine Resources Studies, IPB University, Bogor, Indonesia, [4] Department of Aquatic Product Technology, Faculty of Fisheries and Marine Sciences, IPB University, Bogor, Indonesia, [5] Faculty of Fisheries and Marine Sciences, IPB University, Bogor, Indonesia, [6] Aquaculture and Sea-Ranching, Faculty of Agriculture and Environmental Sciences, University of Rostock, Rostock, Germany, [7] Department of Biology – Genetics, Ecology and Evolution, Aarhus University, Aarhus, Denmark

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***Correspondence:**
Yusli Wardiatno
yusli@apps.ipb.ac.id

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The paper aims to elucidate the physico-chemical characteristics of the shell of mangrove horseshoe crabs (*Carcinoscorpius rotundicauda*) and determine the compilation matrix for the first time. The shell composition matrix of *C. rotundicauda* has never been studied in detail before, especially the shape of the foam, the chemical composition, the functional groups and the mechanical-physical and thermal properties of the shell. Based on this study, the shell structure of the mangrove horseshoe crab has the potential to be used as the base structure for developing bio-foam insulator material in the future. Therefore, the shell of mangrove horseshoe crabs has a unique natural structure in the form of foam. Its robust and elastic structure has the potential for further development for new marine biomaterials. The formation and composition of horseshoe crab shells foam are also believed to be multifunctional in mobility, used for defense mechanisms and thermal stability. The horseshoe crab samples were collected from Pacitan coastal waters, East Java, Indonesia. The research was conducted using physico-chemical and mechanical-physical analysis. The scanning electron microscopy was used in order to clarify the physico-chemical characteristics. The measurements of the mechanical-physical characteristics included density, unit cell size, and water absorption. The tensile strength and compressive strength were analyzed based on the American Society for Testing Material. Thermal resistance was measured by thermal gravimetric analysis. The results showed that the horseshoe crab shells have a unique structure, where chitin, protein and some minerals are the main chemical elements. The combination and major constituents of the horseshoe crab shell material provide strong and plastic mechanical properties with a maximum tensile strength of 60.46 kPa and maximum compressive strength of 110.55 kPa, water absorption of 0.01195 ± 0.001%

and a density value of 0.1545 ± 0.011 g/cm^3 as well as the capability to withstand thermal loads with peak decomposition values of 267.4–823.2°C and thermal stability of 60.59%. Using natural marine biomaterials in the future will be beneficial because it leaves no harmful residues and therefore has environmental advantages and at the same time, it is also more cost-effective.

Keywords: chitin, mechanical properties, shell structure, thermal stability, Chelicerata

INTRODUCTION

In the book "Biological Materials of Marine Origin: Vertebrates" Ehrlich (2010) states that the sea is a material source with great potential for humans in future life. A wide variety and uniqueness characterize marine materials due to marine life diversity; they are conveyed through composition, structure and chemical elements. The engineering of materials originating from the universe evolution for millions of years makes a variety of materials extremely complicated and unique (Jabbari et al., 2014). Additionally, Wegst and Ashby (2004) and Meyers et al. (2008) showed that biological materials are multifunctional because they combine biology, mechanics, and several other functions. Biological materials can even be categorized as polymers, inorganic materials, concentrates, adhesives, and composites (Meyers et al., 2008). The development of marine biological materials is rapidly growing due to the application potential in various life and medical sciences.

Horseshoe crabs are an ancient marine species with attractive functional shapes and behavior. They have descended from Trilobites (Subphylum Trilobitomorpha), which have lived for 445 million years (Chen et al., 2010). Genetically, this species is quite close to Pycnogonida (sea spiders) and Arachnida (spiders, scorpions, harvestmen, mites, etc.) (Edgecombe and Legg, 2014; Giribet, 2018). Kassim et al. (2018) reported that horseshoe crabs (*Carcinoscorpius rotundicauda*) have a body-forming and protective shell with a strong elastic structure. It is formed by the evolutionary process of the hardened material (Joffe et al., 1975).

Indonesia is known to harbor three species of horseshoe crabs, i.e., *Tachypleus gigas* (Müller, 1785), *Tachypleus tridentatus* (Leach, 1819) and *C. rotundicauda* (Latreille, 1802) (Mashar et al., 2017; John et al., 2018a,b). Usually, *C. rotundicauda*, known as the mangrove horseshoe crab, lives in muddy mangrove ecosystems and warm-soft sandy estuaries. In Indonesia, the mangrove horseshoe crabs can be easily found along North Java coastal waters, such as in Indramayu, Subang, Semarang, Demak, Rembang, Sumenep, Bangkalan, Probolinggo, Gresik, and Lamongan. However, the horseshoe crab is experiencing pressure and conservation problems, so it needs research as a baseline for future management (Wang et al., 2020). Current studies on horseshoe crabs in Indonesia cover their biological aspects, such as taxonomy and morphology (Meilana et al., 2016; Sumarmin et al., 2017; Erwyansyah et al., 2018; Aini et al., 2020), reproductive biology (Eidman et al., 1997), feeding habits (Nuraisah et al., 2020), and habitat distribution (Mashar et al., 2017; Erwyansyah et al., 2018; John et al., 2018a,b) solely.

Inspired by nature (bio-inspiration), further materials from horseshoe crabs can be developed in the future, such as different biomaterials like, marine medicines, antioxidants, antifouling and antibacterial materials, etc. (Ismail et al., 2011; Pati et al., 2020). But for this further use, the physico-chemical characteristic informations of the horseshoe crab shells becomes important in the creation of environmentally friendly advanced materials. Mechanical studies on the hard shell (solid cuticle) characteristics of arthropods have been of engineers and biologist interest for five decades (Jensen and Weis-Fogh, 1962; Hepburn and Ball, 1973; Hepburn and Joffe, 1974a,b; Joffe and Hepburn, 1974; Gadgey and Bahekar, 2017). Nevertheless, the compilation matrix of the shell of horseshoe crabs has never been studied before. Additionally, studies are developing regarding horseshoe crab blood benefits and use as a modern pharmaceutical ingredient (Krisfalusi-Gannon et al., 2018).

Chen et al. (2008a) described that a shell of horseshoe crabs consists of 50–60 nm chitin fibers arranged in parallel and layers (nanofibers of chitosan layers). Characteristics of functional groups in the shells of horseshoe crabs show that the shells consist of hydroxyl groups, secondary amides, asymmetric C–H, C–H alkanes, C=O secondary amides and C-aliphatic ether (Kassim et al., 2018). Briefly, the arthropods exoskeleton consists mainly of chitin and is multifunctional, such as supporting the body, resisting mechanical loads, protecting harsh environmental conditions and resistance to desiccation (Neville, 1975; Vincent, 1991, 2002; Vincent and Wegst, 2004; Sanchez et al., 2005; Meyers et al., 2008). Therefore, this study aimed to elucidate the physico-chemical characteristics of the shell of mangrove horseshoe crabs (*C. rotundicauda*) for the first time. The new information on the shells composition matrix of *C. rotundicauda* has never been studied in detail before, especially the shape of the foam, the chemical composition, the functional groups and the mechanical-physical and thermal properties of these shells are a novelty in this baseline study. Based on this study, the shell structure of the mangrove horseshoe crab has the potential to be used as the base structure for developing bio-foam insulator material in the future, such as insulation materials in buildings, vehicle interiors and as blocking materials to block the flow of heat energy.

MATERIALS AND METHODS

Research Location and Examined Crabs

Horseshoe crab samples were taken from local fishermen at coastal waters of Pacitan, north part of East Java – Indonesia, in April and June 2018. Laboratory work was

carried out in January–July 2019 in three laboratories, namely Laboratory of Preservation and Diversification of Aquatic Products, Department of Aquatic Products Technology – Faculty of Fisheries and Marine Sciences, IPB University, Center for Research and Development of Postharvest Agriculture in Cimanggu in Bogor, and Physics Research Center, Indonesian Institute of Sciences in Serpong, Indonesia.

The morphometric measurement of 20 examined crabs *C. rotundicauda* consisted of a length profile of the main body parts, namely prosoma = 65 ± 0.41 mm, ophistoma = 51.15 ± 1.06 mm, and telson = 135 ± 4.32 mm. The frontal margins of the *C. rotundicauda* have a rounded shape with a flat front view and a length of 113.80 ± 1.06 mm. The pedipalps show an identical shape to the pedipalp size = 5.36 ± 0.21 mm and 5.37 ± 0.21 mm. The telson (cross-section) was of triangular shape with obtuse angles, a width of 4.57 ± 0.38 mm and a height of 5.45 ± 0.19 mm. The genital operculum had a round shape with a V pattern and at the posterior end were marginal spines have a uniform shape between the left and right side and size that is marginal right spines have a length of 7.37 ± 0.18 mm to 11.33 ± 0.19 mm and left 7.38 ± 0.20 mm to 11.32 ± 0.18 mm. The total length of *C. rotundicauda* was 259 ± 4.12 mm. The specimens used were males (10) and females (10). The morphometric data can be seen on **Table 1**.

TABLE 1 | Morphometric measurements of mangrove horseshoe crab (*Carcinoscorpius rotundicauda*).

Number	Morphometric characteristics	(mm)
1	Total length	259 ± 4.12
2	Telson length	135.8 ± 4.32
3	Body length	103.8 ± 1.92
4	Prosoma length	65.11 ± 0.41
5	Median ridge length	31.78 ± 0.56
6	Occeli length	30.67 ± 0.84
7	Opthistoma length	51.15 ± 1.06
8	Ventral messel thickness	27.81 ± 0.64
9	Maximum prosoma thickness	113.80 ± 4.02
10	Compoud eyes distance	50.68 ± 0.66
11	Telson's middle high	5.45 ± 0.19
12	Telson mid width	4.57 ± 0.38
13	Length marginal spine I (right side)	11.33 ± 0.19
14	Length marginal spine I (left side)	11.32 ± 0.18
15	Length marginal spine II (right side)	12.51 ± 0.87
16	Length marginal spine II (left side)	12.51 ± 0.86
17	Length marginal spine III (right side)	13.37 ± 0.24
18	Length marginal spine III (left side)	13.41 ± 0.24
19	Length marginal spine IV (right side)	9.92 ± 0.23
20	Length marginal spine IV (right side)	10.00 ± 0.22
21	Length marginal spine V (right side)	7.92 ± 0.22
22	Length marginal spine V (left side)	7.88 ± 0.22
23	Length marginal spine VI (right side)	7.37 ± 0.18
24	Length marginal spine VI (left side)	7.38 ± 0.20
25	Right side chelicerae claw diameter	2.48 ± 0.33
26	Left side chelicerae claw diameter	2.46 ± 0.32
27	Right side pedipalp clamp diameter	5.36 ± 0.21
28	Left side pedipalp clamp diameter	5.37 ± 0.21
29	Right side foot clamp I diameter	5.48 ± 0.22
30	Diameter of the foot clamp I left side	5.47 ± 0.22
31	Diameter of right side foot II foot clamp	3.34 ± 0.17
32	Diameter of foot clamp II left side	3.36 ± 0.17
33	Diameter of foot clamp III right side	3.43 ± 0.28
34	Diameter of foot clamp III left side	3.43 ± 0.28
35	Diameter of IV foot clamp right side	4.52 ± 0.28
36	Diameter of IV foot clamp left side	4.50 ± 0.28

Characterization of the Shell

The research was conducted with physico-chemical and mechanical-physical analysis. The preparation of 20 horseshoe crabs consisted of two main activities; separating the shell/exoskeleton part from the non-shell part and cleaning the shell part from dirt. Cleaning the shell was done using distilled water followed by soaking it into acetone (ratio 1:2) for 24 h to remove impurities. The characterization of 20 horseshoe crab shells began with visualization of the shell and the microstructure analysis of the shell cross-section, referring to the identification technique of Chen et al. (2008a) using scanning electron microscopy (SEM).

The composition of the constituents was identified based on the chemical composition, included analysis of Energy Dispersive X-ray (EDX; Tanasi et al., 2017), water content (AOAC, 2005; item 934.01), protein content (AOAC, 2005; item 955.04), fat content (AOAC, 2005; item 972.28), ash content (AOAC, 2005; item 938.08) and functional groups using FT-IR (Fourier Transform-Infrared) spectrophotometer (ASTM, 2013).

The number of replications for SEM and FT-IR was two for each method used. SEM analysis observed a visual appearance of a cross-sectional incision of a horseshoe crab shell opisthosoma with a structure forming a foam unit cell size of ±70 μm consist of an intermediate layer and an interior core. FT-IR analysis was conducted to determine the sample's chemical structure and the possibilities of interaction between components. Sample preparation by method KBr pellets was a mixture of solid samples with KBr powder (5–10% of the sample KBr powder). The homogeneous mixture was formed by KBr pellets (pills KBr) with the Mini Hand Press. Once formed, the Pill was ready for analysis. The sample was 2.5 × 2.5 cm^2 mounted on the IR card. Spectrum infrared waves were fired through the sample placed in between the spectrophotometer electrode and passed to the computer. Data obtained as a percentage of the absorbance value, with the spectrum's measurement at wave number range 4000–400 cm^{-1}. The results obtained were in the form of a spectrum that appears on the computer.

Scanning electron microscopy- energy dispersive X-ray analysis of horseshoe crab shell was cut crosswise and then was put in microscope slides. The shell structure was then observed using a light polarizing microscope with a magnification of 40 × 10 and photographed using dino lite and saved in JPEG format. XRD analysis, the type of phase and the crystallinity of the petrol shell were analyzed using X-Ray Diffraction (XRD) (PANalytical). Operational conditions equipment namely using a Cu-Kα radiation beam with an angle of 2θ, 20° to 60° and 0.1° resolution, which was operated with an X-ray diffractometer

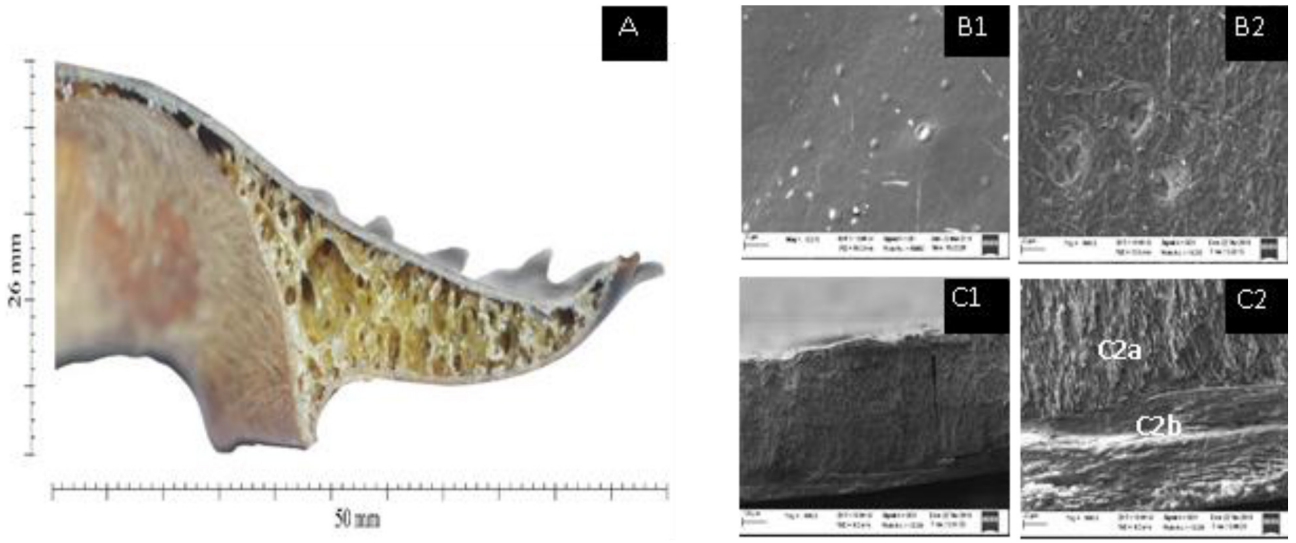

FIGURE 1 | Visual appearance of the incision through the opisthosoma of the shell of mangrove horseshoe crabs (*Carcinoscorpius rotundicauda*) with a foam-shaped structure of ±70 μm **(A)**. The microstructure of the horseshoe crab shell using SEM 500× magnification at the top **(B1)** and bottom **(B2)**. 100× magnification of transverse incision **(C1)**. 500× magnification of transverse incision **(C2)** showing intermediate layer **(C2a)** and interior core **(C2b)**.

according to ASTM (2008). The sample was prepared and as much as 2 mg were placed in a holder measuring 2 × 2 cm^2 on the diffractometer. The voltages are used at 40 kV and the generator current was 30 mA. The starting corner was taken at 5° and the final angle at 80° with a 4° per minute reading speed. The result was a graph of the identified phases based on the intensity and angle of two thetas formed. Determination of the phase refers to the Joint Committee on Powder Diffraction Standard (JCPDS) and calculates the crystallinity degree.

The measurements of the mechanical-physical characteristics included density, unit cell size, and water absorption. It was done following the protocol according to Schmidt and Laurindo (2009), while the tensile strength and compressive strength were analyzed based on ASTM (1991). Thermal resistance was measured by thermal gravimetric analysis (TGA; Klamczynski et al., 2007).

The number of replicates used for physico-chemical analyses was two replicates per method. The testing machine clamped tensile strength, the two ends of the sample. The "start" button was turned on, and the tool pulled the sample to break and note the tensile strength (F) and length after breaking up. After the next sheet was tested, the material's maximum stress could withstand up to its breaking point. Compressive strength, extrudates 2 cm long, was placed on a flat disk. Then, the extrudate was pressed using another dish at a rate of 1 cm per minute. The compression value divided the applied force (kN) value distributed by the surface area sample tested. A high value indicates that the sample was relatively hard, whereas a low value indicates that the sample was soft and easy to compress. As much as 1 g of starch was added to 10 g of distilled water and then stirred for 30 s. Water absorption analysis, the mixture was allowed to stand for 30 min and then centrifuged at a speed of 4,000 rpm for 30 min. Density analysis, the sample was cut into 2 × 2 cm sizes. The mass was known by weighting the pieces with analytical balances. Calculating volume was done by multiplying the length, width and thickness of the piece.

RESULTS

Visual Appearance and Structure of the Horseshoe Crab Shell

The horseshoe crab shell cross-section shows that the cavity structure with strut forming foam created the inside of the horseshoe crab shell, and the strut seems to provide even redistribution of pressure to all parts of the shell. Struts are hollow walls that form open foam cells in the horseshoe crab shell. Struts are described as steel poles that support a bridge but have a stochastic or irregular shape. Cavity cell unit sizes range between ±70 μm (**Figure 1**). Struts on the inside of the shell form irregular angles and has the function as a reinforcement system for the horseshoe crab shell.

Based on the various microstructure views (shooting angle) of the shells, see **Figures 1A–C**, it can be seen that the horseshoe crab shell has a smooth appearance at the top and a rough texture as a link to the foam buffer at the bottom. The transverse incision of the horseshoe crabs shell shows two firm lines showing that the shell is composed of three main layers, which are arranged parallel to each shell, covering the exterior shell, intermediate layer and interior core.

Chemical Composition of the Horseshoe Crab Shell

The chemical composition shows that the shells' main components are protein and crude fiber, i.e., 68.02 and

TABLE 2 | Chemical composition of mangrove horseshoe crab (*Carcinoscorpius rotundicauda*) and some crustacean shells.

Parameters	Horseshoe crab shell[a]	Snow crab shell[b]	Mud crab shell[c]	Crab shell[d]
Moisture (%)	8.50 ± 0.25	72.00 ± 5.8	5.39	–
Protein (%)	68.02 ± 0.36	34.20 ± 2.8	14.11	13.20-20.70
Ash (%)	1.06 ± 0.13	28.50 ± 6.0	57.26	62.90-74.97
Lipid (%)	1.07 ± 0.04	17.10 ± 9.3	2.38	–
Fiber (%)	24.17 ± 0.87	–	–	–

Source: [a]Present study, [b]Yusty et al. (2011), [c]Humaira et al. (2017), [d]Pires et al. (2017).

24.17%, respectively (**Table 2**). This component may be formed during the ecdysis or molting process. The composition of the shell matrix through observations with EDX (**Figure 2** and **Table 3**) shows that the horseshoe crab shell is dominated by carbon (53.54%), oxygen (31.13%), and nitrogen (14.49%). Other constituent elements are chlorine (0.5%), sulfur (0.29%), phosphorus (0.09%), calcium (0.06%), potassium (0.05%), and silicon (0.03%).

The Horseshoe Crab Functional Shell Groups

The absorbent spectrum of horseshoe crabs shell shows the absorption of O–H stretching groups at 3475 cm^{-1}, N–H stretching groups at 3249–3236 cm^{-1}, C–H groups at 2,908–2,866 cm^{-1}, amide II groups at 1564–1488 cm^{-1}, groups of amide III at 1328–1274 $cm^{-1}$and C–O groups at 1081–1029 cm^{-1} (**Table 4**). A comparison of different absorbent spectra from studies on the shells performed on different organisms showed insignificant values and still in the same structure and functional groups (**Table 4**).

Mechanical-Physical and Thermal Characteristics of Shells

The mechanical-physical characteristics of the shell of mangrove horseshoe crabs (*C. rotundicauda*) have a density of 0.1545 ± 0.011 g/cm^3, a water absorption of 0.01195 ± 0.001%, the tensile strength of 0.06 mPa and a compressive strength of 0.11 mPa (**Table 5**). As shown in **Table 5**, the value of the mechanical-physical characteristics of the mangrove horseshoe crab (*C. rotundicauda*) is smaller than in some other crustaceans shells. Mechanical-physical has a correlation characteristic that tends to be positive toward the elongation value (**Figures 3A,B**). In contrast to the thermal characteristics, the mass negatively correlates with temperature (**Figure 3C**).

DISCUSSION

The shell composition matrix of *C. rotundicauda* has never been studied in detail before, especially the shape of the foam, the chemical composition, the functional groups and the mechanical-physical and thermal properties of the shells give a unique insight which can be used in future. Each species in nature appears in various forms; this form is usually influenced by its unique surrounding environment and evolutionary processes (Jabbari et al., 2014). The shell of mangrove horseshoe crabs has a natural structure in arranging of a very strong foam, especially the β-1, 4-glycosidic (chitin) structure, which forms foam and provides resistance to structural damage and thermal degradation is of high value for future natural materials. Due to this very strong and plastic structure,

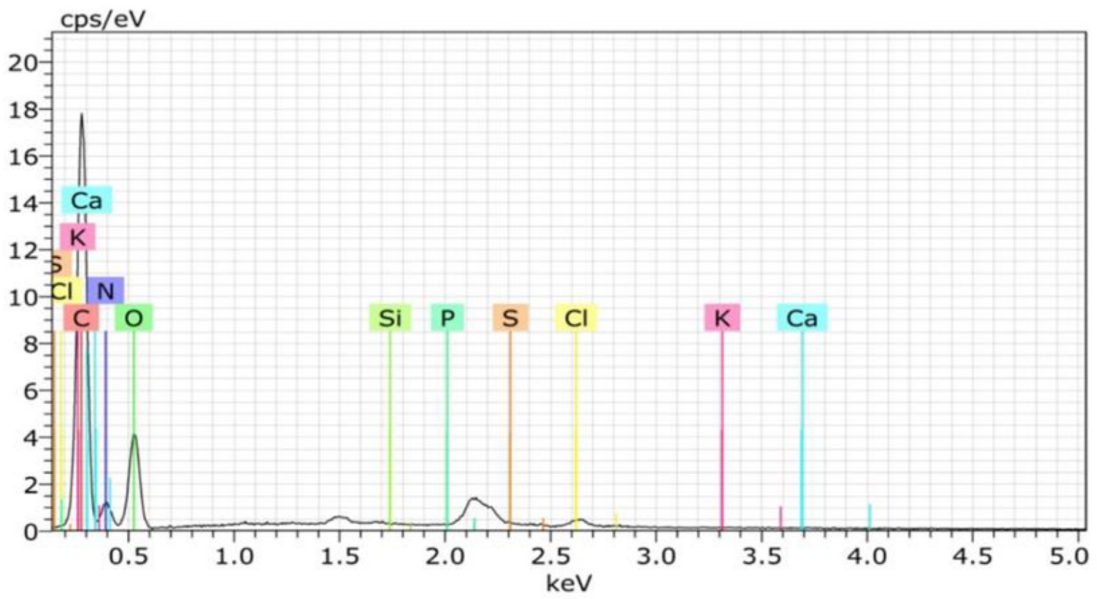

FIGURE 2 | Element compositions (EDX) of horseshoe crab (*Carcinoscorpius rotundicauda*) shells.

TABLE 3 | Element levels of the shells in mangrove horseshoe crabs (*Carcinoscorpius rotundicauda*) and some other crustaceans.

Parameters	Horseshoe crab shell[a]	Crab shell waste from India[b]	Mud crab from Merauke[c]	Crab shell from Cirebon[d]	*Ammonia beccarii* shell[e]
Carbon (%)	53.54	–	19.78	24.19	16.36
Oxygen (%)	31.13	55.94	–	43.83	51.19
Nitrogen (%)	14.49	–	–	–	–
Calcium (%)	0.06	30.18	71.42	27.97	7.94
Sulfur (%)	0.29	–	–	–	0.18
Phosphorous (%)	0.09	9.59	3.98	–	–
Titanium (%)	–	–	–	–	0.18
Chlorine (%)	0.50	1.85	–	–	0.53
Natrium (%)	–	–	–	–	0.44
Magnesium (%)	–	2.44	4.81	1.46	0.56
Potassium (%)	0.05	–	–	–	1.16
Ferric (%)	–	–	–	–	2.36
Aluminum (%)	–	–	–	–	6.22
Silicon (%)	0.03	–	1.16	–	12.88

Source: [a]Present study, [b]Bhattacharjee et al. (2019), [c]Haryati et al. (2019), [d]Nazir et al. (2019), [e]Rositasari et al. (2018).

TABLE 4 | Identification of functional groups and bonds based on the peak FTIR on horseshoe crab shell and other crab shells.

Wave-number (cm^{-1})[a]	Wave-number (cm^{-1})	Structure	Functional group
3475	3477[b]	O–H	Hydroxyl
3249–3236	3258–3101[c]	N–H	Amine
2908–2866	2929–2882[b]	C–H	Alkane
1564–1488	1552[c]	$(RCO)_2NH$	Amide II
1328–1274	1375–1307[c]	$(RCO)_3N$	Amide III
1081–1029	1028[d]	C–O	Carboxyl
852–846	895–890[e]	β-1, 4-glycosidic	Glucose

Source: [a]Present study, [b]Hajji et al. (2015), [c]Pandharipande and Bhagat (2016), [d]Varun et al. (2017), [e]Kumirska et al. (2010).

TABLE 5 | Mechanical-physical characteristics of the mangrove horseshoe crab (*Carcinoscorpius rotundicauda*) and some other crustaceans shells.

Parameters	Horseshoe crab shell[a]	Sheep crab claw shell[b]	Mussel shell[c]	Coconut shell[d]
Density (g/cm^3)	0.15 ± 0.011	–	1.52 ± 0.04	–
Water Absorption (%)	0.01 ± 0.001	–	0.37–10	0.43–0.52
Tensile Strength (mPa)	0.06	9.40 ± 2.60	1.71–2.92	–
Compressive Strength (mPa)	0.11	57.00 ± 10	18.03–41.70	59.33–60.66

Source: [a]Present study, [b]Chen et al. (2008b), [c]Sainudin et al. (2019), [d]Singh and Bhaskar (2013).

it has the potential to be further developed for new marine biomaterials. The combination of constituent elements, such as polysaccharides and minerals in the shell of *C. rotundicauda* needs to be studied through a biomimetic approach as a reference for creating new materials with the same properties from synthetic materials that do not explore excessively against one particular species. The formation and composition of the natural material from the horseshoe crab shell foam are believed to be multifunctional in mobility, used for defense mechanisms and thermal stability. In the future, materials that are strong and able to withstand heat loads can be used as insulation materials in buildings to play a role in reducing thermal loads significantly and effectively to the energy needs of buildings or vehicle interiors and even spacecrafts. Thermal insulation as a building installation element can block the flow of heat energy through energy-saving mechanisms by reducing heat loss and recovery, controlling surface temperature, preventing steam flow, preventing water condensation on cold surfaces and increasing the efficiency of heating-cooling operations. To use natural marine biomaterials will be beneficial because it leaves no harmful residues and therefore has environmental advantages (Silva et al., 2012) and is also more cost-effective (Pati et al., 2020).

Extensive information about this species is published on population structure and morphometry (Koichi Sekiguchi and Shuster, 2009), genetic diversity (Meilana et al., 2016), reproductive system (Brockmann and Smith, 2009), medical applications (Krisfalusi-Gannon et al., 2018), and distribution (Mashar et al., 2017). Horseshoe crabs have a body like a coconut shell, brownish, long spines on the back and hard carapace texture. The shells cover and protect the internal organs of the body (Pratiwi, 1993). The shell hardness seems to be due to naturally occurring constituent elements formed several times during molting (Cousteau, 1975) or through sclerotization. According to Sugumaran (2010), sclerotization is a hardening process of a new cuticle exoskeleton when molting. It involves reactive catecholamine derivatives, which are produced endogenously with structural proteins and chitin fibers. Sclerotization is also known to stabilize the cuticle matrix protein to make it stiffer, harder, more insoluble and more resistant to degradation (Klowden, 2008).

Foam on the horseshoe crabs shell is only found in certain parts, namely the posterior corner of the operculum and all opisthosoma parts. It is concentrated in the marginal spines growth area. The shell structure of horseshoe crabs, which is strengthened by the strut to distribute the pressure evenly to the whole carapace, is believed to have a function as a component for mobility and defense of the horseshoe crab.

The exterior shell forms a Bouligand structure that provides rigidity to the outer side of the shell. The structure is commonly found in arthropod shells (Gadgey and Bahekar, 2017). The structure was proved to repeat, forming the exocuticle and endocuticle (Bouligand, 1972; Giraud-Guille, 1984, 1990; Roer and Dillaman, 1984; Weiner and Addadi, 1997). The intermediate layer consists of a vertical laminate with a thickness of 2–3 μm, which produces elastic characteristics and connects the exterior shell to the interior core. The interior core has a supporting foam structure and a porous shape with a stochastic-patterned strut.

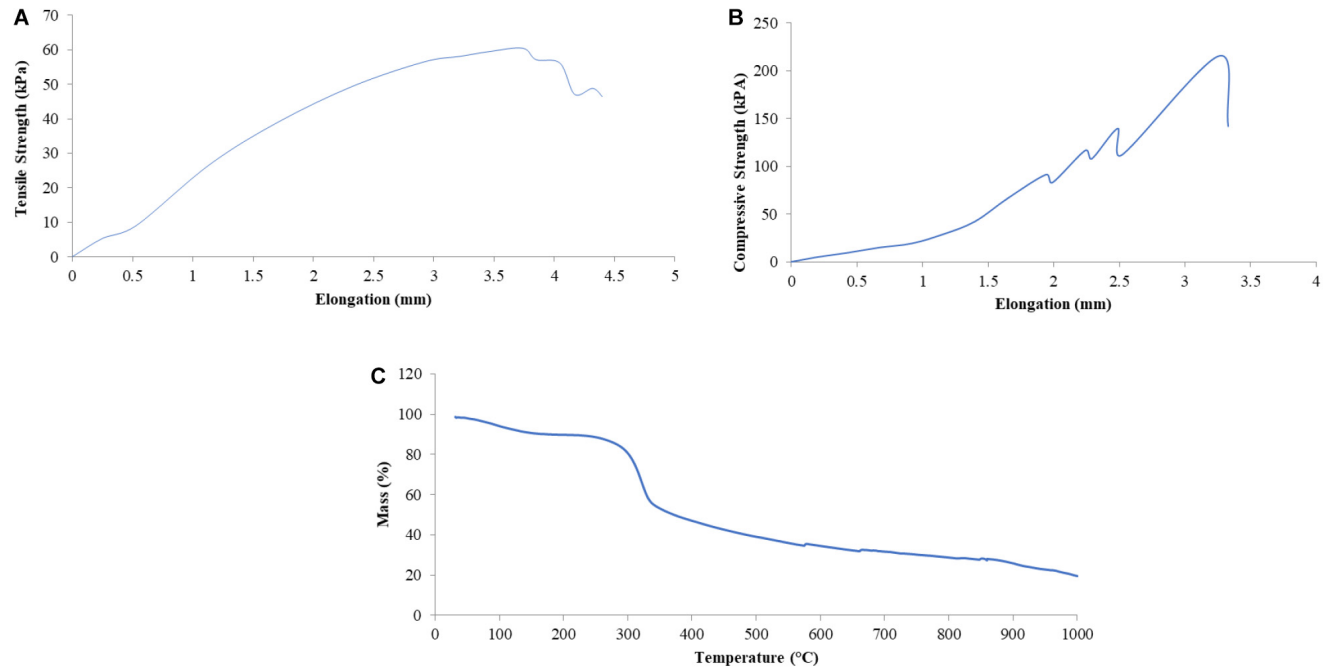

FIGURE 3 | Some mechanical-physical characteristics [Tensile Strength **(A)**, Compressive Strength **(B)**] and Thermal Resistance **(C)** of the shell of mangrove horseshoe crabs (*Carcinoscorpius rotundicauda*).

Forming elements and the foam structure shape are thought to function to provide mechanical strength and thermal stability to the shell. Theoretically, porous structures with strut nuclei are at least seven times stronger than similar open-cell porous; the width of the cavity in the struts further reduces the weight of the shell (Sullivan et al., 2017). In addition to the layer structure of the horseshoe crabs shell, the shell's elasticity and thermal stability are believed to be due to the constituent material presence, i.e., chitin or minerals. Horseshoe crab shells (*C. rotundicauda*) in dry powder have high protein levels, while crude fiber, water, ash and fat are deficient. Generally, the percentage of protein on horseshoe crab shells is 1.99-5.15 times higher than in snow crab shells (34.20%), mud crab shells (14.11%), and crab shells (13.20%) (**Table 2**). This component affects the element composition of carbon in the shell. The carbon content of *C. rotundicauda* is also 2.21–3.27 times higher than in some other crustaceans, such as crab shell waste from India (0%) (Bhattacharjee et al., 2019), mud crab from Merauke (19.78%) (Haryati et al., 2019), crab shell from Cirebon (24.19%) (Nazir et al., 2019), and *Ammonia beccarii* shell (16.36%) (Rositasari et al., 2018; **Table 3**). According to Cardenas et al. (2003), carbon, oxygen and nitrogen atoms in arthropod shells are generally formed from organic chitin compounds and proteins or inorganic salts such as $CaCO_3$. Existing phosphorus is in the form of phosphate ions ($PO4^{3-}$), associated with Ca^{2+} ions to form calcium phosphate, which functions to control the physico-chemical properties of the shell (Bentov et al., 2016).

Sclerotization studies have shown that the marine arthropod shell mineral elements act as nuclei to produce cross-bonds between chitin and protein forming composites. A study conducted by Andersen (2010) has shown that sclerotization produces diverse characteristics with varying thickness, stiffness, elasticity and color specifications. The sclerotization results in the shell are used as protection against environmental changes, like pressure and temperature variations (Cardenas et al., 2003).

Uptake at the wave number 3475 cm^{-1} indicated O–H functional groups. The hydroxy group (O–H) indicates the presence of hydrogen bonds in the sample. Hydrogen bonds produce bands with significant widening. The hydroxy group is one of the most dominant and essential groups for the actual characterization of compounds. The hydroxy group, which is at wave-number 3,400–3,200 cm^{-1}, is a normal polymeric O–H group in the form of stretching (Coates, 2006). The absorption band at the wave-number 3141–3080 cm^{-1} indicates the N–H stretching functional group.

Furthermore, Coates (2006) added that the N–H group indicated amine components in the sample. The absorption band at wave-numbers of 2,908–2,866 cm^{-1} indicated the existence of C–H functional groups. Coates (2006) explained that the C–H group located in the absorption band 2,900–2,880 cm^{-1} belonged to the methylated saturated aliphatic compound. This compound was derived from methane compounds. Also, this compound has four single bonds and one of them is a hydrogen compound. Absorption at wave-numbers 1,564–1,488 cm^{-1} and 1,328–1,274 cm^{-1} indicated the presence of amide II and amide III functional groups. Prabu and Natarajan (2012) explained that the amide group's presence was related to protein compounds in the sample. Absorption of amide groups II and III is ascribed to the N-acetyl or N-methyl groups. Carbohydrates are thought to be the main constituent of the shell acetyl

polymer group. The absorption band at wave-number 1,081–1,029 cm^{-1} indicates the presence of C–O functional groups. Coates (2006) explains that the C–O group indicates a primary alcohol component in the shell.

The shell of horseshoe crabs has its main constituent in chitin polysaccharides with DD% 5.83. Natural chitin is formed mainly as α-chitin and β-chitin (Brunner et al., 2009). The chitin infrared spectrum displays a series of narrow absorption bands, typical of polysaccharide crystal samples. Aung et al. (2018) found a functional group in the FT-IR analysis of crab shells in the form of amide, carbonyl and hydroxyl groups. Pandharipande and Bhagat (2016) stated that the peak of chitin in crab shells from the Nagpur fish market, India was at 3,431–3,430 cm^{-1} (O–H), 3,258–3,101 cm^{-1} (N–H stretching), 1,552 cm^{-1} (amide II), and 1,375–1,307 cm^{-1} (amide III). While the peak of chitin on crab shells in the Sfax market, Tunisia was shown at 3447 cm^{-1} (O–H), 3,267–3,104 cm^{-1} (N–H stretching) and 2,929–2,882 cm^{-1} (asymmetrical and symmetrical C–H) (Hajji et al., 2015). The peak of chitin in crab shell samples from the Russel fish market, India was at 3,443 cm^{-1} (O–H stretching), 3,107 cm^{-1} (N–H stretching), 1,558 cm^{-1} (amide II), 1,314 cm^{-1} (amide III), 1,028 cm^{-1} (C–O stretching) (Varun et al., 2017). Another sign that the compound contains chitin are bending vibrations in the absorption of wave number 852–846 cm^{-1}, which indicates the presence of β-14-glycosidic bonds. Kumirska et al. (2010) explained that the C-H deformation of the β-1.4-glycosidic bond causes a shift in the β-chitin band. Demir et al. (2016) also explained that the wavelength value of 894.91 cm^{-1} represented the stretching of the bonding ring for the β-1.4-glycosidic bond on chitin of the shell of blue crabs (*Callinectes sapidus*).

Referring to the previous visual appearance, the unit size of the shell cells was around 70 μm. Low densities can be caused by the proportion of empty spaces (Yunita and Mahyudin, 2017). Hussein et al. (2011) stated that low-density values were seen from the porous shape of the shell of horseshoe crabs support, which is not uniform due to the agglomeration of the constituent material.

Space on the composite allows water flow to enter capillarization, which results in high water absorption strength (Yunita and Mahyudin, 2017). However, the constituent material in chitin polysaccharides influenced the low water absorption capacity of horseshoe crab shells. Kumar et al. (2016) explained that the main components of the shells are chitin. Chitin chains between one and another are bound by a powerful hydrogen bond between the N-H group of one chain and the C=O group of another adjacent chain. This hydrogen bonding causes chitin to not dissolve in water and to form fibers (fibrils).

The horseshoe crab shell tensile strength produces a robust and plastic pattern with a maximum stress of 60.46 kPa, indicated by the tensile stress and long elongation (**Figure 3A**). Hepburn et al. (1975) elucidated that shell tensile strength is influenced by the polymer of its constituent elements, namely chitin. Chitin is able to form the horseshoe crab shell layer's entire structure, namely Bouligand, laminate and support system (strut), which provides tensile and compressive strength. As the horseshoe crab shells are composed primarily of chitin and also protein, so chitin affords a tensile strength that is useful in curvature. In contrast, proteins may exist as reinforcement for the inorganic phase and might give rise to prestressed material types (Gadgey and Bahekar, 2017). Prestressed material is concrete that is given internal compressive stress such that it can eliminate the tensile stress caused by external loads to a certain extent. The shell water content also influences tensile strength; dry shells show a lower value of flexibility, strength and toughness than the wet shells (Gadgey and Bahekar, 2017).

Horseshoe crab shells produce a rigid pattern with a maximum compressive strength of 110.55 kPa, indicated by gentle compressive stress with short elongation (**Figure 3B**). This value is lower than the compressive strength of sheep or spider crab shells. According to Lombardi et al. (2013), the compressive strength value is influenced by the shell density and thickness and the method used. The density and compressive strength of the shell is directly proportional. The shape of the shell also affects the compressive strength value. The sample curvature can distribute the load throughout the structure resulting in a hard and rigid structure. The compressive strength is also influenced by water content and material density. Materials with high water content will reduce the fragile effect (Gadgey and Bahekar, 2017). However, high density can increase strength due to material density (Lombardi et al., 2013).

Thermal stability is produced by horseshoe crab shells of 60.59% in this study. Their decomposition forms a multi-stage decomposition curve, which means it has undergone three decomposition processes. The first decomposition occurred at a temperature range of 254.7–275.6°C with a peak of decomposition at 267.4°C, the second decomposition occurred at a temperature range of 428.6–448.1°C with a peak of decomposition at 435.9°C and the third decomposition occurred at 811.4–835.8°C with a peak of decomposition at 823.2°C. The first and second decomposition peaks (**Figure 3C**) indicated the degradation of organic compounds in the horseshoe crab shells, such as chitin and protein. Stawski et al. (2008) explained that the temperature of 300–460°C is the temperature range of the occurrence of thermal degradation of chitin polysaccharides from shrimp, crab, krill and squid. The mass loss at temperatures between 200 and 550°C is the temperature range for the degradation of organic and amine groups (Loganathan et al., 2017). Though, the third peak was due to the mineral content in the shell. He et al. (2014) stated the thermal decomposition of calcium carbonate occurs at temperatures around 700°C, with a very large mass reduction approaching 44%, which corresponded to the theoretical decomposition of calcium carbonate. From a thermal point of view, Gbenebor et al. (2016) experimented on the decomposition of crab and shrimp shells according to temperature, and they found the decomposition occurred in three stages. The first decrease occurred at a temperature of 60–110°C due to eliminating water with other volatile components. The second decrease occurred at 316–397°C due to chitin compounds in crab shells and at 321–397°C in shrimp shells. The third reduction of 30.93% in crab shells at 682–729°C and 18.85% in shrimp shells at 665–713°C was caused by the decomposition of $CaCO_3$ to CaO and CO_2. The thermal stability of chitin from crabs was 244% and from shrimp 112.5%. The percentage of $CaCO_3$ elimination can increase thermal stability. These results show that the higher $CaCO_3$ content in the crustacean exoskeleton will reduce the thermal stability of chitin. A shell

with a lower $CaCO_3$ content will produce more stable chitin. Acetyl (CH_3CO) in the acetamide group (CH_3CONH_2) of the chitin structure provides resistance to structural damage and thermal degradation.

CONCLUSION

We herewith determined for the first time the compilation matrix and further physico-chemical characteristics of the shell of mangrove horseshoe crabs (*C. rotundicauda*). The formation and composition of the natural material from the horseshoe crab shell foam are believed to be multifunctional in mobility, used for natural defense mechanisms and thermal stability. The shell of mangrove horseshoe crabs has a hollow structure (foam) with the main chemical elements of chitin, thus providing mechanical-physical properties tensile and compressive strength of the ramps and long elongation. By combining with water absorption strength, density and thermal resistance, the shell structure of the mangrove horseshoe crab has the potential to be used as the base structure to develop bio-foam insulator material (i.e., as thermal insulators), especially the β-1, 4-glycosidic (chitin) structure which forms foam and provides resistance to structural damage and thermal degradation. This is of high value for future materials, for example, as a strong and heat resistant bio-foam insulation material, such as insulation materials in buildings, vehicle interiors, and as blocking material, to block the flow of heat energy against heat loss. Furthermore, biomaterials have a smaller environmental impact than synthetic materials, and here, we expect an improved cost-efficiency. Also, the presented study results can be used as basic information for bio-foam insulator material development, as suggested in the future.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

YW, BR, and NI: conceptualization, methodology, investigation, analysis, resources, data curation visualization and writing – original draft. SK, PF, and FK: supervision and writing – review and editing. All authors contributed to the article and approved the submitted version.

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Tri-Spine Horseshoe Crab Aquaculture, Ranching and Stock Enhancement: Perspectives and Challenges

*Peng Xu[1†], Haiwei Bai[1†], Xiaoyong Xie[2], Chun-Chieh Wang[3], Xing Huang[4], Xueping Wang[4], Mingming Zhang[1], Zhenyu Ye[1], Junhua Zhu[1], Wenquan Zhen[1], Siu Gin Cheung[5], Paul K. S. Shin[5] and Kit Yue Kwan[1]**

[1] Guangxi Key Laboratory of Beibu Gulf Marine Biodiversity Conservation, College of Marine Sciences, Beibu Gulf Ocean Development Research Centre, Beibu Gulf University, Qinzhou, China, [2] Guangdong Provincial Key Laboratory of Fishery Ecology and Environment, Key Laboratory of South China Sea Fishery Resources Exploitation and Utilization, Ministry of Agriculture, South China Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences, Guangzhou, China, [3] Guangxi Key Laboratory of Marine Environmental Science, Guangxi Beibu Gulf Marine Research Center, Guangxi Academy of Sciences, Nanning, China, [4] Guangxi Key Laboratory of Marine Disaster in the Beibu Gulf, Beibu Gulf University, Qinzhou, China, [5] Department of Chemistry, City University of Hong Kong, Hong Kong, China

As a well-known example of "living fossil," horseshoe crabs are ecologically significant macroinvertebrates in coastal and estuarine ecosystems. The tri-spine horseshoe crab, *Tachypleus tridentatus*, has been widely utilized for *Tachypleus* amebocyte lysate production and food consumption since the 1980s, which led to considerable population declines along the west coast of the Pacific Ocean. The declining horseshoe crab population is expected to have ecological and social impacts. Stock enhancement through captive rearing of juveniles is cited as an important alternative to repopulate the native *T. tridentatus*, which in turn supports sustainable resource utilization and research activities. The hatchery production techniques for this species have gradually developed following the mass culture efforts in Japan since the late 1980s. However, the previous studies have primarily concerned the feed types and husbandry conditions to maximize the growth and survival of the juveniles. Little is known about the practicability and effectiveness of releasing large numbers of hatchery-bred individuals through releasing programs. In this review, we (1) summarize the available captive breeding and rearing techniques, (2) discuss the release strategies that could potentially improve the survival of released juveniles, and (3) identify the future opportunities and challenges in establishing technical frameworks to support responsible stock enhancement programs for *T. tridentatus*. The information should benefit future horseshoe crab fisheries management efforts in the attempt to restore the severely depleted populations.

Keywords: captive rearing, release strategy, optimal diet, size at release, tagging

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****Correspondence:***
Kit Yue Kwan
kityuekwan@bbgu.edu.cn

† These authors share first authorship

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INTRODUCTION

Current global fisheries production is severely threatened by overfishing, climate change, and their interactions (Brander, 2007). However, with the advance in aquaculture techniques, this opens up the possibility to release hatchery-reared juveniles to help restore depleted global fishery stocks (Bartley and Bell, 2008; Le Vay et al., 2008; Lorenzen et al., 2010; Han et al., 2016; Taylor et al., 2017). Uses of hatchery releases with different management objectives are referred to as restocking ("to restore depleted spawning biomass to a level where it can once again provide regular, substantial yield"), stock enhancement ("to augment a natural supply of juveniles and optimize harvest by overcoming recruitment limitation"), and sea ranching ("to harvest at a larger size in put-grow-take operations") (Bell et al., 2008). Yet, few releasing programs have claimed their management interventions successful to produce a measurable impact on fisheries stock abundance and yield. Challenges such as producing cost-effective juveniles, developing optimal release strategies and searching for appropriate monitoring and evaluation methods, have been identified (Molony et al., 2005; Wada et al., 2010). A set of elements aimed at developing a responsible approach for releasing programs has been proposed, which emphasizes the equal weight to the dynamics of biological and human components (Blankenship and Leber, 1995; Lorenzen et al., 2010). Lorenzen et al. (2010) also identified common weaknesses in most stock enhancement efforts in coastal systems, including lack of fishery stock assessments and modeling, ignorance in governance framework establishment, rare involvement of stakeholders in program development, and poor integration of adaptive management into stock enhancement plans. Compared to finfish fisheries, some coastal invertebrates may have a higher potential for effective sea ranching and stock enhancement due to their sessile or sedentary characteristic and are more likely to create self-replenishing populations within a relatively localized region (Bell et al., 2006; Gomez and Mingoa-Licuanan, 2006).

A case in point is the potential application of stock enhancement through captive rearing of juveniles as a proactive management tool to restore the dwindling population of the tri-spine horseshoe crab, *Tachypleus tridentatus*, worldwide. Horseshoe crabs are an ancient group of macroinvertebrates that use multiple inshore and estuarine habitats throughout their life cycles: adults spawn on sheltered intertidal flats near the high tide mark, the newly hatched larvae emerge from sediment and enter the sea as plankton, the larvae/juveniles settle and grow on the same or adjacent beaches, and gradually migrate into subtidal areas to reach adulthood (**Figure 1**; Chen et al., 2015; Laurie et al., 2019). Therefore, horseshoe crabs are referred to as the potential sentinel species to indicate the general health status of coastal and estuarine ecosystems (Kwan et al., 2018b).

Horseshoe crabs have a lengthy maturity period. For example, *T. tridentatus* become mature at age 13–14 (instar stage: 17–18) after undergoing 16–17 molts (**Figure 1**; Chen et al., 2010; Hu et al., 2015, for detailed information on instar, age and size). Apart from that, horseshoe crabs also exhibit low dispersal rates, long life span, small home range, and delayed reproductive development (Kwan et al., 2015b, 2020). These life-history features render them vulnerable to overharvesting and habitat loss (John et al., 2018; Laurie et al., 2019). *T. tridentatus* has also been heavily harvested since the 1980s for the production of *Tachypleus* amebocyte lysate (TAL), a substance that quantitatively detects the presence of bacterial endotoxins or fungal contamination in drugs and biomedical devices (Liao and Li, 2001; Liao et al., 2019a). *T. tridentatus* is recently listed as "Endangered (EN)" on the IUCN Red List (Laurie et al., 2019). Considering the increasing demand for pharmaceuticals and biomedical applications in emerging markets, including China, India, and Brazil, mass harvest for wild *T. tridentatus* resources will not cease.

The global decline in *T. tridentatus* populations has attracted increasing interest in searching for appropriate management tools to revert the situation. Despite the introduction of marine protected areas and the establishment of new environmental legislation can be effective, stock enhancement initiative in some cases is considered as a more proactive approach to populate the exploited stocks (Carmichael and Brush, 2012; Wang et al., 2020; Zhu et al., 2020). Releasing programs using hatchery-bred *T. tridentatus* juveniles, mostly the newly hatched first-instars or second-instars, and occasionally adults have been attempted or are underway along the Chinese coast of Fujian, Guangdong, and Guangxi provinces in the Mainland and Taiwan (Hong, 2011; Zhu et al., 2020). In total, more than 7,450,000 juveniles and 2,500 adults of *T. tridentatus* have been used for sea ranching and stock enhancement during 2010–2020, in which 42% of these programs released more than 100,000 individuals each time (**Supplementary Table 1**). Apart from that, every year by joining a conservation education program in Hong Kong, students from 30 selected secondary schools also release the laboratory-reared juveniles under their care at Ha Pak Nai, the most important nursery habitat for the local *T. tridentatus* population (Kwan et al., 2017b). These programs, however, are unlikely to have the desired result given a lack of proper formulation of release strategies, dependable evaluation, and understanding of the ecosystem.

In this review, we summarize the available aquaculture technologies for hatchery production of juvenile *T. tridentatus*. Based on our best knowledge regarding biological and ecological characteristics of the species, release strategies that could potentially improve the survival of released *T. tridentatus* were discussed. Future opportunities and challenges in establishing technical frameworks to support responsible stock enhancement for *T. tridentatus* were also identified. Relevant articles and references were searched and identified mostly through databases and search engines, including Web of Science, Scopus, China National Knowledge Infrastructure, Scholarly and Academic Information Navigator, Japan, and Google Scholar. To include as many studies as possible, reference searching using local names of *T. tridentatus* in varying languages was also conducted. The ultimate goal is to establish effective and economically viable methods for the mass release of environmentally fit juveniles in the attempt to restore the severely depleted *T. tridentatus* populations.

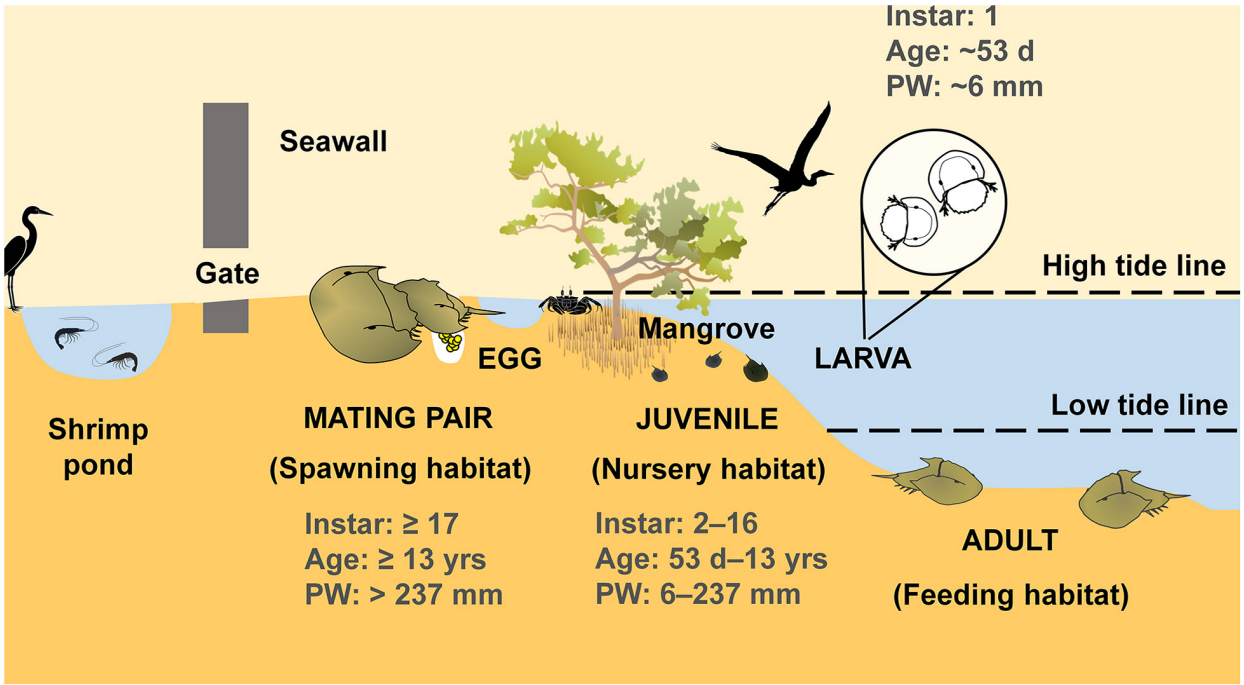

FIGURE 1 | Life cycle and the associated habitats for *T. tridentatus*. PW, prosomal width (size); d, day; yrs, years.

DEVELOPMENT OF HATCHERY PRODUCTION

There is considerable demand in the development of hatchery production technology for rearing horseshoe crab larvae and juveniles due to the decline in horseshoe crab populations (Carmichael and Brush, 2012). However, the capacity of hatcheries to producing a sufficient number of high-quality juveniles in a cost-effective manner is the prerequisite of any releasing activity. Culturing *T. tridentatus* in the laboratory was initiated by the Japanese scientist, Koichi Sekiguchi in 1972 from artificially fertilized eggs with an aim to study the growth and development of juveniles (Sekiguchi et al., 1988). A broader scale of *T. tridentatus* culture efforts has later been attempted in China (e.g., Liang, 1987) and Japan (e.g., Tsuchiya, 2009) since the 1980s. While most previous studies concerned how diet compositions and water quality variables affect the survival and growth of horseshoe crab embryos and juveniles (Carmichael and Brush, 2012; Hu et al., 2013a, 2014, 2018; Liao et al., 2019b), the mass production of cost-effective, high-quality seedstocks has presented a bottleneck in the releasing programs for *T. tridentatus* (Kwan et al., 2014; Zhu et al., 2020). We compiled and discussed the available rearing techniques in maintaining *T. tridentatus* embryos and juveniles in hatcheries with the ultimate goal in defining effective culture conditions (**Figure 2**).

Spawning and Fertilization

The development of *T. tridentatus* aquaculture has been enabled by breakthroughs in artificial breeding. Earlier in the 1940s, Oka (1943) reported his trial on artificial insemination following Patten (1894) to obtain *T. tridentatus* embryos for developmental mechanism study. Sekiguchi et al. (1988) further improved the fertilization rate by spreading the diluted sperm evenly over the eggs after a shorter preparation procedure. The sperm and eggs were collected by cutting the soft tissue on the ventral sides of the prosoma of male and female *T. tridentatus* from the appendage base toward the lateral margin (Chen et al., 2010). Nonetheless, this invasive method would further deplete the remaining population of *T. tridentatus*. Non-invasive electrical stimulation for egg and sperm collection was applied in the Atlantic counterpart, *Limulus polyphemus*. By adopting a similar method, however, a very limited amount of eggs/sperm could be collected from *T. tridentatus* (Sekiguchi et al., 1988; Li, 2008). Alternatively, the natural spawning method has been adopted by keeping amplexed mating pairs of horseshoe crabs in tanks with approximately 10-cm sediment layer underneath (Liang, 1987; Chen and Xia, 2002). The authors also claimed that the released eggs obtained from natural spawning were mostly mature, which can considerably enhance the fertilization rate (Liang, 1987), compared to that from electrical stimulation or artificial insemination.

However, there is relatively little information available regarding the effects of diet and culture conditions on adult *T. tridentatus* survival, breeding, and cellular health. Chen and Xia (2002) reported that natural spawning occurred in breeding tanks during May–July when the mating pairs were fed with shrimp and fish meat, and maintained at water temperature 23–26°C, salinity 25–30, and pH 7.8–8.2. Chow (2019) observed stimulation of *T. tridentatus* spawning when the water salinity

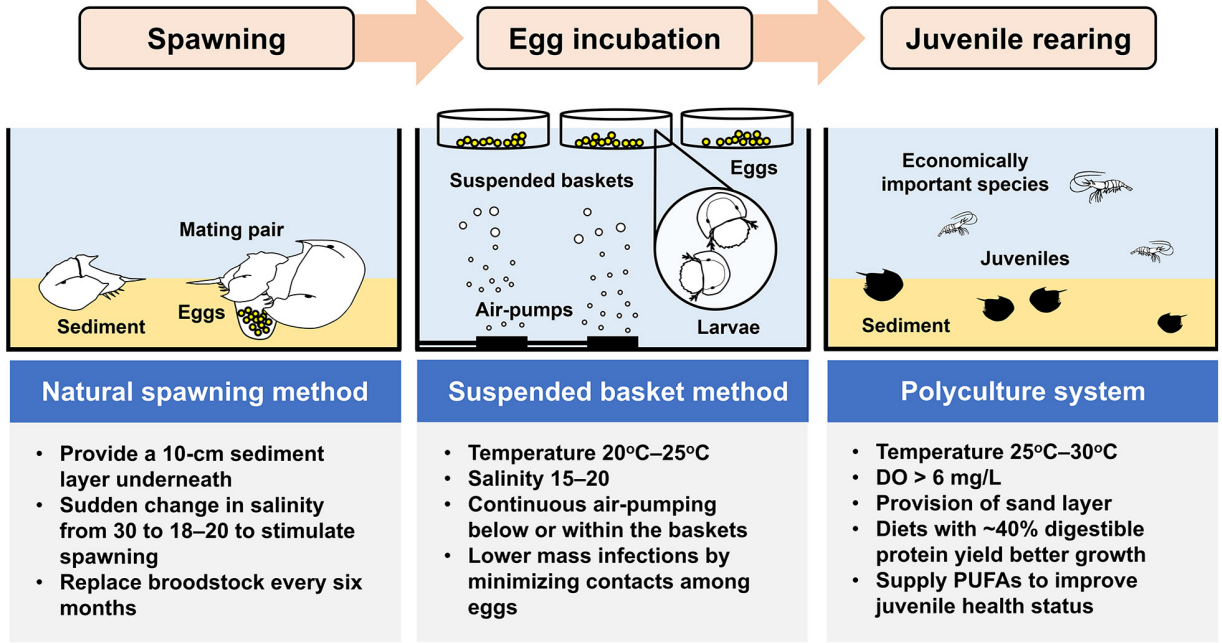

FIGURE 2 | Important techniques and operating procedures on artificial breeding as well as larval and juvenile culture for *T. tridentatus*.

was gradually lowered from 30 to 18–20. A recent study demonstrated that the addition of copper supplements into the feed for adult *T. tridentatus* could improve their immune enzyme activity and hemolymph quality (Xu et al., 2020). Future efforts should be concentrated on developing prototypical nutritional and aquaculture standards for adult horseshoe crab husbandry to support long-term aquaculture management (Tinker-Kulberg et al., 2020a,b,c). Otherwise, the broodstock should be replaced every 6 months. Sustained aquaculture in the facilities has been linked to low survival rates, possibly due to poor nutritional conditions and water quality which would increase their susceptibility to bacterial and parasitic infections (Nolan and Smith, 2009).

Egg Incubation

To maintain relatively stable environmental conditions, the culture of *T. tridentatus* embryos was mostly confined in indoor husbandries or laboratories. In most cases, the fertilized eggs were extracted from the sediment several days after the spawning events, and transferred to an individual culture system for egg incubations. Many authors reported generally higher survival rates (>60%) of fertilized eggs under the following incubation conditions: water temperature 20–35°C, salinity 15–35, pH 8.1–8.3 under continuous aeration with water renewal once or twice a day (Sekiguchi et al., 1988; Li et al., 1999; Li, 2008; Hong et al., 2009, 2011; Chen et al., 2010; Wu et al., 2014; Carmichael and Brush, 2012; Miao et al., 2020).

Despite the fact that most previous studies overlooked the effect of sole environmental factor on fertilization, survival, and hatching of *T. tridentatus* eggs (**Table 1**), Li et al. (1999) found surprisingly high fertilization (>95%) and survival (>98%) rates when the eggs were cultured under salinity ranged 16–33. Li (2008), however, observed interactive effects between temperature and salinity, in which the egg survivorship was deleteriously affected (<30%) at salinity 25 and 30 when the water temperature was kept at 32°C. No hatching was noted during the 90-day experiment in all treatment groups with salinity at 15 (Li, 2008).

Bacterial/fungal infections and dissolved oxygen deficiencies seem to be the most prominent issue in the rearing of horseshoe crab eggs. Faizul et al. (2015) identified four species of bacteria (*Shewanella putrefaciens*, *Bacillus cereus*, *Corynebacterium* sp., and *Enterococcus faecalis*) and fungi (*Aspergillus niger*, *Aspergillus* sp., *Penicillium* sp., and *Gliocladium* sp.), respectively, in infected horseshoe crab eggs. The authors suggested that *S. putrefaciens* and *A. niger* possess primary threats to egg survival and development. While possible treatments to the infections are largely lacking, a 3- to 12-min freshwater bath, and/or addition of chlorine or other disinfectant agents, may be useful in removing the pathogens in hatcheries (Li, 2008; Nolan and Smith, 2009; Shinn et al., 2015). To lower the spread of diseases, it is also important to maintain an appropriately low density of eggs or embryos within each incubator, even though the optimal density information is currently unknown.

Dissolved oxygen (DO) plays a critical role in promoting horseshoe crab embryonic developments (Penn and Brockmann, 1994; Vasquez et al., 2015). Consequently, many attempts were made to maximize the DO concentrations in the culture system for *T. tridentatus* eggs and embryos. Suspended culture, which involves hanging baskets from the surface of culture water, was the most widely reported method to be efficient in egg hatching (Liao, 2011, 2015; Liao et al., 2015). Continuous air-pumping

TABLE 1 | Effects of various environmental factors on fertilization and survival of *T. tridentatus* embryos in hatcheries (–, no data).

Factors	Source	Experimental groups	Day of experiment	Fertilization rate	Survival rate
Salinity	Li et al. (1999)	10 groups ranged 3–70	44	Greater than 95% at 16, 26, and 33	Greater than 98% at 16, 26, and 33
	Li (2008)	15, 20, 25, 30	90	–	No significant difference among salinity groups at 20°C and 25°C, but significantly higher survival rates in salinity 15 and 20, compared to those in salinity 25 and 30 at 32°C
Temperature	Li (2008)	20, 25, 32°C	90	–	20°C and 25°C groups have significantly higher survival rates than 32°C, whereas 20°C and 25°C groups are statistically similar

below or within the baskets where the eggs housed provides more DO. The vigorous airflow can also agitate the eggs constantly to avoid mass infections due to the prolonged contact among eggs. Keeping the eggs/embryos within a moist and sterile sediment layer in husbandries can also be another alternative to be attempted.

Culture of Juveniles

The success of sea ranching and stock enhancement programs is predominantly determined by rearing technologies in the mass production of seedstocks and juveniles for release. However, since most *T. tridentatus* individuals in the releasing programs were the newly hatched first-instar larvae (Zhu et al., 2020; **Supplementary Table 1**), the development of improved culture techniques to produce environmentally fit, older juveniles has been greatly impeded. To date, most available rearing results were derived from laboratory experiments (**Table 2**), in which their practicability in large-scale seedstock production is virtually unknown.

In the culture environment, the first-instar larvae were not fed until after the first post-hatch molt (Carmichael et al., 2009; Schreibman and Zarnoch, 2009; Chen et al., 2010) since the previous studies suggested that the larval digestive system was rather incomplete and appeared to subsist on the yolk of the embryos for nutrition (Botton et al., 1992; Carmichael et al., 2009). For juvenile culture, many studies concerned their growth and survival under varying dietary conditions, except a small amount of research investigated their responses toward different environmental variables such as temperature, DO, light-dark cycle, and substrate type (**Table 2**). Among frozen food sources commonly available for aquarium fish, hatchery-bred first-year juveniles (instars 1–6) fed with brine shrimp, *Artemia* sp. had higher growth and survival rates due to the increased consumption rate (Hu et al., 2013a). Biswal et al. (2016) provided small, chopped earthworms to the second-instar juvenile coastal horseshoe crab, *Tachypleus gigas*, and observed most molted into the fourth-instar within 90 days from the hatching. For older juveniles, clam meat, which has higher crude protein, can significantly promote their growth, compared to sandworms, *Marphysa sanguinea* and brine shrimp that contain indigestible chaete and exoskeleton, respectively (Kwan et al., 2014). Feed size is another vital factor determining juvenile feeding rates and prey sources (Gao et al., 2003). Gaines et al. (2002) found that the prey selection of *L. polyphemus* in varying growth stages depends largely on their mouth size, in which the younger juveniles (instars 2–7) mainly forage on benthic and suspended particulate organic matters (POM), whereas instars 8 or older juveniles shifted their diets to a combination of POM and small benthic invertebrates such as crustaceans and polychaetes. Nonetheless, feeding cultured juveniles with natural diets is costly, and is difficult to be tailored to fulfill the different nutritional requirements in accordance with the ontogenetic development of *T. tridentatus*.

In the search of the optimal diets for hatchery-reared juvenile *T. tridentatus*, Hu et al. (2014) formulated nine artificial diet treatments in different levels of digestible protein and energy. While the fifth-instar juveniles fed with brine shrimp had the highest feeding rate, the formulated diet containing 40% digestible protein yielded the best growth and feed utilization. The authors suggested that excessively high digestible protein diets such as brine shrimp, fish and clam meat, could reduce juvenile feed consumption and pollute the culture environment. Providing the juveniles with artificial feeds using poultry by-products (PB) as well as meat and bone meals (MB) to replace the traditional fish meal were also attempted to lower the production cost (Hu et al., 2018). The experimental results are promising that a mixture of MB and PB in 2:1 resulted in similar growth performance to that fed with fish meals. However, a longer acclimation period may be required with these dry, hard artificial feeds as horseshoe crabs prefer softer food sources in both natural habitats and captive environments (Zhou and Morton, 2004; Kwan et al., 2015a; Razali et al., 2020).

Since the production of high-quality juveniles is essential in any releasing activity, technological development of seed production has gradually shifted its focus from quantity to quality (e.g., Fushimi, 2001). Concerning the health status of cultured juvenile *T. tridentatus*, Kwan et al. (2014) observed a general decline in juvenile hemolymph quality during a 12-week feeding experiment, even though high-protein diets were provided. To further verify the presence of captivity-related stress, Kwan (2016) kept wild juvenile *T. tridentatus* in the laboratory for 3 months under the best husbandry practices. Significant declines in health indicators, including amebocyte density, amebocyte morphological states, and plasma protein level, were consistently found. Such a decrease in juvenile health status may indicate deficiencies of essential diet compositions, movement constraints and/or absence of tidal rhythms in the culture systems (Kwan et al., 2014). To address the problems,

TABLE 2 | Effects of various environmental factors and diet conditions on optimal growth, survival and health status of juvenile *T. tridentatus* in culture environments.

Factors	Source	Instar	Experimental groups	Day of experiment	Growth rate	Survival rate	Feeding rate	Health condition
Temperature	Lee and Morton (2005)	5–9	18–22°C and 28–32°C	60	Considerably higher molting occurrence (50%) in 28–32°C than those (10%) in 18–22°C	Marginally higher (30%) in 18–22°C compared to those (25%) in 28–32°C	–	–
	Liao et al. (2019b)	16	9 groups ranged 0–40°C	7	–	Significantly lower at 40°C	25°C and 30°C are significantly higher	–
Dissolved oxygen	Shin et al. (2014)	4	2, 4, 6 mg L^{-1}	6	Scope for growth: Significantly higher in DO 6 group on 0 d	100% in all treatments	–	–
Substrate	Hong et al. (2009)	1–2	No substrate, sand, sediment	152	Max. 35% PW increase in sand treatment group	>90% in sand and sediment treatment group	–	–
Day/night time	Gao et al. (2003)	13–16	Day time, night time	2	–	–	Optimal 1.4–2.5% d^{-1} at night time	–
Feed type	Hu et al. (2013a)	3	Brine shrimp, opossum shrimp, chironomus larva	100	BW: Significantly higher in brine shrimp group	Significantly higher in brine shrimp group	Significantly higher in brine shrimp group	–
	Hu et al. (2014)	4	Brine shrimp, nine diets in different levels of digestible protein (DP, 36, 40, 43%) and energy (DE, 14, 16, 18 kJ g^{-1})	84	Max. BW in diets with 40% DP, 14 kJ g^{-1} DE	100% in most treatment groups except for diets with 43% DP	Max. in brine shrimp treatment group	–
	Hu et al. (2018)	5	Fish meal with blood meal (FB), meat and bone meal (MB), poultry by-product meal (PB)	84	BW: MB + PB in 2:1 group is significantly higher than 1:1	100% in feeds with FBM, 25% and 50% MB + PB in 2:1, 25% MB + PB in 1:1	MB + PB in 2:1 group is significantly higher than 1:1	–
	Kwan et al. (2014)	7	Brine shrimp, clam, sandworm, clam plus sandworm	84	Significantly higher in clam group	79–96%	Significantly higher in clam group	Unaffected
	Gao et al. (2003)	13–16	Fish, peanut worm, mussel, squid, shrimp	7	–	–	Max. 2% d^{-1} in squid treatment group	–
Feed size	Gao et al. (2003)	13–16	0.3–0.5, 0.5–1.0, 1.5, 2.0 cm	8	–	–	≥1 in 0.3–0.5 cm	–
Diet supplement	Kwan et al. (2017a)	8	Unicellular green algae *Dunaliella tertiolecta* (DT), golden-brown flagellated microalgae *Isochrysis galbana* (IG)	84	Unaffected	93–100%	–	Higher amebocyte viability and granular-spherical amebocyte state with fed with IG
	Kwan et al. (2017a)	8	Microalgae supplement level: 5%, 10% dw	84	Unaffected	93–100%	–	Unaffected

PW, prosomal width; BW, body weight; ind., individual; d, day; dw, dry weight; max., maximum; –, no data. Instar stage of juveniles is referred to Chen et al. (2010).

Kwan et al. (2018a) invented a double-layered water-circulating enclosure, allowing the simulation of the daily ebb and flow tidal conditions. A high survival rate was obtained, as the circulating system can keep good water quality for at least 3 months and prevent disturbance to the juveniles since frequent water renewal is not required. The system also enabled the juvenile horseshoe crabs to accommodate natural feeding activities under low tides. Supplementing diets with microalgal powder with high polyunsaturated fatty acids, notably eicosapentaenoic acids, also improved the hemolymph quality of juvenile *T. tridentatus* (Kwan et al., 2017a).

In terms of environmental variables (**Table 2**), juvenile *T. tridentatus* can survive in a broad range of rearing temperatures (15–30°C), except for the significantly higher mortality that occurred at 40°C (Liao et al., 2019b). It seems that *T. tridentatus* population in the higher latitudes such as that in Japan, is better adapted to the lower temperatures (Shuster and Sekiguchi, 2009; Liao et al., 2019b). The optimal water temperature for growth and survival was observed within the range of 25–30°C. Deficiencies in DO (2 or 4 mg L^{-1}) lowered the scope of growth in juvenile *T. tridentatus*, but their physiological performance can be recovered to the normal level once the

short-term hypoxic conditions were ceased on day 4 (Shin et al., 2014). Gao et al. (2003) showed higher juvenile feeding activities during the night, which is consistent with previous studies, suggesting that horseshoe crabs are nocturnally active (Rudloe, 1979; Wada et al., 2016). Provision of a sand layer in the culture environment that simulates the nursery habitat conditions of juveniles can also effectively enhance their growth and survival by 10% and 42%, respectively (Hong et al., 2009).

Production Modes

The current market demand for mass production of hatchery-bred juvenile *T. tridentatus* is mainly for releasing programs organized by local governments and research institutes (**Supplementary Table 1**; Zhu et al., 2020). In hatcheries, large numbers of fertilized eggs are incubated using suspended baskets or other similar methods within indoor cement ponds (**Figure 2**; Liao, 2011, 2015; Liao et al., 2015). For the third-instar or older juveniles, a very limited amount has been produced from laboratories to serve research (Chen et al., 2010; Kwan et al., 2019) and conservation educational purposes (Kwan et al., 2017b). The production of adults for TAL biomedical industry through captive rearing is potentially technically feasible (Tinker-Kulberg et al., 2020a,b,c). Such an approach could not only reduce the need to harvest wild horseshoe crabs for such bleeding practices, but would also provide a cost-effective indirect approach for the production of seedstock, including the polyculture of juveniles for a limited amount of time for subsequent release for stock enhancement purposes. Such captivity studies have been recently reported with replacement, reduction and refinement as important drivers of change for horseshoe crab conservation (Gorman, 2020). To the best of our knowledge, however, no study has successfully reared the early instar *T. tridentatus* juveniles till adulthood. Sekiguchi et al. (1988) obtained the 10th-instar (prosomal width, PW: 48.4 mm) juveniles after 7 years of laboratory culture, whereas Huang and Tsai (2011) spent only 4 years to successfully rear three 10th-instar juveniles from a total of 12,000 first-instar larvae in research facilities.

To produce more cost-effective juvenile *T. tridentatus* for releasing programs, Chen et al. (2016) proposed to adopt a polyculture system to rear the juveniles with other economically important aquaculture species in husbandries. In this case, the juveniles can be a "by-product" from other rearing activities without the additional investments in husbandry management and maintenance. To examine the practicability of such production mode, a trial on mixed cultivation of juvenile *T. tridentatus* (instars 1–2; 203,000 individuals) with a scavenging species, juvenile spotted Babylon snail, *Babylonia areolata* (338,000 individuals) was conducted in outdoor cement ponds for 150 days (Chen et al., 2016). Despite the fact that the temperature maintenance system was unavailable during the winter and spring, about half of the juvenile *T. tridentatus* molted into the second- or third-instar with an acceptably high survival rate of 33%. Meanwhile, 87% of juvenile *B. areolata* survived through the entire experiment. Chen et al. (2016) explained that the outdoor culture system had provided larger movement space and a relatively stable rearing environment to the juveniles, compared to the typically small experimental tanks within the research facilities. The juveniles can feed on overgrown benthic algae in the outdoor ponds, which are important food sources for these young individuals (Gaines et al., 2002; Carmichael et al., 2009). The polyculture with juvenile *B. areolata* also can enhance the growth performance of juvenile *T. tridentatus* by providing better water quality and culture environment due to their complementing feeding niches. Rearing juvenile *T. tridentatus* with shrimp and other mollusks is also worth to be attempted to identify more practical, cost-effective methods for seedstock production.

The important techniques and operating procedures on artificial breeding, as well as larval and juvenile culture for *T. tridentatus* are summarized in **Figure 2**. All in all, the experimental findings suggest that the optimal rearing techniques of juvenile *T. tridentatus* should be based on their biology and ecology. Therefore, an improved understanding of the released species and ecosystems can compensate for the limitations in providing older captive-bred juveniles and increase the potential success of sea ranching and stock enhancement. While captivity-related and other health issues are largely unsolved, mass production of the juveniles using more natural, ecologically relevant methods, e.g., outdoor ponds with sediment substrates, may be more promising.

RELEASE STRATEGIES

The success of stock enhancement programs depends largely on the survival of the hatchery-bred juveniles to natural habitat conditions. The mortality of released juveniles, particularly those of invertebrates, is typically higher than 60% within the first month (Oliver et al., 2005; Dixon et al., 2006; Purcell and Simutoga, 2008). Thus, potential reasons that can cause high mortality of released juveniles, including predation, habitat conditions, environmental stress, and extreme weather, should be addressed (Lorenzen, 1996; Lorenzen, 2000; Hines et al., 2008; Ceccarelli et al., 2018; Poh et al., 2018). Of these, Bartley and Bell (2008) perceived predation as the key obstacle to the survival of released juveniles. While relevant experimental results in developing optimal release strategy for juvenile *T. tridentatus* are largely lacking, we discuss these based on our understanding of the biology and ecology of juvenile *T. tridentatus* in the estuarine ecosystem.

Selection of Release Sites, Season and Timing

Horseshoe crabs have specific spawning and nursery requirements related to a combination of beach topography and physico-chemical parameters such as dissolved oxygen, chlorophyll a content, total sulfide content, and sediment grain size (Hsieh and Chen, 2009; Vasquez et al., 2015; Cheng et al., 2016; Xie et al., 2020). Therefore, the identification of optimal nursery habitats for releases is the first step. Among the 14 nursery sites distributed along the northern Beibu Gulf, China, a high density of wild juveniles was found in the intertidal areas along the outer fringe of mangrove forest, especially near the outflows of tidal creeks (Xie et al., 2020). In addition, at

the nursery site where seagrass patches are available, areas with higher seagrass coverage and coarser sediment had more abundant juvenile *T. tridentatus* individuals (Xie et al., 2020). Most wild juveniles were found forage actively in areas with surface water with 1–10 mm depth during ebb tides to keep the juvenile book gills wet and enable respiration while feeding under high surface temperatures (Lee and Morton, 2009; Kwan et al., 2020). Based on the above findings, releasing hatchery-produced juveniles at the existing nursery sites on the intertidal areas along the outer fringe of mangroves or near the seagrass patches seems to enhance their survival rate.

Chiu and Morton (2004) demonstrated that juvenile *T. tridentatus* buries in the sediment and remains inactive when the water temperature drops below 20°C. In such cases, decisions related to factors linked to the ecological requirements of the juveniles, including microhabitat, season and timing play critical roles for the release success. To improve the survival of released juveniles, they should be placed on the intertidal areas during low tides in summer when the surface water temperature exceeds 20°C. This can also mimic the spawning period of *T. tridentatus* typically recorded in late spring (Cai et al., 1984) and would coincide with the summer peak in juvenile production.

If there are problems in locating any actively utilized nursery habitats for *T. tridentatus* for releases, the programs can be conducted at any suitable intertidal habitats where the juveniles were known to occur in the past but are now extirpated. However, it is important to ensure the current state of the intertidal areas fits the macrohabitat requirements of the juveniles, e.g., mangroves, seagrass beds, and tidal creeks. For microhabitat conditions, juvenile *T. tridentatus* tends to concentrate in regions where sediment comprised medium-sized (median particle: 0.1–0.5 mm) sand with relatively higher chlorophyll a content (1–4 μg/g) and/or total organic content (0.1–0.7%) (Hsieh and Chen, 2009; Xie et al., 2020), which presumably indicate a higher abundance of food from the marine algae-derived particulate organic matter sources (Gaines et al., 2002). For optimal surface water quality, salinity should range between 12 and 34, dissolved oxygen content 7–9 mg/L, and pH 7.3–7.9 (Hsieh and Chen, 2009; Xie et al., 2020), although Xie et al. (2020) found that sediment physico-chemical conditions, rather than surface water parameters, can better explain the distribution pattern of wild juvenile *T. tridentatus* population.

To date, however, no study has looked specifically at the survival, growth, and behavioral responses of hatchery-reared juvenile *T. tridentatus* at the release sites. During a release trial with 60,000 individuals of cultured first-instar larvae (∼53-day old) in mangrove and seagrass areas at Yuzhouping, Guangxi region, China (Zhu et al., 2020), an actively utilized nursery habitat for *T. tridentatus*, none of the released larvae had been recovered during monthly belt-transect population survey in the following 4 months, which was possibly due to the high predation rates. Size at release is, therefore, another critical component to be considered prior to the releases.

Size at Release

Size at release is one of the key elements in a successful stock enhancement. Natural mortality rates within natural fish (possibly also invertebrate) populations are approximately inversely proportional to their lengths (Lorenzen, 1996). Meanwhile, the predation risk at the released site is inversely related to their age and size. Carmichael et al. (2003) found that only very few *L. polyphemus* in Cape Cod, Massachusetts, United States survived their first year (instars 1–6), in which the cumulative mortality rate can be as high as 99%. Conversely, most juveniles survived to reach adulthood given that they survived through the first year (Carmichael et al., 2003). While there is very limited information regarding key predators of juvenile horseshoe crabs (Botton, 2009), releasing using hatchery-bred juveniles at their sixth instar or older seems to be an optimal decision to minimize potential predation by fish and hermit crabs, and to increase the success of releases.

Releasing undersized juveniles, e.g., the first-instar larvae, is unlikely to be effective, but the effectiveness of releases at small sizes can be increased significantly through the selection of optimal habitats and times as well as acclimation and soft release strategy (refer to sections "Selection of Release Sites, Season and Timing" and "Stress Management"). Apart from that, the fitness of juveniles under prolonged captivity is another area of concern. Despite the development of rearing technologies for Asian horseshoe crabs has been initiated in the 1980s, Kwan et al. (2014) suggested that the present culture models may not guarantee the continuing fitness of juveniles. To maximize the survival rate of released juveniles, it is important to identify the captivity-related issues such as deficiencies in essential dietary components (Kwan et al., 2017a) that are widely present in the current hatchery systems. Nevertheless, it is also important to account for ecological differences between wild and hatchery-produced juveniles (Lorenzen, 2005). Natural mortality rates of hatchery-reared juveniles tend to be substantially higher than those of wild conspecifics of similar size (Lorenzen, 2000; Fleming and Petersson, 2001). Reproductive success of captive-bred salmonids, for example, is generally higher than that of their wild conspecifics (Fleming and Petersson, 2001). However, no strong evidence on their differences in growth was reported (Svåsand et al., 2000; Fleming and Petersson, 2001).

Tagging Methods

The development of cost-effective tagging methods is necessary to identify the released individuals from the wild stock to evaluate the effectiveness of stock enhancement programs (Bartley and Bell, 2008). In fact, little research has focused on selecting appropriate tags and markers for juvenile horseshoe crabs. No tagging experiment was currently conducted on the most commonly released size, the first-instar larvae. For other released size, only physical tags have been attempted. Hu et al. (2013b) marked 300 individuals of the second-instar *T. tridentatus* (∼134-day old; prosomal width, PW ∼8 mm) using visible implant elastomer (VIE, i.e., colored tag; Northwest Marine Technology Inc., WA, United States) and found the 22-d survival rate was 96%. Nonetheless, no information on the juvenile survival rate at the released site was provided. Tag retention is another important consideration when injecting the implants of fluorescent colors under the juvenile carapace. Although the tag retention time in juvenile horseshoe crabs is virtually unknown,

Brennan et al. (2005) observed such tag loss in 2% of tagged snook *Centropomus undecimalis* after 6 months. Kwan et al. (2015b) examined the recovery and movement of a total of 150 individuals of the 7th- to 10th-instar *T. tridentatus* (1–2-year old; PW 31–59 mm) with colored plastic tape, passive integrated transponder tag (PIT; 134.2 kHz, Biomark, WA, United States) and their combinations. The mortalities induced by the three kinds of tags were minimal, and the cumulative recovery rates of the released juveniles within 2 months in summer were ranged from 70 to 82% (Kwan et al., 2015b). The PIT tag seems not to affect the juvenile foraging activity as their utilization distribution area using these three tagging methods was statistically similar.

More tagging studies were conducted in adult horseshoe crabs to investigate their migratory, spawning, and post-bleeding behavior (James-Pirri et al., 2005; Anderson et al., 2013; Bopp et al., 2019). Disk tags were developed by U.S. Fish and Wildlife Service, in which the tagging involves drilling a small hole through the lower back corner of horseshoe crab prosoma. Mattei et al. (2011) found no mortality in tagged adult Atlantic horseshoe crab, *L. polyphemus*, and reported an 11–20% recapture rate within the 44-day field observation. James-Pirri et al. (2005) determined movement patterns of spawning *L. polyphemus* during 2000–2002 with a double T-bar anchor tag (model SHD, Floy Tag Inc., WA, United States). However, the recovery of these conventional passive tags can be labor-intensive and time-consuming, particularly when only a small number of tagged individuals were released to an open area of the appropriate habitat. Ultrasonic transmitters, which allow wider coverage of detection in submerged habitat, were useful in tracking the distribution and movement of adult horseshoe crabs at varying temporal and spatial scales (Schaller et al., 2010; Wada et al., 2016; Owings et al., 2019). Since the transmitter size is much larger and heavier compared to that of conventional physical tags, telemetry technologies are only suitable for adult horseshoe crabs. However, restocking with the adults, should not be encouraged, except for those bled or confiscated from illegal wildlife trade. In fact, all captive adult horseshoe crabs are harvested from the wild stocks because of their lengthy life histories, and thereby impractical to rear them till adulthood.

The search for optimal tagging methods for the first-year juveniles (instar 1–6) is urgently needed, despite the fact that their natural mortality rates are too high (Carmichael et al., 2003) and should not be used for releasing programs. As mentioned earlier, since the selection of release size is a balance between survival rates and production costs, we predict that the use of the first-instar larvae would still be predominant in the releasing programs, unless the hatchery techniques have been largely improved and the production costs can be lowered to an acceptable level. The application of physical tags to these juveniles would be challenging due to their minute body size (PW $<$ 30 mm, wet weight $<$ 2 g), frequent molting (50–290 days for each instar stage), and burying behavior during high tides. The development of biological tags such as genetic markers and some visible morphological characters for the early instar juveniles is worth attempting. Non-invasive genetic markers are developed based on the selection of a rare DNA variant that distinguishes the released batch from the native stock (Romana-Eguia, 2006). Maternally inherited mitochondrial DNA markers and biparentally inherited nuclear DNA markers (such as microsatellite DNA and allozyme electrophoresis) are commonly used and effective for assessing the success of releasing activities and possible loss of genetic variability in both released and wild stocks. Nevertheless, the use of genetic markers can be ineffective when the genetic variation within and between stocks is negligible.

Stress Management

Proper management of stress derived from handling and transport can also improve the survival of released juveniles. Purcell et al. (2006) found that prolonged transport duration (i.e., 12 and 24 h) suppressed the normal sand burrowing behavior of juvenile sea cucumber, *Holothuria scabra*. In addition to transport duration, inappropriate storage temperature and medium, as well as juvenile density during transport can elevate both lethal and non-lethal stress of released invertebrates, which in turn, increase their predation risk upon release (Heasman et al., 2004; Purcell et al., 2006). Unfortunately, to the best of our knowledge, no study has focused on the effects of handling and transport stress on juvenile *T. tridentatus* before release. However, previous studies suggested that health conditions of horseshoe crabs would be affected by hypoxia (Shin et al., 2014), increased temperature (Coates et al., 2012), and aerial exposure originated from transport and holding procedure of biomedical bleeding (Hurton et al., 2009). The changes in water chemistry in transport containers can also be relevant to the survival of released juveniles. Heasman et al. (2004) found increasing temperature, in the combination with elevated ammonia and lowered dissolved oxygen in transport containers, has resulted in substantial mortality of juvenile blacklip abalone, *Haliotis rubra*, during the transport to the release site. Keeping juvenile *T. tridentatus* at cooler and constant temperatures during the transport can be attempted to reduce their metabolic activities, which was proven to be effective in the transport of hatchery-reared grouper, *Epinephelus* larvae (Estudillo and Duray, 2003). Further examinations of an optimal temperature and juvenile density during transport are also necessary for *T. tridentatus*.

Burying behavior is an important measure for juvenile horseshoe crabs to avoid predation. During high tides when large numbers of diurnal predators can access the upper intertidal zones, juvenile horseshoe crabs tend to bury themselves into sediments. Lee and Morton (2009) observed that less than 5% of juvenile *T. tridentatus* emerged from sediments during simulated high tides in a laboratory experiment. In such a case, transport with a thin sediment layer prior to release would be an important approach to lower the transport stress. A similar approach has been undertaken by Juinio-Meñez et al. (2012) showing that sand-conditioned juvenile *H. scabra* took a shorter time burying themselves into the sediment at the released site, which possibly improves their survival in the wild. Alternatively, the juvenile horseshoe crabs can be placed in acclimation cages at the release site for 48 h before the release, in which the technique has been demonstrated successful in improving post-release survival, growth and site fidelity in many fish species such as juvenile winter flounder, *Pseudopleuronectes americanus* (total length:

130–175 mm, Fairchild et al., 2009) and juvenile common snook, *Centropomus undecimalis* (fork length: 76–251 mm, Brennan et al., 2006). Gil et al. (2014) demonstrated that hatchery-produced juvenile meagres, *Argyrosomus regius* had adverse body conditions due to starvation during the first few days after the release, which could cause a high mortality rate.

Genetic Considerations

Genetic factors deserve serious considerations in releasing programs. A study of natural population genetic structure should be conducted once stock enhancement programs have been considered. A systematic review by Kitada (2018) found clear evidence of substantial gene flow from hatcheries, in which the magnitude was attributed to the numbers of broodstock and stocking intensity. Half of 38 empirical studies on marine stock enhancement initiatives demonstrated a reduction in genetic diversity and changes in the population structure of wild populations (Kitada, 2018).

However, no study has investigated the impact of releasing initiatives on the genetic variability of wild horseshoe crab stocks. Sugawara et al. (1988) found no genetic differences among *T. tridentatus* populations from Japanese and Chinese waters. The recent application of amplified fragment length polymorphism on *T. tridentatus* populations from the three coastal provinces in southern China also drew a similar conclusion (Xu et al., 2011). In contrast, at a finer scale, several previous studies also suggested the existence of genetically distinct subdivisions between that on Kinmen Island and Magong Island in Taiwan Strait (Yang et al., 2007), between Ninghai and Danzhou along the Chinese coast (Weng et al., 2013), as well as between eastern and western shores around Kyushu, Japan (Nishida and Koike, 2009).

For *L. polyphemus*, a clear isolation-by-distance model was evident, in which six major zones of genetic discontinuity were identified, including the Gulf of Maine, Mid-Atlantic, Southeast, Florida Atlantic, Northeast Gulf, and Yucatán Peninsula of Mexico (Smith et al., 2017). Within each zone, considerable gene flow was found to occur between geographically proximate localities. The genetic variation patterns may be due to the limited potential of female migration between embayments (King et al., 2005; Swan, 2005) and larval dispersal (Botton and Loveland, 2003).

While the population genetic structure of *T. tridentatus* is insufficiently clear at present, the wild populations are likely to have very low genetic diversity due to their considerably low population sizes and densities. Mass release of seedstocks from a small number of breeders in the hatchery may have substantial effects on the genetic diversity of wild horseshoe crab populations. A large number of parents from geographically proximate estuaries as "genetic repositories" is generally required for the larger stocking rates to maintain genetic diversity in populations (Kitada et al., 2009). Releasing multiple cohorts of juveniles from mating pairs or replacing the breeders regularly can be the alternative way to avoid genetic degradation caused by inbreeding from mass release. Interactions between wild and released stocks in the environment are worth to be investigated in the future.

RECOMMENDATIONS AND FUTURE PROSPECTS

The development of hatchery and stock enhancement technology for horseshoe crabs is new. Compared to other Asian species, there are relatively larger numbers of studies focused on the growth and survival of juvenile *T. tridentatus* under varying husbandry conditions as well as their distribution pattern and habitat characteristics, all of which would benefit the development of the responsible stock enhancement programs. We summarize the important findings from previous studies into a technical framework for guiding the increasing releasing initiatives for the exploited *T. tridentatus* population, especially along the Chinese coast (**Figure 3**).

Despite the recent progress in aquaculture, the mass production of environmentally fit juveniles for releases has been largely impeded by the presence of unidentifiable captivity-related issues due to the knowledge gaps in understanding the growth requirements and complex life histories of *T. tridentatus* in their nursery habitats. The deficiencies in essential fatty acids and tidal rhythms have been identified to contribute to the lowered immune competence under prolonged culture. Other rearing alternatives such as the use of natural sediment from their nursery habitats (with unknown minor essential nutrients and minerals) to replace the commonly applied aquarium sand can be helpful in maintaining the good health and growth performance of juveniles in culture facilities. Further research is also required to explore other possible culture modes, including polyculture with other economically important aquaculture species, to produce *T. tridentatus* seedstocks cost-effectively (**Figure 3**).

Overall, the success of stock enhancement programs must be guided by enhanced knowledge on horseshoe crab biology and ecology, so that the released juveniles can ultimately contribute to spawning biomass. The effectiveness of releases can be increased significantly through responsible release strategies and pre-release acclimation. Release strategies involving the selection of optimal habitats (intertidal areas near mangrove fringes) and timing (during low tides with water temperature exceeds 20°C) with thorough genetic consideration would greatly improve the survival of released juveniles, particularly those at early instar stages. The use of acclimation cages at the release site for the first 48 h or sand conditioning in hatcheries or during the transport would be important to lower natural mortality after releases. Future research should also include the identification of key predators to avoid immediate predation on hatchery-produced juveniles at release sites. Given that efficient tagging methods for the first-year juvenile horseshoe crabs are currently lacking, the development of non-invasive tagging approaches such as genetic markers should be prioritized to evaluate the effectiveness of releasing programs (**Figure 3**). Follow-up monitoring and assessment after the releases are the prerequisite to optimizing the current stock enhancement protocol.

Apart from the releasing program itself, interventions with other management measures, including nursery habitat recovery, fishing pressure reduction and habitat use restriction during peak spawning, would also allow more promising

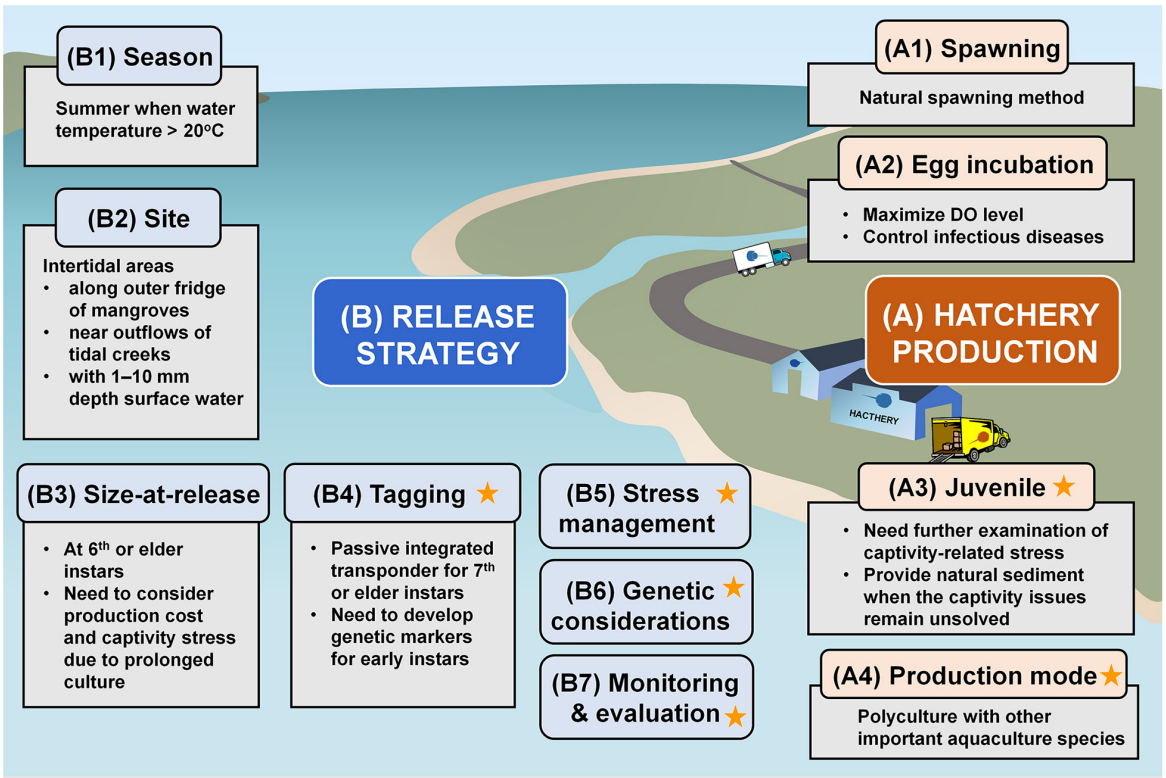

FIGURE 3 | Technical framework for developing the responsible stock enhancement programs for *T. tridentatus* conservation. The star symbols indicate the research gaps which should be focused in future studies. The sources of information are summarized in **Supplementary Table 2**.

conservation outcomes. Identification, mitigation and elimination of human-mediated disturbances at the release site must be addressed prior to the releases. Other institutional, socio-economic and legal factors should also receive equal emphasis. The involvement of local communities and other stakeholders in the releasing programs can reduce the ingrained apathy toward the environment, which often contradicts the community's established practices to consume horseshoe crabs for food and traditional medicine. While there are uncertainties in the potential role of releasing programs, we can expect to gain further insights from continuous research and trial-and-error experience in developing a responsible stock enhancement framework to meet conservation needs for *T. tridentatus*.

AUTHOR CONTRIBUTIONS

PX and HB led the writing and prepared the original draft. MZ and ZY compiled and clustered data from the published studies. XX, C-CW, XH, XW, JZ, and WZ provided input to different writing sections. SC and PS developed the overall review framework and methods. KK supervised, reviewed and edited the final draft, which was proof-read and approved by all authors. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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Potential of Underutilized Marine Organisms for Aquaculture Feeds

Dedi Jusadi†, Julie Ekasari†, Muhammad Agus Suprayudi, Mia Setiawati and Ichsan Achmad Fauzi*

Department of Aquaculture, Faculty of Fisheries and Marine Sciences, Bogor Agricultural University, Bogor, Indonesia

The supply of land-based agricultural products as aquafeed raw materials is challenged by limitations on space and water, and by environmental damage. Marine environments offer a vast opportunity for the expansion of aquaculture, including the production of feed raw materials. Besides fishmeal and fish oil, which are generated from capture fisheries, the use of marine-based feed raw materials from aquaculture production is not yet in common practice. Here, we discuss the potential of underutilized marine organisms that can be cultured by extracting nutrients from their environment and are nutritionally compatible for use as alternative feed materials in aquaculture. We identify marine organisms such as blue and green mussels, *Ulva* spp., and microbial floc that are nutritionally suitable as aquafeed raw material and may further act as bioremediators. However, environmental factors that affect productivity and the risk of pollutant accumulations, which would potentially reduce the safety of aquaculture products for human consumption, may pose challenges to such applications of extractive organisms. Therefore, the development of pretreatment and processing technologies will be critical for improving the nutritional quality and safety of these raw materials for aquafeed production.

Keywords: aquafeed, bioremediation, extractive organisms, macroalgae, shellfish, microalgae

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***Correspondence:**
Julie Ekasari
j_ekasari@apps.ipb.ac.id

†These authors have contributed equally to this work and share first authorship

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INTRODUCTION

Aquaculture is expected to meet the majority of the demand for seafood, given that capture fisheries have been in stagnation for the last few decades (FAO, 2020). However, the development of aquaculture production is challenged by limitations on pivotal resources such as space, water, and feed raw materials. Aquaculture products can be classified into two groups, i.e., fed organisms, which are cultured with the addition of external feed, and unfed organisms, which are cultured without the addition of external feed (Hua et al., 2019; FAO, 2020). At present, most aquaculture activities produce fed organisms that rely heavily on formulated feed. Thus, the increase in aquaculture production has a generally linear relationship with the increase in feed production (FAO, 2020). The inclusion of fishmeal and fish oil, a common source of protein and lipids in aquafeeds in decades, has substantially declined due to rising prices of these products and the sustainability concerns over the harvesting of small pelagic fish used to produce them (Hua et al., 2019). As a result, most raw materials in aquafeed are now agricultural products produced in terrestrial systems where water, space, and other environmental resources have become scarce. The marine ecosystem, on the other hand, offers vast opportunities for the production of produce seafood products and aquafeed raw materials (Gentry et al., 2017).

The intense competition for raw materials due to other human uses, which affects aquafeed supply, is a major motivation for the aquaculture sector to generate its own feed raw materials from marine sources. The demand for high-quality materials for various human-related applications has been increasing, creating more opportunities for the aquaculture sector to produce marine-based high-quality raw materials for various human needs, including other food-producing sectors such as livestock production. Aquafeed raw material exploration should focus on unfed marine organisms, which can act as bioremediators that extract waste nutrients from the environment and convert them into beneficial biomass that may be used as feed raw materials (Agarwal et al., 2020). Here, we discuss the potential use of some underutilized marine organisms as candidates for raw materials in aquafeed, with specific emphasis given to unfed low-trophic-level organisms such as shellfish, seaweed, and microorganisms.

SELECTION CRITERIA FOR AQUAFEED RAW MATERIALS

The following criteria should be considered when selecting appropriate aquafeed raw material: (1) nutritional value relative to the requirement of the cultured animal and its digestibility by the target animal; (2) the presence of antinutritional factors (ANFs) and contaminants; (3) supply reliability; and (4) price volatility (Glencross et al., 2020). The nutritional composition and digestibility of feed materials have synergistic effects on growth outcomes of fed aquaculture species. In addition, the physical and nutritional qualities of raw materials should also include their characteristics during manufacturing processes and how they affect the pellet quality (Turchini et al., 2019). High digestibility ensures high nutrient bioavailability and utilization by the animal. The presence of antinutritional factors, i.e., substances that could interfere with food utilization and negatively affect the health and production of animals, is an important factor determining the nutritional feasibility of a raw material for aquafeed (Francis et al., 2001). In addition, marine-origin raw material may carry the risk of contamination by heavy metals and toxins, with potential direct or indirect adverse effects on the fed organisms and final consumers. In the aquafeed industry, the use of materials with routine and consistent supply is critical to reduce the risks of fluctuations in product quality and specification, cross-contamination, and shortfalls in supply during manufacturing (Glencross et al., 2020). Thus, continuous supply of a particular raw material in bulk quantities should be one of the major considerations when selecting potential raw material for aquafeed. The price volatility of a raw material, which is strongly influenced by its supply and demand, is the main economic factor affecting profitability in aquafeed manufacturing. In the context of raw material production, the supply of a raw material in bulk at an affordable price implies that the culture productivity, i.e., production per unit of area or per unit of water, and the processing cost of the marine-origin raw material should be comparable to that for the production of existing terrestrial-based raw materials. How new raw materials influence the environmental and social sustainability of aquafeeds are also critical considerations in the development of new feed products (Valenti et al., 2018). However, here, we focus on the technical potential of new ingredients as a first step for scoping novel raw materials of interest for the aquafeed industry.

POTENTIAL MARINE UNFED ORGANISMS AS FEED RAW MATERIALS

In this minireview, our focus is mainly given to macro- and microscopic organisms that are high in productivity and can be cultured by using nutrient waste or by-products, either in open marine environments or in enclosures in coastal areas. Based on these criteria, we identify some marine organisms that are potentially useful for aquafeed; these are classified into three groups: of animal, macroalgae, and microscopic origins (**Table 1**).

Animal-Origin Materials

Marine-animal-origin feed raw materials are mostly used as sources of essential amino acids and essential fatty acids for most aquaculture animals. There are at least three animal-origin materials that have the potential to be used as a protein source in aquafeed: mussels, artemia, and amphipods. These animals are low-trophic-level organisms that extract nutrients from primary producers such as microalgae and/or particulate organic matters in the aquatic environment. Mussels such as green (*Perna viridis*) and blue (*Mytilus edulis*) are extractive organisms that grow rapidly in nutrient-rich environments and act as bioremediator agents converting waste nutrients into the protein. Mussels contain considerably high protein [50–70% dry weight (DW)] and lipid (5–16% DW) levels, with comparable essential amino and fatty acids contents to those of fishmeal (Jusadi et al., 2020). A number of studies demonstrated that mussels are a promising protein source in aquafeed, with a reported maximum inclusion level of up to 25% (Weiß and Buck, 2017; Jusadi et al., 2020). From an ecological perspective, mussels have been considered to play some important roles in carbon fixation and mitigation of ocean eutrophication (for a detailed review, see Suplicy, 2020). *Artemia* nauplii have been used as an important live food in almost all aquaculture hatchery productions. However, the supply of *Artemia* nauplii has been heavily reliant on cysts collected from the wild. Thus, many efforts have been undertaken to culture *Artemia* to produce cysts. Moreover, the use of adult *Artemia* as feed has started to gain attention. *Artemia* can be cultured at a relatively high productivity (ca. 2 tons/ha/crop) in shallow ponds by using by-products or waste as their feed (Anh et al., 2009b). The protein content of *Artemia* biomass is relatively high, e.g., a range of 51–61% DW, with a lipid content ranging from 5 to 10% DW (Anh et al., 2009a). Amphipods are another small crustacean that can grow rapidly in nutrient-rich areas. A recent study by Herawati et al. (2020) showed that *Phronima* sp. cultured using microalgae and cow manure could be used as the sole food for Pacific white shrimp postlarvae.

TABLE 1 | Nutritional compositions of some underutilized marine organisms and its utilization as feed raw materials in aquaculture.

Material	Species	Protein (% DW)	Lipid (% DW)	Carbohydrate (% DW)	Fiber (% DW)	Ash (% DW)	Target species	Dietary inclusion (%)	References
Animal origin									
Mussels	*Mytillus edulis*	52–70	7–16	13	1.4	9–11	*Scophthalmus maximus*, *Solea solea*, *Salvelinus alpinus*, *Perca fluviatilis*	25	Mongile et al., 2015; Langeland et al., 2016; Weiß and Buck, 2017; Wagner et al., 2019
	Perna viridis	53.9	11.2		0.1	8.9	*Oreochromis niloticus*	10	Jusadi et al., 2020
Artemia	*Artemia biomass*	50.7–61.4	4.9–9.9		2.5–16.6	25.0	*Macrobrachium rosenbergii*	57.5–100	Anh et al., 2009a
Amphipods	*Phronima* sp.	40.3	15.1	10.0	8.9	17.2	*Litopenaeus vannamei*	100	Herawati et al., 2020
Macroalgal origin									
Chlorophyta	*Ulva lactuca*	11.5–32.2	0.5-6.1	43.5	9.1–15.0	24.4–33.2	*Clarias gariepinus*, *Sparus aurata*, *Oreochromis niloticus*, *Litopenaeus vannamei*	5–25	Abdel-Warith et al., 2016; Shpigel et al., 2017; Suryaningrum et al., 2017; Laramore et al., 2018; Guerreiro et al., 2019
	Ulva rigida	8.0–29.5	0.2–2.0	46.8–50.4	12.3	4.5–26.7	*Sparus aurata*, *Oreochromis niloticus*, *Cyprinus carpio*, *Onchorhyncus mykiss*	5–25	Diler et al., 2007; Ergün et al., 2009; Güroy et al., 2013; Vizcaíno et al., 2016
	Ulva sp.	5.3	0.3–2.7		5.2–5.3	24.7–46.0	*Oreochromis niloticus*, *Litopenaeus vannamei*, *Scatophagus argus*, *Argyrosomus japonicus*	6–25	Silva et al., 2015; Madibana et al., 2017; Qiu et al., 2018; Yangthong and Ruensirikul, 2020
	Caulerpa lentillifera	19.4–29.2	0.8–2.9	44.0–53.5	4.1	16.6–29.6	*Oreochromis niloticus*, *Penaeus monodon*	5–20	Nagappan and Vairappan, 2014; Putri et al., 2017; Putra et al., 2019
	Caulerpa racemosa	17.3–30.0	1.8–2.1	42.7–52.8	3.1–3.3	22.2–26.7	*Penaeus monodon*		Nagappan and Vairappan, 2014; Puspitasari et al., 2019
Rhodophyta	*Gracilaria arcuata*	13.5	7.0			31.9	*Clarias gariepinus*, *Oreochromis niloticus*,	10	Al-Asgah et al., 2016; Younis et al., 2018
	Gracilaria lemaneiformis	18.9	0.5				*Acanthopagrus Schlegelii*, *Pagromus major*	3–15	Xuan et al., 2013, 2019
	Gracilaria pygmaea	16.7	1.0		1.2	29.5	*Onchorhyncus mykiss*	6	Sotoudeh and Jafari, 2017
	Gracilaria cornea	13.5	0.8	39.8		35.6	*Sparus aurata*	25	Vizcaíno et al., 2016
Phaeophyta	*Macrocystis pyrifera*	6.1	0.7	44.2	10.5	31.1	*Litopenaeus vannamei*, *Onchorhyncus mykiss*	1.5–3.3	Cruz-SuÁrez et al., 2009; Dantagnan et al., 2009
	Sargassum horneri	13.2–17.2	0.5–1.3	63.0		11.7–19.4	*Acanthopagrus schlegelii*, *Scophthalmus maximus*	6–10	Shi et al., 2019; Wang et al., 2019
	Sargassum illicifolium	9.2	2.1	33.1	10.3	29.2	*Litopenaeus vannamei*, *Huso huso*	7.5–15	Hafezieh et al., 2014; Yeganeh and Adel, 2019

(Continued)

TABLE 1 | Continued

Material	Species	Protein (% DW)	Lipid (% DW)	Carbohydrate (% DW)	Fiber (% DW)	Ash (% DW)	Target species	Dietary inclusion (%)	References
Microscopic origin									
Microalgae	*Nannochloropsis oculata*	42.2	5.6		0.6		*Litopenaeus vannamei*	25	Gamboa-Delgado et al., 2019
	Nannochloropsis granulata	33.9	27.6	14.4		7.5	*Litopenaeus vannamei*, *Onchorhyncus mykiss*	26–29	Tibbetts et al., 2017
	Nannochloropsis sp.	33–51	18–20			35	*Marsupenaeus japonicus*, *Dicentrachus labrax*, *Scopthalmus maximum*	7–10	Oswald et al., 2019; Qiao et al., 2019; Valente et al., 2019; Adissin et al., 2020
	Chlorella vulgaris	58.0–66.4	4.0–9.6	17.3	3.3	5.1–5.5	*Clarias gariepinus*, *Danio rerio*	0.6–30	Raji et al., 2018; Carneiro et al., 2020
	Schizochytrium sp.	11–16	51–70	19.4		3.8–4.4	*Ictalurus punctatus*, *Pagrus major*, *Salmo salar*	2–11	Li et al., 2009; Kousoulaki et al., 2016; Seong et al., 2019; Katerina et al., 2020
	Tetraselmis suecica	38.7	12.4	44.3			*Litopenaeus vannamei*, *Dicentrarchus labrax*	0.7–12	Messina et al., 2019; Sharawy et al., 2020
	Tetraselmis sp. (defatted)	40.6	1.3			14.6	*Sparus aurata*	10	Pereira et al., 2020
	Isochrysis galbana	23.2	36.6	34.5		1.7	*Trachinotus ovatus*	8.6	He et al., 2018
Cyanobacteria	*Arthospira* sp.	59.4–63.2	2.2–7.0	15.0	1.2–3.2	4.1	*Litopenaeus vannamei*, *Lates calcalifer*, *Onchorhyncus mykiss*, *Salmo salar*, *Clarias gariepinus*	25–50	Burr et al., 2012; Gamboa-Delgado et al., 2019; Raji et al., 2020; Van Vo et al., 2020
Bacteria	Biofloc meal	23.4–49.0	0.3–1.1	18.6–36.4	12.6	13.4–36.6	*Litopenaeus vannamei*, *Penaeus monodon*	12–15.7	Bauer et al., 2012; Simon et al., 2020

Materials of Macroalgal Origin

Some macroalgae (seaweeds) species have been studied intensively as feed raw materials, either as phyco-additives that contribute bioactive compounds such as flavonoids, prebiotics, and carotenoids, or as a source of macro- and micro-nutrients. Seaweeds are also known as effective nutrient biosorbents that remove various nutrients from their surrounding environment. Members of the genus *Ulva* spp. are those seaweeds with greatest potential for aquafeed raw materials. These green macroalgae (Chlorophyta) have a high annual productivity (ca. 838 g C/m^2/year) (Chemodanov et al., 2017) and have the potential to be used as feed material and for other human uses. For instance, with its high total ammonia nitrogen (89%) and phosphate (44%) removal capacity (Kang et al., 2021), *U. pertusa* has the potential to be cultured as a phytoremediator in intensive fish or shrimp ponds, in coastal zones, and/or to be cultivated in integrated multitrophic aquaculture (IMTA) systems (Aníbal et al., 2014). The protein content of *Ulva* spp. may reach up to 32% DW, with a lipid content of <2% DW (**Table 1**). *Ulva* spp. also contains high levels of aspartic acid and glutamic acid as well as alanine and arginine. The apparent digestibility of *Ulva* spp. proteins by rainbow trout and tilapia is 75.6 and 63.4%, respectively (Pereira et al., 2012). Various species of *Ulva* have been studied as a feed material for some aquaculture species, with a maximum inclusion level recorded at 25% (Yangthong and Ruensirikul, 2020). *Gracilaria* sp. is one of the most commonly cultured red algae (Rhodophyta). Members of this genus has been consumed and used to produce agar and can contain protein up to 18.9% DW with a lipid content of <1% DW (Xuan et al., 2019). The protein digestibility of *Gracilaria vermiculophylla* was reported to be about 87.8 and 51.4% in rainbow trout and Nile tilapia, respectively (Pereira et al., 2012). The utilization of *Gracilaria* spp. for aquafeed has been tested in various aquaculture species, with the highest inclusion level reported in European seabass at about 25% (Vizcaíno et al., 2016). Other studies of macroalgal genera in aquafeed have focused on some brown algae (Phaeophyta), such as *Macrocystis* spp., *Ascophyllum nodosum*, and *Sargassum* spp. The inclusion levels of these macroalgae groups, however, were reported to be lower relative to *Ulva* or *Gracilaria*. *Sargassum muticum*, for instance, contains relatively lower protein levels than *Ulva* and *Gracilaria*, at a range of 9–17% DW, with higher protein digestibility in Nile tilapia (71.2%) compared with that of *Gracilaria* sp. (Pereira et al., 2012).

Materials of Microscopic Origin

Materials of microscopic origin are derived from microorganisms such as microalgae, yeast, cyanobacteria, and bacteria. Microalgae contain various essential nutrients such as amino acids, fatty acids, and vitamins as well as bioactive compounds that are beneficial for both aquaculture animals and humans. Studies have recently demonstrated the possibility to generate microalgal biomass using wastewater, which might not suitable for human uses but could be used as a feed material (Dourou et al., 2018, 2020; Malibari et al., 2018). Among the extensively studied marine microalgae, several species that have a high potential as aquafeed raw materials include *Nannochloropsis* spp., *Chlorella* spp., *Schizochytrium* spp., *Tetraselmis* spp., and *Isochrysis* spp. (**Table 1**). *Nannochloropsis* spp. are known as a source of n-3 highly unsaturated fatty acids (HUFAs) that can be cultured with high productivity (33.6–84.0 tons/ha/year) (Griffiths et al., 2012; Chauton et al., 2015). The members of this genus could be used as an aquafeed material with an inclusion level up to 82% (Gbadamosi and Lupatsch, 2018). A recent study showed that the use of defatted *Nannochloropsis oculata* (a by-product of oil extraction for nutraceuticals) and whole cells of *Schizochytrium* sp. to substitute fishmeal and fish oil in Nile tilapia diet resulted in a 48% higher final body weight and 8% lower feed cost per kilogram fish production (Sarker et al., 2020). *Arthrospira* (*Spirulina)* spp. are cyanobacteria with substantial productivity (20–90 tons/ha/year) that has been cultured and used as food and feed supplements (Soni et al., 2017). With the high capacity in removing phosphate (99.97%) and nitrate (81.10%) in water, this group of cyanobacteria has the potential to be cultured in integration with other aquaculture production as a bioremediator (Cardoso et al., 2020). Members of the genus *Arthrospira* are also known for their nutritional benefits. For example, *Arthrospira platensis* is reported to have a significantly high protein content (about 60% DW) (Van Vo et al., 2020) and various high-value bioactive compounds including vitamins, essential lipids, and natural pigments (phycocyanins) (Cuellar-Bermudez et al., 2015). *Arthrospira* spp. have been tested on various aquaculture species with the highest inclusion level recorded in African catfish, at about 30%, and may completely substitute fishmeal use (Raji et al., 2020). Although some marine yeast and bacteria have been identified recently, most of the studies involving these microorganisms as aquafeed raw materials are not specific to marine species. Commercially available bacterial meals are mainly produced from natural gas fermentation by using single or mixed species of methanotrophs (Jones et al., 2020), some of which can also be found in marine environment. Bacterial meal is a single-cell protein that can be used in the diet of various animals including aquaculture species (Øverland et al., 2010). A notable aquafeed raw material is biofloc meal, which mainly consists of a heterogenous mix of bacteria. Biofloc can be generated from fish or shrimp wastewater treatment and has a protein content in the range of 23–49% DW (Dantas et al., 2016). This material could be used in shrimp feed at an inclusion level up to 60% (Bauer et al., 2012; Promthale et al., 2019).

THE CHALLENGES OF UTILIZING MARINE-BASED ORGANISMS FOR AQUAFEED AND STRATEGIES TO ENHANCE UTILIZATION

The utilization of marine-based organisms for feed material is not without challenges. The use of each material is associated with specific challenges that may limit its use in aquafeeds; these include (1) nutritional composition and productivity, which may strongly depend on the environment; (2) risk of contamination by toxins and heavy metals; and (3) presence of antinutritional factors. The productivity and nutritional

composition of macroalgal- and microbial-based materials could be strongly dependent on the nutrient quantity and composition of the water, which are site and season specific (Mohy El-Din, 2019). Likewise, the productivity and nutritional composition of mussels could depend on the quantity of organic matter, microalgal composition, and the presence of stressors in their environment (Martino et al., 2019). This implies that site selection is an essential strategy to maintain high productivity and high quality of marine-based raw materials. Nutrient-rich environments are also associated with the higher possibility of toxin and heavy metal absorption by extractive marine organisms, which may reduce the safety of the raw materials (Torres et al., 2019). A recent study by Jusadi et al. (2020) demonstrated that accumulation of heavy metals in mussel meal could be alleviated by dietary supplementation of fulvic and humic acids at very low concentrations. Fulvic and humic acids are chelating agents that bind heavy metals to prevent their absorption by the fish, thus avoiding the accumulation of heavy metals in aquaculture organisms. van der Spiegel et al. (2013) suggested that some seaweeds may contain some hazards such as ANFs, dioxins, and pesticides that limit their use as feed and/or food materials. Fermentation and biorefinery technologies that have been well-developed in various food technologies could be applied to these materials to improve their nutritional value and to optimize nutrient digestibility as well as eliminate potential hazards (Bikker et al., 2016; Fleurence, 2016). While some of these raw materials, particularly those of macroalgal origin, are typically lower in protein than current sources; the development of protein concentrates for emerging ingredients may help bolster their use in future aquafeeds (Magnusson et al., 2019). Various hydrolytic processes could be applied to these materials to remove possible contaminants such as heavy metals and toxins to ensure their safety for the fed organisms and ultimately for human consumption (Torres et al., 2019).

CONCLUSIONS AND FUTURE DIRECTIONS

Marine-based feed materials are promising raw materials for aquafeed development. From a nutritional point of view, marine-based materials are relatively similar, if not superior, to terrestrial-origin materials. The production of unfed marine-based materials does not require freshwater and may enable the retrieval of waste nutrients from the environment, thus allowing more efficient use of nutrients, reducing the negative impacts of aquaculture on the environment, and promoting the sustainability of marine aquaculture in general. Some of the marine-based feed raw materials are already available commercially, such as seaweeds, microalgae, or bacteria meals; however, the price of these products is still high and is not competitive with conventional feed materials. Thus, more efforts are needed to promote the development of technologies for the production and processing of these materials to enable their commercial use. Further research on the environmental and nutritional requirements of these organisms are needed in order to improve productivity. More studies are also required to elucidate strategies to enhance the nutritional quality of the materials. The development of pretreatment and processing technologies is required to reduce the risks of contamination and antinutritional factors as well as to improve the nutritional quality of the products. Biorefinery technologies that could allow the utilization of all valuable constituents of a raw material in an economically feasible cascading process, with limited to zero waste, could be developed for the efficient use of the raw materials and for the production of high-quality materials for aquafeed production.

AUTHOR CONTRIBUTIONS

DJ was responsible for the conceptualization, data collection, and manuscript preparation. JE was responsible for data collection and manuscript preparation. MAS contributed to the analysis, interpretation of the data, and information as well as providing critical review to the manuscript in particular those sections relating to the criteria of aquafeed raw materials quality. MS was involved in data interpretation and manuscript revision. IF contributed in data acquisition and analysis as well as manuscript revision. All authors contributed to the article and approved the submitted version.

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γ-Irradiated Chitosan From *Carcinoscorpius rotundicauda* (Latreille, 1802) Improves the Shelf Life of Refrigerated Aquatic Products

Siddhartha Pati[1,2,8†], Tanmay Sarkar[3,4†], Hassan I. Sheikh[5], Kaushik Kumar Bharadwaj[6], Pranab Kishor Mohapatra[7], Anil Chatterji[8,9], Bisnu Prasad Dash[1], Hisham Atan Edinur[10,11*] and Bryan Raveen Nelson[8,11*]*

[1] Department of Bioscience & Biotechnology, Fakir Mohan University, Balasore, India, [2] Centre of Excellence, Khallikote University, Berhampur, India, [3] Department of Food Technology and Biochemical Engineering, Faculty of Engineering and Technology, Jadavpur University, Kolkata, India, [4] Malda Polytechnic, West Bengal State Council of Technical Education, Government of West Bengal, Malda, India, [5] Faculty of Fisheries and Food Science, Universiti Malaysia Terengganu, Kuala Nerus, Malaysia, [6] Department of Bioengineering and Technology, Gauhati University, Guwahati, India, [7] Department of Chemistry, C.V. Raman Global University, Bhubaneswar, India, [8] Research Divisions, Association for Biodiversity Conservation and Research, Balasore, India, [9] Aquamarina Research Foundation, Dona Paula, India, [10] Forensic Science Programme, School of Health Sciences, Universiti Sains Malaysia, Kelantan, Malaysia, [11] Institute of Tropical Biodiversity and Sustainable Development, Universiti Malaysia Terengganu, Kuala Nerus, Malaysia

The γ-irradiated horseshoe crab chitosan was used as food coating to extend the shelf life of marine shrimp and fish. Fourier-transform infrared spectroscopy (FTIR), field emission scanning electron microscopy (FE-SEM), and X-ray diffraction (XRD) were used to characterize the γ-irradiated chitosan. After employing control (untreated seafood samples/no preservatives), chemical preservation (treated with 2% glacial acetic acid) and 2% chitosan (0, 10, and 20 kGy γ-irradiation) for the assessment assay, the shrimp (*Penaeus merguiensis*), pomfret (*Pampus argenteus*), and hilsa fish (*Tenualosa ilisha*) samples were examined for pH, thiobarbituric acid reactive substance (TBARS), total viable counts (TVC), and sensory evaluation changes while under 15-day refrigeration at 4°C. The results of FT-IR, XRD, and FE-SEM analysis revealed that irradiated chitosan possessed a crystalline structure with smooth texture on its surface. Analysis of pH, TBARS, TVC, and sensory evaluation demarcated irradiated chitosan with the ability to delay microbial growth and this prolonged the shelf life of refrigerated shrimp and fish. With novelty on γ-irradiated horseshoe crab chitosan use as natural preserving agent, fisheries industries and food packaging practitioners would benefit from its microbial-inert abilities particularly for long distant cold storage transport of packaged marine meats.

Keywords: natural preservative, cold storage, food coating, chitosan, fish, shrimp, sensory evaluation

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Wang Yueqi,
South China Sea Fisheries Research Institute (CAFS), China

***Correspondence:**
Siddhartha Pati
patisiddhartha@gmail.com
Hisham Atan Edinur
edinur@usm.my
Bryan Raveen Nelson
bryan.nelson@umt.edu.my

† These authors have contributed equally to this work

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INTRODUCTION

Seafood contains fatty acids such as omega-3, proteins and minerals which altogether contribute to an ideal diet (Cheung et al., 2010; Kwan et al., 2019). However, these nutritional components are sustenance for microbes during food oxidation (spoilage). Rapid spoilage of food is mainly due to the high moisture and the decomposition of unsaturated fatty acids and free amino acids (Binsi et al., 2015). In fact, unsaturated fatty acids are chemically unstable and easily oxidized (Kilincceker et al., 2009). In the presence of oxygen and temperatures above 4°C, aerobic microorganisms are provided with optimum metabolic conditions to reproduce (Özogul et al., 2004; Li et al., 2020). Therefore, additives that can limit microbial growth are certain to delay the decomposition (fatty acid and lipid oxidation) process and thus, extend the shelf life of food. At present, industries involved with marine produce rely on freezing, salting, chemical treatments and nitrogen or vacuum packaging for long-term storage of their products (Gokoglu, 2019; Hazra et al., 2020; Sarkar et al., 2021).

Freezing (cold storage) will delay microbial growth because it suppresses their enzymatic activities which therefore, locks nutrition in food (Jiang and Lee, 2005; Gonçalves and Gindri Junior, 2009). Yet, freezing alone causes surface dehydration, protein denaturation, and lipid oxidation which negatively impacts flavor, odor, color, and texture of the stored foods (Gao et al., 2014; Duan et al., 2019). Although shelf life of frozen foods is extended with synthetic and artificial additives, these chemicals may cause allergy, health complications or require a secondary natural compound to become edible-safe (Matuska et al., 2006; Gultekin and Doguc, 2013). With such shortcomings, researchers resort to identify suitable biological sources that function as both food additives and preservation (Cao et al., 2012). After a series of extract preparations, chitosan (molecular weight: 50,000–190,000 Da) from crustaceans, insects, and fungi were discovered with antimicrobial ability (Shahidi et al., 1999; Prashanth and Tharanathan, 2007; Friedman and Juneja, 2010). In addition, chitosan derived from natural sources has different viscousness in room temperature, has low toxicity and is biodegradable and biocompatible to various foods (USFDA, 2001; Prashanth and Tharanathan, 2007; Soares et al., 2013).

While heating cleaves the glycosidic bonds of chitosan, reducing its molecular weight can enhance the antimicrobial capabilities (Hao et al., 2021). Therefore, heat-treated or increasing the concentration of naturally sourced chitosan has been an industrial practice to preserve raw (Ouattar et al., 2000; Sagoo et al., 2002; Rabea et al., 2003; Soultos et al., 2008; Raafat and Sahl, 2009; Friedman and Juneja, 2010; Bonilla et al., 2013; Duan et al., 2019; Hu and Gänzle, 2019; Lee et al., 2019) and processed meats on the shelf (Darmadji and Izumimoto, 1994; Georgantelis et al., 2007; Gómez-Estaca et al., 2007; Kim and Thomas, 2007; Kanatt et al., 2008) with additional applications that include long distance transport (Casariego et al., 2008; Cerqueira et al., 2009; Souza et al., 2009; Fernández-Saiz et al., 2013; Zarandona et al., 2021). Yet, the antimicrobial properties of naturally sourced chitosan is selective, depending on its molecular weight, temperature of the storage environment and the pH of the coated food (Devlieghere et al., 2004; Kong et al., 2010; He et al., 2016; Xing et al., 2016). For instance, shrimp chitosan with higher molecular weight (2.3–3.5 $\times$ 10^5 $gmol^{-1}$) offered weaker antimicrobial capabilities than the lower molecular weight horseshoe crab chitosan (1.83 $\times$ 10^5 $gmol^{-1}$) in comparative assays on food oxidation (Zhao et al., 2010; de Queiroz Antonino et al., 2017; Krisfalusi-Gannon et al., 2018; Boudouaia et al., 2019; Pati et al., 2020a).

Researchers learnt that chitosan from marine sources such as shrimp, crab, lobster, krill, and squid possessed different molecular sizes and this influenced the number of functional groups available for antimicrobial capabilities (Younes and Rinaudo, 2015; Tamzi et al., 2020). Since not all marine resources are available throughout the year and their yields vary with catch efforts, researchers explored on the use of heating to reduce the molecular weight of the derived chitosan. It is learnt that heating will de-alkyl chitosan and expose more functional groups whereby, the now less viscous chitosan offers better antimicrobial efficacy (Souza et al., 2010; Ji et al., 2014). Other methods include altering the pH of preserved products to arrest the oxidation process, but this method is less economical and harmful to consumers (Fan et al., 2009). By far, the promising method to reduce chitosan molecular weight is heat irradiation after which several studies made successful comparison between treated and non-treated chitosan to prolong the preservation of frozen meats (Abdeldaiem, 2014; Pati et al., 2016, 2020b; Xing et al., 2016; Hassanzadeh et al., 2017; Lyu et al., 2017; Zhang et al., 2019).

Meanwhile, the mangrove horseshoe crab *Carcinoscorpius rotundicauda* has no commercial importance in India except for indigenous (Noida) preparation into health tonics and pain-relief ointment and the sparring collection for biomedical research (John et al., 2018). Elsewhere with similar opinions, the dried carapace of *C. rotundicauda* is used for bioactive compound research (Alam et al., 2015; Luo et al., 2020; Wardiatno et al., 2021; Xu et al., 2021). With horseshoe crab chitosan, specifically sourced from the dried carapace of *C. rotundicauda* claimed to have the lowest molecular weight than other marine sources, the present study explores on the use of heat radiation [via gamma (γ) irradiation] to further reduce the molecular weight of the derived chitosan for enhanced antimicrobial capability. The chitosan of different molecular weights, recognized as irradiated and non-irradiated, are then coated onto shrimp (*Penaeus merguiensis*), pomfret (*Pampus argenteus*) and hilsa fish (*Tenualosa ilisha*) which have high commercial value throughout Asia (AlMomin et al., 2016; De et al., 2019; Hoang et al., 2020). With novelty on horseshoe crab chitosan being able to display better antimicrobial capabilities after irradiation, the shelf life extension of refrigerated shrimp and fish is assessed using lipid oxidation values, changes to pH and sensory scores within the 15-day storage period as carried out in the present study.

MATERIALS AND METHODS

Materials

A total of three seafood species namely, shrimp (*P. merguiensis*) (each weighing about 30 g, average length 33–38 cm), pomfret

(*P. argenteus*) (each weighing about 650 g, average length 30–35 cm) and hilsa fish (*T. ilisha*) (each weighing about 800 g, average length 30–39 cm) were selected due to their high market and nutritional values (**Figure 1**). All samples were randomly selected and purchased from 3 vendors certified with ISO 9001/HACCP (hazard analysis at critical control point) and seafood good manufacturing practices (SGMP). All the three vendors were located at a fish market in Naya Bazar, Balasore (21.5077° N, 86.9279° E). Simple random sampling was carried out by collecting a total of 342 shrimp samples out of a harvesting population of 50 kg (approximately 1,600 shrimp). In terms of fish, 218 pomfret and 152 hilsa fish were collected out of approximately 550 and 250, respectively. The simple random sampling used for sample collection was based on the margin of error (ME) which was considered as ±0.05 with 95% confidence level (C) at a sample proportion (SP) of 50%. The sample size was determined using the following formula:

$$\text{Sample size} = \text{Population size} \times A/(A + \text{Populationsize} - 1) \quad (1)$$

Where,

$$A = C2 \times SP \times (1 - SP)/ME2 \quad (2)$$

Mangrove horseshoe cab carapace (length 14.4 ± 2.1 cm; width 14.7 ± 1.8 cm) samples were obtained from Bichitrapur mangrove sanctuary (21°35′09.3″N and 87°25′21.3″E) estuary (Balasore, Odisha, India). Each shrimp/fish sample was further divided into five equal parts for the later part of the experimentation. Acetic acid (food grade), hydrochloric acid (HCl), sodium hydroxide (NaOH), potassium bromide (KBr), 1-butanol, and thiobarbituric acid (TBA) were purchased from Merck Life Science Pvt. Ltd. (Mumbai, India). The peptone water was purchased from HiMedia Laboratories Pvt. Ltd. (Mumbai, India), waterproof polyvinyl dichloride (PVDC) bags (50–70 micron of thickness) were purchased from Caprihans India Ltd. (Mumbai, India), while double-distilled (DD) water was prepared in the laboratory.

Preparation of Seafood Samples

The specimens were purchased alive and brought into the laboratory in live condition and were killed by placing the samples in chilled water at 4°C to avoid rigor mortise. A thorough cleaning was carried out using DD H_2O. Water was then allowed to drain and the samples were left to dry prior to dipping in the chitosan solution. Freshly cleaned samples (500 g) of meat/flesh/muscle tissue was cut, weighed and placed separately (Cao et al., 2020).

Pre-processing of Shrimp Samples

The freshly harvested shrimp samples were drained thrice with potable water to reduce its microbial load and the mud adhered 45 min prior to the initiation of the experiment. After draining, the shrimp was processed manually to remove catgut and deshelling. The resulted fleshy part was washed thoroughly with DD water (Tayel et al., 2020). The processing utensils and equipment used were cleaned with high-pressure water jet to maintain the sanitation standard operating procedures (SSOP).

Chitosan Solution Preparation

Chitosan was prepared from the waste carapace of mangrove horseshoe crab (*C. rotundicauda*) according to previous studies (Pati et al., 2018, Pati et al., 2020a) and dried at ambient temperature (30 ± 2°C). Irradiation of chitosan was performed using cobalt (Co-60) source at doses of 10 and 50 kGy. The dose rate used was 10 kGy/h in a gamma cell PX-30 irradiation facility. The chitosan solutions were prepared by taking 10 g of horseshoe crab chitosan and dissolving in 500 ml 2% acetic acid. Each of the above solutions was prepared by stirring the samples for 10 min at 60°C for 1 h and the pH was adjusted to five using NaOH (Xuan and Xuan, 2019).

Sample Dipping

Before coating the samples with chitosan, the collected shrimp/fish samples were sub-grouped into five batches each containing 100 g of sample. Batch A was used as control and consisted of untreated seafood samples (no preservatives). Batch B comprised of samples treated with 2% glacial acetic acid as a chemical preservative method. Batches C, D, and E were treated with different types of 2% chitosan solutions (0, 10, and 20 kGy). Preservation was carried out by dipping the samples in respective chitosan solutions for 1 h. After dipping, samples were allowed to dry for 30 min in an aseptic condition before packaging.

Samples Packing and Storage

Samples were packed into air-sealed PVDC bags before they were numbered and marked with the packaging date. The samples were stored in a refrigerator at 4°C and observed over a 15-day period (Bharathi et al., 2019).

Characterization

Fourier-Transform Infrared Spectroscopy Analysis

The FT-IR spectra of dried samples of irradiated and non-irradiated chitosan biopolymer were recorded using Thermo Nicolet^{TM} 6700; Thermo Fisher Scientific, United States. The observation of the compound was performed by preparing the powdered samples into KBr pellet and dried before subjection to ATR-attenuated FT-IR at 4,000–500 cm^{-1} with sixteen scans being taken at 2 cm^{-1} resolution. Different functional groups against the specific inverse peak of wavenumber (cm^{-1}) had been calculated for the identification of specific functional groups present in the structure of non-irradiated and irradiated samples of the chitosan compound (Jayadevan et al., 2018).

X-ray Diffraction Analysis

The powdered samples of irradiated and non-irradiated chitosan compound were prepared by lyophilization and then subjected to X-ray diffraction (XRD) (Panalytical Diffractometer, X'Pert Pro, United States) at 0.025° (2θ) angle and 52°–45° range with 1.25 s scan time for 1 h with Cu Kα (λ = 1.5406 nm), 45 kV, 30 mA. The crystalline structure and the lattice planes for the changes in the irradiated compound were determined. Different lattice planes against specific diffraction angles were calculated and observed for the identification of the intrinsic details of the structure from the relevant diffractogram (Baran et al., 2015; Sarkar et al., 2020).

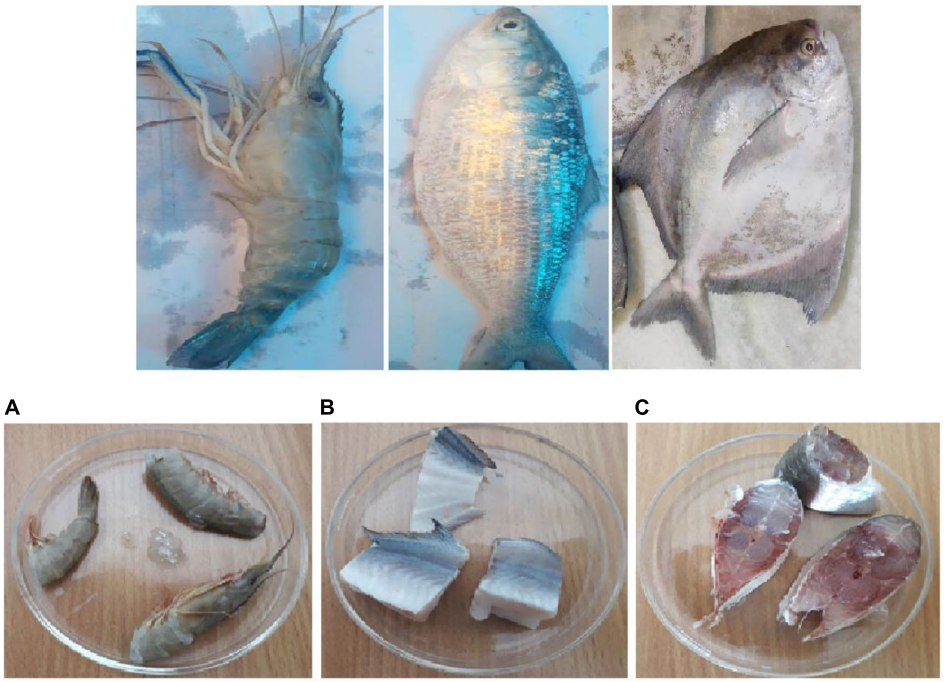

FIGURE 1 | Raw fresh shrimp and fish samples. **(A)** *Penaeus merguiensis*, **(B)** *Pampus argenteus*, and **(C)** *Tenualosa ilisha*.

Field Emission Scanning Electron Microscopy Analysis

The irradiated and non-irradiated chitosan solutions were prepared at 0, 10, and 20 kGy, the cast was then dropped over the coverslip and visualized under scanning electron microscopy (SEM) (Carl ZEISS SMT, Germany) at 20 kV (Bharathi et al., 2019; Lahiri et al., 2021).

Shelf Life Analysis

Shelf life evaluation was conducted based on physio-chemical and biological indicators. Physio-chemical indicators used were pH and thiobarbituric acid reactive substance (TBARS). Total viable counts (TVC) of microbes was adopted as a biological indicator, while sensory evaluation was employed to evaluate the product acceptability and correlate it with physiochemical and biological parameters. Sampling was carried out on days 0, 10, and 15 for all irradiated and non-irradiated samples.

pH Analysis

The pH of the sample was measured using the GB/T method (GB/T 5009.45-2003) with slight modifications. Approximately, 10 g seafood sample was added to 90 ml of distilled water and filtered. Samples were then incubated for 30 min at room temperature. A digital pH meter was used to measure the pH value (Khodanazary, 2019; Lee et al., 2019).

Thiobarbituric Acid Reactive Substance Analysis

Seafood samples (200 mg) were placed in a flask containing 1 mL 1-butanol. Then, 5 mL reagent (200 mg 2-TBA in 100 mL 1-butanol) was added to the sample before gravity-filtering through Whatman No. 1 filter paper. Test tubes containing the assay mixture were vortexed and placed at 95°C for 120 min in a water bath before cooling using air temperature. Development of a pink colored solution indicated that malondialdehyde (MDA) reacted with TBA (Paparella et al., 2016). A spectrophotometer was used to analyze the samples at 532 nm. TBA was calculated as mg MDA/kg sample using Equation (3):

$$\mathrm{TBA} = \left(50 \times \left(\frac{\mathrm{A\ s} - \mathrm{A\ b}}{200}\right)\right) \tag{3}$$

where A_s = absorbance of sample and A_b = absorbance of blank.

Total Viable Counts Evaluation

The TVC of microbes were calculated using the pour-plate method (AOAC, 2012) where 25 g of each seafood sample (in triplicates) were aseptically weighed and homogenized with 225 ml of sterilized 0.1% peptone water for 1 min. The homogenized samples were diluted (1:10) in 0.1% peptone water before 1 ml of the samples were plated onto plate count agar and incubated for 48 h at 35–37°C. Result interpretations were made after colony-forming unit data (CFU/g) were log-transformed (Zhang et al., 2019).

Sensory Evaluation

The sensory analysis of all the batches of meat samples was carried out by 10 semi-trained panelists whom were requested to score every batch of samples. Each panelist scored the characteristics from 1 to 9 (9-point hedonic scale) in terms of color, texture, flavor and overall acceptability of samples (9 = like

extremely, 5 = do not like or dislike 1 = dislike extremely) (Ehsani et al., 2019).

Statistical Analysis

All experiments were run in triplicate and the data obtained were subjected to statistical analysis (one-way ANOVA for comparing results from the five different sample batches). Differences were considered significant at $p < 0.05$. The difference between the mean graphs was constructed using OriginPro8.5.

RESULTS AND DISCUSSION

Characterization

Fourier-Transform Infrared Spectroscopy Analysis

Functional groups were visualized from the wavelengths of 3,435 cm^{-1} (OH and NH_2), 2,922 and 2,871 cm^{-1} (–CH), 1,835 cm^{-1} (–CONH amide I), 1,656 cm^{-1} (NH_2), 1,603 cm^{-1} (–NH), 1,550 cm^{-1} (–NH), 1,245 cm^{-1} (amide-III), 1,160 cm^{-1} (–O), 1,085 cm^{-1} (–CO and –OH), and 1,030 cm^{-1} (–CO and –OH) after FT-IR analysis on irradiated horseshoe crab chitosan (**Figure 2**). Comparatively, the FT-IR analysis revealed functional groups at 3,000–3,800 cm^{-1} (OH, NH_2), 2,850 cm^{-1} (–CH), 1,645 cm^{-1} (–CONH–, amide I), 1,550 cm^{-1} (–NH, amide II), 1,570 cm^{-1} (CN), 1,300 cm^{-1} (amide III), 1,103 cm^{-1} (CO), and 800 cm^{-1} (alkyl) wavelengths. Merging the present and previous opinions (Wang et al., 2016), non-irradiated chitosan has less functional groups and all functional groups are easily detected by the FT-IR. Overall, irradiated chitosan possesses additional –OH (3,070–3,750 cm^{-1}), -NH (3,435.81 cm^{-1}) and –CH (2,988.94 cm^{-1}) functional groups after γ-irradiation instead of –NH groups at the peak wavelengths of FT-IR. With the exposure of γ-irradiation on shrimp chitosan, the deacetylation or degradation of polysaccharides allows free radicals to cross-link and produce new functional groups within a crystalline matrix (Ocloo et al., 2011; Younes and Rinaudo, 2015; Li and Zhuang, 2020). This outcome causes the molecular weight of chitosan to become less (Taşkın et al., 2014). While deacetylation of chitosan is proportional and its molecular weight becomes reduced with the increased dose of γ-irradiation from 10 to 20 kGy, the irradiated chitosan of *C. rotundicauda* (and also *Tachypleus gigas* – c.a. Pati et al., 2020b) was assumed to have a lower viscosity, have increased solubility and therefore possess better antimicrobial capabilities (Ocloo et al., 2011; Xing et al., 2016; Pati et al., 2020a).

X-ray Diffraction and FE-SEM Analysis

Horseshoe crab chitosan generally has a crystalline lattice structure with 10.5°, 20°, and 27° angles (Muley et al., 2019; Pati et al., 2020a). After 10 kGy irradiation, the horseshoe crab chitosan has a constant 10.5° angled crystal lattice and additional curvature, demarcated with 2θ = 10.5°, will become apparent after 20 kGy irradiation (**Figure 3**). While chitosan is a biopolymer, γ-irradiation reduces fibrillar curvatures and shifts the paradigm of its crystalline structures into a fixed 10.5° angle which corresponds to the chitosan becoming compact (Rajeswari et al., 2020). The angling from θ = 10.5° after 10 kGy irradiation into 2θ = 10.5° with 20 kGy irradiation symbolizes the effect of a crystalline structure becoming steep and re-structured, whereby the increasing compaction results to reduction of its molecular weight while this molecule develops into a much smoother and defect-free form (Ling et al., 2018; Pati et al., 2020b). Through a visual inspection, non-irradiated chitosan appears much larger and coarser than irradiated chitosan because of its molecular folding (**Figures 4A–C**). The exposure of γ-irradiation is certain to reorganize chemical bond lengths, giving rise to smaller pore size between the molecules (Muley et al., 2019). However, since the horseshoe crab chitosan becomes compact after γ-irradiation, it possesses more alkyl (–NH) and hydroxyl (–OH) groups, whereby having bonds made between functional groups depicts a stable form of crystal lattice (Ling et al., 2018; Rajeswari et al., 2020).

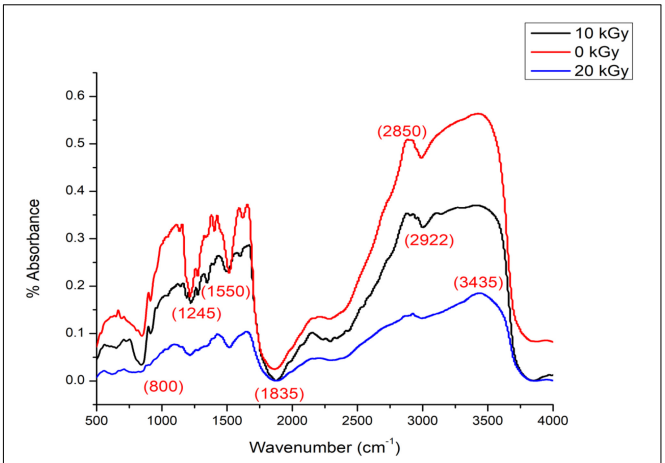

FIGURE 2 | Chitosan with irradiation at three different irradiations (0–20 kGy).

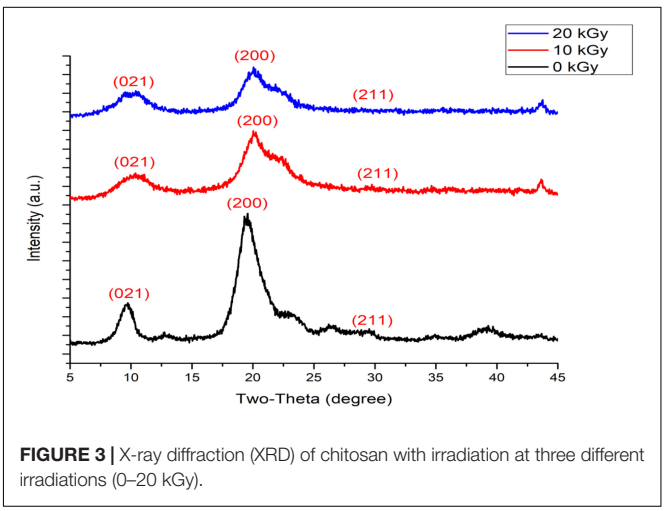

FIGURE 3 | X-ray diffraction (XRD) of chitosan with irradiation at three different irradiations (0–20 kGy).

Shelf Life Analysis

pH Analysis

The exposure conditions for chitosan were essential for its chemical stability, particularly to arrest any underlying microbial

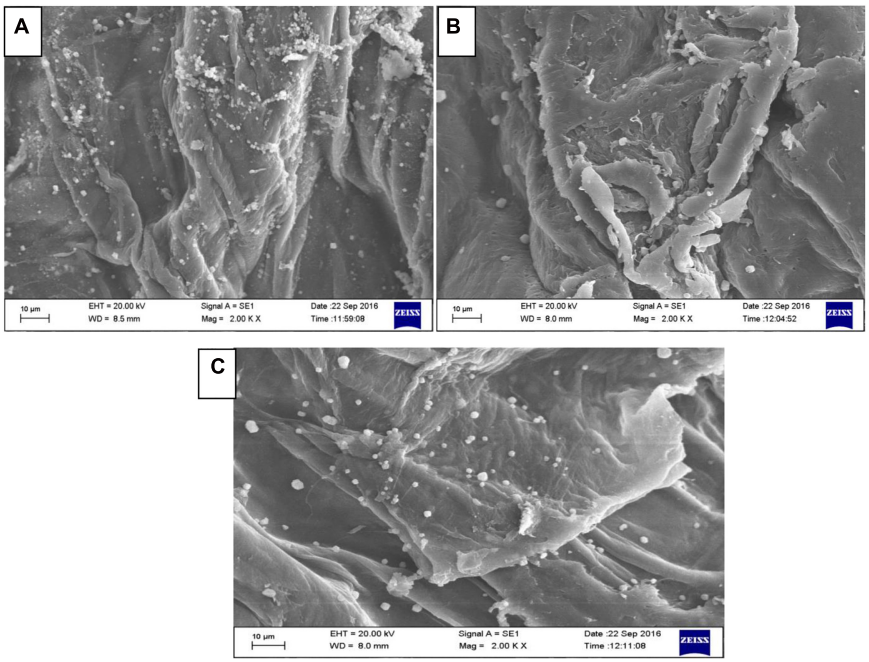

FIGURE 4 | Field emission scanning electron microscopy of chitosan irradiated at three different irradiation ranges **(A)** 0 kGy, **(B)** 10 kGy, and **(C)** 20 kGy irradiation source.

enzymatic activities. With the varying 0.1–0.5 pH ($p < 0.05$) in refrigerated conditions, hilsa fish meat appeared to be slightly acidic with pH 6.3 ± 0.9, the lowest compared to pomfret (pH ∼6.3) and shrimp meat (pH ∼6.9). Within 15 days after the 20 kGy irradiated chitosan coating, the pH of shrimp meat increased by 23.18%, 18.75% in pomfret, and 26.98% in hilsa fish (**Figure 5**). Comparatively, without chitosan treatment (control), the pH of shrimp meat increased by 40.58%, 43.75% in pomfret, and 55.56% in hilsa fish. All refrigerated samples showed an increasing trend of pH for the first 5 days, an observation that conforms to the dissolution of CO_2 (Manju et al., 2007; da Silva Santos et al., 2017; Cao et al., 2020). However, the increasing trend of pH leveled off after 10 days of refrigeration suggests that metabolic enzymatic activities within the meat and among microbes have reached an inactive plateau (López-Caballero et al., 2005). Therefore, the pH of meats would increase very minutely after 10 days of cold-storage, regardless with or without the addition of chitosan (**Figure 5**). However, the freshness, an attribute of moisture and taste locking, pertains to the type of preserving method.

Thiobarbituric Acid Analysis

The oxidation of fatty acids with three or more double bonds results in malonaldehyde production and this compound negatively impacts color, flavor, and odor food when stored for long periods (Jeon et al., 2002). The detection of high TBA beyond 2 mg malonaldehyde/kg in fish meat symbolizes the effects of spoilage (Jeyakumari et al., 2016). In the present study, all meat products without the chitosan coating and with acetic acid coating registered more than 2 mg/kg TBA on day 0 while with the γ-irradiated chitosan coating, shrimp meat contained 0.6 ± 0.03 mg/kg TBA, pomfret contained 0.26 ± 0.01 mg/kg TBA and hilsa fish contained 0.31 ± 0.01 mg/kg TBA (**Figure 6**). After 15 days, the control groups (acetic acid preservation and without any treatment) recorded more than 2 mg/kg TBA in the meat. Meanwhile, samples coated with 20 kGy chitosan had less (0.38 mg/kg) TBA readings than the meats coated with 10 kGy chitosan (1.02 mg/kg TBA) after 15 days of refrigeration (**Figures 5A–C**; $p < 0.05$). This analysis indicated that partial dehydration from 15-day refrigeration allows some fatty acids to oxidize in the meats but, the selection of appropriate preserving agents such as natural (irradiated chitosan) against chemical (acetic acid) is crucial to increase the shelf life quality of the refrigerated meats (Fan et al., 2009; Guizani et al., 2014; Hassanzadeh et al., 2017). The present findings thus show that cold-storage alone as depicted with the control group (without chitosan coating) is proven ineffective to reduce lipid oxidation and therefore, reduces the quality of meat when stored for long durations (Sneddon, 2009).

Total Viable Count

Non-treated meats would attract microbial inhabitants which aid the rotting process. In the food industry, raw salmon (*Salmo salar*), grass carp (*Ctenopharyngodon idellus*), and Wuchang bream (*Megalobrama amblycephala*) filets intended for long-term storage are added with chitosan so that delay to microbial metabolism would decrease their proliferation rate (López-Caballero et al., 2005; Gómez-Estaca et al., 2010; Souza et al., 2010; Fernández-Saiz et al., 2013; Yu et al., 2017). This practice, by using 10 and 20 kGy irradiated chitosan, maintained the TVC

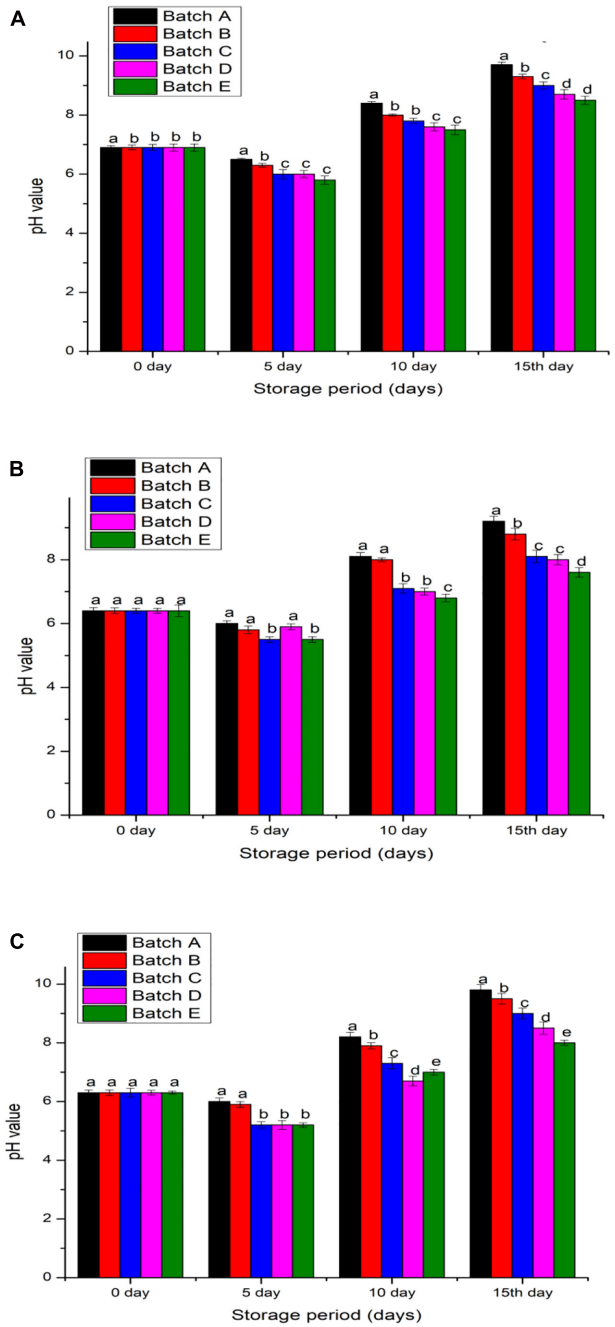

FIGURE 5 | pH value of samples **(A)** *Penaeus merguiensis*, **(B)** *Pampus argenteus*, and **(C)** *Tenualosa ilisha.* Batch A: normal untreated, Batch B: treated with 2% glacial acetic acid, Batches C, D, and E were treated with different types of 2% chitosan solutions (0, 10, and 20 kGy). Value is provided as mean ± SE ($n = 3$). The same letters (a–e) over the bar plot stands for insignificant difference ($p > 0.05$).

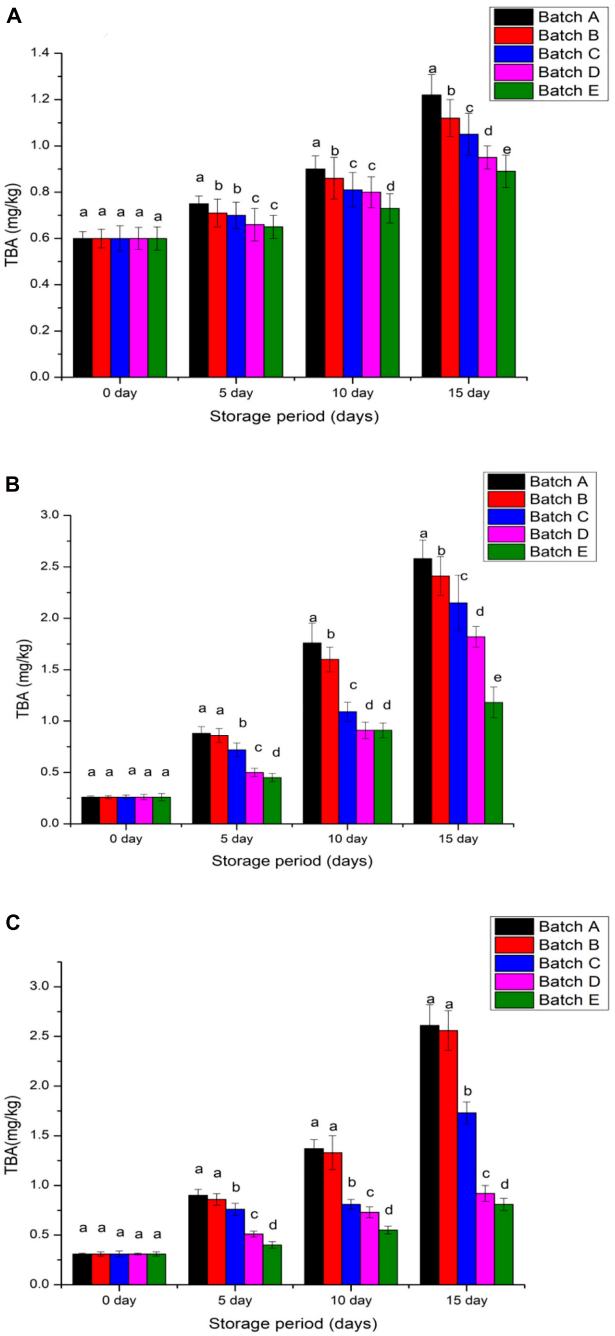

FIGURE 6 | Thiobarbituric acid value of each samples **(A)** *Penaeus merguiensis*, **(B)** *Pampus argenteus*, and **(C)** *Tenualosa ilisha.* Batch A: normal untreated, Batch B: treated with 2% glacial acetic acid, Batches C, D, and E were treated with different types of 2% chitosan solutions (0, 10, and 20 kGy). Value is provided as mean ± SE ($n = 3$). The same letters (a–e) over the bar plot stands for insignificant difference ($p > 0.05$).

of pomfret, and hilsa fish below 6.3 log CFU/g, an acceptable food safety benchmark (Jeyakumari et al., 2016; **Figure 7**). It was difficult to sustain minimum viable counts in shrimp because on day 0, non-treated samples recorded 4.3 log CFU/g, acetic acid treated samples reached 5.7 log CFU/g, while chitosan treated samples exhibited 4.36 log CFU/g viable counts. All chitosan treated and non-treated shrimps had viable counts that gradually increased within the 15-day refrigeration period

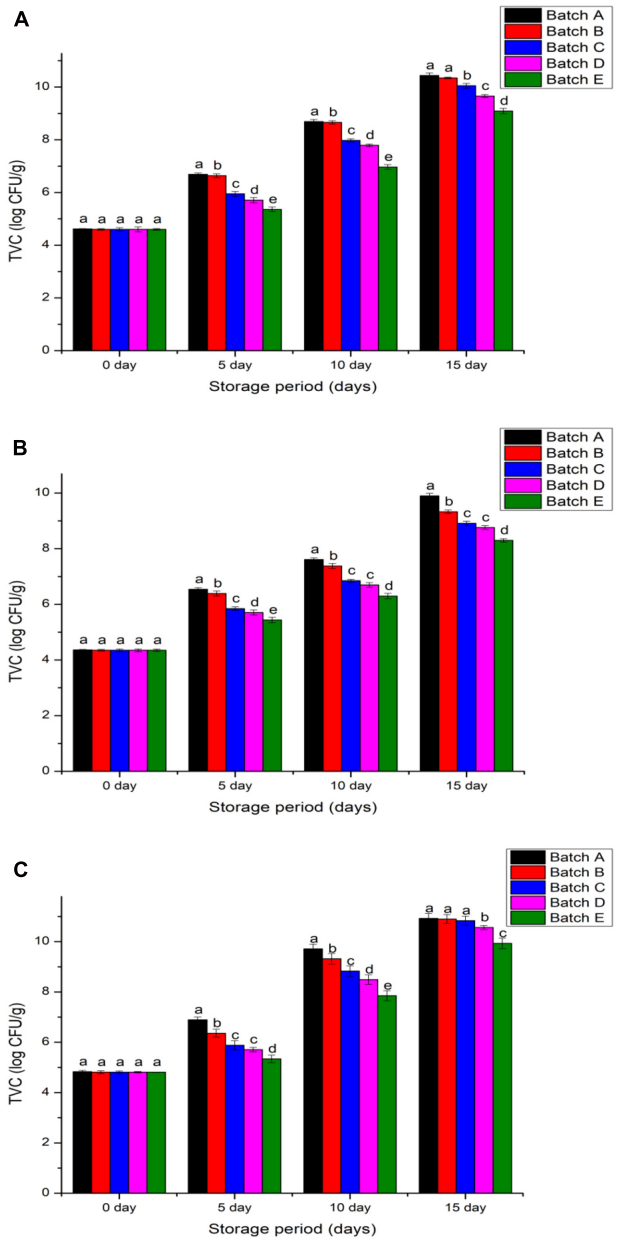

FIGURE 7 | Total viable counts of samples **(A)** *Penaeus merguiensis*, **(B)** *Pampus argenteus*, and **(C)** *Tenualosa ilisha.* Batch A: normal untreated, Batch B: treated with 2% glacial acetic acid, Batches C, D, and E were treated with different types of 2% chitosan solutions (0, 10, and 20 kGy). Value is provided as mean ± SE ($n = 3$). The same letters (a–e) over the bar plot stands for insignificant difference ($p > 0.05$).

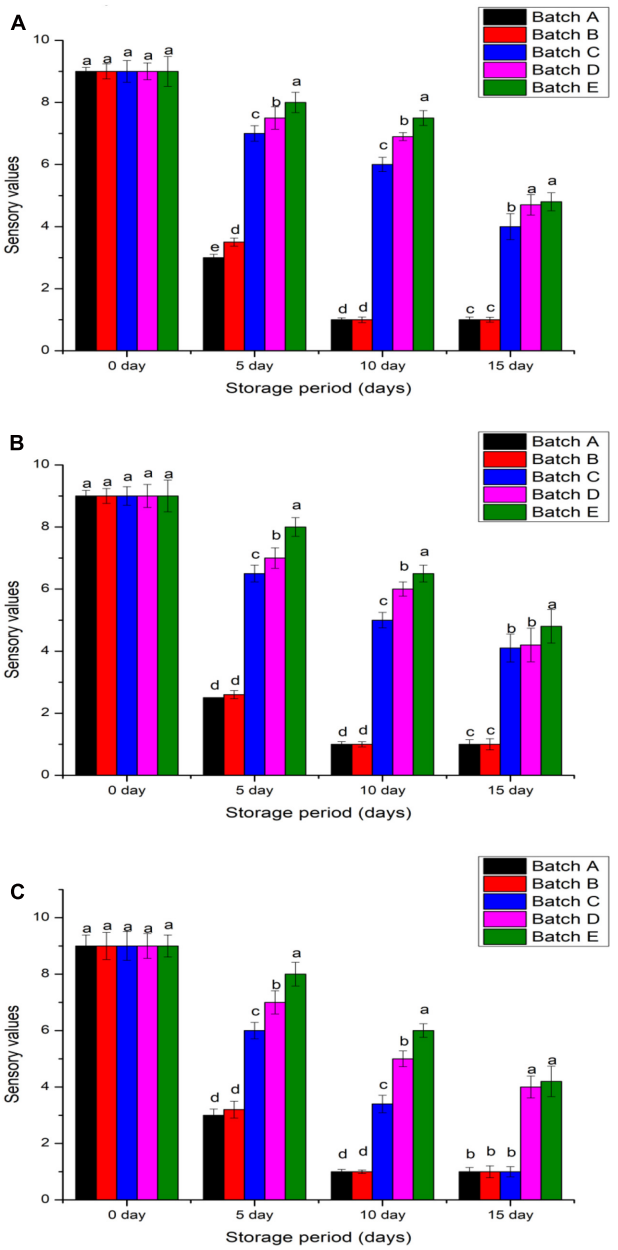

FIGURE 8 | Sensory scores of samples **(A)** *Penaeus merguiensis*, **(B)** *Pampus argenteus*, and **(C)** *Tenualosa ilisha.* Batch A: normal untreated, Batch B: treated with 2% glacial acetic acid, Batches C, D, and E were treated with different types of 2% chitosan solutions (0, 10, and 20 kGy). Value is provided as mean ± SE ($n = 3$). The same letters (a–d) over the bar plot stands for insignificant difference ($p > 0.05$).

but, samples treated with chitosan (10 and 20 kGy) did not exceed 8 log CFU/g in comparison to non-treated samples that exceeded 10 log CFU/g ($p < 0.05$). Moreover, the rate (days) of viable count growth was delayed in 20 kGy irradiated chitosan than when 10 kGy chitosan was used to coat the meats, which suggest that additional –NH and –OH groups in the further-compacted crystal lattice of 20 kGy chitosan demanded more microbial metabolic energy to break the bonds (Tsai et al., 2004; Song et al., 2011).

Sensory Evaluation

The sensory quality of food products revolves with texture, odor, gloss and an appeal (non-stale) to reach customer satisfaction. During refrigeration, the non-treated and acetic acid treated

meats changed their color from gray to gray–white with an odor score that immediately reduced and ranged 4.71–4.92 in 5 days before greatly reducing to <1.0 in 15 days (**Figure 8**). It was different for hilsa fish, shrimp and pomfret that received the chitosan coat because after 5 days, their odor scores were 6.39–6.63 and the score only reduced to 5.04 (for 20 kGy irradiated chitosan) and 4.83 (for 10 kGy irradiated chitosan) within 15 days of refrigeration. It is understood that refrigeration alone is ineffective after 5 days because microbial action to decay the meat will produce a putrid odor (Yu et al., 2017). However, adding a preservative such as (non- or irradiated) chitosan is sufficiently promising to delay the decay of meats and this maintains their odor score above five, which is the baseline score for safe-to-consume shrimp and fish (Yao et al., 2015).

CONCLUSION

The effects of γ-irradiated horseshoe crab chitosan on the shelf life of fish and shrimp were evaluated based on microbial activity, pH, TBA, and sensory qualities. It is understood that (10 and 20 kGy) irradiated chitosan had the lowest molecular weight ($\sim 1.83 \times 10^5$ $gmol^{-1}$), possessed additional –NH and –OH functional groups and the 10.5° angles between bonds in the crystal lattice maintained the chitosan within a compact structure and assured this new polymer resistance against microbial action. Overall, high dose (20 kGy) irradiation developed a more effective chitosan polymer for preserving marine meats to successfully extend the shelf life of marine meats beyond the capabilities of refrigeration. The present findings indicate that horseshoe crab chitosan benefits the postharvest industry during long-term transportation of raw meats because coating foods with the irradiated version of this polymer can maintain the freshness and prolong the food shelf life for at least 15 days.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

SP designed and performed the experiments and participated in the interpretation of the results and the writing of the manuscript. BN, TS, HE, HS, SB, PM, and KB interpreted the results and edited the manuscript. AC and BD supervised and discussed the research and edited the manuscript. All authors contributed to the realization of the manuscript, have read and agreed to the published version of the manuscript.

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Microbiomes of Healthy and Bleached Corals During a 2016 Thermal Bleaching Event in the Upper Gulf of Thailand

Heru Kusdianto[1], Chitrasak Kullapanich[2,3], Matanee Palasuk[2,3], Suppakarn Jandang[1], Kobchai Pattaragulwanit[2], Jamal Ouazzani[4], Suchana Chavanich[1,5], Voranop Viyakarn[1] and Naraporn Somboonna[2,3]**

[1] Reef Biology Research Group, Department of Marine Science, Faculty of Science, Chulalongkorn University, Bangkok, Thailand, [2] Department of Microbiology, Faculty of Science, Chulalongkorn University, Bangkok, Thailand, [3] Microbiome Research Unit for Probiotics in Food and Cosmetics, Chulalongkorn University, Bangkok, Thailand, [4] Centre National de la Recherche Scientifique, Institut de Chimie des Substances Naturelles ICSN, Gif-sur-Yvette, France, [5] Department of Marine Science, Faculty of Science, Center of Excellence for Marine Biotechnology, Chulalongkorn University, Bangkok, Thailand

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***Correspondence:**
Naraporn Somboonna
Naraporn.S@chula.ac.th
Voranop Viyakarn
Voranop.V@chula.ac.th

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Global warming has caused elevated seawater temperature and coral bleaching, including events on shallow reefs in the upper Gulf of Thailand (uGoT). Previous studies have reported an association between loss of zooxanthellae and coral bleaching. However, studies on the microbial diversity of prokaryotes and eukaryotes (microbiome) as coral holobionts are also important and this information is still limited in the uGoT. To address this shortcoming, this report provided baseline information on the prokaryotic (bacteria and archaea) and eukaryotic microbes of healthy and bleached colonies of four prevalent corals *Acropora humilis*, *Acropora millepora*, *Platygyra sinensis*, and *Porites lutea* and surrounding seawater and sediments, using 16S and 18S rRNA gene next-generation sequencing. Both prokaryotic and eukaryotic microbes showed isolated community profiles among sample types (corals, sediment, and seawater) (ANOSIM: $P < 0.001$, $R = 0.51$ for prokaryotic profiles and $P < 0.001$, $R = 0.985$ for eukaryotic microbe profiles). Among coral species, *P. sinensis* showed the most diverse prokaryotic community compared with the others (ANOSIM: $P < 0.001$, $R = 0.636$), and *P. lutea* showed the most diverse eukaryotic microbes ($P = 0.014$, $R = 0.346$). Healthy and bleached corals had some different microbiomes in species and their prevalences. For instance, the significant increase of Alphaproteobacteria in *P. sinensis* resulted in reduced prokaryotic community evenness and altered potential metabolic profiles (i.e., increased amino acid metabolism and genetic information processing and transcription, but decreased prokaryotic functions in cell motility, signaling, and transduction). For eukaryotic microbes, the loss of the algal *Symbiodinium* (colloquially known as zooxanthellae) in bleached corals such as *P. lutea* resulted in increased Chromista and Protista and, hence, clearly distinct eukaryotic microbe (including fungi) communities in healthy vs. bleached colonies of corals. Bleached corals were enriched in bacterial pathogens (e.g., *Acinetobacter*, *Helicobacter*, *Malassesia*, and *Aspergillus*) and decreased coral-beneficial prokaryotic and eukaryotic microbes (e.g., Rhizobiales

and *Symbiodinium*). Additionally, this study identified microbiome species in bleached *P. lutea* that might help bleaching recovery (e.g., high abundance of Rhizobiales, Oceanospirillales, Flavobacteriales, and Alteromonadales). Overall, our coral-associated microbiome analyses identified altered diversity patterns of bacteria, archaea, fungi, and eukaryotic microbes between healthy and bleached coral species that are prevalent in the uGoT. This knowledge supports our ongoing efforts to manipulate microbial diversity as a means of reducing the negative impacts of thermal bleaching events in corals inhabiting the uGoT.

Keywords: coral bleaching, coral reefs, microbiome, bacteria, fungi, small eukaryotes, next generation sequencing

INTRODUCTION

Coral reefs represent one of the most productive and biodiverse ecosystems on earth, when normalized per unit area, than any other marine environments (Hatcher, 1990). Coral reefs provide many ecosystem services including being the major marine nutrient resources (carbon and nitrogen supplies) and habitat to many diverse marine organisms. Additionally, coral reefs are a source of bioactive compounds and provide coastal protection against waves and floods (Rosenberg et al., 2007; Burke et al., 2012; Bourne et al., 2013). However, during the past few decades, coral reefs have been extensively endangered by anthropogenic activities, directly (e.g., overfishing and coral collection) and indirectly (primarily by greenhouse gas pollution) (Hughes et al., 2003; Harvey et al., 2018). Accumulated greenhouse gas pollution affects UV radiation and global climate change (elevated atmosphere and seawater temperatures) and subsequently causes thermal coral bleaching events (Lesser et al., 2004; Burke et al., 2012; Bourne et al., 2013). When seawater temperature is too warm, corals expel their algal holobionts, of which one of the most predominant is *Symbiodinium*. *Symbiodinium*, alternatively known as zooxanthellae, are single-cell photosynthetic dinoflagellates that live in symbiosis with corals and several marine invertebrates (Hoegh-Guldberg and Smith, 1989). *Symbiodinium* spp. provide photosynthetic food to corals and protect corals from pathogens by competing against pathogens for food and space on coral bodies (Lesser et al., 2013). When *Symbiodinium* is expelled, corals become discolored (white) due to the absence of colored photosynthetic pigments from *Symbiodinium*; this is referred to as "coral bleaching" and importantly results in loss of food that is normally produced by *Symbiodinium* to feed the coral (Baker et al., 2008). Consequently, coral growth, reproduction, resistance to disease and stress, and survivability decline (Baker et al., 2008). These thermal bleaching events are considered the most problematic coral situation worldwide, including in the upper Gulf of Thailand (uGoT) that first recorded a thermal bleaching event in 2006 in Sattahip District, Samae San Island, Chon Buri Province (Chavanich et al., 2009).

Thermal events are the most well-known events that cause coral bleaching, but other factors may be involved, such as seawater acidification [which could indirectly be caused from greenhouse gas (CO_2) pollution] (Anthony et al., 2011), increased levels of sediment that cover corals, smothering them and/or blocking sunlight (Peters, 1984), and dysbiosis of the coral-associated microbiome (Ritchie, 2006; Bourne et al., 2008a). In addition to *Symbiodinium*, corals live symbiotically with prokaryotic (bacteria and archaea) and eukaryotic microbes (Rohwer et al., 2002; Rosenberg et al., 2007). Scientists reported that coral prokaryotes, in particular, bacteria, are diverse and include species that are able to either provide food *via* photosynthesis, acquire and decompose organic and inorganic nutrients, and/or produce antibiotics and antioxidants to boost immunity of corals and promote resistance against pathogens and environmental stress (including coral bleaching). Coral-symbiotic bacteria also compete with coral pathogens for space and nutrients (Lesser et al., 2004; Rosenberg et al., 2007; Lema et al., 2012; Webster et al., 2016, Webster and Reusch, 2017).

For coral-associated fungi, some reports suggested their symbiotic roles in coral skeletal biomineralization (Le Campion-Alsumard et al., 1995), nitrogen fixation (Wegley et al., 2007), and UV protection (Dunlap and Shick, 1998), while others act as pathogens such as *Aspergillus sydowii* (Geiser et al., 1998; Smith and Weil, 2004), *Rhytisma acernium*, and *Stephanocoenia intersepta* (Sweet et al., 2013; Meyer et al., 2016). Other eukaryotic microbes, besides *Symbiodinium*, have been less documented, but they likely play major roles as photosynthesis and food providers, and/or coral resilience support to environmental stresses (Kramarsky-Winter et al., 2006; Harel et al., 2008) and disease (Bourne et al., 2008b; Sweet et al., 2013) is possible.

Profiling coral-associated prokaryotic and eukaryotic microbes (microbiome) is an important step required to understand coral holobionts and how, or if, these communities regulate coral health (e.g., against thermal bleaching). In Thailand, coral-associated bacteria and fungi microbiome studies have been limited. Thailand coral reefs are fringe type with three dominant coral genera: *Acropora*, *Platygyra*, and *Porites* (Phongsuwan et al., 2013). During the past few decades, corals have been reported to be continuously reduced in abundance, aerial coverage, and general health. For example, in Mun Island (Rayong Province) and Chang Island (Trat Province), the reported decreases ranged from approximately 37.4% in 1995 to 33.3% in 2006 and 22.2% in 2011, with more severe declines noted in 2011 related to thermal bleaching events

(Phongsuwan et al., 2013; Pengsakun et al., 2019). Because the declines in corals have been continuous and are growing worse with time in the uGoT, we undertook studies aimed to reveal the healthy and bleached, coral-associated microbiome profiles to support restoration of Thailand coral reefs.

This report utilized 16S and 18S rRNA gene next-generation sequencing (NGS), to firstly identify both prokaryotic and eukaryotic microbe communities associated with healthy and bleached corals that are dominant in the uGoT (i.e., *Acropora humilis*, *Acropora millepora*, *Platygyra sinensis*, and *Porites lutea*), along with the microbiomes of the surrounding seawater and sediments, during a thermal bleaching event in 2016. Our studies included comparative alpha and beta community diversity analyses, correlation analyses, and comparisons of metabolic potentials of prokaryotic communities at three study sites in the uGoT [Tao Mo Island (T), Khao Ma Cho (M), and Samae San Island (S), in Sattahip District, Chon Buri Province]. Overall, we consider the microbiome knowledge gained from this study to be a crucial part of our understanding of coral reef health in the uGoT, which over time, will help us devise microbe-mediated strategies to protect corals from thermal bleaching events.

MATERIALS AND METHODS

Sample Collections

Samples including coral species *A. humilis* (AH), *A. millepora* (AM), *P. sinensis* (PS), and *P. lutea* (PL), as well as sediment (S) and seawater (W), were collected from Tao Mo Island (T) (12°38′35.2″N, 100°51′43.3″E), Khao Ma Cho (M) (12°35′50.4″N, 100°56′52.5″E), and Samae San Island (S) (12°34′30.33″N, 100°57′29.55″E), in Sattahip District of Chon Buri Province, Thailand, during a midday of the great global thermal bleaching event in June–July 2016 (**Figure 1**). During the period of sample collections, the seawater temperature was approximately 32°C (minimum 30.66°C and maximum 33.74°C) at all three sites. For each coral species, healthy (H) and bleached (B) colonies were collected. Healthy and bleached coral colonies were determined *via* their appearance (i.e., white color for bleached coral) by on-site marine scientist divers (Chavanich, Jandang, and Viyakarn) (Bulan et al., 2018a,b). At least three independent colonies of each species were sampled in each location. For each sample, a fragment approximately 5 cm in length and 5 cm in diameter was collected. The distance between each sampled colony was approximately 5 m. After a sample was collected underwater, it was placed in a plastic bag individually. For sediment samples, at least three samples (approximately 50 g of each) were collected just below each of the sampled coral colonies. Similar to the sediment samples, at least 3 L of seawater samples were collected directly above each of the sampled coral colonies. All samples were transported immediately to the laboratory and stored at −20°C. The abbreviations of the samples are as follows: sample (AH, AM, PS, PL, S, or W) followed by coral condition (H or B) (for coral), site (S, M, or T), and independent replicate number (1, 2, or 3). For instance, PLHS1 and PLBS3 represent coral *P. lutea* at Samae San Island of healthy replicate 1 and bleached replicate 3, respectively.

DNA Extraction

Metagenomes were extracted using Power Soil DNA Isolation Kit (for coral and sediment samples) and Power Water DNA Isolation Kit (seawater samples) (MoBio, Carlsbad, CA, United States), following the manufacturer's instructions and previous literature (Bulan et al., 2018a,b). The coral sample was ground using sterile mortar and pestle, and 1 g of coral and 1 g of sediment were used. For seawater, 2.5 L was filtered using a sterile 0.22-μm filter membrane (Merck Millipore, Burlington, MA, United States) and the filtered membrane was used. The quality and quantity of the extracted metagenomic DNA were checked by agarose gel electrophoresis (0.55% agarose gel w/v) and NanoDrop spectrophotometry, respectively.

16S and 18S rRNA Gene Library Preparation and Next-Generation Sequencing

Libraries of the V4 region of the 16S rRNA gene (for bacteria and archaea) and the V9 region of the 18S rRNA gene (for fungi and other small eukaryotes) were prepared by polymerase chain reaction (PCR) according to Caporaso et al. (2012). The universal prokaryotic primers 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) and the universal eukaryotic primers Illumina_Euk_1391F (5′-GTACACACCGCCCGTC-3′) and lllumina_EukBr (5′-TGATCCTTCTGCAGGTTCACCTAC-3′), with appended 5′ Illumina adapter and 3′ Golay barcode sequences, were used, respectively. Each 25-μl PCR reaction comprised 1 × EmeraldAmp® GT PCR Master Mix (TaKaRa, Shiga, Japan), 0.3 μM of each primer, and 75 ng of the metagenomic DNA. For the 16S rRNA gene, the PCR conditions were 94°C for 3 min and 30 cycles of 94°C for 45 s, 50°C for 60 s, and 72°C for 1 min 30 s, followed by 72°C for 10 min. For the 18S rRNA gene, 10 μM of mammal-blocking primer (5′-GCCCGTCGCTACTACCGATTGGIIIIITTAGTGAGGCCCT3S pC3-3′) (Caporaso et al., 2012) was also included in the PCR recipe, and the PCR conditions were 94°C for 3 min, followed by 30 cycles of 94°C for 45 s, 65°C for 15 s (for mammal blocking primer), 57°C for 30 s (for universal eukaryotic primers), 72°C for 90 s, followed by 72°C for 10 min. Triplicate PCRs were performed and pooled for each sample to prevent stochastic bias. Amplicons of ∼381 bp (16S rDNA) and ∼260 bp (18S rDNA) in length were excised from agarose gels. The amplicons were purified using GF-1 Gel Extraction Kit (Vivantis Technologies Sdn Bhd, Selangor, Malaysia) and quantified with Picogreen using Qubit dsDNA HS assay kit (Invitrogen, Eugene, OR, United States) and Qubit 3.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA, United States). Each barcoded sample (200 ng) was pooled and sequenced on MiSeq 300 NGS platform (Illumina, San Diego, CA, United States) at OMICS Science and Bioinformatics Center, Faculty of Science, Chulalongkorn University (Bangkok, Thailand).

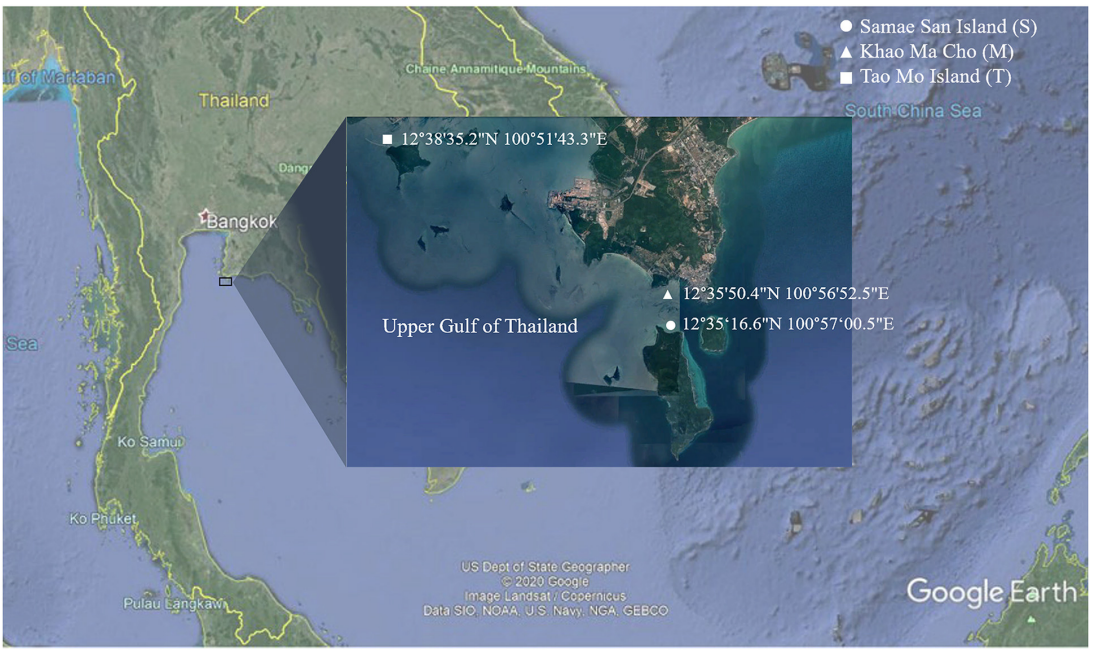

FIGURE 1 | Map representing sampling sites of Samae San Island, Khao Ma Cho, and Tao Mo Island, in the upper Gulf of Thailand.

Bioinformatic and Statistical Analyses

Sequences were processed according to Mothur's standard operating procedures (SOP) (Schloss et al., 2009). For data cleaning, reads containing (i) ambiguous bases, (ii) >1 mismatch base in the primer region, (iii) >10 homopolymer, (iv) sequence length < 100 bp, and (v) chimera sequence were removed. Silva databases (version 1.32) were used to align the sequences and remove contaminated sequences (i.e., mitochondria and chloroplast sequences). Sequences that belong to corals were also removed for 18S rDNA sequences. For taxonomic classification, 16S and 18S rDNA sequences were classified prokaryotic and eukaryotic operational taxonomic units (OTUs) using Silva databases (version 1.32). Alpha diversity (Good's coverage to estimate a sequencing coverage, Chao1 richness, and Shannon diversity indices) and beta diversity [thetayc dissimilarity index and non-metric multidimensional scaling (NMDS)] at the genus level were determined using Mothur (Schloss et al., 2009). Correlation analysis was performed by RStudio using VEGAN package (Oksanen et al., 2019). Potential metabolic function of the community was predicted using PICRUSt (version 1.1.4) following established protocols (Langille et al., 2013). The metabolic functions were categorized by Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. For correlations between coral-associated prokaryotic and eukaryotic genera, the analyses were based on positive (or negative, or no) correlation between the corresponding genera relative frequency percentages.

Mean and standard deviation (mean ± SD) were computed. For statistical analysis, Student's *t*-test and analysis of similarities (ANOSIM) were used to test for significant differences between and among groups ($P < 0.05$) for alpha and beta diversities, respectively. The significant differences for potential metabolic function were tested by White's non-parametric *t*-test. Data visualization and statistical analyses were conducted using Microsoft Excel, metastats (Mothur), and RStudio version 1.3.1093[1].

RESULTS

16S and 18S rRNA Gene Sequencing and Their Alpha Diversities

Quality scores (Q30) of 88.3% for 16S and 89.8% for 18S rRNA gene sequences were retrieved from the NGS runs. Note that Q30 represents an average sequence error rate of 1 in 1,000 or a corresponding base call accuracy of 99.9%; a higher Q30 percentage thereby infers a higher base call accuracy, and Illumina NGS runs should have Q30 score above 70% (Kastanis et al., 2018). After the Mothur's SOP for quality read process, a total of 1,468,626 quality reads for prokaryotic sequences and 8,005,841 quality reads for eukaryotic microbe sequences were retrieved. The average quality reads per sample were 34,967 and 190,615 for prokaryotic and eukaryotic microbes, respectively. These numbers of quality reads per sample were considered sufficient sequencing depth, because they resulted in the computed Good's coverage indices at genus-level OTUs to be all above 99%, except AHBT2 (97.36%) and PSHM1 (97.32%) prokaryotic communities (**Supplementary Tables 1A,B**). To prevent sequencing depth bias, every community profile was normalized to the same sequencing depth (6,846 quality

[1]https://rstudio.com/products/rstudio/download/

sequences per 16S rRNA gene sample and 18,503 quality sequences per 18S rRNA gene sample) for analyses.

The alpha diversity indices of the samples across different sample types (corals, sediment, and seawater) were compared using Chao and Shannon indices (**Supplementary Table 1** and **Figure 2**). Overall, the diversity of prokaryotes was found to be much greater than that of eukaryotic microbes for coral samples (**Supplementary Table 1**: prokaryotes avg. 437.25 ± 186 OTUs, eukaryotic microbes avg. 58.63 ± 27.46 OTUs) and sediments (prokaryotes avg. 650.33 ± 30.25 OTUs, eukaryotic microbes avg. 178.11 ± 18.42 OTUs). For seawater, the diversity of prokaryotes remained higher but was closer to that of eukaryotic microbes (prokaryotes avg. 329.56 ± 24.33 OTUs, eukaryotic microbes avg. 217.33 ± 24.99 OTUs).

The greatest relative prokaryotic OTU richness (Chao index) was observed in sediment samples, followed by corals, and seawater represented the least OTU richness. Statistical tests demonstrated significant differences in the prokaryotic OTU diversity between the sediments and the other sample types (ANOSIM: $P < 0.01$). OTU richness varied among coral species: two *Acropora* had relatively greater prokaryotic diversity than *Porites* and *Platygyra* (**Figure 2A**: avg. Chao index of *A. humilis* was 705.63 ± 109.85, *A. millepora* 689.19 ± 94.85, *P. sinensis* 397.72 ± 42.0, and *P. lutea* 458.96 ± 119.44). Significant differences were determined between *A. humilis* vs. *P. sinensis* (*t*-test: $P = 0.0002$), *A. humilis* vs. *P. lutea* ($P = 0.003$), *A. millepora* vs. *P. sinensis* ($P < 0.001$), and *A. millepora* vs. *P. lutea* ($P = 0.0021$). Nonetheless, OTU evenness (Shannon indices) was found to be relatively similar among samples. No significant differences in Shannon indices were observed between the sediments (avg. 4.08 ± 0.11) and the other samples (3.45 ± 0.33) ($P > 0.01$), highlighting an evenness of individual distributions of the prokaryotic OTUs in each sample (**Figure 2B**: avg. Shannon index 3.28).

For eukaryotic microbes, seawater (**Figures 2C,D**: avg. Chao index 262.22 ± 44.44, avg. Shannon index 2.31 ± 0.53) and sediment (avg. Chao index 207.06 ± 20.41, avg. Shannon index 2.14 ± 0.11) samples had relatively greater alpha diversities than corals (avg. Chao index 74.19 ± 33.27, avg. Shannon index 0.57 ± 0.35). The statistically significant differences were observed when comparing the seawater and sediment samples against the coral samples, in both OTU richness (ANOSIM: $P < 0.01$) and evenness ($P < 0.01$). Furthermore, healthy coral samples showed lower alpha diversity than bleached coral samples, for instance, avg. Shannon index of healthy corals was 0.30 ± 0.21 and bleached corals was 0.84 ± 0.23 (**Supplementary Table 1** and **Figure 2**). Statistically significant increases in both OTU richness and evenness between healthy and bleach conditions were found for three coral species: Chao1 indices for *A. humillis* (*t*-test: $P = 0.027$, given mean ± SD was 53.89 ± 6.91 for healthy and 114.28 ± 24.19 for bleached), *P. sinensis* ($P = 0.009$, given mean ± SD was 46.06 ± 12.62 for healthy and 98.76 ± 32.48 for bleached), and *P. lutea* ($P = 0.03$, given mean ± SD was 56.94 ± 8.42 for healthy and 116.78 ± 39.19 for bleached) and Shannon indices for *A. humillis* ($P = 0.014$, given mean ± SD was 0.49 ± 0.07 for healthy and 0.81 ± 0.12 for bleached), *P. sinensis* ($P = 0.006$, given mean ± SD was 0.24 ± 0.07 for healthy and 0.77 ± 0.15 for bleached), and *P. lutea* ($P = 0.009$, given mean ± SD was 0.05 ± 0.003 for healthy and 1.00 ± 0.429 for bleached).

Community Compositions and Beta Diversity Analyses of Prokaryotic Communities

The percent relative abundance of prokaryotic phylum compositions was found to be relatively close within the same sample types and (for corals) the same coral genus. The three corals (*A. humilis*, *A. millepora*, and *P. sinensis*) and sediment samples shared dominant prokaryotic phyla: Proteobacteria (**Figure 3A**: avg. 62.33 ± 15.93%) followed by Bacteroidetes (avg. 12.81 ± 7.02%). Seawater samples displayed different compositions: Cyanobacteria (avg. 48.62 ± 7.04%) followed by Proteobacteria (avg. 22.61 ± 2.77%). It is worth noting that the prokaryotic compositions were relatively consistent among sites for sediment and seawater samples (**Figure 3A**). This finding may support the close sediments and seawater environments among T, M, and S sites, yet a more diverse prokaryotic (in particular bacterial) compositions for corals may partly be owning to the genera of corals.

Prokaryotic diversity was analyzed in detail by class, order, and species compositions, between bleached and healthy conditions, and across coral species (**Figures 3B–D**). Bacterial orders Rhizobiales and Rhodobacterales were found shared across all coral species and were among the top 20 most abundant in all cases, suggesting their important functions in corals. The prokaryotic compositions showed more variation between coral genera than the variations observed between healthy vs. bleached conditions. For example, relatively high percentages of class Alphaproteobacteria were found in *P. sinensis* (**Figure 3B**: healthy 39.01% and bleached 64.26%). Differences in diversity between healthy and bleached corals were observed, but these differences were non-statistically significant when analysis was conducted at the genus level (**Figure 3D**): *A. humillis* (ANOSIM: $P = 0.21$, $R = 0.26$), *A. millepora* ($P = 0.20$, $R = 0.11$), *P. sinensis* ($P = 0.10$, $R = 0.96$), and *P. lutea* ($P = 0.10$, $R = 0.89$). In addition, some classes that were reported in both healthy and bleached corals were found to be populated by different genera: for example, predominant Alphaproteobacteria in *P. sinensis* contained different genera between healthy and bleached conditions. Bleached *Acropora* species exhibited heightened proportions of bacterial genera *Acinetobacter* and *Helicobacter*. For bleached *P. sinensis*, Actinobacter and unclassified genera of class Alphaproteobacteria, class Oxyphotobacteria, and order Rhizobiales were instead found to increase, whereas unclassified genera of family Stappiaceae, family Rhodobacteriaceae, and class Gammaproteobacteria decreased. For bleached *P. lutea*, unclassified genera of BD2-11 terrestrial group, A4b, order Dadabacteriales, and bacteria of subgroup10 were found to increase, whereas unclassified genera of class Gammaproteobacteria, phylum Proteobacteria, class Oxyphotobacteria, class Bacteroidia, and SAR202 clade decreased.

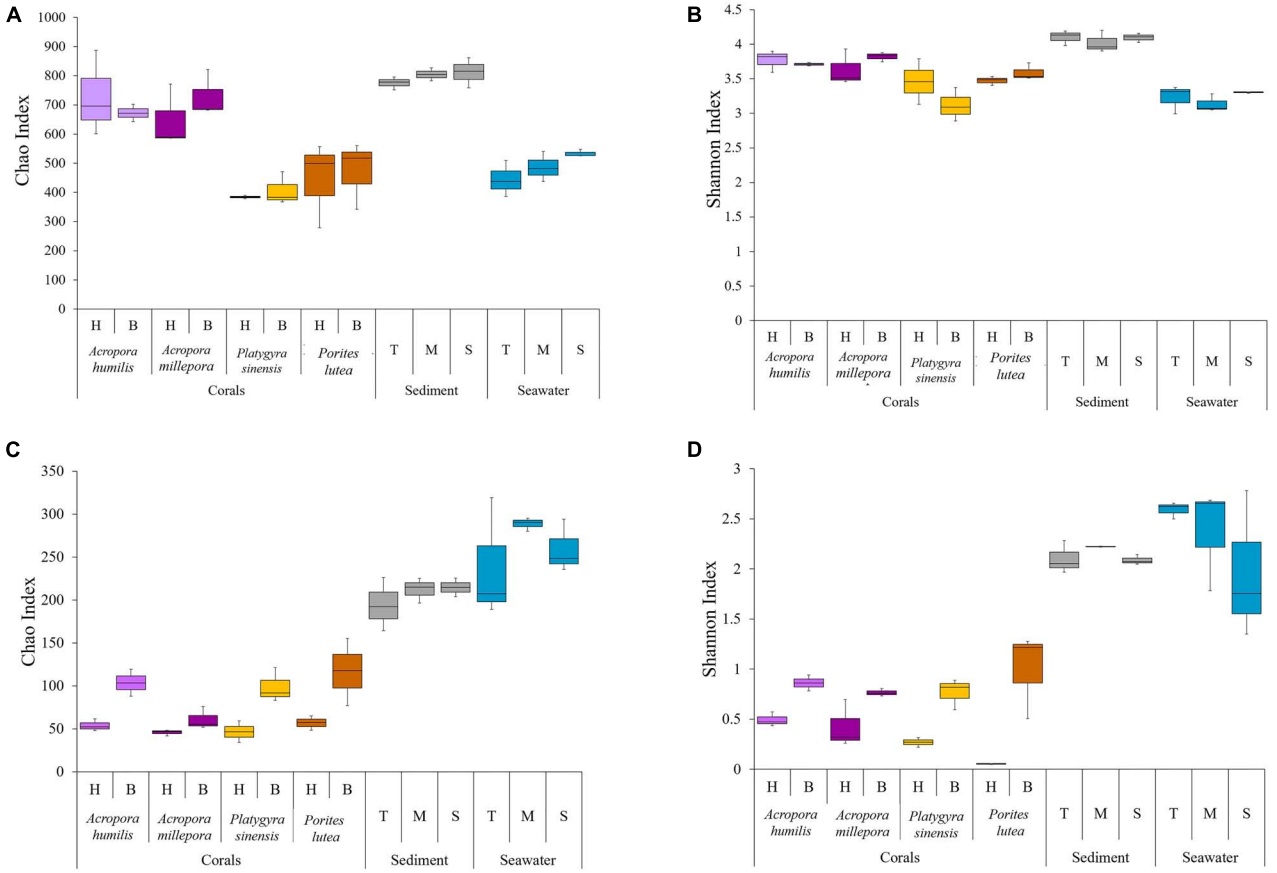

FIGURE 2 | Alpha diversity indices of **(A,B)** prokaryotic Chao and Shannon and **(C,D)** small eukaryotic Chao and Shannon.

Non-metric multidimensional scaling demonstrated clearly the community profile differences across coral genera and that *A. humilis* and *A. millepora* positions overlapped. Bleached *P. sinensis* demonstrated the most separation relative to their profiles in healthy samples (**Figure 4A**); this separation was largely due to the increased proportions of unclassified Alphaproteobacteria and Desulfobacterales along with decreases of the orders Rhizobiales, Rhodobacterales, Vibrionales, and Oceanospirillales (**Figure 3**). Correlation analysis further revealed the prokaryotic taxa contributing to each direction of the community; for example, *P. sinensis* was enriched with Alphaproteobacteria and Rhizobiales (consistent with **Figure 3** results), while Proteobacteria was abundant in healthy, but not in stressed, *P. sinensis*. For *Acropora* and *Porites* genera, other specific bacteria taxa were associated with the changes in healthy vs. bleached corals (**Figure 4A**).

Beta Diversity Analyses of Eukaryotic Microbe (Fungi and Small Eukaryote) Communities

The microbial 18S rRNA gene NGS yielded eukaryotic microbe profiles of fungi and small eukaryotes in the kingdoms of Chromista (avg. 61.58 ± 9.44%), Animalia (26.59 ± 3.41%), Plantae (0.41 ± 0.16%), and Protista (0.33 ± 0.09%) (**Figure 5A**). While sediments showed similar eukaryotic microbe compositions at all sites, seawater and corals showed community variations among sites especially at the S site. Corals *A. humilis*, *A. millepora*, and *P. lutea* demonstrated clearly different eukaryotic microbe compositions at the phylum level between healthy and bleached conditions compared with *P. sinensis*. A single-cell eukaryotic Dinoflagellata (algae) represented the most predominant phylum among corals and sediments. Of note was that the sediment eukaryotic microbe profiles contained many unclassified phyla. Next, we analyzed the relative abundances of important coral organisms of the genus *Symbiodinium* (phylum Dinoflagellata) (**Figure 5B**), simple eukaryotic Chromista (which contain photosynthetic organelles) and Protista (most of which contain photosynthetic organelles) (**Figure 5C**), and fungi (**Figure 6**). Comparing healthy and bleached corals, *Symbiodinium* was significantly reduced in most bleached corals, especially in bleached *P. lutea*: *A. millepora* (*t*-test: $P = 0.04$, given mean ± SD was 90.59 ± 3.85% for healthy and 82.06 ± 0.42% for bleached), *P. sinensis* ($P = 0.004$, given mean ± SD was 96.89 ± 0.38% for healthy and 88.41 ± 1.59% for bleached), and *P. lutea* ($P = 0.016$, given mean ± SD was 99.64 ± 0.02% for healthy and 65.94 ± 11.91% for bleached) (**Figure 5B**). The 10 topmost

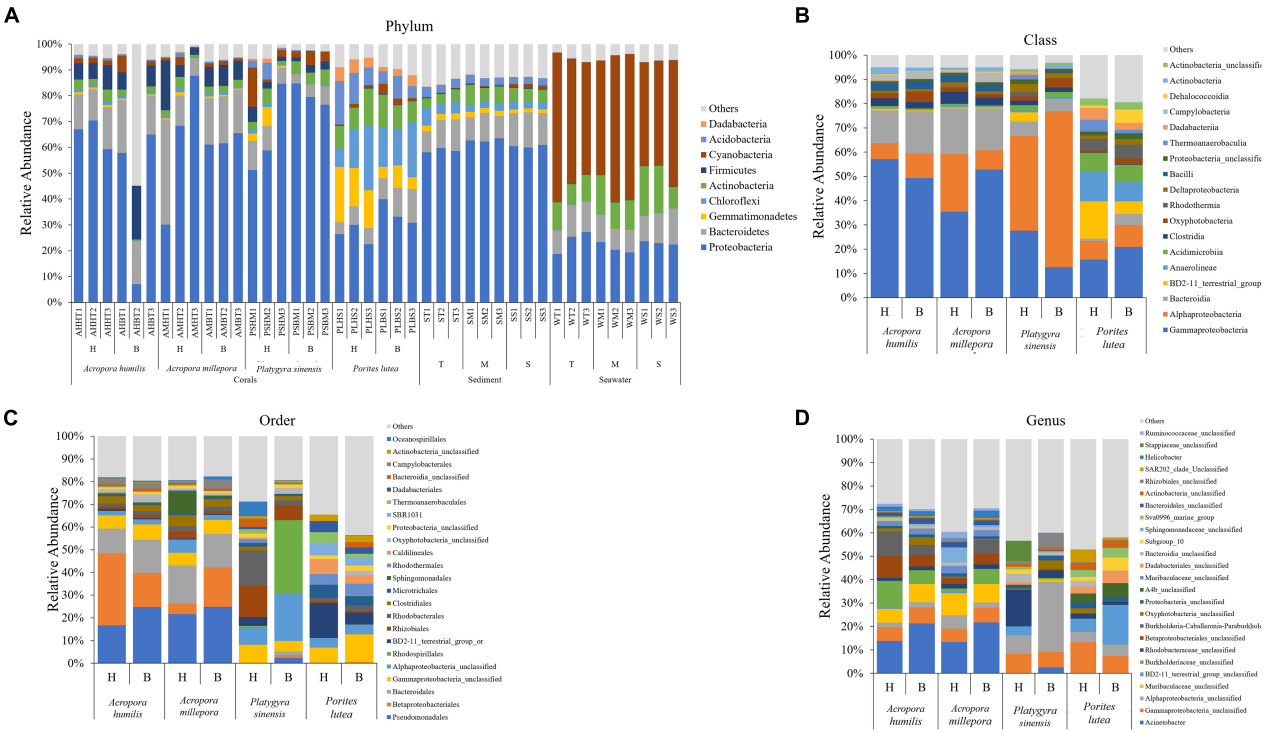

FIGURE 3 | Percent relative abundance of prokaryotic communities analyzed at the **(A)** phylum, **(B)** class, **(C)** order, and **(D)** genus levels, respectively. Others represent genera with <1% abundance. For operational taxonomic units (out) classification where the genus could not be identified, the deepest classification level was given (g, genus; f, family; c, class; o, order, respectively).

abundances of Chromista–Protista genera in corals were analyzed, and interestingly, the bleached corals had the higher number of unclassified genera of Dinophyceae and Alveolata and the genus *Navicula* (diatom) than those in their healthy corresponding coral species. Different Chromista–Protista OTUs showed fluctuation patterns across coral conditions, coral genera, and species. For instance, the unclassified genera of Suessiaceae, Phaeophyceae, Stramenopiles, and Embryophyta tended to be high in coral *Acropora* spp. The abundance of unclassified Gymnodiniphycidae genus was reduced in bleached *A. humilis* and *A. millipora* but increased in bleached *P. sinensis* (**Figure 5C**).

Analyzing the microbial community of bleached coral clusters showed that the bleached eukaryotic microbe cluster had rather community structure disparity from the healthy eukaryotic microbe cluster, especially for bleached *P. lutea* (**Figure 4**). Relatively higher frequencies of *Symbiodinium* showed positive correlation with healthy coral eukaryotic communities (**Figure 5B**). The correlation analysis also revealed that Demospongiae and Clionaida were statistically responsible for the separation of bleached from healthy corals, in particular, *P. lutea* (**Figure 5B**). Bacillariophyceae, Suessiaceae, Dinophyceae, and Gymnodiniphycidae were responsible for the correlation in the other bleached coral samples (**Figures 4A**, **5C**).

For fungi communities, phyla Ascomycota and Basidiomycota were generally predominant in all samples. For healthy *P. lutea*, the single dominant Basidiomycota were unique (**Figure 6A**). *P. lutea* demonstrated the most fungal community dissimilarity between healthy and bleached conditions, followed by *P. sinensis*, *A. millipora*, and *A. humilis*, in order (**Figure 6**). For fungi, the same genus of corals did not always show consistent changes. Some findings of the differing fungi between healthy vs. bleached *A. humilis* were opposed to those in *A. millepora*: for instance, genera *Malassezia* (class Malasseziomycetes), unclassified Mucorales, and unclassified Ascomycota. The changes in bleached *A. humilis* shared commonality with those in bleached *P. sinensis* and *P. lutea* (**Figure 6C**). Correlation analysis of merely the fungi communities demonstrated no distinct community separations between healthy and bleached conditions, somewhat because of relatively high abundances of *Saccharomyces* and *Malassezia* in *P. sinensis* and the unclassified genus of Basidiomycota in *P. lutea* (**Figures 4C**, **6C**).

Functional Potentials of Healthy and Bleached Coral-Associated Prokaryotic Communities

Functional potentials estimated from prokaryotic communities demonstrated bacterial functions in various categories, including membrane transport, amino acid and carbohydrate metabolisms, replication and repair, energy metabolism, translation, metabolism of cofactors and vitamins, xenobiotic biodegradation and metabolism, cellular processes and signaling, and lipid metabolism (**Figure 7A**; Hewson and Fuhrman, 2004; Tout et al., 2014). Nevertheless, the comparing pairs between healthy and

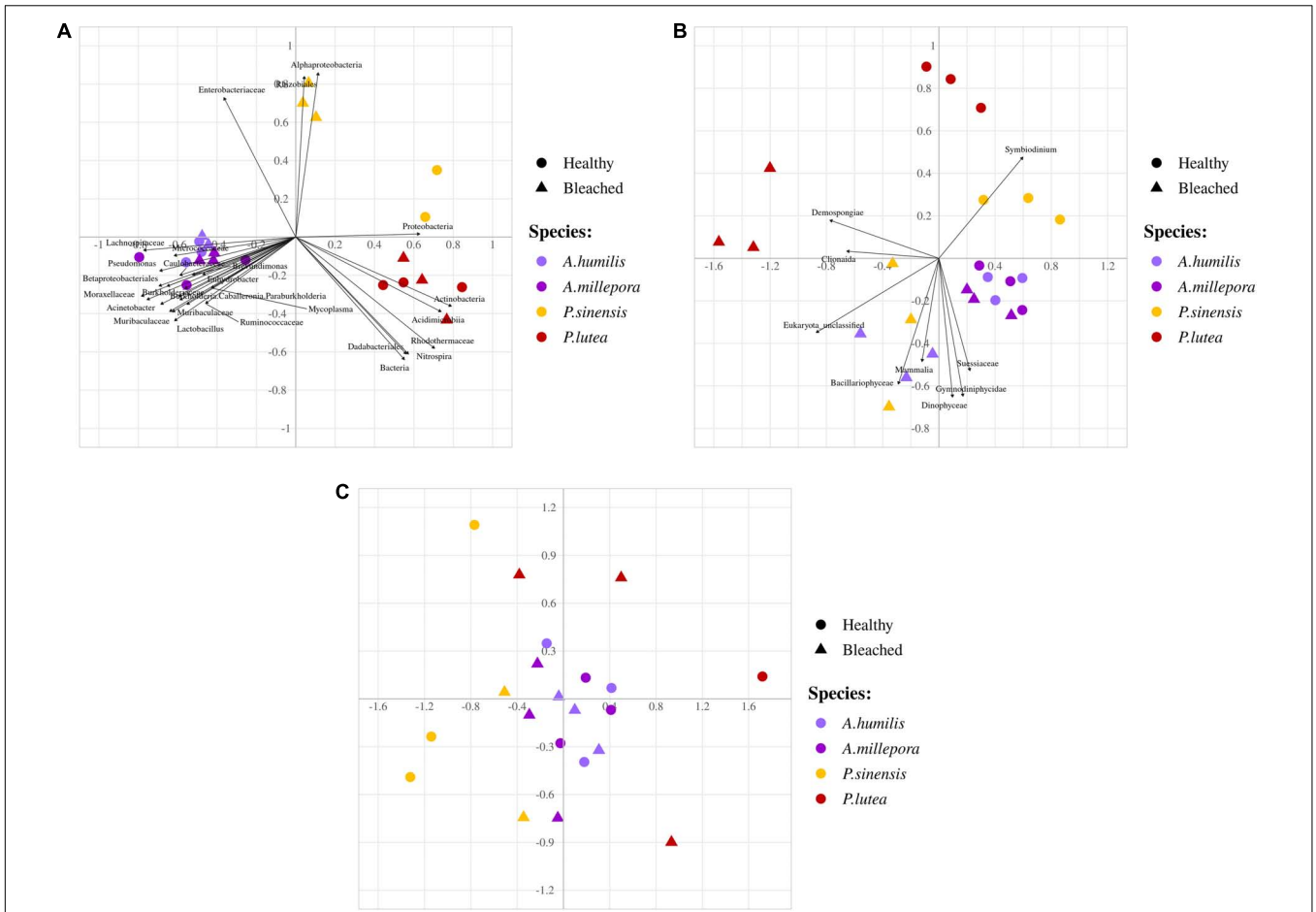

FIGURE 4 | Non-metric multidimensional scaling analyses of **(A)** prokaryotes, **(B)** fungi and small eukaryotes, and **(C)** fungi communities at genus level OTUs. Arrows indicate significant correlation analysis of prokaryotic taxa to samples ($P < 0.05$).

bleached coral genera showed statistically significant differences of function frequencies involving immune system diseases for bleached *Acropora*. There were also statistical differences of function frequencies in cell motility, amino acid and nucleotide metabolism, cellular processes and signaling, genetic information processing, signal transduction, transcription, biosynthesis of secondary metabolites, transport and catabolism, and various body systems for *Platygyra* and xenobiotics biodegradation, nucleotide metabolism, transcription, and biosynthesis of secondary metabolites for *Porites* (*t*-test: $P < 0.05$) (**Figure 7B**). These differences in functional attributes highlighted key differences in bacterial community structure in healthy vs. bleached conditions of each coral genus.

Correlations Between Coral-Associated Prokaryotic and Eukaryotic Genera

As prokaryotes and eukaryotes were reported to interact in a coral holobiont system (Bernasconi et al., 2019a,b; Matthews et al., 2020), the coral-associated prokaryotic and eukaryotic genera were analyzed for correlation of prevalence. Although the statistics for the overall correlation between prokaryotic and eukaryotic communities were found not significant, many genera between prokaryotes and eukaryotes were found to be statistically significantly correlated (**Supplementary Figure 1**: green color). An unclassified eukaryotic genus of Suessiaceae showed relatively positive correlation to most number of bacterial genera, and the greatest positive correlation was determined between the unclassified eukaryotic genus of Suessiaceae and an unclassified prokaryotic genus of Lachnospiraceae ($r = 0.719$, $P = 0.000033$). This unclassified genus of Suessiaceae showed a strong positive correlation with other bacteria genera, such as *Acinetobacter* ($r = 0.675$, $P = 0.00017$), *Helicobacter* ($r = 0.672$, $P = 0.00019$), an unclassified genus of Muribaculaceae ($r = 0.683$, $P = 0.00013$), and an unclassified genus of Bacteroidales ($r = 0.667$, $P = 0.00023$). The pattern of these bacterial genera correlations with the unclassified genus of Suessiaceae was also observed with some other eukaryotes, including an unclassified genus of Stramenopiles, an unclassified genus of Embryophyta, and an unclassified genus of Dinophyceae. On the other hand, certain genera of bacteria (e.g., an unclassified genus of BD2-11 terrestrial group, *Candidatus nitrosopumilus*, and unclassified genera of Dadabacteriales and Nitrosopumilaceae) showed negative

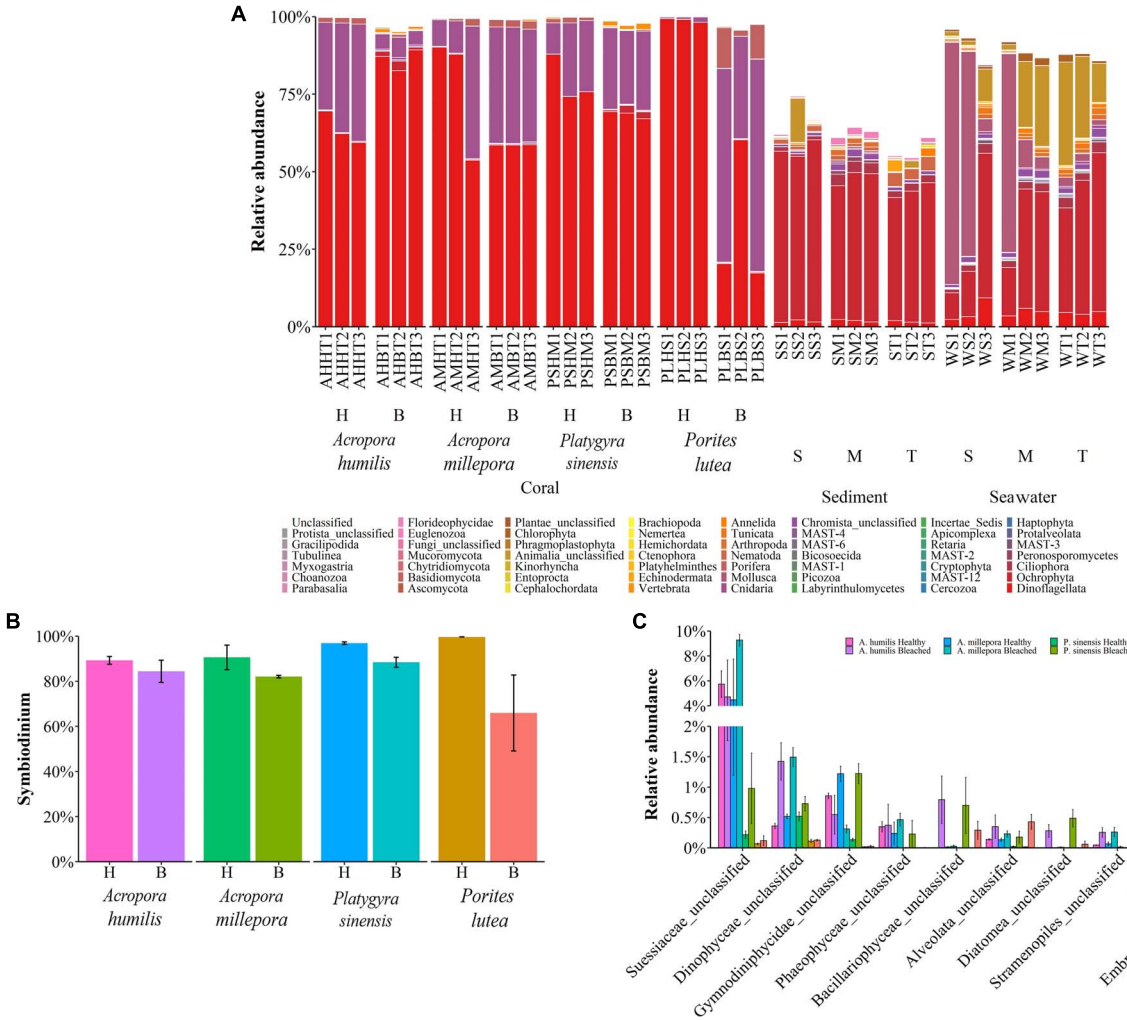

FIGURE 5 | Percent relative abundance of **(A)** eukaryotic phyla across corals, sediments, and seawater; **(B)** *Symbiodinium*; and **(C)** simple eukaryotic Chromista and Protista across coral species.

correlation to most eukaryotic genera except *Symbiodinium* (**Supplementary Figure 1**). The strongest negative correlation was found between an unclassified genus of Dinophyceae and *Candidatus nitrosopumilus* ($r = -0.458$, $P = 0.023$).

DISCUSSION

Coral holobionts (i.e., microbiome) are known to be specific to sites and coral species and to be altered in bleached vs. healthy corals (Rohwer et al., 2002; Pootakham et al., 2017, 2018). Previously, Roder et al. (2014) reported a higher abundance of coral pathogenic bacteria in bleached corals, and Littman et al. (2011) reported a shift in bacterial metabolisms (e.g., virulence genes, metabolisms of fatty acids, proteins, phosphorus, and sulfur) in bleached corals relative to healthy corals (Littman et al., 2011). The prokaryotic and eukaryotic microbe microbiomes associated with healthy and bleached coral species in the uGoT have not been extensively studied, yet microbial and small organisms play important roles in coral symbiosis in the uGoT, with important implications for coral health. Coral microbiomes play roles in providing nutrients for coral growth and reproduction and synthesize bioactive compounds that promote coral resistance to pathogens, as previously reported (Rosenberg et al., 2007; Wegley et al., 2007; Lesser et al., 2013; Webster and Reusch, 2017). We also know that greenhouse gas pollution has caused seawater temperature to rise; this shift in temperature has affected the microbiome of corals worldwide. This study firstly revealed both the prokaryotic and eukaryotic microbe microbiomes representing healthy and bleached samples of the four prevalent coral species (*A. humilis*, *A. millipora*, *P. sinensis*, and *P. lutea*) in the uGoT and also the microbiomes of underlying sediments and surrounding seawater over the corals. Samples were taken during the global thermal bleaching event in 2016 when significant bleaching occurred in the UGoT. Results reported here lay a firm foundation for understanding

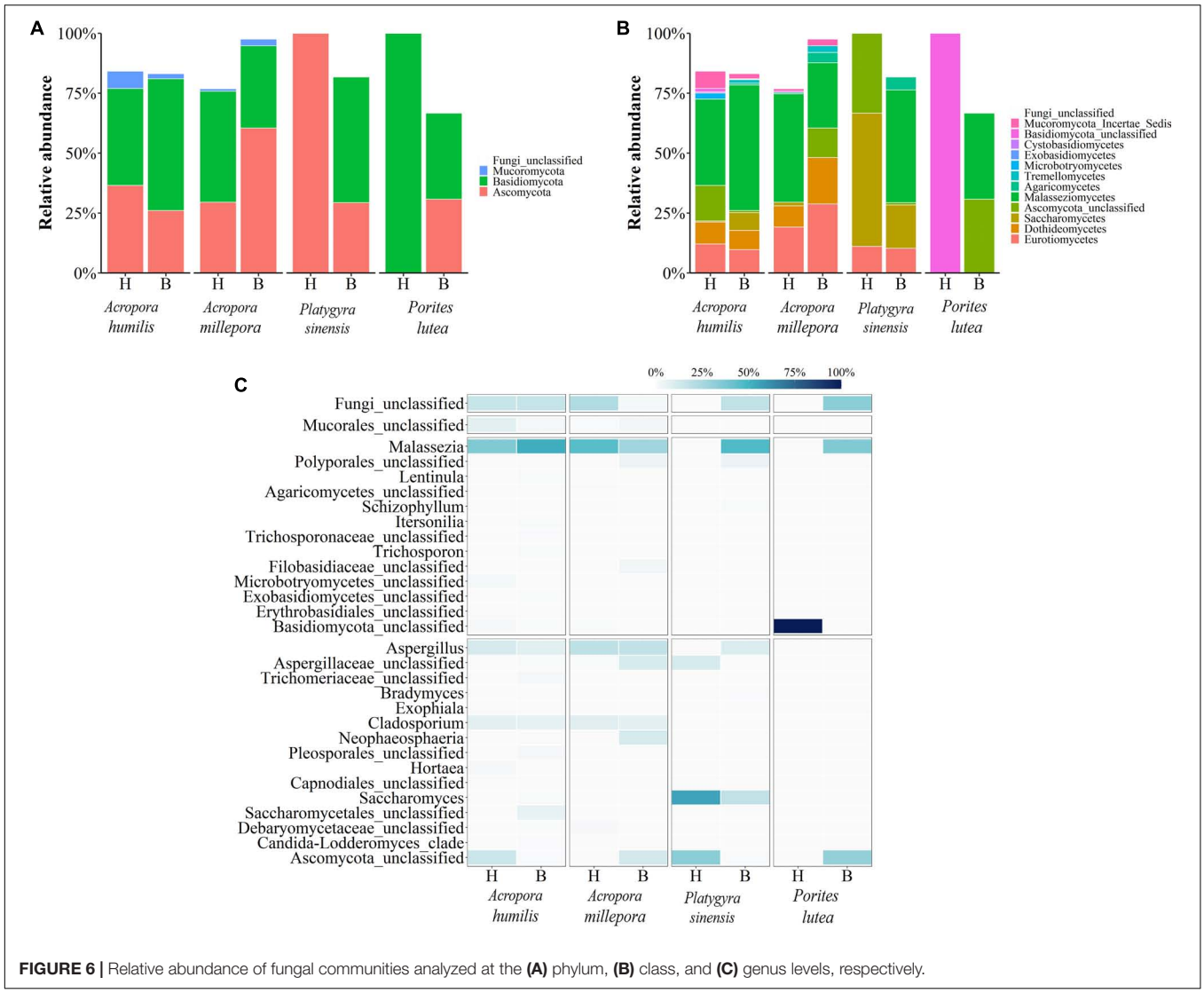

FIGURE 6 | Relative abundance of fungal communities analyzed at the **(A)** phylum, **(B)** class, and **(C)** genus levels, respectively.

the coral microbiome in the UGoT, understanding how thermal bleaching events result in alterations in the microbiome and, with additional research, how it may be possible to promote coral recovery and health in future thermal bleaching events in the uGoT through manipulation of the coral microbiome.

In this study, different prokaryotic and eukaryotic microbiomes were found across different coral genera in healthy vs. bleached conditions. The greatest alpha diversity of prokaryotic OTUs was observed in sediments beneath the corals, which previous research reported the sediment may affect coral rates of growth, photosynthesis, larval settlement and survival, coral bleaching, and mortality (Tuttle et al., 2020). Sediment bacteria could play a role providing and/or recycling nutrient resources for coral growth/reproduction. In contrast, fungi and small eukaryotes had higher OTU diversity in seawater, which might be because these organisms are more independent on a requirement of surface area (i.e., coral surface) for colonization; also, some seawater small eukaryotes can harvest food from the water column like bacteria (Nielsen and Risgaard-Petersen, 2015; Mincer et al., 2016).

Gardner et al. (2019) previously reported that *Acropora muricata* and *Acropora gemmifera* are more bleaching sensitive than *P. lutea*, and raised a hypothesis that the high bacterial diversity found in *Acropora* may negatively affect bleaching resistance. In general, our results support this hypothesis, as we found that bleached corals exhibited a higher degree of both prokaryotic and eukaryotic microbe Shannon indices (which include both OTU richness and evenness) than healthy corals, consistent with previous reports that claimed an association between increased community diversity and corals undergoing a bleaching event (McDevitt-Irwin et al., 2017; Gardner et al., 2019; Sun et al., 2020).

Analyzing the beta diversity of the compositions of prokaryotes (and also fungi and other small eukaryotes) and the corresponding statistical analyses supported the general trends seen previously, namely, that microbiomes

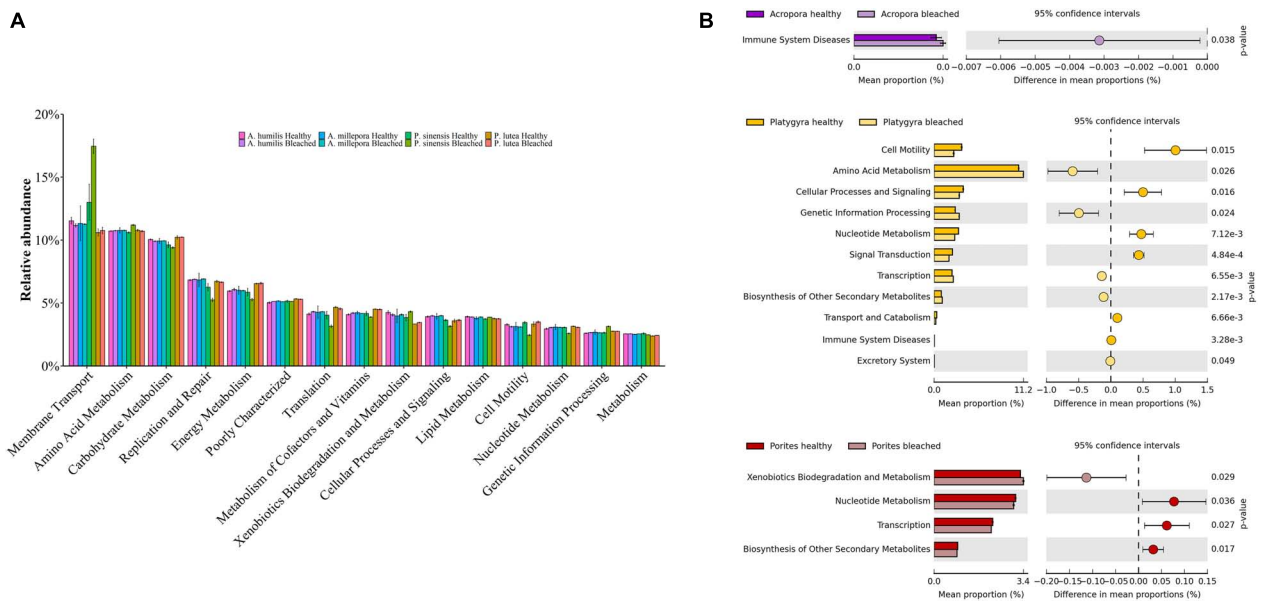

FIGURE 7 | Functional potentials of **(A)** healthy and bleached coral-associated prokaryotic communities and **(B)** corresponding prokaryotic community pairs between healthy and bleached coral genera.

were specific to sample types (corals vs. sediment vs. seawater) (Bulan et al., 2018a,b). The NMDS analyses further suggested that the compositions of prokaryotes (and also fungi and other small eukaryotes) were clustered separately by coral genera. For some coral genus microbiomes (i.e., **Figures 4A,B**, prokaryotic and eukaryotic microbe communities in healthy vs. bleached *P. sinensis*), bleached and healthy conditions showed diversified microbiomes.

The percent relative abundances and NMDS correlation analyses revealed differences in healthy and bleached samples, of each coral species. For instance, Rhizobiales, bacteria with high functions in nitrogen fixation (Lema et al., 2014), were high in bleached *P. sinensis*. Nonetheless, healthy and bleached *Acropora* demonstrated very close prokaryotic community clustering, a surprising finding since some bacteria, such as *Acinetobacter*, were found in diseased corals including dark spot syndrome disease (Sweet et al., 2013; Meyer et al., 2016). Other shared common altered species in bleached *Acropora* and *P. sinensis* included a decrease of beneficial bacteria, such as Rhizobiales, which Gardner et al. (2019) suggested was associated coral bleaching resistance. Interestingly, bleached *P. lutea* and *P. sinensis* shared higher prevalence for three of four beneficial bacteria taxa: Oceanospirillales (Kirkwood et al., 2010; Raina et al., 2016), Flavobacteriales (Kelly et al., 2014), Alteromonadales (Ceh et al., 2013), and Desulfobacterales (Gobet et al., 2012). Bleached *Acropora* species had higher prevalence for just two of four of these beneficial bacteria taxa. Note that Oceanospirillales could play a role in dimethylsulfoniopropionate (DMSP) degradation and antimicrobial compound production, which may be important during bleaching events (Kirkwood et al., 2010; Raina et al., 2016); we found these bacteria statistically higher in bleached *A. millepora* (*t*-test: $P = 0.021$) and *P. lutea* ($P = 0.039$) lending strength to the hypothesis that Oceanospirillales could play a role to support recovery from coral bleaching. Other orders of bacteria have diverse functional attributes; Flavobacteriales are energy scavengers from organic debris (Kelly et al., 2014), Alteromonadales are denoted as nitrogen fixers (Ceh et al., 2013), and Desulfobacterales are denoted as organic sulfate recyclers (Gobet et al., 2012). In our study of the uGoT, the communities of bleached corals tended to have higher percentages of Flavobacteriales (*t*-test: significant statistic for *A. humilis*, $P = 0.025$), Alteromonadales (significant statistics for *A. millepora* and *P. sinensis*, $P = 0.015$), and Desulfobacterales (significant statistics for *P. sinensis*, $P = 0.018$). Overall, our findings suggested that there are alterations of coral microbiome during bleached events, with the specific pattern of changes related to each coral examined. Increases of Vibrionales, Alteromonadales, and Rhizobiales were previously reported in stressed corals (Bulan et al., 2018b; Tout et al., 2015; McDevitt-Irwin et al., 2017). Vibrionales were previously reported to increase following a rise in seawater temperature, and some species of this bacterial order may act as coral pathogens (Kushmaro et al., 1998; Bulan et al., 2018a; Tout et al., 2015). The bacteria compositions in bleached *P. lutea* of our study were consistent with the bleached *P. lutea* collected at the Andaman Sea (Pootakham et al., 2017, 2018); both uGoT and Anadaman Sea *P. lutea* had high percentages of Rhizobiales, Oceanospririllales, and Rhodobacterales in their microbiome.

An analysis of prokaryotic function potentials, estimated from the prokaryotic community composition, has shown that a variety of essential prokaryotic metabolic functions (such as membrane transport, amino acid and carbohydrate metabolisms, replication and repair, and energy metabolism) remained conserved following bleaching events (Badhai et al., 2016; Bulan et al., 2018a); however, the relative frequencies of the functions were sometimes found different in bleached vs.

healthy corals. In our study, there were a significant increase of human immune system disease functions of bacteria in bleached *Acropora* species and the increase of xenobiotics biodegradation and metabolism in bleached *P. lutea*. Of note, there was a significant increase of metabolic function in bleached *Acropora* species, suggestive of the inflammatory disease state, which might play a role in supporting resistance to bleaching events. Although the immune system disease functional category of prokaryotes that we found belongs in human disease, corals are complex mutualisms with multiply associated microbiota and small eukaryotes, and our finding may support the modern concepts of learning immune responses in invertebrates and a coral holobiont immunity homeostasis (Palmer, 2018a,b; Takagi et al., 2020). Nevertheless, the interpretation on this finding remains to be elucidated.

Moreover, fungi, *Symbiodinium*, and Chromista–Protista were analyzed in this study, as these microbes have been shown to have important relationships in coral symbiosis (Falkowski et al., 1984; Mieog et al., 2009). There is a well-known relationship for corals and *Symbiodinium*, e.g., the loss of *Symbiodinium* is associated with increased seawater temperatures and, thus, coral bleaching (Salih et al., 1997; Gardner et al., 2019). We too observed a reduction of *Symbiodinium* in all bleached coral species, with the largest reduction in *P. lutea*. Additionally, we have identified differing *Symbiodinium* clades in *P. lutea* (clade C15 in *Porites* and clade C3 in *Acropora* and *Platygyra*) following the established clade naming of Fisher et al. (2012). These differing *Symbiodinium* clades might have different heat resistance. Perhaps, *P. lutea* had a less heat-resistant *Symbiodinium* clade, so this coral species have adapted to heat stress through its prokaryotic (in particular bacteria) and eukaryotic microbe diversity. Following the loss of *Symbiodinium*, scientists have also observed that other fungi and small eukaryotes (Chromista–Protista) replace *Symbiodinium* in terms of providing photosynthesis and coral covering functions (Fine et al., 2005; del Campo et al., 2016; Bernasconi et al., 2019a,b). Consistent with this "replacement" hypothesis, our analysis also found increased abundances of unclassified genera in Dinophyceae and Alveolata and genus *Navicula* in all bleached coral species. We suggest that Dinophyceae and Alveolata could provide photosynthesis (Gómez, 2012; Kim et al., 2013) and the diatom *Navicula* could replace nitrogen and phosphate recyclings (Kwon et al., 2013), for nutrients in corals that have lost *Symbiodinium* during a bleaching event. Moreover, the correlation analysis between coral-associated prokaryotic and eukaryotic genera highlighted a uniqueness of *Symbiodinium* that conferred a positive correlation to the otherwise prokaryotic genera that had negative correlations to other eukaryotic genera. Also, the correlation analysis revealed other eukaryotic genera that may be of importance to coral health and are actually phylogenetically associated with *Symbiodinium*, such as Suessiaceae, Stramenopiles, Dinophyceae, and Dinoflagellata (Pochon et al., 2014; Liu et al., 2018; Yorifuji et al., 2021).

In summary, this study firstly revealed both prokaryotic and eukaryotic microbiomes of four prevalent coral species in the uGoT and their surrounding niches (sediment and seawater) and compared healthy and bleached colonies of corals. Independently, triplicate sequencings per sample demonstrated that bleached *Acropora*, *Platygyra*, and *Porites* microbiomes were diverse. Overall, our findings were generally consistent with earlier work, but there were some key differences in our samples from the uGoT relative to reports for the same coral species in other geographic locations. We suggest that these geospecific differences in microbiomes in healthy or bleached conditions involve differences in marine biogeography, consistent with other results from corals in the uGoT (Somboonna et al., 2017). The results presented here will help lay a foundation to help minimize coral bleaching and/or maximize coral restoration following bleaching events; as long-term goals, we are actively working on techniques and strategies to improve beneficial microbiome members that would help protect or restore functions in heat-stressed corals. Finally, we note that this study was based on a single time point and a single bleaching event; we are gathering other coral microbiome bleaching event data to help us better understand which core microorganisms support bleaching resistance and coral reef restoration in the uGoT.

DATA AVAILABILITY STATEMENT

Nucleic acid sequences in this study were deposited in an NCBI open access Sequence Read Archive database, accession number SRP291375 for 16S and 18S rDNA sequences.

AUTHOR CONTRIBUTIONS

HK, CK, and MP did molecular biology experiments and data analysis. CK helped draft the manuscript. SJ and SC collected samples. KP, JO, SC, and VV conceived of the study. SC and VV helped revise the manuscript. NS conceived of the study, coordinated the experiments and data analysis, and wrote the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2021.643962/full#supplementary-material

Supplementary Figure 1 | Pearson's correlations between coral associated prokaryotic and eukaryotic genera. Green color indicates positive correlation between species, red color indicates negative correlation, and white color indicates no correlation.

Supplementary Table 1 | Number of sequencing reads, Good's coverage and alpha diversity indices of coral, sediment, and seawater samples of **(A)** 16S rDNA and **(B)** 18S rDNA profiles, at genus level OTUs.

Supplementary Table 2 | Taxonomy names along the number of OTUs and sequences in coral, sediment, and seawater samples of **(A)** 16S rDNA and **(B)** 18S rDNA profiles.

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Effects of Light Intensity and Wavelength on the Phototaxis of the *Crassostrea gigas* (♂) and *Crassostrea sikamea* (♀) Hybrid Larvae

Xuekai Zhang[1†], Chao Fan[1†], Xingzhi Zhang[2], Qiongzhen Li[2], Yangchun Li[1] and Zhaoping Wang[1]*

[1] Fisheries College, Ocean University of China, Qingdao, China, [2] Guangxi Key Laboratory of Aquatic Genetic Breeding and Healthy Aquaculture, Guangxi Academy of Fisheries Sciences, Nanning, China

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***Correspondence:**
Zhaoping Wang
zpwang@ouc.edu.cn

† These authors have contributed equally to the work

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Light sensitivity is important for marine benthic invertebrates, and it plays a vital role in the oysters settling. Generally, the emerging of eyespot is a signal of oyster larvae settling, while like most of the other coastal species, the oysters are threatened by artificial light pollution. *Crassostrea gigas* and *Crassostrea sikamea* are two oyster species naturally distributed in China, and their hybrids are potential material for oyster cross-breeding. Therefore, we investigated the phototaxis of hybrid eyespot larvae and eyeless larvae under different light intensities and wavelengths to uncover how light affects their behaviors. The results indicated that hybrid oyster larvae had positive phototaxis to specific light intensity and wavelength. We further concluded that 5 lx was the positive phototaxis light intensity for the eyeless hybrid larvae, and that the acceptable light intensity range of the eyespot hybrid larvae expanded to 5–10 lx, but no higher than 15 lx; besides, the hybrid larvae behaved negatively to the light over 25 lx. The present study also suggested the positive effects of green light on larvae gathering and the induction of red light on eyespot larvae settling. In conclusion, our study may contribute to the understanding of phototaxis of hybrid oyster larvae, as well as the further perspective of light pollution on benthic communities and coastal system restoration.

Keywords: phototaxis, light intensity and wavelength, eyespot larvae, RGB color model, hybrid oyster

INTRODUCTION

The environment has an attractive impact on the development of behavioral patterns, evolution, and morphology (Brown and Braithwaite, 2005). Light, as one of the most basic environmental factors, is the compelling one that has massive ecological functions. Generally, light is an energy signal that directly or indirectly affects the growth and metabolism of organisms. In the process of biological evolution, many animals have gradually formed a biological clock to the rhythmic changes of light that are largely mediated by the occurrence of the photosensitive organ (Naylor, 1999; Gaston et al., 2017; Hobbs et al., 2021).

In nature, animals have evolved various delicate light sensors to adapt to the environment. The pediveliger larva is the last pelagic phase before oysters settling, during which the oyster larvae develop foot and also a pigmented eyespot. Eyespot is the simplest animal eye that is composed of sensory cells and shading pigment cells (Jékely et al., 2008). Ultrastructural studies on the epidermal eyespots of *Microstomum lineare* and *Oncholaimus vesicarius* show that pigment cells envelop the processes of the sensory cells, which indicates that the pigmented eyespots have dual functions of photoreceptors and chemoreceptors (Burr and Burr, 1975; Palmberg et al., 1980). Eyespot structure is characteristic of the pelagic larvae for invertebrates in the ocean, though eyespots cannot form images but enable animals to sense the direction of light that mediates the phototaxis of larvae (Thorson, 1964; Jékely et al., 2008).

Light is an electromagnetic radiation that occurs over an extremely wide spectrum, ranging from 10^{-2} nanometers to meters. Visible light narrowly ranges from approximately 700 nm for red light to 400 nm for violet light in the broad spectrum. Light intensity represents the amplitude of light with the same wavelength, and light wavelength is a property of light that determines the colors of the light (Sliney, 2016). For most marine invertebrates, light can influence the development of their pelagic larvae. A previous study on the response of 141 different oceanic larvae to light suggested that 82% of these larvae respond positively to light, but too much light caused photonegative behaviors (Thorson, 1964). In terms of light wavelength, invertebrate larvae also have notable features of wavelength-dependent phototaxis (Kim et al., 2021). Baker and Mann (1998) suggested that planktonic invertebrates are usually unresponsive, or only weakly responsive, to long-wavelength lights, but the *Crassostrea virginica* larvae can respond to most of the visible lights.

As a mixture of visible light, ultraviolet, and infrared, sunlight is susceptible to plankton and dissolved organic matters in water, which results in a variety of light intensities and colors on different water layers (Blaxter, 1968). Previous studies indicated that the phototactic behaviors of plankton are highly related to the absorption pattern of different wavelengths by eyespot (Forward and Cronin, 1979; Marsden, 1988; Kim et al., 2018). It was reported that light wavelength ranging from 500 to 650 nm can be efficiently absorbed by the eyespot of *Crassostrea gigas* larvae, especially in the pigmented area (Kim et al., 2021). The RGB color is an additive color model of which the three primary colors (TPCs; red, green, and blue) are added together in various ways to reproduce a broad array of colors (Ibraheem et al., 2012). By this color model, computers can visualize what the human does in the hue and lightness of colors. According to the principle of liquid crystal display (LCD) processing, displays can emit 700 (red, R), 546.1 (green, G), and 435.8 nm wavelength (blue, B) (Trussell et al., 2005) that offers a reliable artificial light source for the zooplankton phototaxis research.

Recently, light pollution has sparked scientific interests in many ways, one of which is the negative effect on the biological processes of the marine environment. Although the negative impacts of artificial light have been reported in the marine environment, it remains largely unknown how marine organisms in coastal areas had been impacted (Davies et al., 2014; Bolton et al., 2017; O'Connor et al., 2019). As the dominant species in the intertidal zone, the oysters occupy a precise niche in the coastal ecosystem that is also a potential indicator of light pollution. Besides, the oyster is a worldwide aquaculture shellfish with high commodity value as a protein source. The wild hybrid of *C. gigas* and *Crassostrea sikamea* has been identified in Suncheon Bay, Korea (Hong et al., 2012) and the northern Ariake Sea, Japan (Hedgecock et al., 1999), where *C. gigas* and *C. sikamea* natively live together. Unsurprisingly, both *C. gigas* and *C. sikamea* are native species in China, and they are complementary to each other in ecological habits (Wang et al., 2013). As a potential aquaculture variety, the biological characteristics of *C. gigas* and *C. sikamea* hybrids need to be further explored (Gaffney and Allen, 1993; Xu et al., 2019).

In this study, we firstly investigated the phototaxis of the *C. sikamea* and *C. gigas* hybrid larvae in terms of visible light intensity and light wavelength. Then, we analyzed the relationship between computerized TPCs and the phototaxis of hybrid larvae by the pathway analysis.

MATERIALS AND METHODS

Eyespot Larvae Recruitment

The *C. gigas* and *C. sikamea* were collected from oyster farms located in Rushan, Shandong (36.41°N, 121.36°E) and Beihai, Guangxi (21.46°N, 109.39°E), respectively, in China. Oyster broodstocks were collected before March 2019, then identified by restriction fragment length polymorphism (RFLP)–PCR, and spawned in May 2019. Indoor maturation was deployed to ensure the synchronization of gametes. The hybridization can only be complete between *C. sikamea* eggs and *C. gigas* sperms, but not the opposite way (Banks et al., 1994; Xu et al., 2019).

Daily management of the hybrid larvae referenced to the procedure described by Xu et al. (2019). In brief, the fertilized eggs were hatched for 20 h, and hybrid larvae were reared in sand-filtered seawater and initially fed with *Isochrysis galbana*; when the mean shell height of hybrid larvae exceeded 120 μm, a mixture of *I. galbana* and *Platymonas subcordiformis* was added. In the experiment, the eyespots of hybrid larvae emerged around day 24.

Light Source Calibration

Calibrating of Light Intensity and GMTPC

The light source images were built by Adobe Photoshop (PS; Adobe Inc., Delaware, United States; **Figure 1A**) based on geometric mixed TPCs (GMTPC). The default quantized value of R/G/B (255/255/255) was defined as 100%, then graded the GMTPC image by 10%, and measured the light intensity of each gradient. After that, the fitting formula was calculated statistically (**Table 1**).

Calibrating of Light Intensity With TPCs and GMSCs

The light source images based on TPCs and geometric mixed secondary colors (GMSCs) were also built by PS. For the TPCs, we defined the default quantized values of R = 255, G = 255, and B = 255 as 100%; similarly, the GMSCs quantized in

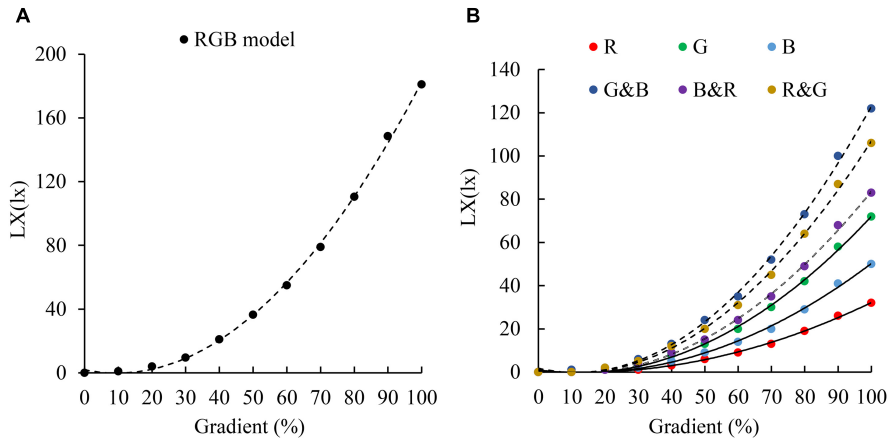

FIGURE 1 | Light intensity adjustment for RGB color model on TFT-LCD display. **(A)** The quadratic fitting line of light intensity and GMTPC gradients. **(B)** The quadratic fitting lines of light intensity with TPCs and GMSCs gradients.

TABLE 1 | The fitting formula of light intensity with the different gradients of GMTPC, TPC, and GMSC.

Color index		Quadratic fitting formula	R^2
GMTPC		$Y_{GMTPC} = 0.0223X^2 - 0.4280X + 1.9091$	$R^2 = 0.9991$
TPCs	Red	$Y_{(R)} = 0.0042X^2 - 0.1079X + 0.5455$	$R^2 = 0.9979$
	Green	$Y_{(G)} = 0.0094X^2 - 0.2282X + 1.0280$	$R^2 = 0.9987$
	Blue	$Y_{(B)} = 0.0067X^2 - 0.1742X + 0.8811$	$R^2 = 0.9975$
GMSCs	Green and blue	$Y_{(G\ and\ B)} = 0.0135X^2 - 0.2935X + 1.2168$	$R^2 = 0.9986$
	Blue and red	$Y_{(B\ and\ R)} = 0.0107X^2 - 0.2491X + 1.0280$	$R^2 = 0.9986$
	Red and green	$Y_{(R\ and\ G)} = 0.0135X^2 - 0.2935X + 1.2168$	$R^2 = 0.9987$

R^2 represents the coefficient of determination, which reflects the proportion of all the variation that can be explained by the regression relationship, similarly hereinafter.

R/G = 255/255, G/B = 255/255, and B/R = 255/255 were defined as 100%, then graded the TPC and GMSC images by 10%, and measured the light intensity. After that, the fitting formulas were calculated statistically (**Figure 1B** and **Table 1**).

Experimental Design

This study was deployed in a dark room, and each experiment was repeated three times. The hybrid larvae were collected at day 26 with a 200-micrometer sieve with a mean shell height of 307.73 ± 29.63 μm. In this condition, we obtained a mixture of hybrid eyespot larvae and eyeless larvae, of which the eyespot can be easily distinguished under a microscope. Hybrid oyster larvae were placed and aerated in a 20-liter bucket as a larvae pool, and the larvae density was approximately 57.5 ± 8.0 ind/ml. The first and second phases were light intensity experiments. The first phase was set to determine the lighting time and narrow the range of light intensity. The second phase was set to find the specific intensity light that hybrid larvae positively respond to. Based on the above results, the third phase explored the phototaxis of hybrid larvae to various wavelengths at a certain intensity.

The Photosensitivity Experiment of Larvae to Light Intensity

In terms of the first phase, light intensities were graded at 25/50/75/100 lx, and the quantized RGB values were calculated by the fitting formula "Y_{GMTPC}" in **Table 1**, then converting parameters into images by PS (**Figure 2B** and **Table 2**). After that, 300 ml of larvae was transferred into a Petri dish from the larvae pool, then stirred, and left for 5–10 min to ensure that the hybrid larvae were evenly distributed in the Petri dish. Turning on the display, hybrid larvae were sampled after 30 and 60 min, respectively.

The range of light intensity was narrowed to 0–25 lx in the first experiment. Thus, the light intensities in the second phase were graded at 0/10/15/20/25 lx, and the quantized RGB values are calculated by the fitting formula "Y_{GMTPC}" in **Table 1**, then converting parameters into images by PS (**Figure 2C** and **Table 2**). The hybrid oyster larvae were only sampled after 60 min of lighting in this section. All larvae were distinguished and counted under a microscope.

The Photosensitivity Experiment of Larvae to Different Wavelengths of Light

The second experiment finalized the optimum light intensity as 5 lx. In terms of the third phase, the TPCs and GMSCs were fixed, and the quantized RGB values were calculated by the fitting formulas in **Table 1**. The fixed parameters were converted into images by PS (**Figure 2D** and **Table 3**). The hybrid larvae were deployed and transferred in the same way as that of section "The

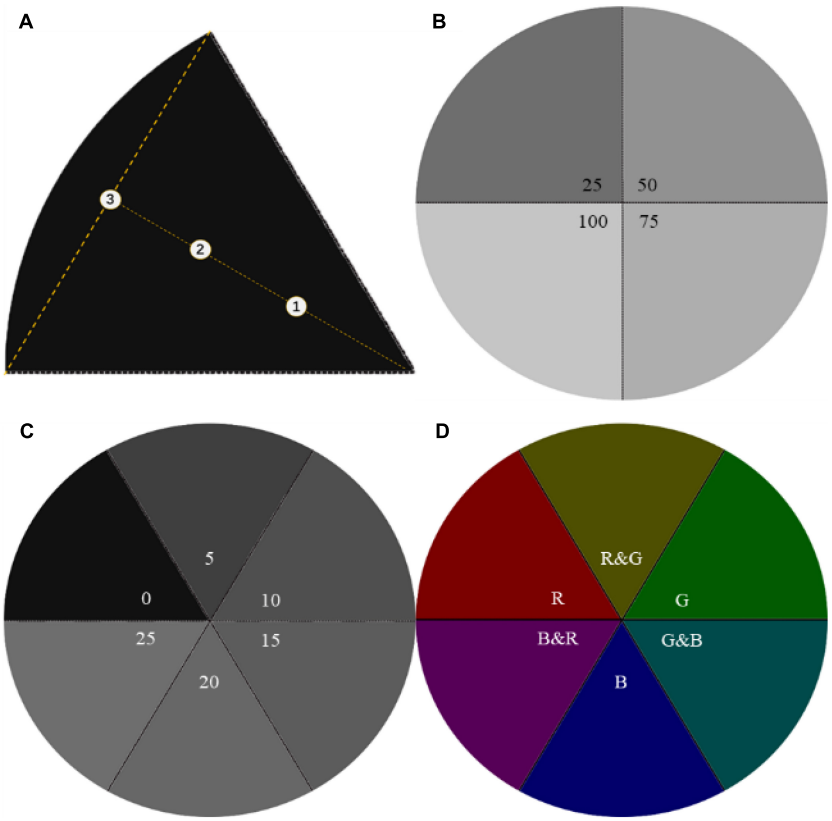

FIGURE 2 | Sampling points and light source images. **(A)** The markers ①②③ are the sampling points on the centerline in a sector, and ①② is the trisection of the vertical. **(B)** Light intensity of GMTC at 25/50/75/100 lx in the first phase. **(C)** Light intensity of GMTC at 0/5/10/15/20/25 lx in the second phase. **(D)** The three primary colors (TPCs) and the geometric mixed secondary colors (GMSCs) at 5 lx in the third phase.

Photosensitivity Experiment of Larvae to Light Intensity." Then, the display was turned on and the larvae were sampled after 60-minute lighting. All larvae were distinguished and counted under a microscope.

Data Collection

The lights were emitted by light-emitting diodes (LEDs) inside of the thin-film transistor-liquid crystal display (TFT-LCD, B156HAN02.1; AU Optronics Co., China), RGB (vertical strip), maximum brightness (250 cd/m^2), contrast (800:1), and gamma value (2.2); light intensity was measured by a smart sensor photometer (AS813; Arco Electronics Ltd., China) in lux, with an accuracy of ± 5%rdg; the size of the Petri dish is φ1,500 mm, with a depth of 31 mm, and made of borosilicate; larvae were sampled with 1,000 μl Eppendorf pipettes in the middle layer of water. Three sampling points were set in each sector, where points were located on the centerline of equal intervals (**Figure 2A**).

TABLE 2 | The gradient and quantized RGB values of fixed light intensity for the first phase and second phase.

Color index	Phase	LX (lx)	Gradient (%)	Quantized value of R/G/B
GMTPC	First	25	43	110/110/110
		50	57	145/145/145
		75	68	174/174/174
		100	77	197/197/197
	Second	0	7	18/18/18
		5	25	64/64/64
		10	31	79/79/79
		15	36	92/92/92
		20	40	103/103/103
		25	43	110/110/110

Statistical Analysis

Normality and homogeneity of variance assumptions for statistical data were tested using the Shapiro–Wilk and Bartlett tests, respectively (with R package "car" version 3.0-8). A: Permutational multivariate analysis of variance (PERMANOVA) with 1,000 permutations per analysis based on Euclidean distance was performed to evaluate the changes in larvae distribution and proportion after light stimulation, and PERMANOVA pairwise comparison tests were run if necessary (Anderson, 2014). PERMANOVA was performed with R package "vegan 2.5-7" version 4.0.3. B: One-way analysis of variance (ANOVA) was carried out to test the difference in larvae counts between sectors. Where significant effects were found, Duncan's new multiple

TABLE 3 | The gradient and quantized RGB values of the TPCs and the GMSCs at 5 lx for the third phase.

Phase	Color index	Color/wavelength	LX (lx)	Gradient (%)	Quantized value of R/G/B
Third	TPCs	Red/700 nm	5	48	122/0/0
		Green/546.1 nm	5	36	0/92/0
		Blue/435.8 nm	5	41	0/0/105
	GMSCs	Red and green/700 and 546.1 nm	5	34	79/79/0
		Green and blue/546.1 and 435.8 nm	5	31	0/74/74
		Blue and red/435.8 and 700 nm	5	29	87/0/87

range comparison was performed to compare the difference between groups with R package "agricolae" version 1.3-3. C: Path analysis (Gauss–Doolittle algorithm) was run to analyze the coefficient of light wavelength to larvae phototaxis with R package "agricolae" version 1.3-3. D: Pearson concordance index analysis was performed to analyze the correlation between larvae distribution and TPCs with R package "agricolae" version 1.3-3. Computations were running in R version 4.0.3 (64 bit) for Windows, and all statistical analyses were carried out at a significance level of $p < 0.05$.

RESULTS AND DISCUSSION

How Does Light Intensity Affect the Phototaxis of Hybrid Oyster Larvae?

The results showed that both distributions of eyespot and eyeless hybrid oyster larvae were significantly affected by light at 25/50/75/100 lx with time ($p < 0.001$). The distribution change of total larvae and eyespot larvae could be explained at a relatively high level by time ($R^2 = 0.968$ and $R^2 = 0.922$, respectively). The subsequent PERMANOVA pairwise comparisons test showed that the *F*-value for the comparison of 0 and 60 min was greater than that of the other two comparisons, indicating that the polarized level of total larvae and eyespot larvae distribution was increased at 60 min; on the contrary, the level of polarized distribution for eyeless larvae was reduced (**Table 4**). Similar to the first phase, the distribution of total larvae, eyespot larvae, and eyeless larvae changed significantly under 0/5/10/15/20/25 lx in the second experiment ($p < 0.01$). The *F*-value showed that the distribution of eyespot larvae changed visibly, and *F*-values were ordered as $F_{(totallarvae)} > F_{(eyespotlarvae)} > F_{(eyelesslarvae)}$ (**Table 5**). The changes in larvae distribution suggested that hybrid larvae were sensitive to light, and that the response could be traced within only 30 min and intensified later.

For the first phase, massive larvae with eyespot moved to the area at 50 lx light after 30 min where eyespot larvae density was significantly higher than the other sectors. However, there was no significant difference in the distribution of eyeless larvae compared between the 25 lx sector, 50 lx sector, and 75 lx sector. In terms of total larvae, when the lighting time extended to 60 min, the density of total larvae amounted to 205.0 ± 17.5 and 224.0 ± 10.0 (per 1.5 ml) in the 25 lx sector and 50 lx sector, respectively, resulting in no significant difference between the two sectors (**Table 6** and **Figure 3A**). As the extension of lighting time, there was no significant difference in the distribution of eyespot larvae between the 25 lx sector and 50 lx sector ($p > 0.05$; **Table 6**). The results showed that the density of eyeless larvae was positively correlated with light intensity in the 30-min group (except for the 100 lx sector); interestingly, the correlation was reversed in the 60-minute group (**Figure 3A**).

In general, zooplankton will migrate to the depth layer during daylight to avoid the threats of visual predation and surfacing at night to feed (Medcof, 1955; Forward, 1988; Hobbs et al., 2021); thus, many of them evolved to adapt to low light environments and cannot endure high-intensity light. Kim et al. (2014) found that reproduction of *Brachionus plicatilis* will be negatively affected by light over 0.5 W/m^2, and Tielmann et al. (2017) reported that larval *Sander lucioperca* had significantly higher natural mortality when reared under a light intensity of 2,500 lx. Similarly, in the present study, in terms of total larvae when the light intensity was over 25 lx and lighting time exceeded 30 min, oyster larvae would move to the weakest light area that was the evidence of negative phototaxis.

In the second phase, the 5 lx light sector gathered the most larvae of 192.0 ind (±23.5 ind per 1.5 ml), which was significantly higher than the other light intensities ($p < 0.01$); the highest density of eyespot larvae was sampled in the 10 lx sector (118.0, ± 4.0 ind per 1.5 ml), and there was no significant difference from the 5 lx sector; eyeless larvae density under the 5 lx light (87.5, ± 32.5 ind per 1.5 ml) was also significantly higher than the other intensities ($p < 0.01$; **Table 7** and **Figure 3B**). Based on the above results, we gave the following conjecture and interpretation that hybrid oyster larvae had positive phototaxis to a certain range of light intensity, but it could reverse to be negative if the light intensity exceeded a critical intensity.

TABLE 4 | PERMANOVA pairwise comparison analysis for the distribution difference in eyespot larvae, eyeless larvae, and total larvae of the hybrid oyster with time.

Metric	Pairwise	*F*-value	R^2	*p*-value	*p*-Adjusted A
Eyespot larvae	0 vs. 30 min	75.270	0.883	0.002	0.006
	30 vs. 60 min	23.628	0.703	0.003	0.009
	0 vs. 60 min	189.146	0.950	0.003	0.009
Eyeless larvae	0 vs. 30 min	26.087	0.723	0.001	0.003
	30 vs. 60 min	17.703	0.639	0.002	0.006
	0 vs. 60 min	16.428	0.622	0.002	0.006
Total larvae	0 vs. 30 min	167.915	0.944	0.001	0.003
	30 vs. 60 min	56.417	0.849	0.002	0.006
	0 vs. 60 min	1439.290	0.993	0.004	0.012

TABLE 5 | PERMANOVA analysis for the distribution difference in eyespot larvae, eyeless larvae, and total larvae of the hybrid oyster with time.

Phase	Pairwise	Metric	Sum of sq.	Mean sq.	*F*-value	R^2	*p*-value A
First	0 vs. 30 vs. 60 min	Eyespot larvae	0.397	0.198	88.299	0.922	0.001***
		Eyeless larvae	0.637	0.318	19.816	0.725	0.001***
		Total larvae	0.391	0.195	229.530	0.968	0.001***
Second	0 vs. 60 min	Eyespot larvae	0.355	0.355	231.490	0.959	0.002**
		Eyeless larvae	0.319	0.319	9.441	0.486	0.003**
		Total larvae	0.286	0.286	181.020	0.948	0.003**

*R^2 represents the degree to explain the differences among groups. Metrics marked with asterisks significantly differed (Signif. codes: 0, ***; 0.001, **; 0.01), similarly hereinafter.*

A previous study inferred that the invertebrate larvae had positive phototaxis to light at a low intensity and negative phototaxis to light at a high intensity (Forward, 1976), which was in conjunction with our findings. On this basis, we had a further conclusion that 5 lx intensity light was the positive phototaxis light for eyeless hybrid larvae, and that the optimal intensity for eyespot larvae was between 5 and 10 lx, but no higher than 15 lx. In a nutshell, the presumed optimal light intensity ranged from 0 to 15 lx for hybrid oyster larvae, and the light over 25 lx might cause negative phototaxis.

How Do Light Wavelengths (TPCs) Affect the Phototaxis of Hybrid Oyster Larvae?

Previous studies suggested that the sensitivity wavelength of zooplankton could be predicted by the efficient absorbance of light wavelengths by eyespot (Kim et al., 2013, 2014, 2018). The eyespot of *C. gigas* had a higher absorbance in the range of visible light, from 500 to 650 nm, and the highest absorbance of wavelength was at 620 nm (Kim et al., 2021), which meant that oyster larvae were more sensitive to green light (wavelength at 546.1 nm) among TPCs. In the present study, the PERMANOVA analysis of the third experiment showed that light wavelength could significantly affect the distribution of hybrid oyster larvae under the same light intensity ($p < 0.01$). Differences in the distribution of eyespot larvae, eyeless larvae, and total larvae could be highly explained by time changes ($R^2 = 0.986$, $R^2 = 0.989$, and $R^2 = 0.995$, respectively). Besides, the PERMANOVA analysis also showed that the number of attached larvae was significantly related to light wavelength ($p < 0.01$; **Table 8**). The above results further proved that hybrid oyster larvae were sensitive to light wavelength.

TABLE 6 | The density distribution of eyespot larvae, eyeless larvae, and total larvae of the hybrid oyster with time and light intensity (25/50/75/100 lx).

Metric	LX (lx)	Time		Δ
		30 min	60 min	
Eyespot larvae	25	105.0 ± 9.5^{b}	141.5 ± 6.5^{a}	36.5
	50	131.0 ± 11.0^{a}	167.0 ± 28.5^{a}	36.0
	75	81.0 ± 18.0^{c}	52.0 ± 6.0^{b}	−28.5
	100	8.5 ± 2.0^{d}	2.5 ± 1.5^{c}	−6.0
	p-Value B	$7.74e^{-06}$	$3.03e^{-06}$	
Eyeless larvae	25	57.0 ± 43.5^{a}	63.5 ± 7.0^{a}	6.0
	50	74.0 ± 10.0^{a}	57.0 ± 39.0^{a}	−17.0
	75	93.0 ± 14.5^{a}	25.0 ± 7.5^{ab}	−68.0
	100	1.0 ± 1.5^{b}	3.0 ± 2.0^{b}	1.5
	p-Value B	0.00724	0.0201	
Total larvae	25	162.0 ± 38.0^{b}	205.0 ± 17.5^{a}	43.0
	50	205.0 ± 11.0^{a}	224.0 ± 10.0^{a}	19.0
	75	174.0 ± 6.0^{ab}	77.0 ± 3.5^{b}	−97.0
	100	10.0 ± 1.0^{c}	5.5 ± 0.5^{c}	−4.0
	p-Value B	$9.87e^{-06}$	$3.74e^{-10}$	

The density data show the number of eyespot larvae, eyeless larvae, and total larvae per 1.5 ml. "Δ" represents the difference in the number of larvae between the 30-min group and the 60-min group.

The absorption of visible light in aquatic environments led to the common assumption that aquatic organisms sensed and adapted to penetrative blue/green light but little or no response to red light (Fortunato et al., 2016). Coincidentally, the green light patch and the green-blue light patch gathered the most eyespot larvae and eyeless larvae in the study, which was significantly higher than the other patches ($p < 0.01$). The eyespot larvae were significantly gathered by red-green light but less than that of green light and green-blue light ($p < 0.01$), and eyeless larvae were also relatively highly gathered in the red-green patch. These results indicated that green light caused positive phototaxis of hybrid oyster larvae. Surprisingly, the settling data suggested that though the red-green patch did not gather the most larvae, it collected a massive amount of settled larvae (20.0, ± 3.5 ind), which were significantly higher than the other patches (**Table 9**). Thus, we gave a bold assumption that red light had the function of settling induction to eyespot larvae, and that green light had a certain attraction to both eyespot larvae and eyeless larvae resulting in the largest number of hybrid larvae settled onto the red-green patch. The promotion of red light on eyespot larvae settling was also proposed in the study of *C. gigas* (Kim et al., 2021). Recently, four rhodopsin-like superfamily genes were identified in the genomic study of *C. gigas* (Wu et al., 2018), which provided us with another perspective on it.

The relationship between TPCs (red, green, and blue) and hybrid larvae distribution was deployed by path analysis (**Table 10**). The result showed that green light and red light could be two key points to the distribution of eyespot larvae and eyeless larvae ($p < 0.01$). Besides, red light and blue light were significantly correlated with the settling of oyster larvae ($p < 0.05$).

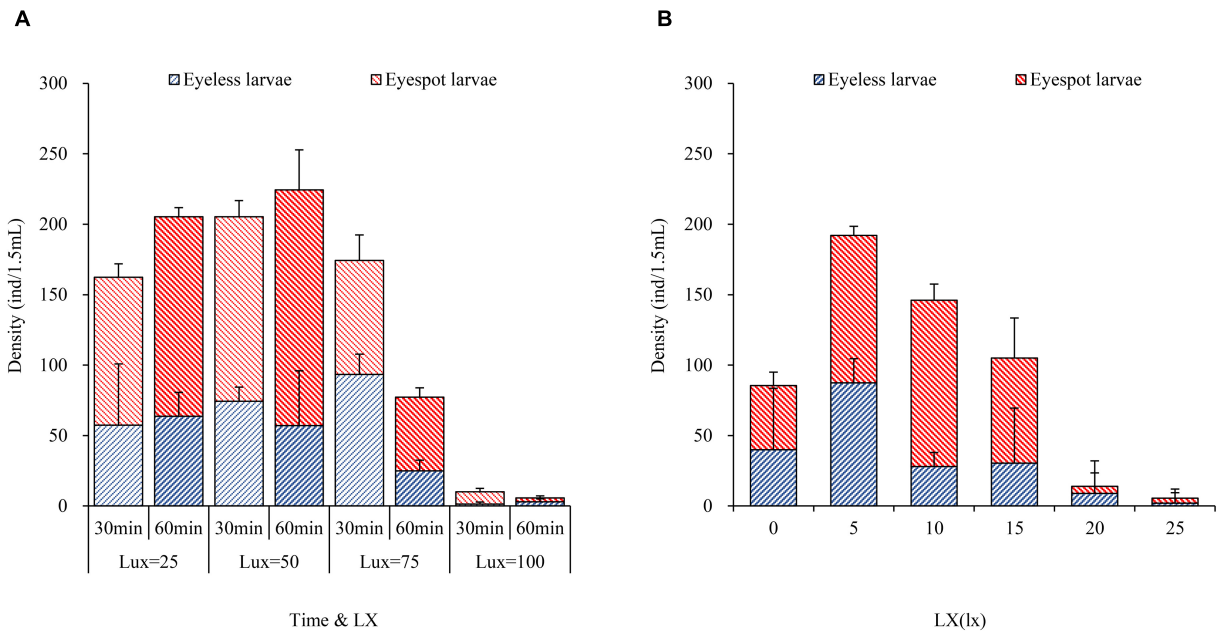

FIGURE 3 | The bar plots of hybrid oyster larvae distribution to light intensity. **(A)** The density of eyespot larvae and eyeless larvae with time and light intensity. **(B)** The density of eyespot larvae and eyeless larvae with light intensity.

The direct-acting and indirect-acting coefficients of red light on total larvae distribution were negative ($P_{0.j} = -0.50$, $P_{i.j} = -0.17$), but positive for green light ($P_{0.j} = 0.48$, $P_{i.j} = 0.29$), and total larvae distribution was poorly determined by blue light. In terms of eyespot larvae, the distribution was positively related to green light ($P_{0.j} = 0.51$), and red light had a negative attraction on them ($P_{0.j} = -0.66$). Similar to eyespot larvae, red light and green light had opposite effects on eyeless larvae ($P_{0.j} = -0.68$, $P_{0.j} = 0.74$; $p < 0.01$; **Table 10**).

In terms of larvae settling, green light had no significant effect on hybrid larvae settling, but red light was positively related to it, which meant that red light might be a factor in inducing hybrid oyster settling. Besides, blue light was negatively related to larvae settling, and the direct-acting coefficient on larvae settling was −0.37, which suggested that blue light could be an inhibiting factor to eyespot larvae settling ($p < 0.01$, **Table 10**). Oyster spat collection is an important process of oyster seedling (Taylor et al., 1998; Funo et al., 2019; Poirier et al., 2019), while oyster spat collection is unstable in the wild, as it is largely dependent on environmental situation (Absher, 2016). Many studies on oyster aquaculture had tried to enhance spat collection by developing tools (Holliday et al., 1993; Devakie and Ali, 2002; Buitrago and Alvarado, 2005; Arini and Jaya, 2011). Our findings demonstrated that red light had the function of inducing oyster larvae settling and green light had a certain attraction to oyster larvae that could be a guideline in oyster spat collection.

TABLE 7 | The density distribution of eyespot larvae, eyeless larvae, and total larvae of hybrid oysters under 0, 5, 10, 15, 20, and 25 lx light.

LX (lx)	Eyespot larvae	Eyeless larvae	Total larvae
0	45.5 ± 6.5^{c}	40.0 ± 14.5^{b}	85.5 ± 10.5^{c}
5	104.5 ± 9.0^{a}	87.5 ± 32.5^{a}	192.0 ± 23.5^{a}
10	118.0 ± 4.0^{a}	28.0 ± 14.5^{b}	146.5 ± 11.0^{b}
15	74.5 ± 16.5^{b}	30.5 ± 29.0^{b}	105.0 ± 12.5^{c}
20	5.0 ± 2.5^{d}	9.0 ± 4.0^{b}	14.0 ± 1.5^{d}
25	3.5 ± 2.0^{d}	2.0 ± 0.0^{b}	5.5 ± 2.0^{d}
p-Value B	$2.16e^{-09}$	0.00275	$2.27e^{-09}$

On a global scale, the oyster is distributed widely in tropical and temperate waters, predominantly coastal, and occupying the intertidal estuaries, marshes, and bays (Bayne, 2017). Oyster reefs provide important habitat for a marine animal assemblage that is both ecologically interesting and important to the estuarine food web (Luckenbach et al., 1999; Baggett et al., 2015)9). In the past century, the extent and intensity of nighttime illumination have dramatically increased such that it has substantial effects on the biology and ecology of species in the wild (Cinzano et al., 2001; Longcore and Rich, 2004), resulting in coastal habitats adjacent to populated areas becoming particularly vulnerable to light pollution (Gaston et al., 2013; Tamir et al., 2017). Considering the conclusion of the present study, light intensity and wavelength could be a potential tool for coastal habitat restoration, due to the function of specific light on oyster larvae gathering and settling induction. Surely, more studies need to be done for the comprehensive assessment of artificial light and coastal ecosystems.

CONCLUSION

To our knowledge, the present research firstly reported the impacts of light intensity and artificial TPCs on the phototaxis

TABLE 8 | PERMANOVA analysis for the distribution difference in eyespot larvae, eyeless larvae, and total larvae and the number of attached larvae of hybrid oysters with time.

Phase	Pairwise	Metric	Sum of sq.	Mean sq.	*F*-value	R^2	*p*-Value A
Third	0 vs. 60 min	Eyespot larvae	0.898	0.898	711.510	0.986	0.001
		Eyeless larvae	0.845	0.845	912.850	0.989	0.002
		Attached larvae	0.158	0.158	23.416	0.701	0.004
		Total larvae	0.878	0.878	2121.300	0.995	0.007

TABLE 9 | The density distribution of eyespot larvae, eyeless larvae, and total larvae and the number of attached larvae of the hybrid oyster to each TPC and the GMSCs patch (LX = 5 lx).

RGB color	Eyespot larvae	Eyeless larvae	Attached larvae (per patch)	Larvae
Red	6.0 ± 2.0^{c}	6.5 ± 0.5^{b}	13.0 ± 3.5^{b}	12.5 ± 2.5^{c}
Red and green	16.0 ± 2.5^{b}	7.5 ± 5.0^{b}	20.0 ± 3.5^{a}	23.5 ± 7.0^{b}
Green	112.0 ± 4.5^{a}	74.0 ± 2.5^{a}	7.0 ± 1.0^{c}	186.0 ± 4.0^{a}
Green and blue	108.0 ± 11.5^{a}	72.5 ± 4.0^{a}	2.0 ± 1.5^{d}	181.0 ± 7.5^{a}
Blue	2.5 ± 1.0^{c}	6.0 ± 1.5^{b}	10.0 ± 3.0^{bc}	8.5 ± 2.5^{c}
Blue and red	5.0 ± 1.0^{c}	2.5 ± 2.5^{b}	7.0 ± 1.5^{c}	7.5 ± 1.5^{c}
p-Value B	$4.63e^{-12}$	$9.35e^{-13}$	$3.49e^{-05}$	$4.41e^{-15}$

TABLE 10 | The path analysis for the distribution difference in eyespot larvae, eyeless larvae, and total larvae and the number of attached larvae of the hybrid oyster to the TPCs.

Metric C		Correlation coefficient ($r_{0.j}$) D	Direct-acting ($P_{0.j}$)	$r_{0.j} = P_{0.j}$	Indirect-acting ($P_{i.j}$)			
					Red	Green	Blue	Σ
Eyespot larvae	Red	−0.66**	−0.47	0.3113		−0.23	0.04	−0.19
	Green	0.78***	0.51	0.4007	0.21		0.05	0.27
	Blue	−0.18	−0.11	0.0203	0.18	−0.25		−0.07
Eyeless larvae	Red	−0.68**	−0.56	0.3807		−0.18	0.06	−0.12
	Green	0.74**	0.41	0.3019	0.25		0.08	0.33
	Blue	−0.15	−0.17	0.0250	0.21	−0.20		0.02
Attached larvae	Red	0.54*	0.38	0.2060		0.02	0.14	0.16
	Green	−0.03	−0.04	0.0011	−0.17		0.18	0.01
	Blue	−0.50*	−0.37	0.1865	−0.14	0.02		−0.13
Total larvae	Red	−0.67**	−0.50	0.3369		−0.22	0.05	−0.17
	Green	0.77***	0.48	0.3706	0.23		0.06	0.29
	Blue	−0.17	−0.13	0.0221	0.19	−0.23		−0.04

"$r_{0.j} \times P_{0.j}$" represents the contribution of x_j to R^2. Metrics marked with asterisks "" indicates a significant correlation between color and larvae distribution, and "*" represents $p < 0.05$; "**" represents $p < 0.01$; and "***" represents $p < 0.001$.*

of the *C. gigas* (♂) and *C. sikamea* (♀) hybrid larvae. The result showed that the hybrid oyster larvae had positive phototaxis to specific light intensity and wavelength, and that this ability was not a unique characteristic of eyespot larvae, which was also observed in eyeless larvae. When the eyespot appeared, the acceptable light intensity range of the hybrid larvae was expanded. The present study also suggested the potential positive effects of the green light on oyster larvae gathering and settling induction of the red light. In conclusion, our study may contribute to the understanding of the phototaxis of hybrid oyster larvae, as well as the further perspective of light pollution on the benthic communities and coastal system.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

XuZ, CF, and ZW gathered, analyzed and interpreted data, discussed the results and co-wrote the manuscript. XiZ, QL, and YL contributed constructively ideas for the work. All authors contributed to the article and approved the submitted version.

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Aquaculture Impacts on China's Marine Wild Fisheries Over the Past 30 Years

Kangshun Zhao[1,2], Min Zhang[3], Kang Wang[1,2], Konghao Zhu[1,2], Congjun Xu[1,2], Jiayi Xie[1,2] and Jun Xu[1,4,5]**

[1] Donghu Experimental Station of Lake Ecosystems, State Key Laboratory of Freshwater Ecology and Biotechnology of China, Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan, China, [2] University of Chinese Academy of Sciences, Beijing, China, [3] Hubei Provincial Engineering Laboratory for Pond Aquaculture, College of Fisheries, Freshwater Aquaculture Collaborative Innovation Center of Hubei Province, Huazhong Agricultural University, Wuhan, China, [4] State Key Laboratory of Marine Resource Utilization in South China Sea, Hainan University, Haikou, China, [5] Laboratory for Marine Fisheries Science and Food Production Processes, Qingdao National Laboratory for Marine Science and Technology, Qingdao, China

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***Correspondence:**
Jun Xu
xujun@ihb.ac.cn
Min Zhang
zhm7875@mail.hzau.edu.cn

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China is the world's largest producer of aquaculture and capture fisheries. How this country develops its aquaculture sector and whether such development can relieve pressure on wild fisheries remain a contentious issue in the past and for the future. This study aims to provide a broad assessment on the impact of aquaculture development in different periods on marine wild fisheries on the basis of aquaculture and marine wild fish catch data from all the coastal provinces of China. China's aquaculture and capture fisheries have undergone substantial changes. From 1989 to 2002, China's aquaculture, especially mariculture, had a strong relationship with marine wild fisheries. However, from 2003 to 2018, the impact of mariculture was weakened, whereas that of freshwater aquaculture had increased. Although aquaculture still puts pressure on marine wild fisheries, China's aquaculture is currently moving toward sustainable development pattern with low input and high output. These results provide the first statistical evidence on the effects of aquaculture development on marine wild fisheries and contribute to the sustainable management of China's aquaculture and marine capture fisheries.

Keywords: aquaculture, marine wild fisheries, forage fish, fishery management, sustainable development

INTRODUCTION

China has the largest capture fisheries and aquaculture production worldwide and accounted for 19.2% of global marine capture fishery production and 61.5% of global aquaculture production in 2016 (FAO, 2018). Aquaculture in China has developed rapidly in the past few decades, leading to country being the predominant aquaculture producer in the world since the 1990s. China's aquaculture industry has recently received many critiques on the grounds of the environment (Biao and Kaijin, 2007; Herbeck et al., 2013; Zeng et al., 2013) and the excessive usage of marine wild fisheries resources (Cao et al., 2015). For example, Cao et al. (2015) expressed that China consumes a mass of fishmeal and forage fish (e.g., anchovy, sardine, and herring) due to its

huge aquaculture industry and thus consequently diminishes the world's wild fish resources. However, many peer experts such as Tang et al. (2016) and Han et al. (2018) disagreed with their negative views. These scholars have described the structure of China's aquaculture in recent decades, the trends of fishmeal usage, and the efforts made by the Chinese government in protecting wild fisheries and sustainable development of fisheries. Their findings supported that the future world aquatic food would mostly be supplied from aquaculture (Cressey, 2009), which is destined to reduce the demand on wild fisheries (Han et al., 2018). Their standpoints are not without reasons. China contributes more than 60% to the global aquaculture output and will account for 38% of the fish supply for world's human consumption by 2030 (FAO, 2014); however, the expenses would constitute only 25–30% of the world fishmeal (Han et al., 2018). Hambrey (2017) also reported the importance of aquaculture development for the Global Sustainable Development Goals.

How China develops its aquaculture sector and whether such development can relieve pressure on wild fisheries remain a contentious issue in the past and for the future (Chiu et al., 2013; Cao et al., 2015). Both sides have their own views, and neither side has directly explored the statistical relationship between China's aquaculture and marine wild fisheries resources. No dispute arises on the existence of an inevitable link, positive or negative, between China's aquaculture and wild fish resources in the past decades. Whether China's efforts to aquaculture industries (such as alternative protein sources, improved feed manufacturing techniques, and decreased inclusion of fishmeal in aquafeeds) has relieved pressure on wild fisheries in recent decades must be analyzed. The relationships between the development of China's aquaculture and the demand of marine wild fisheries resources are poorly understood. In this study, all the coastal provinces of China (accounts for 68.9% of the total aquaculture production in China) (Ministry of Agriculture and Rural Affairs of the People's Republic of China, 1990–2019) were selected as the research object to explore the impact of aquaculture development in different periods on marine wild fisheries resources under the background of the rapid development of China's fishery industry over the past 30 years.

On the basis of literatures and prior knowledge, a conceptual meta-model (**Figure 1**) was designed and used as the basis for testing the relationship between aquaculture and marine wild fisheries. Fishery gross product (FGP) was predicted to drive mariculture production (MAP), freshwater aquaculture production (FAP), and marine edible economic fish production (EFP) (De Silva and Turchini, 2009; Zhao et al., 2018). MAP and FAP were predicted to drive EFP and marine forage fish production (FFP) (Funge-Smith et al., 2005; De Silva and Turchini, 2009; Li et al., 2011; Chiu et al., 2013; Cao et al., 2015; Szuwalski et al., 2017; Han et al., 2018). MAP and FAP were predicted to accelerate each other (Funge-Smith et al., 2005; Li et al., 2011). Finally, EFP was predicted to drive FFP (Szuwalski et al., 2017; Zhang et al., 2020). This research is classified as a broad assessment, because the complex relationship between aquaculture structure, fisheries resource utilization and the unidentified "nei" species (not elsewhere included or unidentified species)/trash fish are not considered.

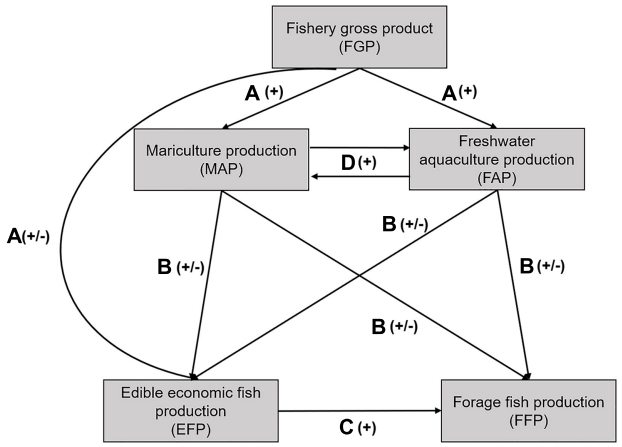

FIGURE 1 | Conceptual diagram or metamodel of hypothesized influences on marine wild fisheries. Solid black arrows depict known causal effects among variates. Arrow labels correspond to exemplar references **(A)** De Silva and Turchini (2009) and Zhao et al. (2018), **(B)** Cao et al. (2015), Chiu et al. (2013), De Silva and Turchini (2009); Han et al. (2018), Li et al. (2011), Funge-Smith et al. (2005) and Szuwalski et al. (2017), **(C)** Szuwalski et al. (2017), and Zhang et al. (2020), **(D)** Li et al. (2011) and Funge-Smith et al. (2005). Parenthetic signs next to black arrow labels indicate that the relationship is expected to be positive (+), negative (–), or variable (±).

MATERIALS AND METHODS

Data Set

Marine wild fish catch data were collected from the Ministry of Agriculture and Rural Affairs of the People's Republic of China (1990–2019). Twenty-eight marine fish species or groups in the China Seas were reported according to province in the Chinese National Fishery Statistics Yearbook. Since 1989, 16 species or species groups have been reported continuously (**Supplementary Table 1**), accounting for over 84% of the reported fish species catch over the past three decades and indicating more comprehensive species recorded than those before 1988. Thus, the catch data of 16 marine wild fish species or species groups were collected in 11 coastal province administrative regions (Tianjin, Hebei, Liaoning, Shanghai, Jiangsu, Zhejiang, Fujian, Shandong, Guangdong, Guangxi, and Hainan) to reflect the demand of marine wild fish resources from 1989 to 2018 (**Supplementary Figure 1**). According to human uses, 16 marine wild fish species or species groups were divided into two categories: high-valued edible economic fish (i.e., Japanese scad, large yellow croaker, small yellow croaker, soiny mullet, pike conger, ilisha, grouper, snapper, hairtail, golden threadfin bream, mackerel, pomfret, and black scraper) and low-valued forage fish (i.e., anchovy, sardine, and herring). These three forage fish species are the most typical and mainly used as the raw material to process fishmeal in China, and a small amount is used for direct feeding (Yu et al., 2020). Although these species cannot represent all the utilized wild fisheries resources, the fishing practices in China are largely indiscriminate (∼50% trawl fisheries) and there is little discard because a market exists for a wider range of species and sizes than are typically

salable in other locales (Costello et al., 2016; Costello, 2017; Szuwalski et al., 2017). Thus, these 16 recorded fish species were chosen as indicator species for the demand of marine wild fisheries resources.

Mariculture production and FAP variables were selected to represent aquaculture development in China. These values were obtained for each province from Ministry of Agriculture and Rural Affairs of the People's Republic of China (1990–2019). FGP measures the size of the fishery economy and represents the development of China's fishery industry, and its data were extracted from National Bureau of Statistics of China (1990–2019).

Statistical Analysis

Stratigraphically constrained hierarchical clustering (SCHC) (Grimm, 1987) offers a suitable alternative for the detection of shifts in multivariate space and was used in this study to detect significant compositional shifts at the province-level of marine wild fish catches. According to the shifts, the impact of aquaculture on marine wild fisheries resources was analyzed in different periods. The method used is based on cluster analysis with the constraint that clusters are formed from the hierarchical agglomeration of annually adjacent assemblages. This clustering technique aims to assign cases to homogeneous groups (i.e., clusters) in which the constraint that encompasses each group is contiguous in time. Such hierarchical agglomeration depends on the local, not the entire, sequences; hence, the positions of splits will not change even when the sequence of data is truncated. This analysis method can be used to break the time series into contiguous blocks of time with similar assemblages (Legendre and Legendre, 2012). R packages "rioja" (Juggins, 2014) and "vegan" (Oksanen et al., 2013) were applied to conduct SCHC and broken-stick test, respectively.

Structural equation modeling (SEM) was used to determine the pathways underlying the effects of aquacultural predictors on marine wild fish catches in the context of fishery industry development. SEMs can be used to evaluate cause-and-effect relationships among discrete variable (Grace et al., 2012) and can simultaneously test system-level hypotheses that are expressed as complex networks of interrelationships among variables (Grace, 2006). Before the models were built, all variables were rescaled to 0–1 to cope with their large differences in scales. All continuous variables were tested for normality, and non-normal variables were root transformed to approach normal distribution (Birk et al., 2020). In reality, sites that are proximate are likely to share similar characteristic. Within a site, observations closer in time are likely to be more similar than those that are farther apart. Given that all the related data were non-independent in time (i.e., year) and site (i.e., province), piecewise SEM was used and specified using a list of structured equations, which can be built using most common linear modeling approaches in R. Each response was fitted to a generalized linear mixed model (GLMM) with Gaussian distribution. For each component model, year and province indicated the random effects to account for temporal autocorrelation and spatial autocorrelation (Birk et al., 2020; Liu et al., 2020). SEM was conducted using "piecewise SEM" (Lefcheck, 2016), "nlme," and "lme4" packages. Fisher's C test ($0 \leq$ Fisher's C/df ≤ 2 and $p > 0.05$) was employed to confirm the goodness of the modeling results. All statistical analyses were performed in R version 3.6.3 for Windows (R Core Development Team, 2020).

RESULTS

Shift Dynamics of Marine Wild Fish Catch

Shifts detected using the shift detection algorithm were partially supported by the SCHC analysis. According to the total sum of shift counts per province, seven statistically significant assemblage breaks occurred from 1996 to 2013, and the major break was 2003 (**Figure 2**). Hence, 2003 was selected as the node, and the marine wild fish catches were divided into two period over the past 30 years (**Figure 3**). In the first period (i.e., from 1989 to 2002), all species or species groups fluctuated and increased, except for the catch of black scraper that was decreased. During the second period (i.e., from 2003 to 2018), the catch of all three forage species or species groups all declined. Other edible economic fish also showed a downward trend, such as ilisha, Japanese scad, hairtail, pomfret, and black scraper. The catch of large yellow croaker, golden threadfin bream, So-iny mullet and mackerel greatly fluctuates. For all fish species or species groups, the catch continued to decline from 2017 to 2018 (**Figure 3**).

Impact of Aquaculture Development on Marine Wild Fisheries

From 1989 to 2002, the relationship between all variables was significantly positive, except for FGP to EFP, FAP to EFP, and EFP to FFP (**Figures 4A,C**). In the second period, the development of fishery economy negatively affected the EFP and still positively affected the output of aquaculture (**Figures 4B,D**). Different from that in the first period, the MAP in the second period was not significantly correlated with the EFP and FFP. Furthermore, the FAP positively affected the EFP, but the effect on the FFP was changed to negative. The EFP had a positive influence on the FFP from 2003 to 2018 and had been strengthened over the past three decades. A significant positive relationship was found between the MAP and FAP, except for the effect of FAP on the MAP from 2003 to 2018 (**Figures 4A–D**).

China's Aquafeeds and Aquaculture Production and Fishmeal Usage From 1989 to 2018

From 1989 to 2005, the volume of imported fishmeal showed a fluctuating upward trend, but tended to be stable from 2006 to 2018. The trend of domestic fishmeal is similar to that of imported fishmeal, which increased first and then slightly decreased, but the transition year has been advanced to around 2000 (**Figure 5A**). However, China's aquafeeds and aquaculture production have continued to grow rapidly from 1989 to 2018 (**Figures 5A,B**).

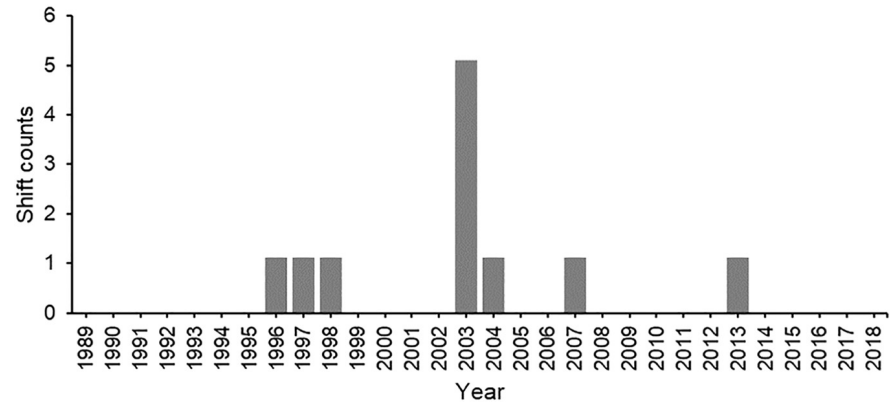

FIGURE 2 | Summary of significant compositional shifts at province-level detected by stratigraphically constrained hierarchical clustering. The histogram shows significant shifts counts for each year from 1989 to 2018.

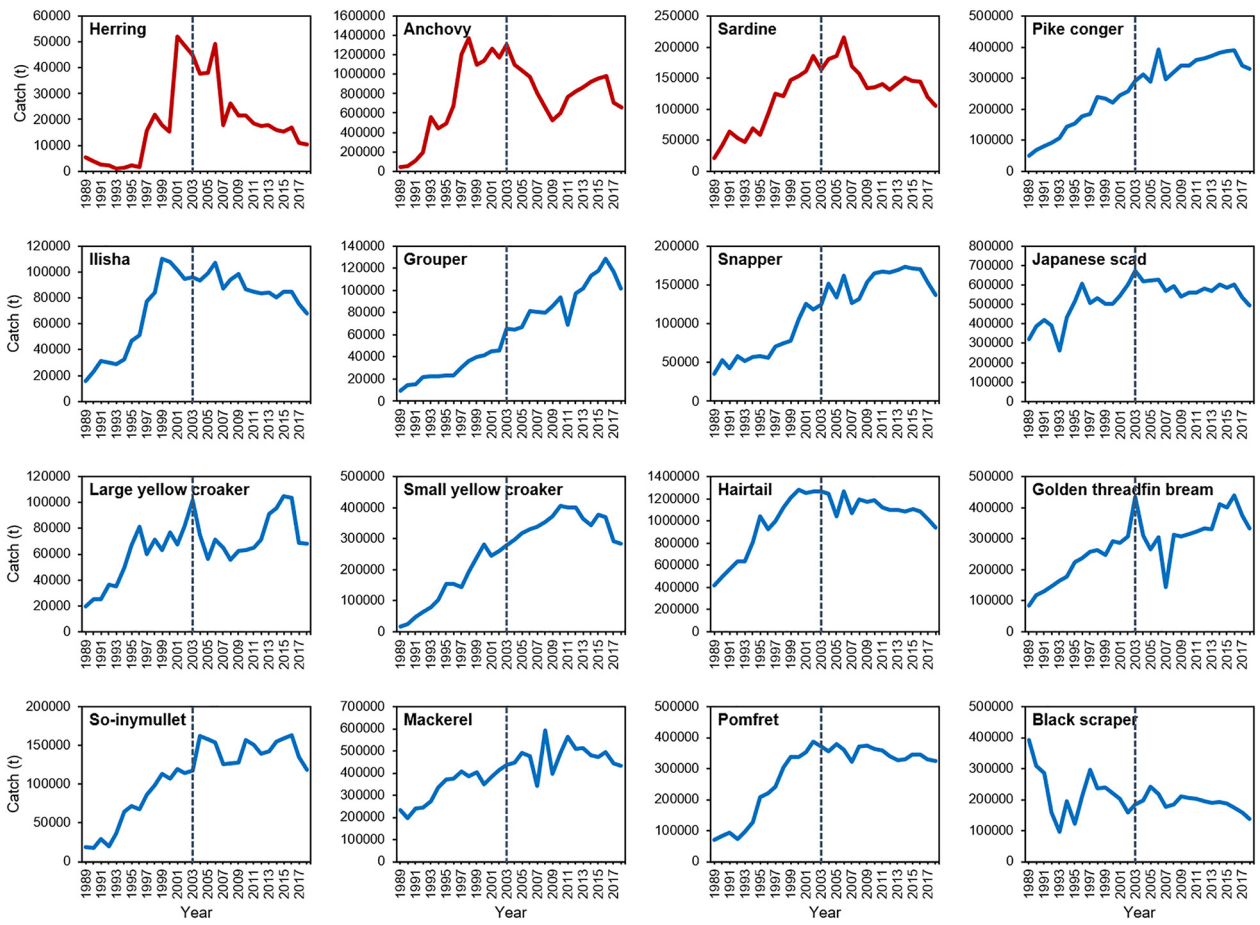

FIGURE 3 | Trends in catch of 16 species or species groups over the past 30 years in China. The trend line is red for forage fish and blue line for edible economic fish. The dotted black line is the dividing line between the two periods, located at 2003.

DISCUSSION

Farming has become the predominant form of food fish supply, which is similar to all other staples (De Silva, 2012). China, currently the major producer of farmed food fish, has produced this product more than the rest of the world combined every year since 1991 and contributed the most to the growth in the world's per capita availability of food fish (FAO, 2018). The ensuing

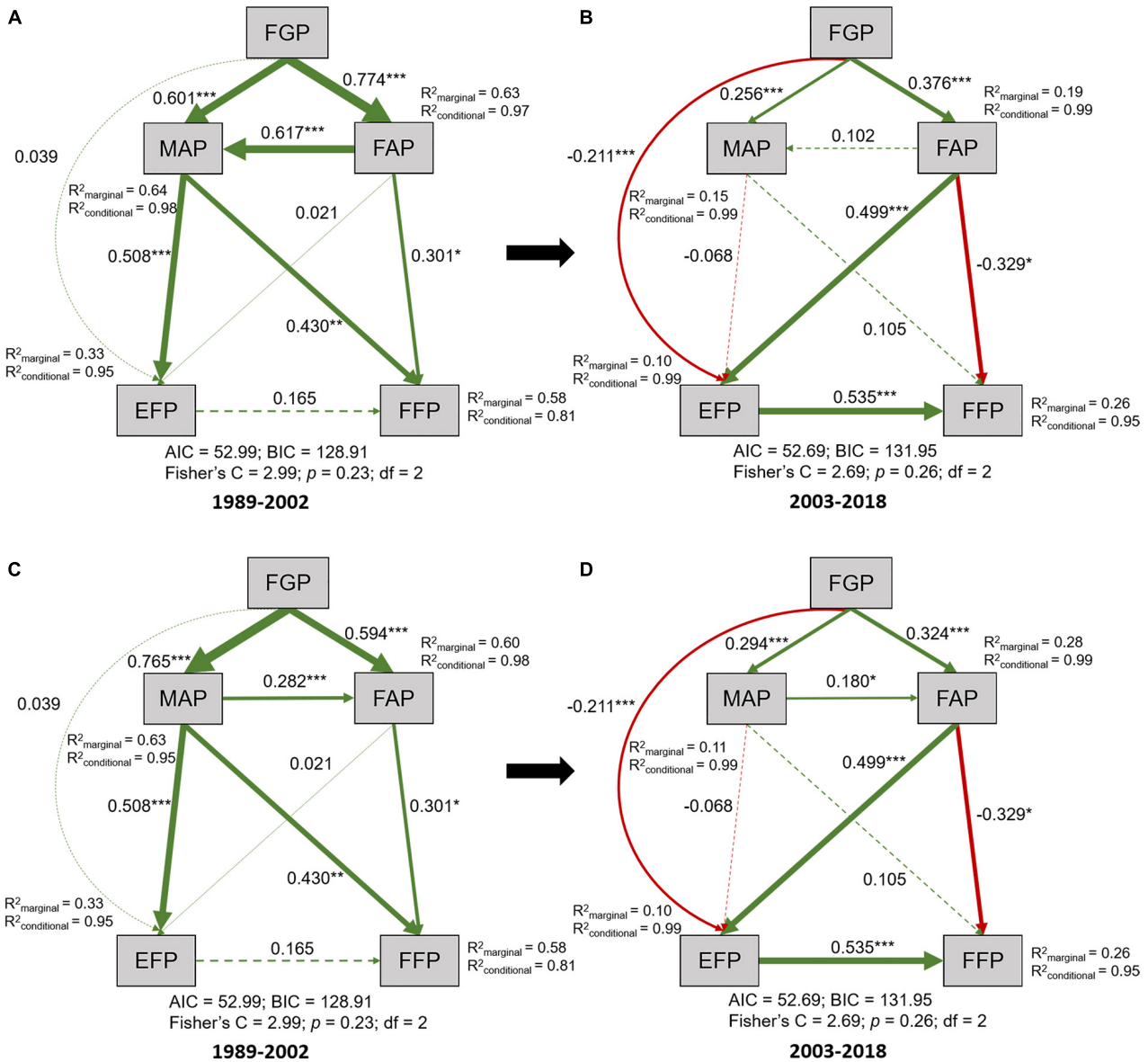

FIGURE 4 | Piecewise structural equation modeling (SEM) describing the effects of aquaculture development on marine wild fisheries in two periods. The first period was from 1989 to 2002 **(A,C)** and the second period was from 2003 to 2018 **(B,D)**. Path thicknesses have been scaled based on the magnitude of the standardized regression coefficient, which adjacent to arrows are indicative of the effect size of the relationship. Positive and negative effects among variables are depicted by green and red arrows, respectively, the dashed arrow indicate a not significant ($p > 0.05$) effect. Conditional R^2 denote the proportion of variance explained by the included predictors without accounting for random effects. Marginal R^2 denotes the proportion of variance explained by the included predictors by accounting for random effects. Significance levels of each predictor are $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. FGP, fishery gross product; MAP, mariculture production; FAP, freshwater aquaculture production; EFP, edible economic fish production; FFP, forage fish production.

debate is whether China's aquaculture is relieving the pressure on marine wild fisheries resources or is continuously inducing damages. This study aims to evaluate the impact of China's aquaculture development on marine wild fisheries resources and provides reference for the sustainable development of marine capture fisheries and aquaculture industries.

In the first study period, the development of the fishery economy has promoted the increase in aquaculture production, which further enhanced the increase in the FFP (**Figures 4A,C**). Fish farming is influenced by various human demands, socioeconomic development, and policies (Cao et al., 2015; Zhao et al., 2018). Zhao et al. (2018) reported that the rapid economic growth in China and the increasing food demand have promoted aquaculture development. In 1986, the Fishery Law of the People's Republic of China formally established the principle of "giving priority to aquaculture with simultaneous efforts in aquaculture, fishing and processing, taking measures in accordance with local conditions, with respective priorities,"

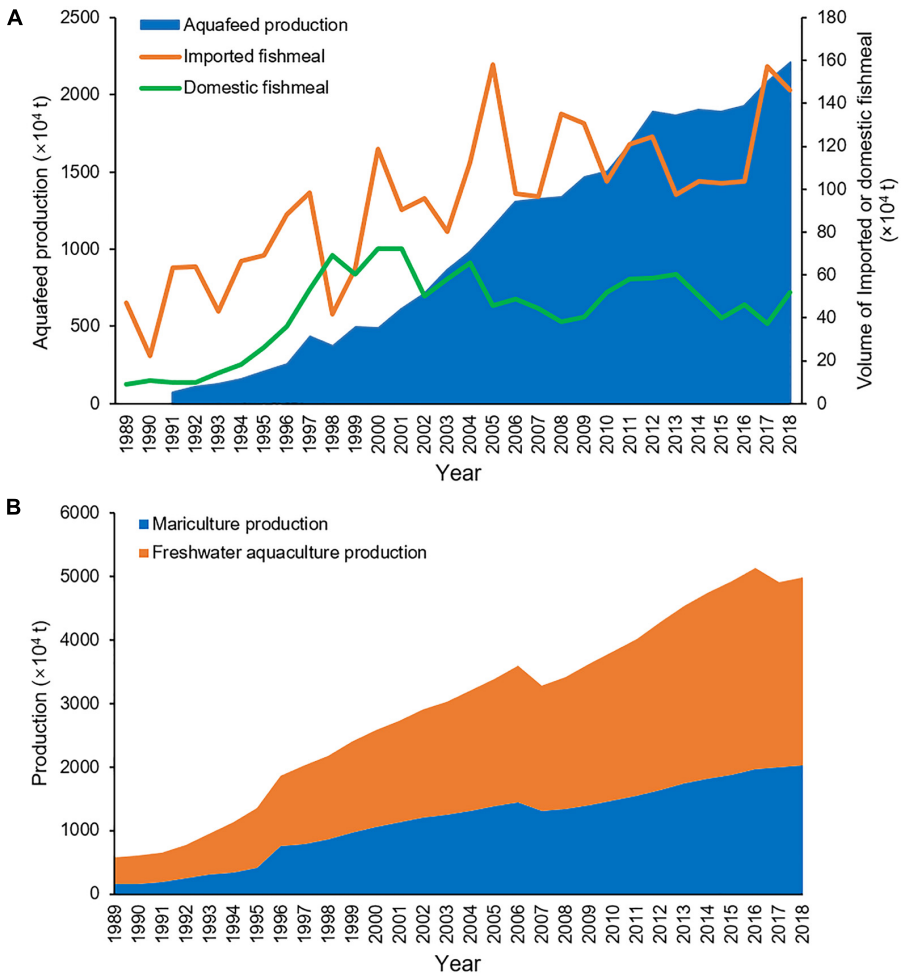

FIGURE 5 | The production of aquafeeds, aquaculture and fishmeal usage over the past 30 years in China. Based on data from China Feed Industry Yearbook (1991–2019), Fishery Statistics Yearbook (1990–2019a) and FAO (2021). Trends in production of aquafeeds, and volume of imported and domestic fishmeal **(A)**; Trends in production of mariculture and freshwater aquaculture **(B)**.

thereby marking the official determination of China's "giving priority to aquaculture" policy in fishery (Standing Committee of the National People's Congress, 1986). This policy promoted the rapid growth of China's aquaculture industry (Li et al., 2011). In 1988, China became the only country in the world where aquaculture production exceeds the marine wild catch and thus has replaced capture fisheries as the major fishery activity.

In the meantime, the consequent high demand for high-quality feed stimulates the development of fishmeal manufacturing and feed industry (Funge-Smith et al., 2005; Li et al., 2011). The product processing, logistics, and market expansion in aquaculture have also been accelerated (Li et al., 2011). Therefore, freshwater aquaculture and mariculture have mutual promotion effects (**Figures 4A,C,D**). The development of the feed industry further induced the private sector to engage in the farming of high-valued species (e.g., shrimp, large yellow croaker, and perch) in the late 1980s and early 1990s. Domestic fishmeal production was increased fourfold from 100,000 tons in 1992 to 400,000 tons in 2002, with production peaking in 1999 at 755,000 tons (Funge-Smith et al., 2005). A mass of low-valued fish/trash fish was captured and used for direct feeding and fishmeal processing, and the marine finfish culture had the highest usage of low-valued fish/trash fish (Funge-Smith et al., 2005; De Silva and Turchini, 2009). Fishing practices in China are largely indiscriminate (Szuwalski et al., 2017), and many forage fish fishing fleets also catch plenty of edible economic species. Therefore, the MAP can also have a substantial positive effect on the EFP (**Figures 4A,C**).

In the second period, the relationship among fishery economy, aquaculture production, and the catch of marine wild fish was changed. No substantial correlation with the MAP was found for the EFP and FFP (**Figures 4B,D**), which does not indicate that the mariculture did not depend on any marine wild fisheries resources because many of the trash fish and "nei" species used for direct feeding and fishmeal processing were not considered here (Cao et al., 2015). Trash fish are generally a mix of species of varying sizes and minimum commercial value and therefore often not suitable for human consumption. Funge-Smith et al. (2005)

and De Silva and Turchini (2009) estimated China's high-valued mariculture uses to approximately 3 million tons of trash fish each year for direct feeding. But the weakening of the relationship between mariculture and marine wild fish catches is a clear sign that mariculture is becoming less dependent on forage fisheries, which has been proved by many facts. China's mariculture is mainly composed of mollusk and algae that require no external feed inputs. The main fishmeal fed species in mariculture are crustaceans, sea bass, flatfish, large yellow croaker, grouper, and other marine fish, which only account for approximately 5.5% of the total aquaculture production (Tang et al., 2016; Han et al., 2018). In recent decades, the trophic level of crustaceans has decreased from 3.5 in 1985 to 2.89 in 2014 due to the increase in the proportion of compound aquafeeds and the decrease in trash fish feeding. The trophic level of fish also decreased from 4.5 in 1985 to 3.77 in 2014, and that of other species also decreased from 3.00 in 1985 to 2.31 in 2014 (Tang et al., 2016). Increased knowledge on the digestive processes and nutritional requirements of farmed species and on the processing of raw material combined with the use of alternative proteins has led to impressive reductions in fishmeal inclusions in aquafeeds and rise in feed conversion ratios in cultured species in China (Zhou et al., 2015; Han et al., 2018). Moreover, the cultured species that previously only feed on low-valued fish/trash fish, for example, large yellow croaker, can also feed on compound aquafeeds, and the proportion of aquafeeds is continuously increasing (Li et al., 2013, 2021; Zhang et al., 2016). These findings explain that aquaculture production in China continues to grow, whereas the usage of imported and domestic fishmeal has remained relatively stable (**Figures 5A,B**; Han et al., 2018).

Owing to the use of compound aquafeeds, the trophic level of freshwater aquaculture also decreased (Tang et al., 2016). In 2014, 35.2% of China's FAP was obtained from non-fed species (fish, mollusk, and algae). More than 82% of finfish in freshwater aquaculture are filter feeding, herbivorous, and omnivorous and thus do not depend on fishmeal to any significant extent (Han et al., 2018). However, Chiu et al. (2013) believed that these fish are cultured in large volumes, and even low inclusion rates add up to a substantial portion of fishmeal demand. The results indicate that FAP had a significant negative effect on the FFP and a significant positive effect on the EFP (**Figures 4B,D**), thus suggesting that the demand for marine fisheries resources has not been reduced so far due to the large volume of freshwater aquaculture (**Figure 5B**). With aquaculture development, the feed value of forage fish (e.g., anchovy) has been recognized since the 1990s. From the 1990s to the beginning of 2000s, in order to supply a large part of the fish used for livestock/fish feed, and the FFP has increased dramatically (**Figure 3**; Yu et al., 2020). However, since the early 2000s, forage fish stocks have declined remarkably due to long-term overfishing (Zheng et al., 2014; Zhang et al., 2020), and the production has been seriously reduced as a result (**Figure 3**). Similarly, the negative effect of FGP on EFP suggests that the economic upsurge in China has increased the living standards and consumption of high-valued seafood species (De Silva and Turchini, 2009) and caused most edible economic fish species or species groups resources to be unstable and thus decline in this period. Besides, the positive effect of the EFP on FFP changed from non-significant to significant, implying that the forage fish bycatch increased substantially. This finding was consistent with the changes in the domestic marine catch composition from a predominance of relatively large-sized, high-valued, high trophic level, demersal species to multiple small, low-valued, short-lived, and low trophic level (Liu and De Mitcheson, 2008; Shen and Heino, 2014; Cao et al., 2017; Zhang et al., 2020).

However, China is a world leader in low trophic level aquaculture production, and its aquafeeds and aquaculture production have continued to grow without the need to increase the imported and domestic fishmeal usage from around 2000 to 2018 (**Figures 5A,B**; Shepherd and Jackson, 2013; Han et al., 2018). Since 2000, only 50–60% of the fishmeal consumed annually in China was used for aquaculture, and 35–38% for pig and poultry. Moreover, aquatic products processing wastes have accounted for 25–35% of the raw materials in the fishmeal and fish oil manufacturing, and freshwater fishmeal showed an increasing trend with freshwater fish production (Yu et al., 2020). Cao et al. (2015) estimated that fish processing wastes could meet almost half, and potentially two-thirds, of China's current demand for fishmeal in aquafeeds. Given that Asian fishmeal manufacturing increasingly tends to use byproducts from the fish food industry, the dependence on low-valued fish/trash fish for fishmeal production could be further decreased (De Silva and Turchini, 2009). Furthermore, our results also demonstrate a shift in dependence on marine wild fisheries from mariculture to freshwater aquaculture over the past 30 years (**Figure 4**). Although our results indicated that there is still a great demand for marine fisheries resources due to the large quantity of freshwater aquaculture (**Figures 4B,D**, 5B), but low trophic level fish are the main species in freshwater aquaculture, with trophic level from 2.0 (around 65%) to 3.5 (around 35%) accounting for around 100%. In China, aquaculture production is dominated by plants, non-fed mollusks and fish, herbivores and omnivores, which have a very low Fish In, Fish Out ratio and showed an overall downward trend, particularly for freshwater aquaculture (Tacon and Metian, 2008; Byelashov and Griffin, 2014; Tang et al., 2016; Han et al., 2018; FAO, 2021). Consequently, China's aquaculture is transforming to a sustainable development pattern with low input and high output.

From the early 2000s to the present, fishery development policies have undergone great changes in fishing and aquaculture industries. The fishery-related policies aim to control the fishing capacity and resource utilization intensity, and the aquaculture policies have shifted from focusing on quantity growth to quality improvement. China has seriously considered a ban on the direct use of low-valued fish in aquaculture and has promulgated "Outline of China Aquatic Organism Resources Conservation Action" in 2006 and "Several Opinions on Promoting the Sustainable and Healthy Development of Marine Fishery" in 2013 (Han et al., 2018). The 13th Five-Year Plan (FYP) echoes the call for "prioritizing ecosystem and promoting green development," which breaks from the earlier balancing act of "paying equal attention to the development of production and ecological

conservation" stated in the 12FYP. A series of initiatives has been taken since 2016 to improve the mechanisms and methods for marine fishery management. The 13FYP introduced concrete objectives: by 2020, China will decommission 20,000 motorized marine fishing vessels accounting for at least 1.5 million kilowatts of main engine power (double-control target) and reduce the total domestic fishing production to <10 million tons (from ∼13 million tons in 2015) (Ministry of Agriculture, 2016). After 13FYP proposed the updated double-control targets and the first total yield limit, the Ministry of Agriculture issued the *Notice on Further Strengthening the Management of Domestic Fishing Vessels and Implementing the System for Managing Total Marine Fisheries Resources* in February 2017, which outlined China's central agenda for fisheries reforms. To meet these targets, the Chinese government has invested extensive efforts into reviewing and reforming its legislation, institutional arrangements, and governance of marine capture fisheries (Su et al., 2020). These measures have resulted in a sustained and substantial decline in total domestic fishing production from 2017 to 2018 (**Figure 3**). Obviously, China's national development philosophy has gradually shifted from emphasizing economic growth to highlighting ecological protection, and the fisheries resource conservation has become a top priority of marine fishery management in China.

CONCLUSION

This study provides the first large-scale statistic evidence on the links between China's aquaculture and marine wild fish catches changes over the past 30 years. Although aquaculture still puts pressure on marine wild fisheries resources, this practice provides food for China and the world and reduces the huge demand for marine capture fisheries. Results revealed that China's aquaculture is transforming to a sustainable development pattern with low input and high output, and this shift has started to exhibit positive effects. Such evidence is important for evaluating the impact of China's aquaculture on marine wild fisheries resources and contributes to the sustainable management of China's aquaculture and marine capture fisheries.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

JX and KSZ conceived the study. JX, MZ, KW, KHZ, CJX, JYX, and KSZ collected the data and analyzed the data. KSZ, KW, KHZ, CJX, JYX and MZ drafted the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2021.710124/full#supplementary-material

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Heat and Hypoxia Exposure Mediates Circadian Rhythms Response *via* Methylation Modification in *Apostichopus japonicas*

Jiong Wu [1,2,3], *Weiwei Zhang* [1,2,3]* *and Chenghua Li* [1,2,3,4]*

[1] *Key Laboratory of Applied Marine Biotechnology, Ministry of Education, Ningbo University, Ningbo, China,* [2] *Collaborative Innovation Center for Zhejiang Marine High-Efficiency and Healthy Aquaculture, Ningbo University, Ningbo, China,* [3] *School of Marine Sciences, Ningbo University, Ningbo, China,* [4] *Laboratory for Marine Fisheries Science and Food Production Processes, Qingdao National Laboratory for Marine Science and Technology, Qingdao, China*

As global warming progresses, heat and hypoxia are gradually becoming important factors threatening the survival, reproduction, and development of marine organisms. To determine the effect of heat and hypoxia on *Apostichopus japonicus*, whole genome methylation of the respiratory tree was determined under heat, hypoxia, and heat-hypoxia conditions [designed as heat stress treatment (HT), hypoxia treatment (LO), and heat-hypoxia combined treatment (HL) groups]. The number of differentially methylated regions (DMRs) under three treatments was determined based on the Venn diagram. The network of the DMRs associated with promoters that were co-existed under the three conditions showed that circadian rhythm was involved based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses. Circadian rhythm-related genes, *CRY1a*, *CRY1b*, *CLC*, and *TIM*, decreased in LO and HL groups, while *CRY1a*, *CRY1b*, and *BMAL1* increased in the HT group. Bisulfite sequencing PCR (BSP) showed that the methylation levels of CpG island regions in the promoters of *CRY1a* and *CRY1b* were upregulated in HT, LO, and HL groups, leading to the decreased promoter activity of *CRY1a* and *CRY1b*. RNAi of *CRY1a* and *CRY1b* led to increased enzyme activities of two energy-related enzymes, pyruvate kinase (PK) catalyzing the rate-limiting step in glycolysis, and ATPase hydrolyzing ATP to ADP, which were also increased under the three tested conditions. Thus, it was concluded that *A. japonicus* may respond to the heat, hypoxia, and heat-hypoxia stresses *via* the DNA methylation of heat, hypoxia, and heat-hypoxia stresses via the DNA methylation of CpG islands of circadian rhythm-related genes, which increased the activity of energy-related enzymes.

Keywords: ***Apostichopus japonicus*, DNA methylation, heat, hypoxia, circadian rhythm**

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Yunwei Dong,
Ocean University of China, China
Lina Sun,
Chinese Academy of Sciences (CAS), China

***Correspondence:**
Weiwei Zhang
zhangweiwei1@nbu.edu.cn
Chenghua Li
lichenghua@nbu.edu.cn

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INTRODUCTION

Heat stress is a negative factor affecting the production and health of cultured animals, endangering the development of animal husbandry, thus causing serious economic losses and its impacts have been deteriorated due to global warming (St-Pierre et al., 2003; Quinteiro-Filho et al., 2010). In practice, heat stress tends to accompany hypoxia stress commonly, and the increased temperature is always following decreased dissolved oxygen (DO) (Parthasarathy et al., 1992). Therefore, heat and hypoxia are two related stressors that can threaten the cultured animals alone or in synergy

(McBryan et al., 2013; Crozier and Hutchings, 2014; Jenny et al., 2016). Beyond a specific critical temperature, the organisms can expand their passive caloric range by increasing the anaerobic metabolic capacity, inducing molecular protective mechanisms and minimizing metabolic costs through metabolic inhibition (Pörtner et al., 2017). When organisms are continuously exposed to heat and hypoxia, the organisms exhibit many physiological and molecular responses to cope with these stresses, one of which is heat acclimation (Carter et al., 2005; Hung et al., 2005; Magalhães et al., 2010).

Heat acclimation is regulated by epigenetic factors from previous work, such as DNA methylation and histone methylation (Horowitz, 2016). DNA methylation regulates cellular response by transferring methyl to the C5 position of cytosine to form 5-methylcytosine (m5C) with no change in sequence. DNA sequence can be modified and reversed by intracellular and extracellular stimuli such as heat, hypoxia, pollutants, and nutrients (Baylin, 2006; Byun and Baccarelli, 2014). Previous researches have shown that the DNA methylation profiles changed under heat stress (Horowitz, 2016). DNA methyltransferases (DNMTs), such as DNMT1 and DNMT3, have been proven in response to heat stress before the formation of heat acclimation, indicating a relationship between methylation modification and heat stress (Dai et al., 2017, 2018). The pieces of evidence have shown that changes of CpG methylation profile in HSP70 distal promoter region in the chicken and high mitochondrial calcium content in *Rattus norvegicus* may respond to heat acclimation (Assayag et al., 2012; Kisliouk et al., 2017). Data on mitochondrial DNA methylation in human diseases suggest that DNA methylation may play a potential role under the harmful environmental stress (Iacobazzi et al., 2013). Epigenetic-related metabolites produced by mitochondria play an essential role in processes of epigenetic and nuclear transcription, such as histone modification, chromatin remodeling, and nucleosome localization (Shaughnessy et al., 2014). In general, DNA methylation can help organisms to adapt to environmental changes through different adjustment methods.

Apostichopus japonicus are rich in nutrition and medicinal value and distributed across the northwest coast of the Pacific Ocean (Yang et al., 2005; Yuan et al., 2006). The echinoderms are more sensitive to environmental stress than other marine organisms, such as ascidians, mollusks, and anthozoans (Riedel et al., 2012). The heat and hypoxia stresses in summer far exceeded the optimal conditions for the growth and survival of *A. japonicus*, resulting in high mortality (Wang et al., 2015; Xu et al., 2016). To see the response of *A. japonicus* through DNA methylation, the whole-genome DNA methylation of the respiratory tree of *A. japonicus* under heat, hypoxia, and heat-hypoxia stresses were determined. Circadian rhythms drive 24-h cycles in the physiology and behavior of organisms, and it enables organisms to synchronize their internal biology with their external environment to maximize fitness and survival (Yerushalmi and Green, 2009; Martinez-Bakker and Helm, 2015). Many studies have shown that changes in abiotic environmental conditions, such as heat, hypoxia, and toxic chemicals can affect circadian rhythms (Pittendrigh and Caldarola, 1973; Lim et al., 2006; Claudel et al., 2007; Svendsen et al., 2014). In the present study, the methylation changes of promoters of the circadian rhythm-related genes under the heat, hypoxia, and combined stresses, and their probable function were further explored.

MATERIALS AND METHODS

Animals

The *A. japonicus* in the experiment were collected from the Shandong Oriental Ocean Technology Co., Ltd., of Yantai, China, and the *A. japonicus* is 9.5–11 cm in length and 3.5–4.5 cm in width with a wet weight of 100 ± 5 g. After being weighed, *A. japonicus* were kept at 16 ± 0.4°C with 5 mg/L DO for a week. We used 5 mg/L DO because it is the level of DO in seawater with aeration at 26°C, which was coincided with the fact that DO in the living aquarium of the *A. japonicus* in summer is maintained at 5–6 mg/L. During acclimation and experiment, the remaining feed was removed daily. For the stress conditions, 26°C was used for heat stress and 2 mg/L DO was used for hypoxia stress, for the fact that the *A. japonicus* experienced the limits of temperature at 26°C and 2 mg/L DO in summer in its important local living environment of northern Yellow sea and Bohai sea, as well as the definition of hypoxia by the committee on Environment and Natural Resources at the National Science and Technology Council in 2000 (Huang et al., 2012; Liu et al., 2014; Huo et al., 2019). Then, *A. japonicus* were divided into four groups. The standard control (NC) group was maintained in seawater with 5 mg/L DO at 16°C. The hypoxia treatment (LO) group was maintained in seawater with 2 mg/L DO at 16°C. The heat stress treatment (HT) group was maintained with 5 mg/L DO at 26°C. The heat and hypoxia combined treatment (HL) group was maintained with 2 mg/L DO at 26°C.

Under the experimental conditions, the environmental temperature was gradually increased from 16 to 26°C at the rate of approximately 2°C/h using a 100 W heating rod. The environmental DO level was reduced from 5 to 2 mg/L at the rate of approximately 1 mg/L/h to simulate environmental stress *via* air-N_2 gas flow adjustment system, which fills the seawater with oxygen and nitrogen continuously with real-time DO probe detection (Cao et al., 2019). The initial time is defined as the time when the stress group reaches the specified stress condition.

Histology Observation

The respiratory tree, as the unique respiratory organ of *A. japonicus*, plays an important role in respiration and metabolism (Huo et al., 2017). Thus, in this experiment, respiratory tree was chosen to study the mechanism under heat and hypoxia stress. The respiratory tree of *A. japonicus* from each group (NC, HT, LO, and HL) were collected, which were put into paraformaldehyde solution, then in 70% ethanol. The samples were dehydrated in ethanol at a graded series of 70, 75, 85, 95, and 100%, and then embedded in paraffin after rinsing with xylene. The tissue was cut into 7 mm sections and then stained with H&E (Xu et al., 2015). The tissue sections were observed with light microscopy (Nikon Corporation, Japan).

Whole-Genome DNA Methylomics

DNA Extraction and Preparation

After exposure to heat, hypoxia, and heat–hypoxia stress for 48 h, the respiratory trees of six *A. japonicus* from each group (NC, HT, LO, and HL) were taken and mixed in liquid nitrogen. Genomic DNA was isolated from *A. japonicus* respiratory tree using a TIANamp Genomic DNA kit (Tiangen Biotech, Beijing, China). DNA quality was detected by 1% agarose gel electrophoresis.

Library Preparation and Quantification

A total of 5.2 μg of genomic DNA was fragmented to 200–250 bp by sonication using a Bioruptor (Diagenode, Belgium), then the blunt-ending, dA addition to 3′-end, and adaptor ligation. Bisulfite converted from ligated DNA was treated with EZ DNA Methylation-Gold Kit (ZYMO Research, CA, USA) which made the template DNA Illumina HighSeq4000 (BGI, Shenzhen, China) was used for the sequencing of ligated DNA. Sequence data have been deposited at the NCBI BioProject database under accession PRJNA748843.

Methylomic Data Analysis

After sequencing, the raw reads were analyzed by BGI programs by removing contamination, adaptor sequences, and low-quality reads to get the clean data. The clean data were mapped to the genome of *A. japonicus* by whole genome bisulfite sequence mapping program (BSMAP), followed by merging the mapping results and removing the duplication reads (Xi and Li, 2009). The methylation levels in the NC, HT, LO, and HL groups were detected by covering each mC by the total reads covering that cytosine.

Differentially Methylated Region Analysis

The differentially methylated regions (DMRs) were identified by comparison of the treatment groups and the NC group in *A. japonicus* using windows that contained at least five CpG (CHG or CHH) sites with a two-fold change in methylation level and Fisher's test $P \leq 0.05$. The degree of difference of a methyl-cytosine (mCG, mCHG, and mCHH) was calculated while comparing the methylation level of DMRs in different groups by CIRCOS (Krzywinski et al., 2009).

The DMR-related genes were mapped to gene ontogeny (GO) terms in the database for the GO enrichment analysis (http://www.geneontology.org/) to find the significantly enriched GO terms by the hypergeometric test. The Kyoto Encyclopedia of Genes and Genomes (KEGG) identifies significantly enriched metabolic pathways or signal transduction pathways, and the calculating formula is the same as that in GO analysis (Kanehisa et al., 2008).

Bisulfite Treatment of Genomic DNA

Genomic DNA of each group (NC, HT, LO, and HL) was treated with an EZ DNA methylation Gold^{TM} kit (ZYMO Research, California, USA), and the unmethylated cytosine (C) in the sequence was transformed into uracil (U). In the process of PCR amplification, all uracil (U) was transformed into thymine (T), and the bisulfite sequencing PCR (BSP) products were sequenced to determine whether the CpG site was methylated. All the primers used for BSP are shown in **Table 1**.

TABLE 1 | Primers used in this study.

Primers	Sequences
PRIMERS FOR BSP	
CRY1aproF	TTTGGAAGTTTTTTGTATTTTTTG
CRY1aproR	AATCACTACTAATAAAAAACCTAATATTAT
CRY1bproF	GGTTATTTGAGGGGTTTGTAAAATTA
CRY1bproR	CCACAAAAATTCATTAAAAATCATAAAC
CLCproF	TATTAGAAGTTTTTGAATATGGTGATTTAG
CLCproR	ATTAATCATACCAATCCTCATAAACC
BMAL1exonF	TATGAAAAAGGTTTTTAAATAAATTTATAG
BMAL1exonR	ATCTTCATATATTACACAACCATCC
PRIMERS FOR RT-PCR	
BMAL1-F	TTACTGTGCCGAATGCTAATGA
BMAL1-R	GGAATCCCAGATGAGTGGAAATA
CRY1a-F	CTGGATACATCAGGTGGCTCG
CRY1a-R	ACTGGGCAGAAACAGGACGG
CRY1b-F	TCGGCTGTCTTTCATCGC
CRY1b-R	TCCCTCTGCCCATCTTTTC
CLC-F	TTACTGTGCCGAATGCTAATGA
CLC-R	GGAATCCCAGATGAGTGGAAATA
TIM-F	TCAGTGCGTTTCTGTCTCCC
TIM-R	CTGGCATTCGCTTCGGT
β-actin-F	CCATTCAACCCTAAAGCCAACA
β-actin-R	ACACACCGTCTCCTGAGTCCAT

Methylation Modification and Double Luciferase Experiment

To study the effect of methylation level in promoter region on the promoter activity, the CpG free vector was used as described previously (Klug and Rehli, 2006). All the primers used for plasmid construction are shown in **Table 1**. The sequence of all methylation sites of putative cryptochrome-1a (*CRY1a*) was screened by BSP and was further used to construct the recombinant plasmid CpGCRY1apro-1. The sequence of all the methylation sites of putative cryptochrome-1b (*CRY1b*) and the sequence of 84 methylation sites were screened by BSP and were further used to construct the recombinant plasmid CpGCRY1bpro-1 and CpGCRY1bpro-2, respectively. CpGCRY1apro-1, CpGCRY1bpro-1, and CpGCRY1bpro-2 were separately transfected into EPC cells and tested for the promoter activities using the dual luciferase test to the protocol of the CpG Methyltransferase (M.SssI) kit (Thermo Fisher Scientific, MA, USA).

Real-Time Quantitative PCR

The respiratory trees of *A. japonicus* from each group (NC, HT, LO, and HL) were taken with three biological replicates after exposure to stress for 0, 6, and 18 h. After that, RNA was extracted to detect the expression of circadian

rhythm-related genes. Total RNA was isolated from respiratory trees with the Trizol total RNA Kit (Takara Bio, Dalian, China) and treated with DNase I (Sigma, NY, USA) to remove genomic DNA. The concentration was detected *via* Nanodrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA). According to the TaKaRa M-MLV RTase cDNA synthesis kit, 1 μg of RNA was used for cDNA synthesis (TaKaRa Bio, Dalian, China). The β-actin of *A. japonicus* was used as the internal standard. All the primers used for real-time quantitative PCR (qRT-PCR) are shown in **Table 1**. The baseline was set automatically by the software to maintain consistency. The expression levels of the circadian rhythm-related genes were determined by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

Enzyme Activity Measurement

Considering the fact that pyruvate kinase (PK) is the rate-limiting enzyme catalyzing the final step in glycolysis, converting phosphoenolpyruvate (PEP) to pyruvate while phosphorylating ADP to produce ATP and ATPase is a kind of enzyme that catalyzes the hydrolysis of ATP to ADP, the enzyme activities of PK and ATPase were measured because both of which could reflect the energy metabolism of the organism (Lunt and Vander Heiden, 2011). ATPase and PK enzyme activities were measured in the *A. japonicus* collected from groups of NC, HT, LO, and HL. After 24 h of treatment under heat, hypoxia, and heat-hypoxia stress, the respiratory tree was quickly placed into liquid nitrogen, and the cell lysate was obtained after grinding. The BCA Protein Assay Kit detected the protein concentration. PK activity and ATPase activity were detected using the pyruvate kinase assay kit and the ATPase assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

To see if the *CRY1a* and *CRY1b* gene were involved, dsRNA *CRY1a* and dsRNA *CRY1b* were applied. The dsRNA was obtained using the MEGAscript™ T7 Transcription Kit (Thermo Fisher Scientific, MA, USA) by primers whose production was about 500 bp, with the T7 promoter sequence added in the forward primer. The designed fragment did not overlap with the quantitative verification fragment. In the study, 50 μl *CRY1a* dsRNA or *CRY1b* dsRNA were injected into *A. japonicus* using a 1 ml syringe. The expressions of *CRY1a* and *CRY1b* after interference were performed to verify its success interference rate. The *A. japonicus* were divided into the *CRY1a* interference group and *CRY1b* interference group. After interference, the enzyme activity of ATPase and PK was measured after 24 h of treatment under heat, hypoxia, and heat–hypoxia stresses.

Statistical Analysis

Statistical analysis was performed using the two-tailed Student's *t*-test. One-way ANOVA was applied to determine the differences between the control and the experimental groups. Any significant difference relative to the control for each time point was indicated using $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

RESULTS

Changes in the Morphology of the Respiratory Tree Under Standard Control (NC), Heat Stress Treatment (HT), Hypoxia Treatment (LO), and Heat-Hypoxia Combined Treatment (HL) Groups

When the *A. japonicus* was reared in seawater in the groups of heat stress treatment (HT), hypoxia treatment (LO), and heat-hypoxia combined treatment (HL), the morphology of the respiratory tree changed dramatically. Using light microscopy, the structural changes in the respiratory tree were observed (**Figure 1A**). The size of the muscular layer marked the overall outline of the respiratory tree. In the standard control (NC) group, the thickness of connective tissue was high, and the dendritic lining epithelium was well-distributed and well-organized. The atrophy of connective tissue and dendritic lining epithelium was observed in all three groups. The thickness of connective tissue decreased, and the degeneration of connective tissue was the most obvious in HL group among all the three treatment groups. The dendritic lining epithelium and the dendritic lining epithelium, distributing on the inner side of connective tissue, continued to be shrinking in all three treatment groups.

Genome-wide DNA Methylation Patterns of *A. japonicus*

To further understand the molecular mechanism after the heat, hypoxia, and heat-hypoxia stresses, the genome-wide DNA methylation patterns of *A. japonicus* were investigated. Approximately 4% of all genomic C sites were methylated. Methylation in the DNA of *A. japonicus* was found to exist in three sequence sites: CG, CHG, and CHH (where H is A, C, or T), and 25.522% CG, 0.585% CHG, and 0.597% CHH was methylated in the mapping reads. A higher rate of methylated sites was present in the structure gene regions of internal exon and internal introns. Among all the regions, the average level of methylation in promoter regions was the lowest (**Figure 1B**). The DNA methylation in the other three treatment groups (HT, LO, and HL) showed the same characterization in the control sample.

DMR Analysis

DMRs were detected to characterize the differences in whole-genome DNA methylation levels among NC and HT, LO, and HL groups, respectively. A total of 43,929, 39,820, and 43,901 DMRs were separately identified in the three treatment groups compared with the NC group, which were mainly related to the methylation of CG sites (**Table 2**). DMRs could be divided into two contents, one kind located inside the structural gene regions (named DMRs associated with genes), and another kind located inside the promoter region (named DMRs associated with promoters). Approximately 19% of DMRs were identified as DMRs associated with the gene, and 8% of DMRs were identified as DMRs associated with the promoter (**Figure 1C**).

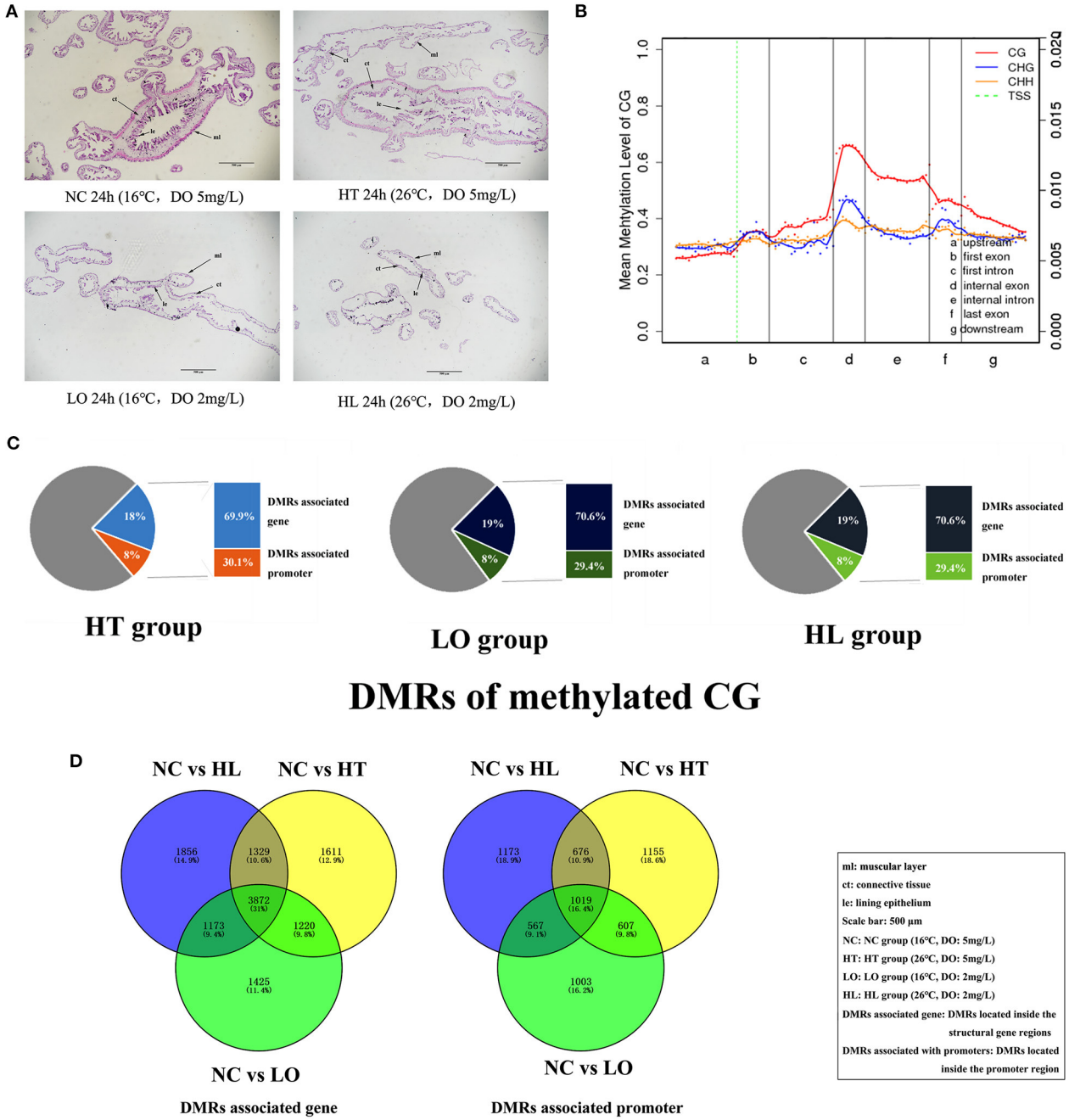

FIGURE 1 | (A) Tissue section of the respiratory tree in *A. japonicus* under heat stress (26°C, 5 mg/L DO), hypoxia stress (16°C, 2 mg/L DO), heat–hypoxia stress (26°C, 2 mg/L DO), and NC group (16°C, 5 mg/L DO) for 24 h. ct, connective tissue; le: lining epithelium; ml, muscular layer. Scale bar 500 μm. **(B)** Canonical DNA methylation profiles of the entire transcriptional units of the NC group. **(C)** Pie chart proportion of DMR corresponding genes, promoters, and other regions. **(D)** Venn diagram of the DMRs in the NC, HT, LO, and HL groups.

The DMRs associated with genes and DMRs associated with promoters were analyzed by GO and KEGG. The GO analysis showed that DMRs associated with genes or promoters were significantly enriched in the biological process of cellular process, metabolic process, cellular component of membrane, membrane part and molecular function of binding, and catalytic activity in all the three groups of HT, LO, and HL (**Figure 2**). Kyoto Encyclopedia of Genes and Genomes analysis showed that these DMRs associated with promoters were enriched in a notch signaling pathway and circadian rhythm in the groups of HT, LO, and HL (**Figure 3A**). The DMRs associated with genes were significantly enriched in ubiquitin-mediated proteolysis,

TABLE 2 | DNA methylation patterns of *A. japonicus* of the four groups (CN, HT, LO, and HL group).

Samples	Clean reads	Clean bases (BP)	Total mapped reads	Mapping rate (%)	Bisulfite conversion rate (%)	DNA methylation sites compared with NC			gDMR associated with gene			gDMR associated with promoter		
						CG	CHG	CHH	CG	CHG	CHH	CG	CHG	CHH
CN	166294858	24944228700	88773790	53.38	99.59									
HT	168344670	25251700500	84274018	50.06	99.62	43842	11	76	8032	2	33	3457	2	5
LO	178772156	26815823400	90073198	50.38	99.63	39745	4	71	7690	0	28	3196	0	2
HL	151900758	22785113700	80187234	52.79	99.61	43810	6	85	8230	1	33	3435	0	6

lysosome, and other glycan degradation in the groups of HT and HL, while significantly enriched in apoptosis and fatty acid metabolism in the groups of LO (**Figure 3B**).

Through the analysis of the Venn diagram of the DMRs in the groups of NC, HT, LO, and HL, 3,872 DMRs associated with genes and 1,019 DMRs associated with promoters were identified in all of the three treatments groups simultaneously (**Figure 1D**). Then, further analysis of the network of signal pathways of the 3,872 DMRs associated with genes and 1,019 DMRs associated with promoters were performed through KEGG enrichment (**Figures 4**, **5**). The network of DMRs associated with genes was enriched in a big category, such as endocrine resistance, Th1 and Th2 cell differentiation, thyroid hormone signaling pathway, and dorso-ventral axis formation, which may take functions with notch signaling pathway. The network of DMRs associated with promoters was mainly enriched into two categories. One category consisted of the notch signaling pathway and the mRNA surveillance pathway. The other category consisted of the circadian rhythm, the calcium signaling pathway, the oxytocin signaling pathway, and the apelin signaling pathway. The circadian rhythm was first enriched in KEGG in all the treatment groups of DMRs associated with promoters from the results above. The circadian rhythm was further enriched in the network of signal pathways *via* the KEGG enrichment of DMRs in all of the three treatments groups simultaneously selected from the Venn diagram. In addition, circadian rhythm took the functions as a center and regulated peripheral signaling pathways in the network of DMRs associated with promoters. So, circadian rhythm was chosen for further study.

Relative Expression of Circadian Rhythm-Related Genes Under Heat and Hypoxia

Five circadian rhythms-related genes were identified from the genome of *A. japonicus*: *CRY1a*, *CRY1b*, Putative aryl hydrocarbon receptor nuclear translocator like 1 (*BMAL1*), Clock (*CLC*), and Timeless (*TIM*). The expression of circadian rhythm-related genes was studied after heat, hypoxia, and heat–hypoxia stress for 0, 6, and 18 h (**Figure 6A**). At the HT group, *BMAL1* increased significantly and peaked at 6 h (6.88-fold of the control, $P < 0.01$). The expressions of *CRY1a* and *CRY1b* reached peaked at 18 h (2.56- and 2.63-fold, respectively, $P < 0.05$). There was no significant difference in *CLC* and *TIM*. In the LO group, the expression of *CRY1a* decreased continuously and reached the lowest level at 18 h (0.55-fold of the control, $P < 0.05$). However, the expression of *CRY1b*, *CLC,* and *TIM* decreased at 18 h, while *BMAL1* had no changes. The expressions of *CRY1a*, *CRY1b*, *BMAL1*, *CLC*, and *TIM* were significantly decreased in the group of HL ($P < 0.05$).

Methylation of the Promoters of Rhythmic Genes

Among the five circadian rhythms-related genes, *CRY1a*, *CRY1b*, and *CLC*, *BMAL1* possessed hypermethylated CpG island regions when identified through the whole genome DNA methylomics.

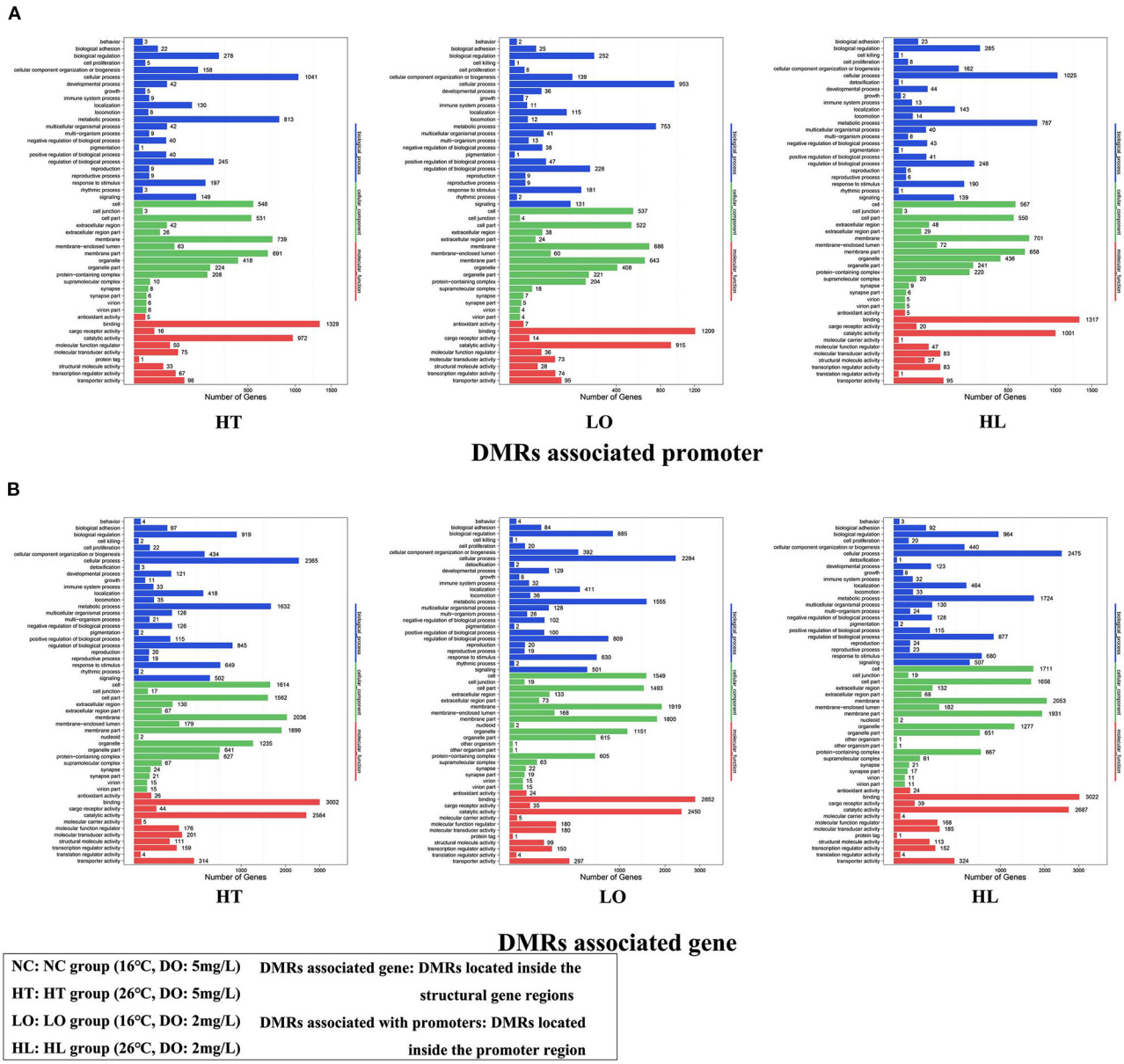

FIGURE 2 | (A) Gene ontogeny (GO) assignments of DMRs associated promoters of HT, LO, and HL groups compared with NC group. **(B)** GO assignments of DMRs associated genes of HT, LO, and HL groups compared with NC group. The abscissa represents the number of genes or promoters associated with methylation, and the ordinate represents the GO terms. Different colors represent the three GO terms, such as cell components, biological processes, and molecular functions.

There are four high CpG islands as follows: *CRY1a* promoter region (site 299378–site 299529), *CRY1b* promoter region (site 52083–site 52112), *CLC* promoter region (site 3142–site 3218), and *BMAL1* exon 1 region (site 280973–site 281011). The methylation sites of *CRY1a*, *CRY1b*, *BMAL1,* and *CLC* in these regions were identified under heat, hypoxia, and heat–hypoxia stresses (**Figure 7A**). The methylation level of *CRY1a* CpG island region showed significant differences under heat, hypoxia, and heat–hypoxia stresses compared with the group of NC. The probability of methylation was 30% in the HT group and about 10% in the LO group, and 10% HL group compared with the control group. The probability of methylation of *CRY1b* CpG island region did not change, except for the methylation of site 84 significantly increased in the group of HT, LO, and HL, with about 30% probability of occurrence. The methylation level of *BMAL1* and *CLC* did not change significantly compared with the NC group. The results of the dual-luciferase assay showed that when CpGCRY1apro-1 or CpGCRY1bpro-1 was methylated, the promoter activity containing the CpG island region decreased significantly ($P < 0.05$, **Figure 7B**). In addition, the activity of

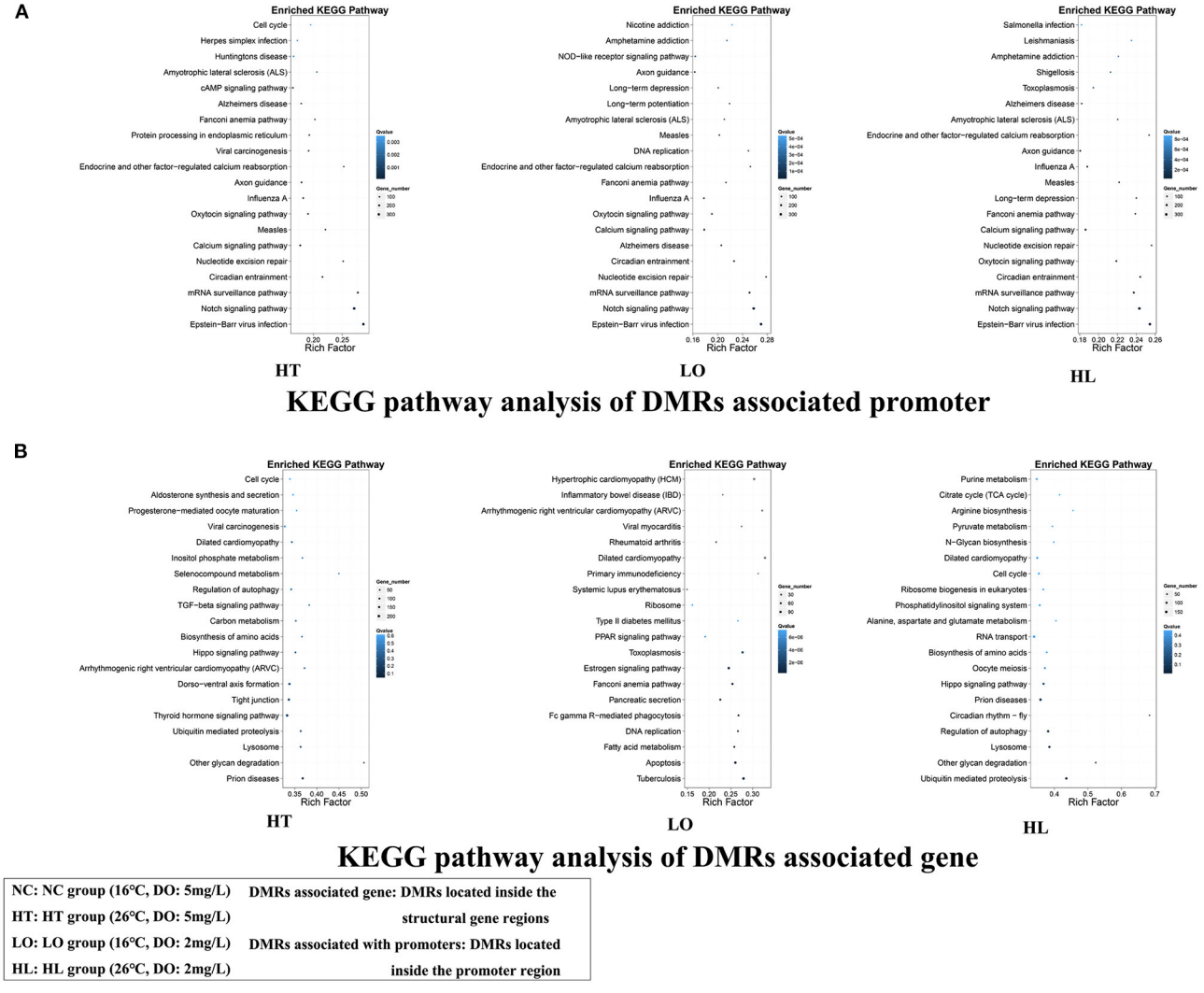

FIGURE 3 | (A) Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis of DMRs associated promoters of HT, LO, and HL groups compared with NC group. **(B)** KEGG pathway analysis of DMRs associated genes of HT, LO, and HL group compared with NC group. The abscissa represents the proportion of the number of DMRs belonging to a specific KEGG pathway to the whole number of differentially expressed genes, and the ordinate represents the specific KEGG pathway.

CpGCRY1bpro-1 and CpGCRY1bpro-2 decreased to a similar extent after methylation.

Function of CRY1a and CRY1b Under Heat, Hypoxia, and Heat–Hypoxia Stress

The response of aquatic species to heat and hypoxia is to enter a state of low metabolism, reduce metabolic rate, suspend development and reproduction, and survive in the critical ecological changes. The activities of two enzymes, PK and ATPase, which dominated the energy levels, were detected under the three tested conditions (**Figure 6B**). Compared with the enzyme activity of the normal *A. japonicus* in the NC group (39.4 U/mgprot), the PK activity in the HT group and HL group increased significantly ($P < 0.05$), but there was no significant difference in the LO group ($P > 0.05$). Compared with the ATPase activity of *A. japonicus* in the NC group (102.3 U/mgprot), the ATPase activity increased significantly in the HT group (141.3 U/mgprot, $P < 0.001$). In comparison, it decreased in the LO group (66.1 U/mgprot, $P > 0.05$), and there was no significant difference in the ATPase activity in the HL group.

To see if *CRY1a* and *CRY1b* were involved in the energy generation, *CRY1a* and *CRY1b* were interfered *in vivo*. The PK activity in the interference groups was significantly increased under all three conditions ($P < 0.001$). In *the CRY1a* interference group, the highest PK activity was found in the HT group (84.5 U/mgprot), followed by the HL and LO groups. In the *CRY1b* interference group, the highest PK activity was found in the HL group (122.4 U/mgprot), followed by the HT group and LO group. The PK activity in the dsRNA *CRY1b* group was higher than that in the dsRNA *CRY1a* group. Compared with the control group, the ATPase activity of the interference groups was also significantly increased ($P < 0.001$). Among the

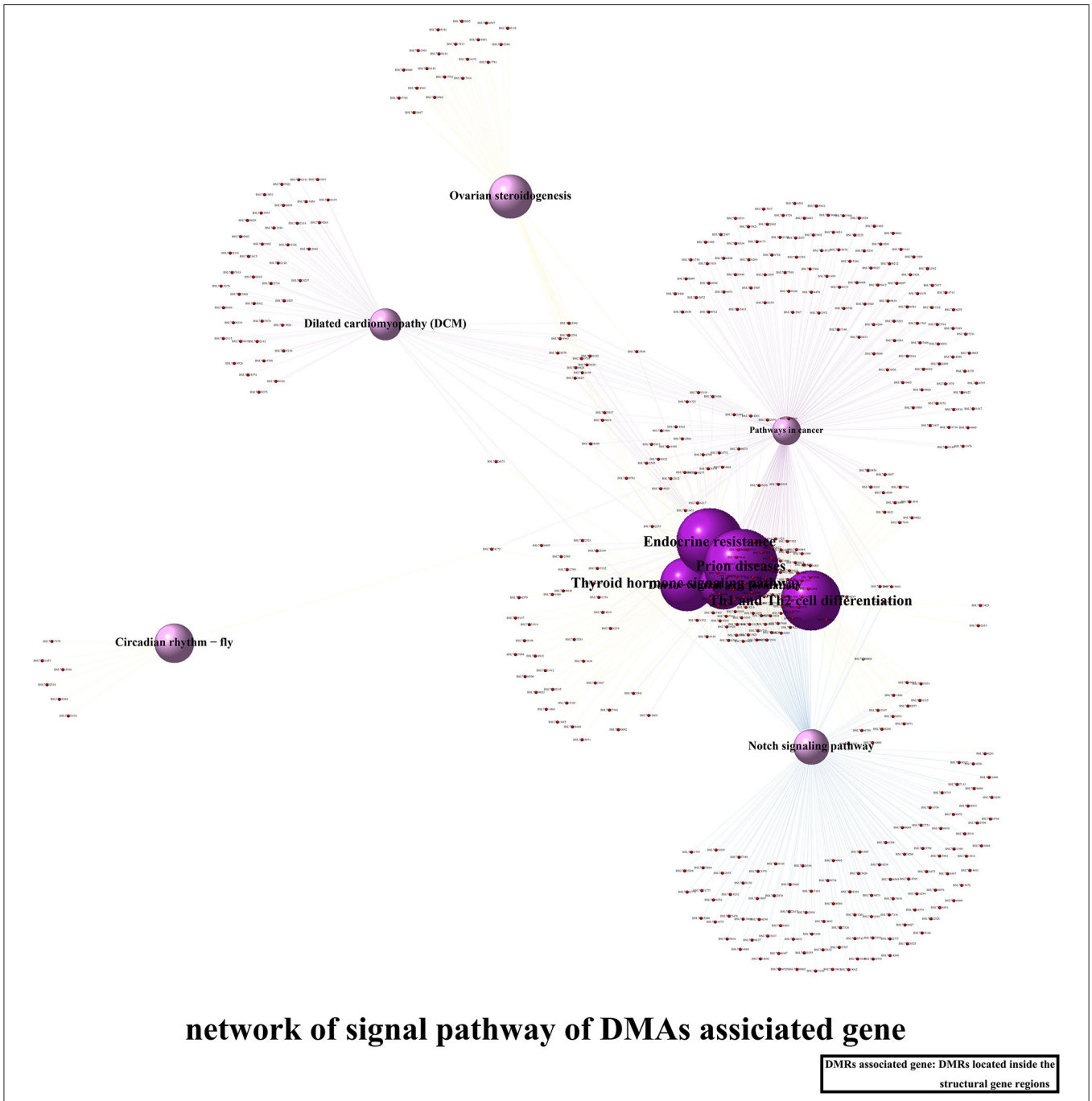

FIGURE 4 | Network of signal pathways *via* the KEGG enrichment of DMRs associated genes in all of the three treatments groups simultaneously selected from Venn diagram.

CRY1a interference groups, ATPase activity was highest in the HT group (883.6 U/mgprot). Among the *CRY1b* interference groups, ATPase activity was highest in the HL group (874.3 U/mgprot) (**Figure 6B**).

DISCUSSION

From the results of the respiratory tree became smaller and shrunk with small diffuse debris suffering from heat and hypoxia stress, it suggested that the respiratory tree was sensitive to heat and hypoxia, and can well-reflect the changes of environmental stress on the respiratory tree (Huo et al., 2018). After analyzing various methylation patterns (CG, CHG, and CHH) of whole-genome DNA in the respiratory tree of *A. japonicus*, most DNA methylation occurred at CpG sites, which was consistent with the methylation happened in mammals (Lister et al., 2011; Tomizawa et al., 2011; Ziller et al., 2011). It was shown that 19% of DMRs associated with genes and 8% of DMRs

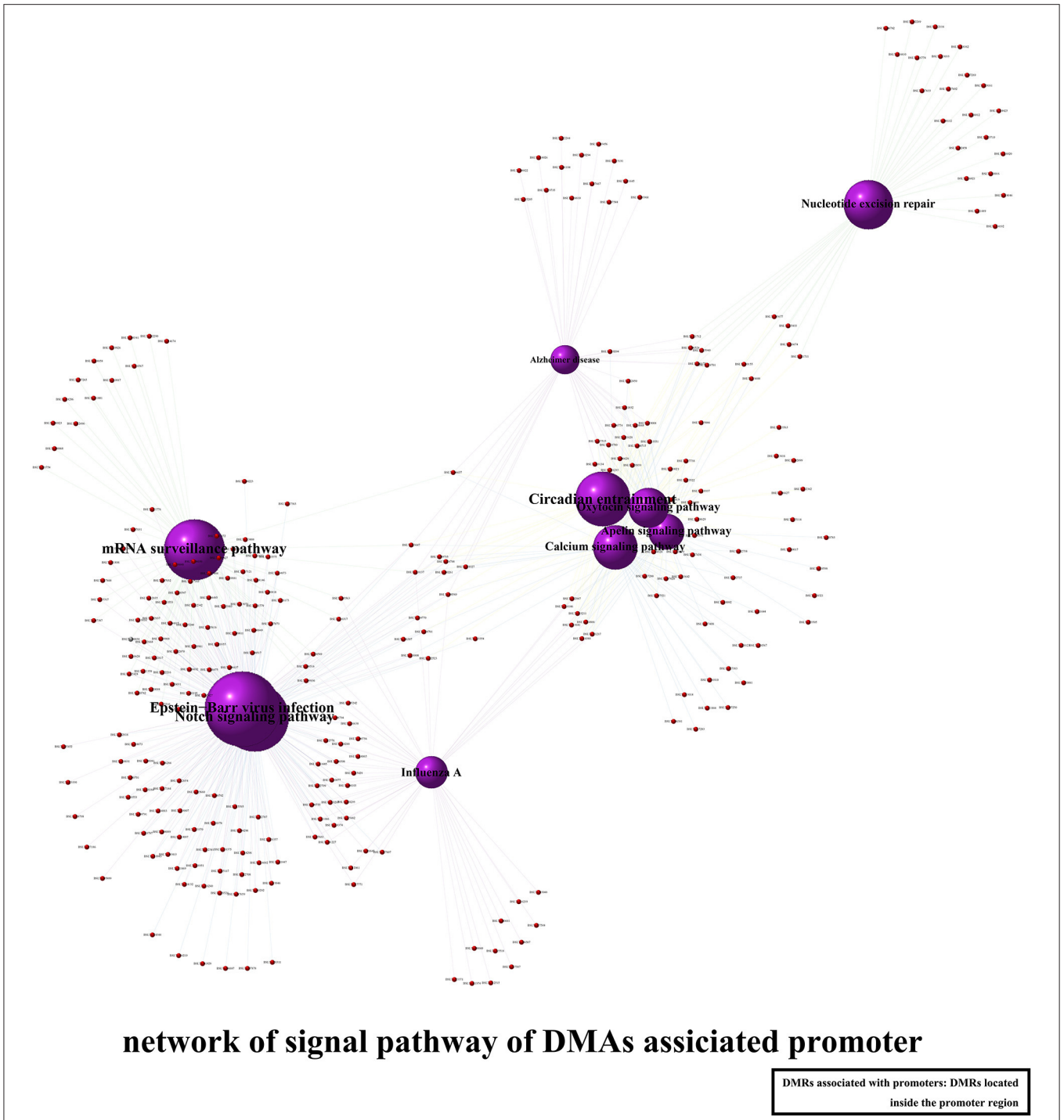

FIGURE 5 | Network of signal pathways *via* the KEGG enrichment of DMRs associated promoters in all of the three treatments groups simultaneously selected from the Venn diagram.

associated with promoters might significantly influence the gene expression. DMRs localized in the internal sequence of the gene, such as internal exon and internal introns, which may affect gene expression *via* changes in chromatin structure or transcription efficiency (Lorincz et al., 2004; Klose and Bird, 2006; Suzuki and Bird, 2008). DMRs located in promoter regions which were detected in this study, the activity of CpG regions in promoters of *CRY1a* and *CRY1b* decreased modified by *in vitro* methylation. There are two explanations for the mechanism of DNA methylation maintaining promoter

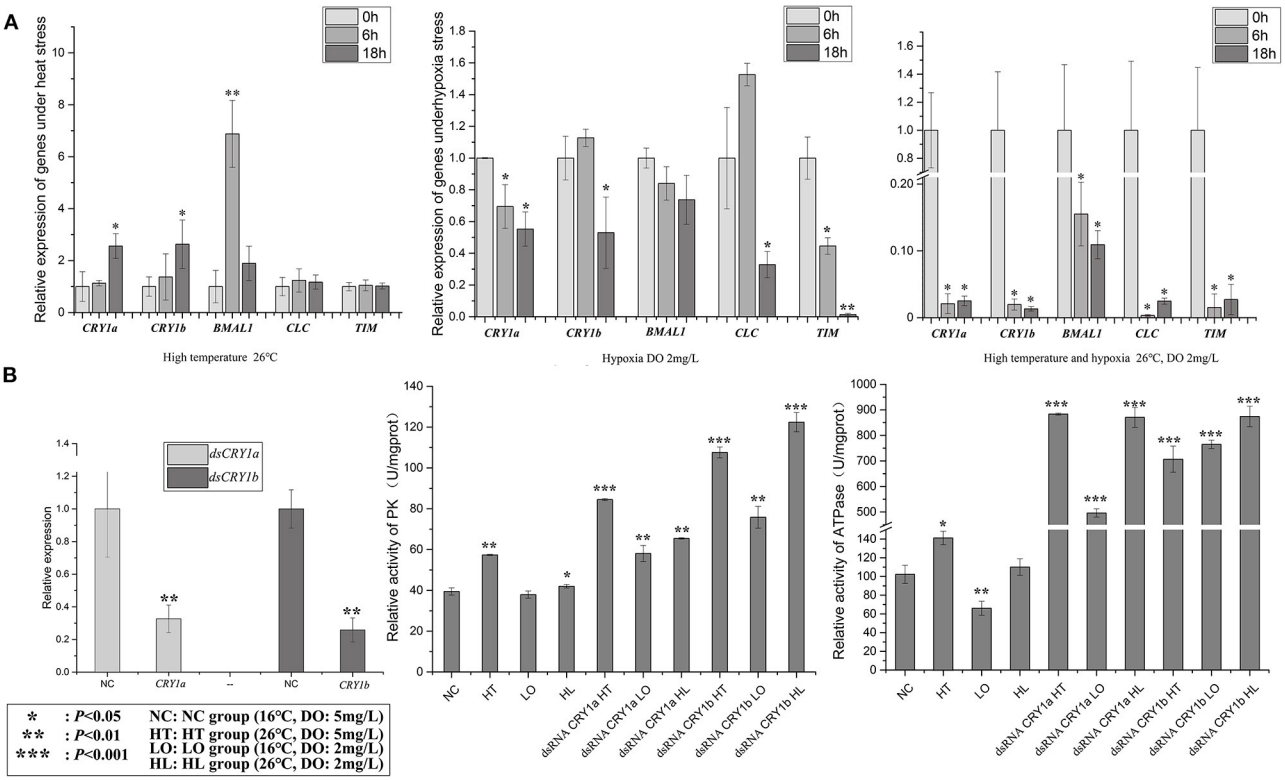

FIGURE 6 | (A) The relative expression of genes of circadian rhythm under heat stress, hypoxia stress, and heat–hypoxia stress. **(B)** The relative expression of *CRY1a* and *CRY1b* after interference and relative activity of PK and ATPase under heat stress, hypoxia stress, and heat–hypoxia stress for 24 h and the interference groups of dsRNA *CRY1a* and dsRNA *CRY1b* under heat stress, hypoxia stress, and heat–hypoxia stress for 24 h.

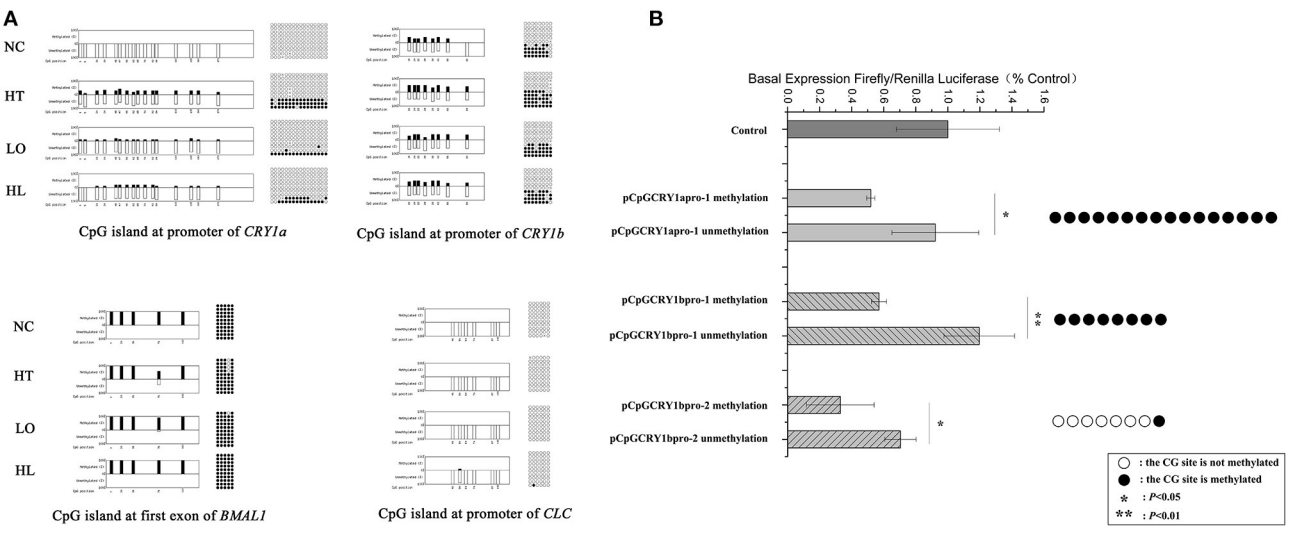

FIGURE 7 | (A) Methylation sites of *CRY1a*, *CRY1b*, *BMAL1*, and *CLC* under heat stress, hypoxia stress, and heat–hypoxia stress. The histogram represents the proportion of methylation and unmethylation, black represents methylation and white represents unmethylation. Dot graph: each row represents a single clone, 10 replicates. Each dot represents a CpG dinucleotide, the white dot represents unmethylated CpG, and the black dot represents methylated CpG. **(B)** Promoter activity of *CRY1a*, *CRY1b,* CpGCRY1apro-1, and CpGCRY1bpro-1 selected all methylated sequences from **(A)**, and CpGCRY1bpro-2 selected the methylation sequence at site 84 from **(A)** (the sequence was screened by repeated experiments).

silencing. First, methylation changes chromatin structure and inhibits transcription factors and co-transcription factors, thereby reducing the gene expression. Second, DNA methylation can recruit methyl binding proteins (MSPs) to inhibit chromatin state through interaction-maintained promoter silencing (Campanero et al., 2000; Joulie et al., 2010; Deaton and Bird, 2011). The results of GO and KEGG of DNA methylation status of promoter and gene regions showed that notch signaling pathway, Th1-Th2 pathway, apelin pathways, and circadian rhythm were enriched, which were all regulated by circadian rhythm (Arjona and Sarkar, 2006; Henley et al., 2009; Cai et al., 2018).

Furthermore, Li discovered *Klf2* and *Egr1* exerted the effects through a clock gene-controlled process through genome sequence analysis under heat and hypoxia stress (Li et al., 2018). It indicated that circadian rhythm might act as a manager to regulate numerous signaling pathways in response to heat and hypoxia in *A. japonicus*. Although global DNA methylation has been analyzed in the respiratory tree of *A. japonicus* by Guo (Guo et al., 2013) and Zhao (Zhao et al., 2015), only the differences in overall DNA methylation levels between the respiratory tree and other tissues were discussed without defined pathways analysis. This study is the first to systematically explore the DNA methylation under heat, hypoxia, and simultaneous heat–hypoxia stresses.

In this study, the expression of *CRY1a*, *CRY1b*, *BMAL1*, *CLC*, and *TIM* in HT, LO, and HL groups suggested that circadian rhythm may play an important role in *A. japonicus* response to environmental stressors. The expressions of *CRY1a* and *CRY1b* increased in the group of HT, which were consistent with the study that heat upregulated the expression of *CRY* (Ji et al., 2018) but were inversely proportional to the results of DNA methylation inhibiting the activity of CpG regions in our experiment. In comparison, decreased expression of *CRY1a* and *CRY1b* in the groups of LO and HL were consistent with results of DNA methylation inhibiting the activity of CpG regions in our experiment. Studies have shown no correlation between promoter DNA methylation and transcriptional activity of some genes (Grimm et al., 2013; Vucic et al., 2014; Yoo et al., 2015). Additionally, we only selected 200 bp CpG island regions for promoter activity detection, which cannot represent the whole promoter activity.

Moreover, promoter DNA methylation can also be involved in regulating the expression of other distal genes (Bell et al., 2011; Yoo et al., 2015). Then we compared the stress conditions of the HT group and groups LO and HL. The cause of this phenomenon may be hypoxia stress. The study showed that hypoxia stress increased HIF-1 α, which decreased *CRY* content to some extent (Dimova et al., 2019). The circadian rhythms may be destroyed in extremely harsh environments, such as heat–hypoxia. Therefore, DNA methylation is not a single negative regulatory relationship with gene expression, and we need to further study the mechanism of methylation modification on gene expression of *CRY1a* and *CRY1b*.

To study the relationship between circadian rhythm and energy metabolism, PK and ATPase enzyme activities in groups of NC, HT, LO, and HL were detected, which were consistent with the expressions of *CRY1a* and *CRY1b*. The expression level and enzyme activity increased in the HT group while decreasing in LO and HL groups. In addition, the activity of PK and ATPase increased significantly after interfering with *CRY1a* and *CRY1b,* which suggests that *CRY1a* and *CRY1b* may inhibit PK activity and ATPase to some extent. As lower basal metabolic rate in heat acclimation organisms, circadian rhythm may contribute to the formation of heat acclimation by regulating the metabolizing enzyme activity. Studies have shown that heat stress delays the circadian rhythm of subsequent activities for several hours under constant temperature and dark conditions, compared with flies without heat stress (Sidote et al., 1998). It is suggested that heat may cause the rhythm delay of *CRY1a* and *CRY1b*, leading to dynamically change in activities of PK and ATPase to satisfy the energy demand of the body or a result of metabolic compensation (Lemos et al., 2003).

In general, this study provided a comprehensive analysis of DNA methylation in *A. japonicus*, and circadian rhythm was determined to be involved in response to the heat, hypoxia, and heat–hypoxia stresses *via* DNA methylation. Therefore, the results of this study might provide new clues for deciphering the response of *A. japonicus* to the global warming signals *via* the circadian rhythm and methylation modification.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/, PRJNA748843.

AUTHOR CONTRIBUTIONS

JW performed the experiments, interpreted the data, and wrote the manuscript, and WZ and CL participated in the experimental design, interpreted the data, and supervised the research project. All authors approved the final draft.

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Metagenomic Analysis of Bacterial Communities and Antibiotic Resistance Genes in *Penaeus monodon* Biofloc-Based Aquaculture Environments

Xieyan Chen[1], Zihao He[1], Jichen Zhao[1], Minze Liao[1], Yuan Xue[1], Jianing Zhou[1], Rowena Hoare[2], Sean J. Monaghan[2], Na Wang[3], Huanying Pang[1,4] and Chengbo Sun[1,5]**

[1] College of Fisheries, Guangdong Ocean University, Zhanjiang, China, [2] Institute of Aquaculture, University of Stirling, Stirling, United Kingdom, [3] Chinese Academy of Inspection and Quarantine, Beijing, China, [4] Guangdong Provincial Key Laboratory of Pathogenic Biology and Epidemiology for Aquatic Economic Animal, Key Laboratory of Control for Disease of Aquatic Animals of Guangdong Higher Education Institutes, Zhanjiang, China, [5] Guangdong Provincial Laboratory of Southern Marine Science and Engineering, Zhanjiang, China

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Teresa Maria Coelho Baptista,
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Ana Teresa Gonçalves,
GreenCoLAB, Portugal

***Correspondence:**
Huanying Pang
phying1218@163.com
Chengbo Sun
suncb@gdou.edu.cn

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Biofloc technology (BFT) is one of the most promising technologies in global aquaculture for the purpose of improving water quality, waste treatment, and disease prevention in intensive aquaculture systems. However, characterization of the microbial species and antibiotic resistance potentially present in biofloc-based aquaculture environments is needed. In this study, we used high-throughput sequencing technology to comprehensively compare the bacterial communities in mariculture ponds of *Penaeus monodon* (*P. monodon*), by testing of water, biofloc, and intestine of *P. monodon*. Operational taxonomic units (OTUs) cluster analysis showed that the nine samples tested divided into 45 phyla and 457 genera. *Proteobacteria* was the dominant bacteria in water, biofloc and prawn intestine. In biofloc and intestine, the *Ruegeria* (2.23–6.31%) genus represented the largest proportion of bacteria, with *Marivita* (14.01–20.94%) the largest group in water. Microbial functional annotation revealed that in all the samples, genes encoding metabolism were predominant. The antibiotic resistance gene annotation showed the highest absolute abundance of *patB*, *adeF*, *OXA-243*, and *Brucella_suis_mprF* from *Proteobacteria*. *PatB* (11.33–15.01%), *adeF* (15.79–18.16%), *OXA-243* (35.65%), and *Brucella_suis_mprF* (10.03%) showed the highest absolute abundance of antibiotic resistance genes in water, biofloc, and intestines, respectively. These findings may greatly increase our understanding of the characteristics of the microbiota of shrimp biofloc-based aquaculture systems and the complex interactions among shrimp, ambient microflora, and environmental variables. It provides a reference basis for policy on breeding, environmental safety, and maintaining food safety in the production of *P. monodon*.

Keywords: metagenomic analysis, antibiotic resistance genes, bacterial community, *Penaeus monodon*, biofloc

INTRODUCTION

With the pollution of the marine aquatic environment and the decline of the wild fishing industry, aquaculture plays an increasingly important role in the sustainable supply of food. *Penaeus monodon* (*P. monodon*) (giant tiger shrimp) aquaculture accounts for a significant proportion of seafood consumption and is the second most farmed variety of shrimp worldwide (FAO, 2020).

However, bacterial diseases such as death by early mortality syndrome (EMS), acute hepatopancreatic necrosis disease (AHPND), and hepatopancreatic necrosis syndrome (HPNS) have reduced shrimp production by nearly 40%, resulting in global losses of over 1 billion dollars per year for shrimp aquaculture (Flegel, 2012; Lee et al., 2015; Huang et al., 2016). Furthermore, the misuse and unmonitored discharge of antibiotics has led to the proliferation and spread of antibiotic resistance genes (ARGs) and antibiotic-resistant bacteria in the aquatic environment, leading to poor outcomes for aquaculture and the surrounding environments (Pruden, 2014; Shlrene et al., 2020). The occurrence of ARGs, as environmental contaminants, in aquatic environments is an emerging public health concern, attracting global attention (Pruden et al., 2006; Sui et al., 2016; Dang et al., 2017).

As an environmentally friendly and economical aquaculture model, biofloc technology has continued to attract attention in aquaculture systems around the world (Wilson et al., 2006; Yoram, 2007; Mishra et al., 2008). A biofloc-based aquaculture system is a microbial treatment based on the composition of abundant microflora. In this system, chemoautotrophic and heterotrophic bacteria are involved in the formation of biofloc, which also include fungi, protozoa, zooplankton, and microalgae, which decompose food remnants and animal waste, remove excess ammonia and nitrogen from the water, and maintain a stable nutrient level (Yoram, 1999; Wei et al., 2020). Animal productivity is intimately linked to health and the gut microbiome is becoming increasingly recognized as an important driver of cultivation success (Corey et al., 2020). Besides, effective microorganisms can increase survival rates of shrimp and decrease the feed conversion ratio (Huang, 2009). In addition, biofloc can be used as a supplementary food source for animals, which can improve the growth performance and immunity of cultured organisms (Julie et al., 2014; Kim et al., 2015). Biofloc technology (BFT) cannot only enhance growth performance of an organism, elevate digestive enzyme activity, and strengthen antioxidant status, but also has been shown to improve immune response through the probiotic and immunostimulant effects of the microorganisms present in the biofloc (Yu et al., 2020).

In fact, most eukaryotes maintain close mutualistic relationships with microorganisms that are, in most cases, linked to their nutrient acquisition and thereby crucial for their performance and survival (Stéphane et al., 2015). There have been few studies of the bacterial communities and ARGs of biofloc (Surapun et al., 2020). In order to understand the optimal conditions for establishing and maintaining a biofloc, it is necessary to characterize the microorganisms present. Culture-dependent approaches present a limitation to characterizing the complete microbial population present in biofloc. It has been estimated that the cultivation approach commonly reveals only a small portion of the bacterial population present, of which only 0.1% has characterized (Zhou et al., 2019). Microbial ribosomal RNA (rRNA) genes can be detected and sequenced directly from water or biofloc without the need for culture. In this study, we used metagenomic sequencing to comprehensively compare the structure and function of bacterial communities in water, biofloc, and intestinal samples from a biofloc-based aquaculture system of *P. monodon* and analyzed the ARGs therein. This study provides data to support future improvements of the biofloc-based aquaculture system of *P. monodon*, which will inform the policies of breeding, environmental safety, and assist in maintaining food safety for this valuable species.

MATERIALS AND METHODS

Pond Conditions

The *P. monodon* culture ponds used in this study were 3 m × 4 m, with a depth of 1.5 m and water depth of 1.2 m, the seedling size was 1.0 ± 0.1 cm, and the seedling density was 500 shrimp seeds/m^3. The culture was according to the biofloc-based aquaculture environments. Glucose was used to adjust the C/N (carbon to nitrogen ratio) and sodium bicarbonate was used to adjust the total alkalinity and pH.

Sample Collection and Extraction of Total DNA of Microbial Flora

Samples were collected on June 15, 2020 from three shrimp biofloc ponds, located at Donghai Island Marine Biological Research Base, Guangdong Ocean University, Zhanjiang, Guangdong, China. At the time of sampling, the culture time of shrimp was 150 days and the average body length and body weight of puffers were 11.25 ± 0.79 cm and 19.67 ± 3.84 g, respectively. To fully reflect the pond condition, each intestinal sample randomly captured the intestines of 60 shrimps from a pond and pooled them into one sample. Water and biofloc samples were collected at five locations in the pond (the four diagonal and middle locations of the pond) and mixed into one sample. The suspended matter was settled by standing water samples for 1 to 2 h. The supernatant was filtered through a membrane (0.45 μm, Jinteng) with a diaphragm vacuum pump (give make and model of pump). Each filter membrane enriched with microorganisms was installed into a Biofil 50-ml centrifuge tube and labeled A1, A2, and A3, respectively. Biofloc samples of 25 ml were taken from the biofloc pool and labeled B1, B2, and B3, respectively. The intestinal samples were dissected by sterile scalpel and the entire intestine was removed under sterile conditions. The intestinal segments were immediately placed on ice in a RNA-free tube and labeled C1, C2, and C3, respectively. Each intestinal sample tube was placed in liquid nitrogen, transported to the laboratory, and stored at −80°C prior to DNA extraction. During 150 days of the study, no disease occurred in the shrimp and no antibiotics or drug treatments were applied to the ponds. The three ponds use water from the sea at the farming site. Hengxing shrimp compound feed was used throughout the whole process and the same management was carried out during the breeding period. The genomic DNA of water, biofloc, and

intestinal samples was extracted by the cetyltrimethylammonium bromide (CTAB) method (Khalid et al., 2021) and the purity and concentration of DNA was measured by agar gel electrophoresis. DNA samples were diluted to 1 μg/μl with sterile water.

Physicochemical Parameters of Water Quality

Water quality indicators were tested at the same time as sampling. The pH and temperature were measured *in situ* with Shanghai Sanxin SX620 measuring pen. Concentrations of nitrite (NO_2^-N), nitrate (NO_3^-N), ammonia (NH_4^+-N), and total alkalinity (TA) were determined by the chemical method (Lei, 2006).

16S Ribosomal RNA Gene Amplification and Sequencing

To investigate the microbial communities of water, biofloc, and intestinal samples, the 16S rRNA gene was amplified using the diluted genomic DNA as the template and a set of universal primes, 16S V3-V4 primer 515F (5′- GTGCCAGCMGCCGCGGTAA-3′) and 806R (5′-GGACTACHVGGGTWTCTA AT-3′). All the PCR reactions were carried out with 15 μl of the Phusion® High-Fidelity PCR Master Mix (New England Biolabs, America), 2 μM of forward and reverse primers, and about 10 ng of template DNA. The PCR reaction procedures were as follows: thermal cycling consisted of initial denaturation at 98°C for 1 min, followed by 30 cycles of denaturation at 98°C for 10 s, annealing at 50°C for 30 s, and elongation at 72°C for 30 s and finally, 72°C for 5 min. A total of 5 μl of each PCR product was separated on a 2% agarose gel (Sangon Biotech, Shanghai, China) containing 0.1 μg ml^{-1} ethidium bromide in 0.5× Tris-acetate-EDTA (TAE) buffer (20 mM Tris and 10 mM acetic acid) (Servicebio, Wuhan, China). 100–2,000 bp DNA ladder (Sangon Biotech, Shanghai, China) was used as molecular size marker. PCR products were mixed in equidensity ratios. Then, this mixture of PCR products was purified with the Qiagen Gel Extraction Kit (Qiagen, Germany, United Kingdom). The purified PCR products were used for library preparation and high-throughput sequencing on a MiSeq sequencer (Novogene Illumina, Tianjin, China). The sequencing data were deposited into the Sequence Read Archive under accession number PRJNA758114.

Analysis and Processing of 16S Ribosomal RNA Gene Sequencing Data

Paired-end reads were assigned to samples based on their unique barcode and truncated by cutting off the barcode and primer sequence. Paired-end reads were merged using Flash (FLASH) (Magoč and Salzberg, 2011), a very fast and accurate analysis tool, which was designed to merge paired-end reads when at least some of the reads overlap the read generated from the opposite end of the same DNA fragment and the splicing sequences were called raw tags. Quality filtering on the raw tags was performed under specific filtering conditions to obtain the high-quality clean tags (Bokulich et al., 2013) according to the Quantitative Insights Into Microbial Ecology (QIIME) (Caporaso et al., 2010) quality-controlled process. The tags were compared with the reference database (Silva database), using UCHIME (a new program that detects chimeric sequences with two or more segments) algorithm (Robert et al., 2011) to detect the chimera sequences, remove the chimera sequences (Brian et al., 2011), and finally get effective tags.

Sequence analysis was performed by Uparse software (Edgar, 2013). Sequences with ≥97% similarity were assigned to the same operational taxonomic units (OTUs). Representative sequences for each OTU were screened for further annotation. For each representative sequence, the Silva database (Quast et al., 2013) was used based on the Mothur algorithm to annotate taxonomic information. Multiple sequence alignment with improved accuracy and speed (MUSCLE) (Edgar, 2004) (version 3.8.31[1]) software was used to perform rapid multiple sequence alignment and obtain the phylogenetic relationship of all the OTUs representative sequences. The data of each sample were normalized and the sample with the least amount of data was used as the standard. The subsequent alpha diversity analysis and beta diversity analysis are based on the data after normalization. The differences among groups were analyzed by the one-way ANOVA with multiple comparisons of the Tukey's honestly significant difference (HSD) test using the SPSS software version 17.0, SPSS 17.0. $p < 0.05$ was considered as statistically significant.

Metagenome Gene Amplification and Sequencing

The methods of quality control for DNA samples: degree of DNA degradation and potential contamination were monitored on 1% agarose gels. DNA concentration was measured using the Qubit® dsDNA Assay Kit in Qubit® 2.0 Fluorometer (Life Technologies, California, United States). The optical density (OD) value was between 1.8 and 2.0 and DNA contents above 1 μg were used to construct the library. A total amount of DNA per sample was used as input material for the DNA sample preparations. Sequencing libraries were generated using the NEBNext® Ultra DNA Library Prep Kit for Illumina (NEB, Ipswich. Massachusetts, United States) following the recommendations of the manufacturer and index codes were added to attribute sequences to each sample. The DNA sample was fragmented by sonication to a size of 350 bp and then DNA fragments were end-polished, A-tailed, and ligated with the full-length adaptor for Illumina sequencing with further PCR amplification. Finally, PCR products were purified (AMPure XP System, Hangzhou, China) and libraries were analyzed for size distribution by the Agilent 2100 Bioanalyzer System and quantified using real-time PCR.

Metagenome Sequencing, Data Preprocessing, and Assembly

Preprocessing of the raw data obtained from the Illumina HiSeq sequencing platform was conducted with Readfq (V8[2]) to acquire

[1]http://www.drive5.com/muscle/

[2]https://github.com/cjfields/readfq

the clean data for subsequent analysis. The specific processing steps were as follows: reads with low-quality bases were removed (default quality threshold value ≤38) above a certain portion (default length of 40 bp), reads with N base that reached a certain percentage were removed (default length of 10 bp), and reads with shared the overlap above a certain portion with adapter were removed (default length of 15 bp). Considering the possibility of host contamination might exist in samples, clean data needed to be blast to the host database, which default using Bowtie2.2.4 software (Bowtie2.2.4[3]) to filter the reads that are of host origin. The Bowtie parameters (Karlsson et al., 2012, 2013) were: end-to-end, sensitive, I 200, and X 400.

Clean data were assembled and analyzed by SOAPdenovo software (version 2.04) (Luo et al., 2015), used MEGAHIT software (version 1.0.4-beta) to assemble clean data, and then disconnect the assembled scaftigs from the N connection and make scaftigs without N (Mende et al., 2012; Nielsen et al., 2014; Qin et al., 2014). The clean data of all the samples were compared with each scaffold through Bowtie 2.2.4 software to obtain unused paired end (PE) readings (Qin et al., 2014). Combined the unused reads of all the samples in the previous step and then used SOAPdenovo (version 2.04)/MEGAHIT (version 1.0.4-beta) software for mixed assembly, the parameters were the same as single assembly; disconnect the mixed assembly from the N connection scaffolds and got scaftigs. Filter all the fragments that were shorter than 500 bp in scaftigs for statistical analysis. The genes with reads ≤2 in each sample were filtered to obtain the final gene catalog (Unigenes) for subsequent analysis.

Metagenome Sequencing Data Analysis

According to the number of mapped reads and the length of genes, the abundance information of each gene in each sample was counted (Qin et al., 2010; Fredrik et al., 2012). Basic information statistics, core-pan gene analysis, sample correlation analysis, and gene number Venn diagram analysis are all based on the abundance of each gene in each sample in the gene catalog. Double index alignment of next-generation sequencing data (DIAMOND) (Buchfink et al., 2015) software (version 0.9.9) was used to blast Unigenes into bacteria, fungi, archaea, and viruses sequences extracted from the National Center for Biotechnology Information (NCBI) non-redundant (NR) database. For the final alignment results of each sequence, since each sequence may have multiple alignment results, the result of E-value ≤minimum E-value × 10 (Julia et al., 2014) was selected as the least common ancestor (LCA) algorithm applied to Metagenome Analyzer (MEGAN) (Daniel et al., 2011) system classification of software to ensure the species annotation information of the sequence. According to the LCA annotation results and the gene abundance table, a table containing the number and abundance information of each sample in each classification level (boundary, phylum, class, order, family, genus, and species) was obtained. Linear discriminant analysis Effect Size (LEfSe) analysis was performed by LEfSe software (the default linear discriminant analysis (LDA) score is 3) (Nicola et al., 2011). DIAMOND software (version 0.9.9) was used to blast Unigenes into the Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa et al., 2006, 2014) functional database. According to the function annotation results and the gene abundance table, the predicted functional pathways of each sample microbiota were obtained.

Resistance Gene Annotation

The Resistance Gene Identifier (RGI) software was used to align Unigenes with the Comprehensive Antibiotic Resistance Database (CARD)[4] (Mcarthur et al., 2013; Martínez et al., 2014; Jia et al., 2017) with the parameter set to Blastp (BLASTP), E-value ≤1e−30. According to the comparison result, the relative abundance of the antibiotic resistance ontology (ARO) in all the genes was calculated as parts per million (ppm). According to the abundance of the ARO, the abundance bar graph and the number difference between the resistant genomes are displayed. In the

[3]http://bowtie-bio.sourceforge.net/bowtie2/index.shtml

[4]https://card.mcmaster.ca/

TABLE 1 | Alpha diversity metrics of microbiotas in water, biofloc, and intestine of shrimp sampled from three biofloc ponds.

Sample name	Observed species	Shannon	Simpson	Chao1	Ace	Good's coverage
AS.1	1176	6.320	0.947	1282.757	1338.824	0.994
AS.2	1233	6.607	0.956	1343.228	1386.914	0.994
AS.3	1073	6.106	0.933	1149.790	1225.910	0.995
BS.1	1537	7.467	0.969	1680.477	1762.483	0.992
BS.2	1249	6.954	0.962	1334.532	1367.683	0.995
BS.3	1206	6.859	0.959	1300.776	1355.316	0.995
CS.1	1000	5.410	0.900	1096.049	1165.578	0.995
CS.2	1140	7.049	0.972	1229.851	1284.471	0.995
CS.3	1298	7.205	0.977	1397.124	1481.553	0.994
Mean ± S.D						
AS	1160.667 ± 81.095[a]	6.344 ± 0.251[b]	0.945 ± 0.012[a]	1258.592 ± 98.957[a]	1317.216 ± 82.648[a]	0.994 ± 0.0004[a]
BS	1330.667 ± 179.979[a]	7.093 ± 0.327[a]	0.963 ± 0.005[a]	1438.595 ± 210.155[a]	1495.161 ± 231.591[a]	0.994 ± 0.0014[a]
CS	1146.000 ± 149.091[a]	6.555 ± 0.994[a]	0.949 ± 0.043[a]	1241.008 ± 150.847[a]	1310.574 ± 159.592[a]	0.994 ± 0.0005[a]

AS, water sample; BS, biofloc sample; CS, intestinal sample. Rank each group in ascending order, with the largest mean labeled "a" and the largest and least significant differences labeled "a" until a significant difference was labeled "b".

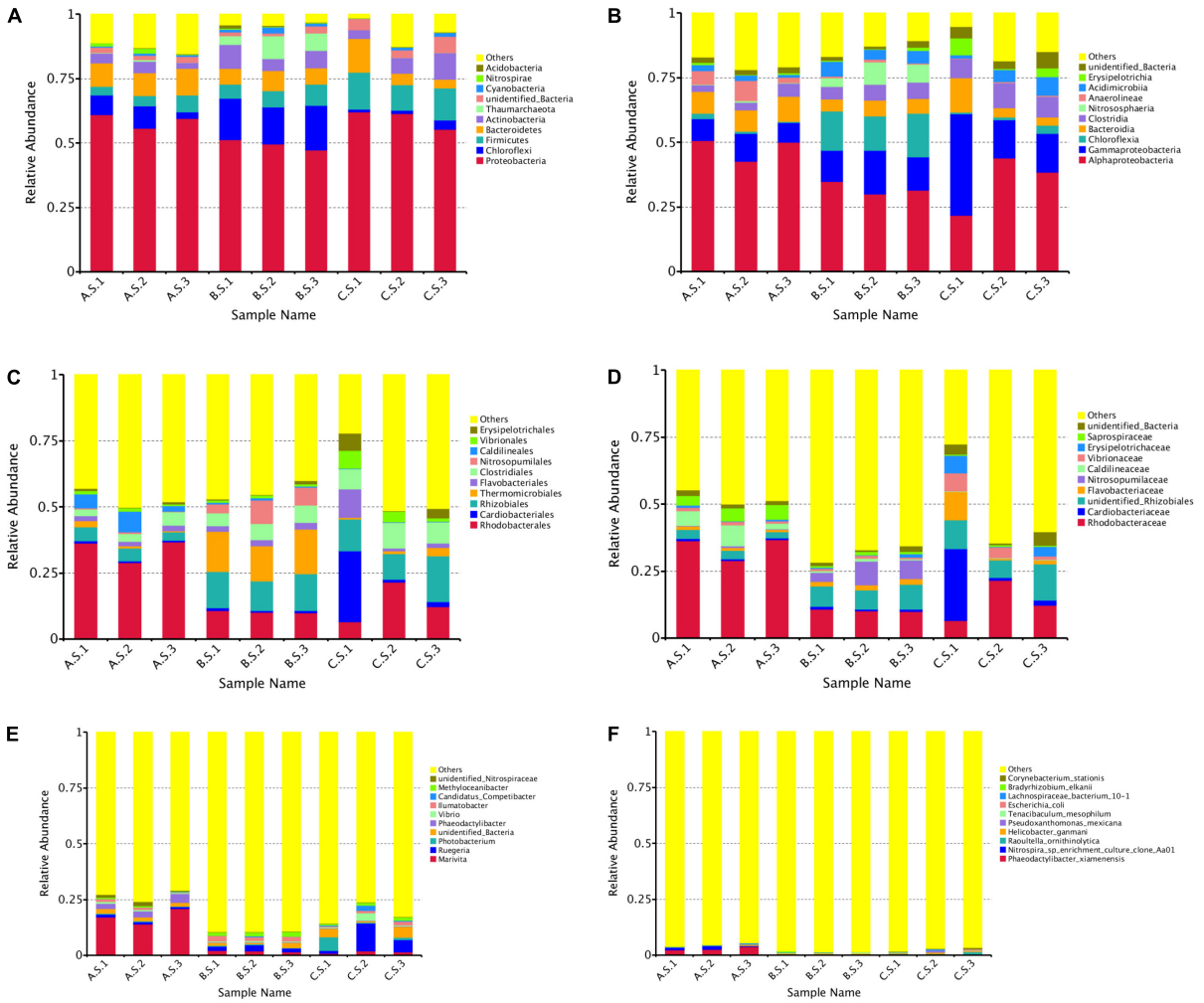

FIGURE 1 | Relative abundances of the dominant bacteria in each sample obtained from 16S rRNA gene sequencing **(A)** (phylum), **(B)** (class), **(C)** (order), **(D)** (family), **(E)** (genus), and **(F)** (species). AS, BS, and CS were water, biofloc, and intestinal samples of shrimp sampled from biofloc ponds. AS, water sample; BS, biofloc sample; CS, intestinal sample; 16S rRNA, 16S ribosomal RNA.

same way, the abundance distribution of resistance genes in each sample, the species attribution analysis of resistance genes, and the resistance mechanism of resistance genes were analyzed.

RESULTS

Sequencing Results

The 16S rRNA sequencing results of 72,444–95,756 raw tags, 70,791–93,569 clean tags, and 47,842–62,966 effective tags were obtained from the nine samples (three water: AS, three biofloc: BS, and three intestinal samples: CS). As shown in **Table 1**, Good's coverage revealed that 99.23–99.49% of species were obtained in all the nine samples. The Shannon and Simpson indexes, Chao1 together with abundance-based Coverage Estimator (ACE) values of the nine samples varied from 5.410 to 7.467, 0.900 to 0.977, 1096.049 to 1680.477, and 1165.578 to 1762.483, respectively. The Shannon index of BS and CS was significantly higher than that of AS ($p < 0.05$), indicating that the bacterial abundance of BS and CS was the highest of all the three samples and BS was higher than CS. Although the difference was not always statistically significant, biofloc had the highest species abundance, taking into account various indicators.

Taxonomic Profiles of Microbiota in Shrimp Biofloc Ponds

According to the OTUs clustering results, species information and species-based abundance distribution of the nine samples were obtained. **Figures 1A–F** depict species abundance detected at different taxonomic levels. In total, 45 identified phyla were observed and ten phyla were identified at an abundance >1%. As shown in **Figure 1**, in AS, *Proteobacteria* (55.67–61.04%), *Bacteroidetes* (8.89–10.26%), *Chloroflexi* (2.61–8.74%), *Firmicutes* (3.46–6.54%), and *Actinobacteria* (2.24–4.46%) were the most abundant phyla. *Chloroflexi* (14.26–17.30%) and *Actinobacteria*

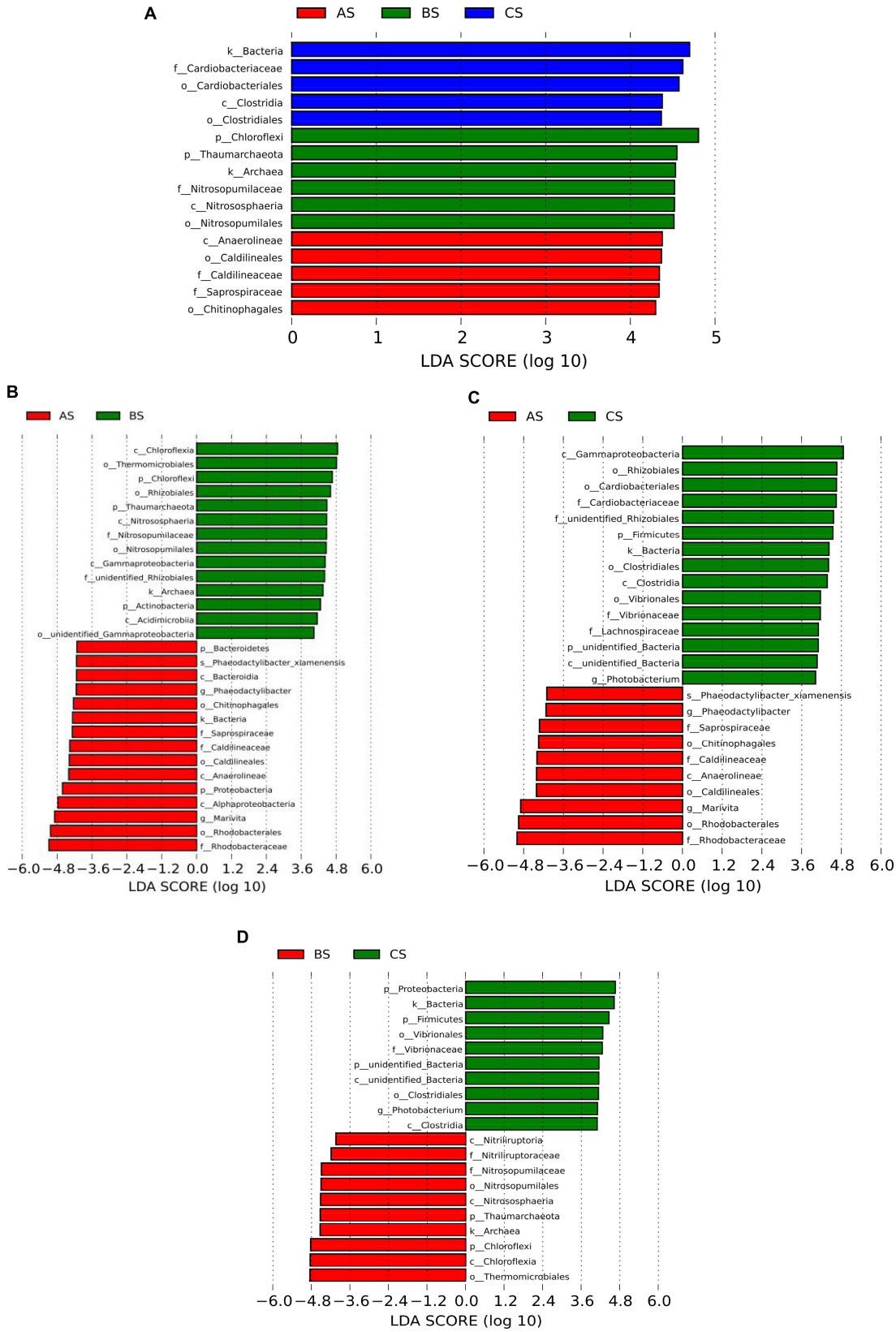

FIGURE 2 | LEfSe analysis among AS vs. BS vs. CS **(A)**, AS vs. BS **(B)**, AS vs. CS **(C)**, BS vs. CS **(D)**. Only taxa with a LDA value higher than 4 are shown. AS, water sample; BS, biofloc sample; CS, intestinal sample.

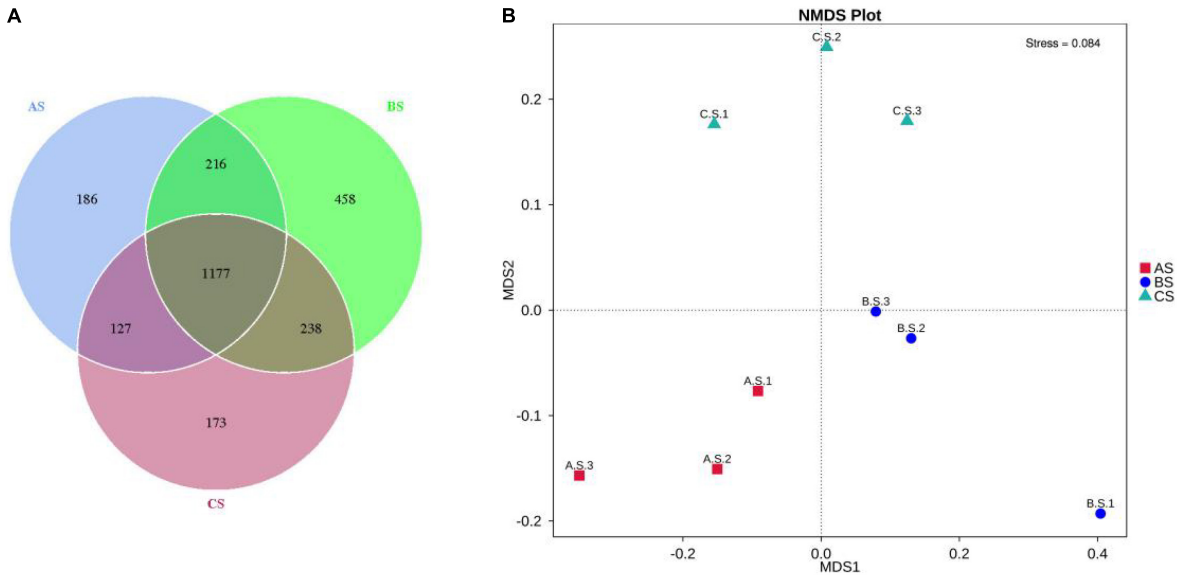

FIGURE 3 | Venn diagram of microbial communities at OTUs level among AS, BS, and CS **(A)**, NMDS analysis **(B)** among microbial communities (n = 9). AS, water sample; BS, biofloc sample; CS, intestinal sample, OTUs, operational taxanomic units.

(4.81–9.25%) were the second and third dominant phylum after *Proteobacteria* (47.31–51.32%) in BS. In CS, the top three abundant phyla were *Proteobacteria* (55.41–62.15%), *Firmicutes* (10.01–14.28%), and *Bacteroidetes* (3.43–13.27%).

At the class level (**Figure 1B**), *Alphaproteobacteria* (21.84–50.74%) was the most abundant class in AS, BS, and CS. *Gammaproteobacteria* (7.53–39.17%) was the second most abundant in AS and CS, but ranked third in BS. *Chloroflexia* (13.16–16.87%) was the second most common phylum in BS. In **Figure 1C**, *Rhodobacterales* (28.88–36.76%), *Caldilineales* (2.14–7.84%), and *Rhizobiales* (3.14–5.32%) were the top three most abundant order in AS. *Thermomicrobiales* (13.14–16.85%), *Rhizobiales* (11.16–13.91%), and *Rhodobacterales* (9.92–10.78%) were the most abundant order in BS. *Rhodobacterales* (6.49–21.55%), *Rhizobiales* (9.89–17.25%), and *Cardiobacteriales* (0.96–26.89%) were the most abundant orders in CS. *Rhodobacteraceae* (6.49–36.76%) was the most abundant family in AS, BS, and CS (**Figure 1D**). When the OTUs were considered at the genus level, a high diversity of microbes was identified. A total of 457 genera were detected in all the samples. In BS and CS, the genus level accounting for the largest proportion was *Ruegeria* (2.23–6.31%) and *Marivita* (14.01–20.94%) in AS (**Figure 1E**). The top 10 dominant genera were *Marivita*, *Ruegeria*, *Photobacterium*, unidentified_Bacteria, *Phaeodactylibacter*, *Vibrio*, *Ilumatobacter*, *Candidatus_Competibacter*, *Methyloceanibacter*, and unidentified_Nitrospiraceae.

LEfSe analysis was used to reveal specific taxa associated with each sample. As shown in **Figure 2A**, LEfSe analysis was conducted between AS, BS, and CS (**Figure 2**). AS relevant taxa were *Bacteroidetes*, *Proteobacteria*, *Bacteroidia*, *Anaerolineae*, *Alphaproteobacteria*, *Phaeodactylibacter*, and *Marivita*. BS relevant taxa were *Chloroflexi*, *Thaumarchaeota*, *Actinobacteria*, *Chloroflexia*, *Nitrososphaeria*, *Gammaproteobacteria*, and *Acidimicrobiia*. CS relevant taxa were *Firmicutes*, unidentified_Bacteria, *Gammaproteobacteria*, *Clostridia*, and *Photobacterium*. Orders and family were differentially abundant among samples as shown in **Figures 2A–D**.

The OTUs results were obtained according to the clustering. **Figure 3A** shows the common and unique OTUs among different groups. The percent of OTUs shared by AS and BS, AS and CS, and BS and CS were 57.99, 61.60, and 59.23%, respectively, implying AS and CS shared more OTUs. Simultaneously, the nonmetric multidimensional scale analysis (NMDS) analysis (**Figure 3B**) demonstrated that samples were classified into three large groups. In terms of potential pathogenic *Vibrio*, there were six unique to AS, five unique to BS, and six unique to CS. The total shared number of *Vibrio* species in the three groups was 37.

Function Annotation Profiles of Microbiota in Shrimp Biofloc Ponds

DIAMOND software was used to compare the functional differences of the microbiota between shrimp intestines, water, and biofloc (**Figures 4A–F**). The relative abundance of functional pathways based on the KEGG annotation is shown in **Figure 4**. The functional pathways found to have higher relative abundance were mainly related to metabolic functions (especially amino acids and carbohydrates). Shrimp intestinal microbiota was found to have distinct functional pathways from the microbial communities in water and biofloc. The intestinal microbiota of shrimp was found to have different functional pathways from the microbial communities in water and biofloc. It is worth noting that the antimicrobial function in the predictive KEGG resistance pathway was found to be the most abundant and is lower in CS than AS and BS. The same was found to best true for infectious

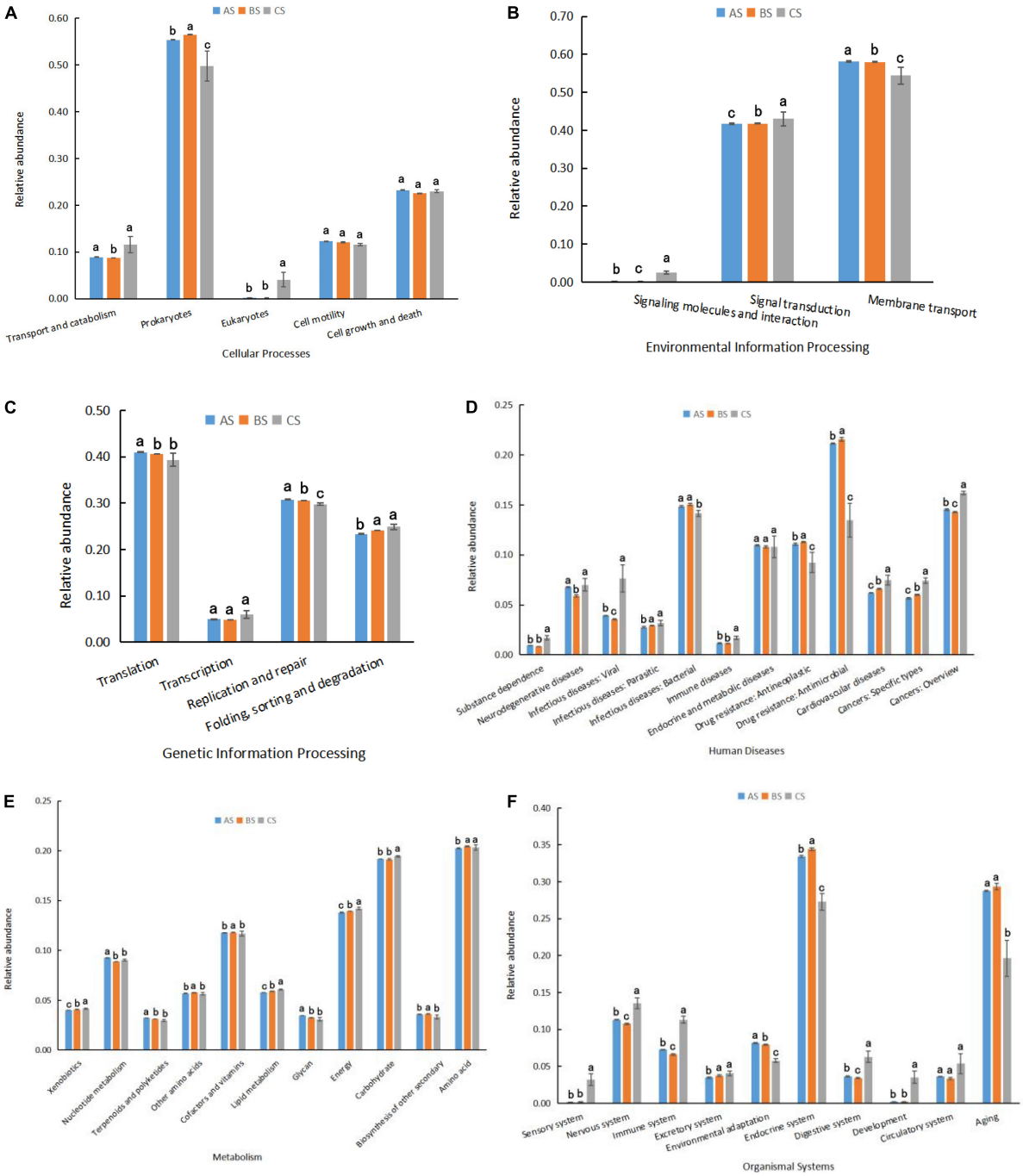

FIGURE 4 | (A–F) Predicted functional pathways for microbiotas in shrimp rearing water (AS), biofloc (BS), and intestine (CS). Means with different superscripts are significantly different ($p < 0.05$) within each functional pathway. AS, water sample; BS, biofloc sample; CS, intestinal sample.

bacteria, which suggests that there is a link between these two pathways (**Figure 4D**).

Resistance Gene Annotation of Microbiota in Shrimp Biofloc Ponds

Bacterial ARO annotation results showed that ARO species of AS, BS, and CS were significantly different (**Figure 5A**). In this study, the relative abundance of total ARGs of AS, BS, and CS was 3.76–3.92 $\times$ 10^{-4} ppm, 2.46–2.77 $\times$ 10^{-4} ppm, and 4.93–5.82 $\times$ 10^{-4} ppm, respectively. Among all the samples, the abundance of ARGs found in the intestine was the highest.

As shown in **Figure 5B**, in AS, the ARO with the highest absolute abundance is *patB* (11.33–15.01%). In BS, the ARO with the highest absolute abundance is *adeF* (15.79–18.16%), while in CS, the AROs with the highest absolute abundance are *OXA-243* (35.65%) and *Brucella_suis_mprF* (10.03%). The most

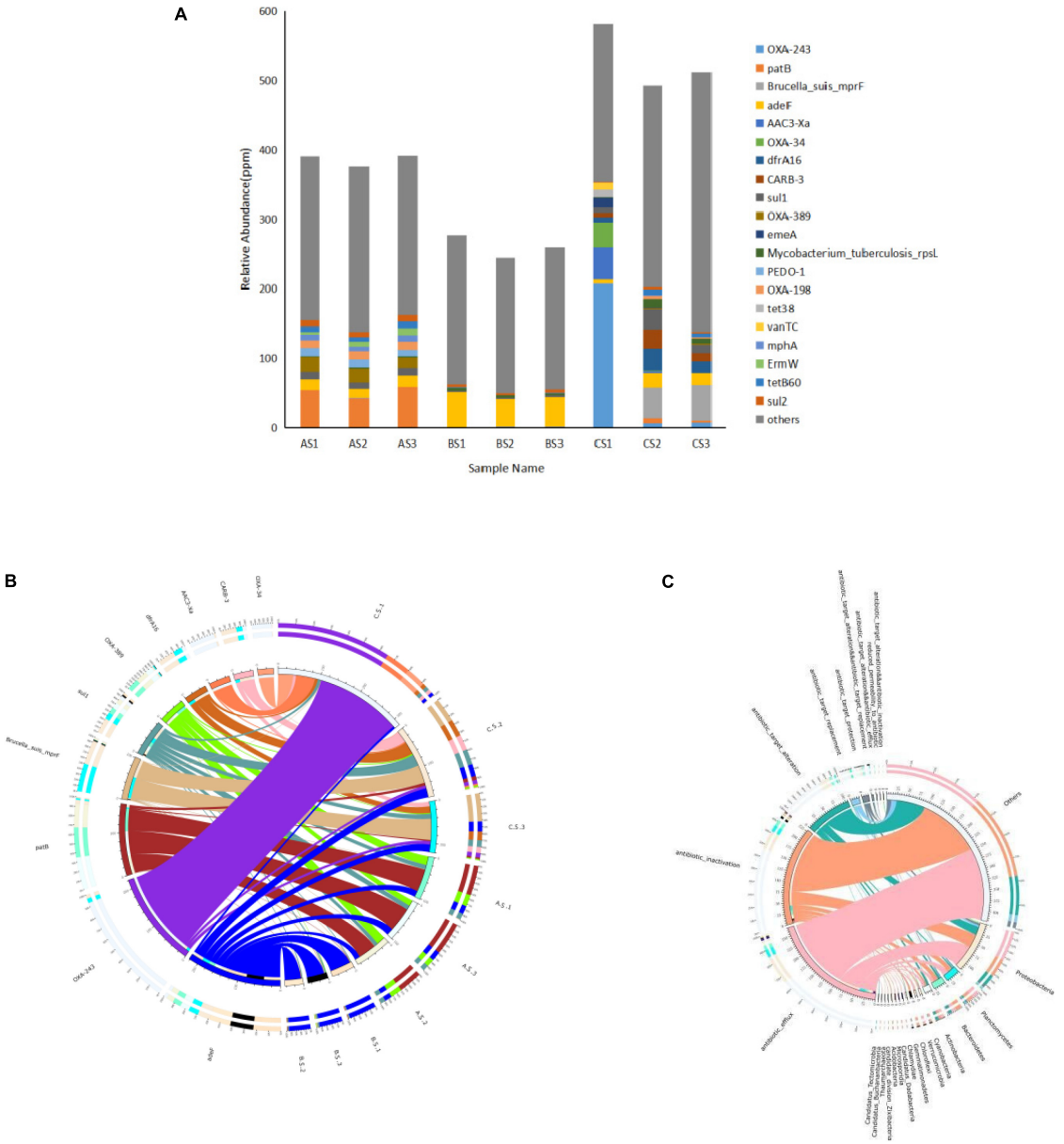

FIGURE 5 | (A–C) Figure 5A represents the relative abundance of all the genes in each sample (unit ppm, the result of magnifying the original relative abundance data by 106 times). **Figure 5B** represents the ARO with the maximum abundance of top 10 (the circle diagram is divided into two parts, the sample information on the right and ARO information on the left). Different colors in the inner circle represent different samples and AROs, with the scale being relative abundance and the unit being PPM. **Figure 5C** represents an overview of resistance mechanisms and species (the circle diagram is divided into two parts). The right side shows the information of species at the level of phylum and the left side shows the information on resistance mechanism. The inner circle shows the resistance mechanism of different species and resistance with different colors and the scale shows the number of genes. On the left is the sum of the number of resistance genes in the species with this resistance mechanism and on the right is the sum of the number of resistance genes in the species with different resistance mechanisms. AS, water sample; BS, biofloc sample; CS, intestinal sample.

resistance mechanisms found were related to antibiotic efflux, with 270 resistance genes, followed by 229 resistance genes for the inactivation of antibiotics. Proteobacteria was found to have 118 resistance genes (**Figure 5C**).

The antibiotic resistance mechanisms of the ARGs detected in the testing samples included efflux proteins, β-lactamase, resistance nodulation division (RND) transporters, reductase, and defensin. The bacterial ARO in AS was mainly derived from *Proteobacteria* (31%), *Bacteroidetes* (14%), and *Actinobacteria* (6%). The bacterial ARO in BS was mainly derived from *Proteobacteria* (39%), *Bacteroidetes* (10%), and *Planctomycetes* (7%). The bacterial ARO in CS was mainly derived from *Proteobacteria* (39%), *Planctomycetes* (6%), and *Actinobacteria* (6%) (**Figure 6**).

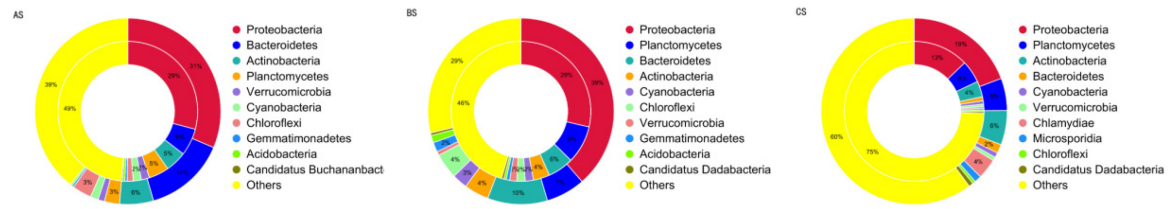

FIGURE 6 | Species identification analysis of AROs in AS, BS, and CS (the inner circle is the species distribution of ARO and the outer circle is the species distribution of all the genes in the group). AS, water sample; BS, biofloc sample; CS, intestinal sample.

DISCUSSION

In this study, high-throughput sequencing and metagenomic analysis were used to investigate the diversity of bacterial communities and ARGs in three *P. monodon* biofloc ponds in Guangdong Province, China. Metagenomic analysis is a culture-independent molecular approach, which uses the generated metagenomic sequences to search, annotate, and predict targeted genes (Soucy et al., 2015). A total of 45 phyla and 457 genera were identified. Our results revealed that biofloc has the highest alpha diversity metric as shown by the Shannon index, consistent with the levels in *Fenneropenaeus merguiensis* (banana shrimp) cultured using biofloc technology (Wang et al., 2020). *Proteobacteria* was the predominant phylum in all the water, biofloc, and intestinal samples, consistent with the levels in environmental samples collected from *Marsupenaeus japonicus* (Japanese tiger prawn) ponds (Alexia et al., 2020), freshwater crab aquaculture environments (Fang et al., 2019), co-cultured oyster, and *Penaeus vannamei* (*P. vannamei*) (whiteleg shrimp) (Wang J. et al., 2019) and *P. vannamei* infected with white spot syndrome virus (Zeng et al., 2020). Besides *Proteobacteria*, other phyla such as *Bacteroidetes*, *Chloroflexi*, *Firmicutes*, and *Actinobacteria* were also abundant in the *P. monodon* biofloc aquaculture environment and the bacterial taxa showed more abundant species diversity compared to other aquaculture environments (Wobus et al., 2003; Gilbert et al., 2012; Tang et al., 2014). In human health, Na-Ri et al. (2015) proposed Proteobacteria as a potential diagnostic feature for ecological disorders and increased risk of disease. It is worth noting that *Proteobacteria* is the dominant phylum in crab and fish aquaculture farms and different aquaculture models (Colombo et al., 2016). These data reinforce the importance of carefully treating aquaculture wastewater before discharge into the environment to reduce the transfer of *Proteobacteria* to humans. In addition, *Proteobacteria* species richness was not the determinant of microbial community distribution in the pond (Liu et al., 2020; Shen et al., 2020). According to this study, microbial taxa might be the potential hosts of ARGs (Chen et al., 2017). Therefore, *Proteobacteria* may be a potential host for ARGs in biofloc-based aquaculture environments.

Resistance genes are ubiquitous in both the human gut microbes and other environmental microbes and infections caused by antibiotic-resistant bacteria are a major threat to public health (Ross et al., 2020). Aquaculture is known to be a reservoir of antibiotics and ARG (Syeda et al., 2020). As a new aquaculture model, it is necessary to evaluate the safety and environmental impact of biofloc-based aquaculture models by analyzing the ARGs within them. The successful application of metagenomics in environmental pollution tracking paves the way for the establishment of a new accurate source tracking framework, which greatly simplifies the ARGs variant-based source tracking process (Li et al., 2020). The relative abundance of total ARGs in aquaculture systems based on biofloc was about 2.46–5.82 $\times$ 10^{-4} ppm, which was similar to that of coastal industrial mariculture (0.27–4.55 $\times$ 10^{-4} ppm) (Wang et al., 2018). In this study, the relative abundance of total ARGs of water, biofloc, and intestinal was 3.76–3.92 $\times$ 10^{-4} ppm, 2.46–2.77 $\times$ 10^{-4} ppm, and 4.93–5.82 $\times$ 10^{-4} ppm, respectively. The relative abundances of ARGs were highest in the intestinal samples, which indicated that the shrimp intestines might be a more favorable habitat for aquaculture bacteria carrying ARGs (Su et al., 2018). The biofloc samples had the highest numbers in terms of species richness of bacteria; however, the bacteria in bioflocs carried fewer ARGs. Therefore, biofloc may be the key to controlling the concentration of ARGs in biofloc-based aquaculture. A total of 483 ARGs were identified in the biofloc-based aquaculture ponds in this study. This result was significantly lower than urban hospital and adjacent urban and suburban communities, which had 643 ARGs in airborne samples (He et al., 2020). However, the number of ARGs in biofloc-based aquaculture ponds was higher than that found in the river reservoir system (N24°210–25°060, E115°000–115°470) used for drinking water (242 ARGs) (Chen et al., 2019) and shrimp ponds in Maoming Guangdong Province (217 ARGs) (Zhou et al., 2019).

In this study, quinolone resistance gene *patB* was the most abundant ARGs in water, tetracycline resistance gene *adeF* was the most abundant ARGs in biofloc, and cephalosporin resistance gene *OXA-243* and peptide resistance gene *Brucella_suis_mprF* were the most abundant ARGs in intestines, which contrast with the findings of some previous studies (Gao et al., 2012; Huang et al., 2017; Su et al., 2017). It has been shown that the ARGs carried by culturable bacteria are different during shrimp production in aquatic environments (Zhou et al., 2020). In natural water sediment (from the Pearl River), peptide resistance gene *bacA*, tetracycline resistance gene *tet39*, and sulfonamide resistance gene *sul1* were the most abundant (Zhang et al., 2018).

Antibiotic resistance genes also have the characteristics of "propagation and persistence" (Mao et al., 2015). The

transmission of the ARGs as a genetic element can be mediated by genetic mechanisms such as conjugation, transduction, and transformation (Soucy et al., 2015; Ma et al., 2017; Zhao, 2021). In particular, the ARGs in aquaculture environments can be transferred horizontally among microbes and ultimately to fish pathogens (Shah et al., 2012). In addition, the ARGs can also be discharged into the aquatic environment by livestock, agricultural, or aquaculture wastes *via* natural food chains (Liu et al., 2019). This poses a serious threat to human health, e.g., prolonged morbidity and increased mortality (Ashbolt, 2013; Chamosa et al., 2017). In most circumstances (even without the presence of excess inducers such as antibiotics or heavy metals), plasmids (Hall et al., 2017) and mobile genetic elements (MGEs) (e.g., class 1 integrons) commonly exist in aquatic environments (Gaze et al., 2011), which may cause dissemination of the ARGs in aquatic environment without the presence of antibiotics (Wang Y. et al., 2019). Under the condition of zero exchange water and no antibiotic addition in biofloc-based aquaculture ponds, the ARGs are likely to be transmitted through plasmids and MGEs. Resistant bacteria and genes when entering a healthy organism tend to proliferate, therefore forming a resistance module against the antibiotic within the organisms (Singer et al., 2016). The results of this study and previous studies on biofloc-based aquaculture systems indicate that the system can effectively reduce emissions of the ARGs to the coastal environment due to its low sewage discharge. But, once it is necessary to discharge aquaculture wastewater, more advanced wastewater treatment systems are needed.

CONCLUSION

In this study, the bacterial composition of water, biofloc, and shrimp intestine sampled from biofloc-based aquaculture ponds was found to be different. Species abundance of biofloc was the highest by combining various indexes. The common dominant phyla were *Proteobacteria*, *Bacteroidetes*, *Chloroflexi*, *Firmicutes*, and *Actinobacteria*. Overall, our results suggest that biofloc-based aquaculture is rich in opportunistic pathogen-associated taxonomic groups that may host ARGs associated with critically important antibiotics used in human medicine.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: NCBI (accession: PRJNA758114).

AUTHOR CONTRIBUTIONS

XC, ZH, JZ, ML, YX, JZ, RH, SM, NW, HP, and CS researched data for the article, made substantial contributions to discussions of the content, wrote the article and reviewed and edited the manuscript before submission. All authors contributed to the article and approved the submitted version.

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First Draft Genome of a Mud Loach (*Misgurnus mizolepis*) in the Family Cobitidae

Younhee Shin[1†], *Eun Soo Noh*[2†], *Ji-Hyeon Jeon*[1], *Ga-Hee Shin*[1], *Eun Mi Kim*[2], *Young-Ok Kim*[2], *Hyeongsu Kim*[3], *Hyungtaek Jung*[4] *and Bo-Hye Nam*[2*]

[1] Research and Development Center, Insilicogen Inc., Yongin-Si, South Korea, [2] Biotechnology Research Division, National Institute of Fisheries Science, Busan, South Korea, [3] Advanced Aquaculture Research Center, National Institute of Fisheries Science, Changwon, South Korea, [4] School of Biological Sciences, The University of Queensland, St Lucia, QLD, Australia

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Terengganu, Malaysia

***Correspondence:**
Bo-Hye Nam
nambohye@korea.kr

[†] *These authors have contributed equally to this work*

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INTRODUCTION

Fish in aquatic environments generally obtain oxygen from the water. Mud loaches inhabit muddy swamps, ponds, and rice fields subject to periodic drying. The respiratory systems of freshwater fish subject to drought have adapted to enable cutaneous/air respiration via other organs. Loaches can breathe in water or soil depending on the dissolved oxygen content (Park et al., 2001). Mud loach aquaculture in freshwater is common in South Korea, Taiwan, China, and Japan. The mud loach *Misgurnus mizolepis* belongs to the family Cobitidae and is widely used in basic biological research. It can live in soil and water and survive in human wastewater, such as ditches and septic tanks; it is also useful for harvesting antimicrobial peptides. Controlling disease outbreaks in aquaculture systems, such as the 2012 *Aeromonas sobria* outbreak that caused 61% mortality in 2 days, is a major challenge. This devastated fisheries aquaculture production; the production in the previous 5 years averaged 766 tons and was valued at $ 7.2 M (https://www.kostat.go.kr/). Considering the above, and to expand genetic research to preserve this species, we generated a draft genome for *Misgurnus mizolepis*. Presently, only the mitochondrial genome of this species is available (Lee, 2016) and no nuclear genomes for the family Cobitidae have been reported.

Value of the Data

This *M. mizolepis* genome is the first reference genome for molecular studies in the family Cobitidae. It should be useful for comparative analyses among or within species in the genus *Misgurnus* or closely related genera in the family Cobitidae, and could enhance the genome selection process in molecular breeding.

MATERIALS AND METHODS

Sampling and Genomic DNA and RNA Preparation

Four 1-year-old wild *M. mizolepis* were supplied by Inland Aquaculture Research Center, National Institute Fisheries Science (NIFS), Changwon, South Korea in April 2019, at Buk-myeon, Jeongeup, South Korea. Abdominal muscle tissue was removed aseptically from one specimen as per the NIFS ethical committee provided instruction (2018-NIFS-IACUC-03) and dipped in liquid nitrogen for genomic DNA (gDNA) and RNA preparation; liver, abdominal muscle, and brain tissues were taken from the other three specimens for RNA extraction. The DNA and RNA isolation and sequencing were conducted by DNALink (Seoul, South Korea).

Genomic DNA and Transcriptome Sequencing

The gDNA and RNA were isolated from the samples by the DNeasy Animal Mini Kit and RNeasy Animal Mini Kit (QIAGEN, Hilden, Germany), respectively. The isolated gDNA sequenced with PacBio Sequel platform (Pacific Biosciences of California, Menlo Park, CA, USA), by capturing a 240-min movies for each SMRT cell. The RNA from the same individual was converted into cDNA using the SMARTer PCR cDNA Synthesis kit and subjected for the above steps for SMRTbell™ library preparation (except for fragmentation), and then sequenced with the PacBio Sequel platform. Similarly, another portion of the isolated gDNA and RNA from three different tissue samples of the three biological replicates was used to prepare sequencing libraries with the stranded Illumina paired-end (PE) protocol, using the TruSeq Nano DNA Prep Kit and TruSeq Stranded mRNA LT Sample Prep Kit (Illumina, San Diego, CA, USA), respectively. The Illumina NovaSeq6000 sequence machine used with desire size of DNA and RNA fragments.

Sequencing Read Preprocessing and Genome Size Estimation

The DNA and mRNA sequences from illumina sequencer were subjected to pre-processing steps involving adapter and quality trimming (Q20), with subsequent contaminant removal for DNA sequences. The adapter and quality trimming processes were conducted using Trimmomatic-0.32 functions (Bolger et al., 2014), and microbial contaminants were removed using Bowtie2 with specific in-house database constructed for bacterial, viral, and marine metagenomes. The processed DNA sequences from the Paired end library were subjected to genome size estimation using the *k*-mer based method (Shin et al., 2018). The *k*-mer frequencies (*k*-mer size = 21) were received by Jellyfish v2.0 (Marçais and Kingsford, 2011) and calculated using the below formulas: Genome Coverage Depth (CD) = (*k*-mer CD) × Average Read Length (ARL)/(ARL – *k*-mer size + 1) and Genome size = Total Base Number/Genome CD.

De-novo Genome Assembly and Scaffolding

Error correction for the complete sequence processed with SMRT Analysis v2.3, and imported into a diploid-aware FALCON (Chin et al., 2016) genome assembler used to assemble long contigs from the PacBio reads. Additionally, assembled contigs subjected to polishing by Quiver method to reduce the base call errors (Chin et al., 2013). Further, contigs were used to assess the genome completeness with BUSCO v5.0 (Simão et al., 2015). The reference BUSCO dataset was actinopterygii_odb10. The quality of the assembled genome was assessed by short-read mapping to the draft assembly with Bowtie2. Finally, the assembled contigs were scaffolded based on 25 chromosomes of the stone loach *Triplophysa tibetana* genome (GCA_008369825.1), which belongs to suborder Cobitoidei, using RagTag v2.0.1 (Alonge et al., 2019). The unknown sequences between contigs in a scaffold were filled with 100 bp of N.

De-novo Repeats Identification Process

Repeat regions in assembled genome were predicted using the *de-novo* method and categorized into repeat subclasses; *de novo* repeats estimation for *M. mizolepis* was conducted using RepeatModeler, which incorporated with methods as RECON (Bao and Eddy, 2002), RepeatScout (Price et al., 2005), and TRF (Benson, 1999). Those modeled repeats were sub-categorized using the Repbase v20.08 database as a reference (Bao et al., 2015), and the repeats were masked using RepeatMasker v4.0.5 with RMBlastn v2.2.27+.

Gene-Prediction and Annotation

Genes from the genome of *M. mizolepis,* predicted by an gene prediction pipeline developed in house, which incorporated an evidence-based gene modeler, an *ab-initio* gene modeler, and a consensus gene modeler. After gene prediction, functional annotation was conducted for the consensus genes. Initially, sequenced transcriptomes from the Illumina Novaseq6000 were mapped to the draft repeat-masked draft genome using STAR (Dobin et al., 2013) and assembled transcriptome used for genome-guided Trinity (Grabherr et al., 2011; Haas et al., 2013). Full-length transcript sequences were also generated from high-fidelity PacBio Sequel cDNA sequences using IsoSeq3 (Pacific Biosciences of California, Menlo Park, CA, USA). The *de novo* assembled transcriptome and full-length transcript sequences were then subjected to the following steps. To train the *ab-initio* and evidence-based gene models, which include Exonerate (Slater and Birney, 2005) and AUGUSTUS (Stanke et al., 2006), with several genomes were used for gene prediction (**Supplementary Table 4**). Finally, the transcripts and predicted evidence-based gene and *ab-initio* models were subjected to a "consensus gene modeler" to produce the final gene and transcript models. The consensus transcripts were subjected to functional annotation using biological databases (NCBI-NR, Swiss-Prot, Gene Ontology, and KEGG databases) using BLAST+ v2.6, OmicsBox v1.4 and Trinotate v3.2 (Bryant et al., 2017).

Gene Expression Profiling

The pre-processed RNA-Seq reads from the liver, muscle, and brain tissues of three biological replicates were mapped to the coding sequences of the predicted genes using Salmon v1.4 (Patro et al., 2017). Genes with NumReads (estimated read counts) values greater than five and transcript per million (TPM) values > 0.3 in one or more tissue-specific group(s) were counted as expressed. Differentially expressed genes (DEGs) were identified using edgeR v3.30 in the TCC v1.28 R package (Robinson et al., 2010; Sun et al., 2013), with a threshold of 2 for $\log_2$ fold-change values and 0.05 for false-discovery rates (FDRs) in the pairwise control-case comparisons.

Mitochondrial Genome Assembly and Annotation

Pre-processed DNA short reads, including organelle sequence reads, were used to assemble the *M. mizolepis* mitochondrial genome (mitogenome) using NOVOPlasty v4.2 (Dierckxsens et al., 2017), assisted by the reference *M.*

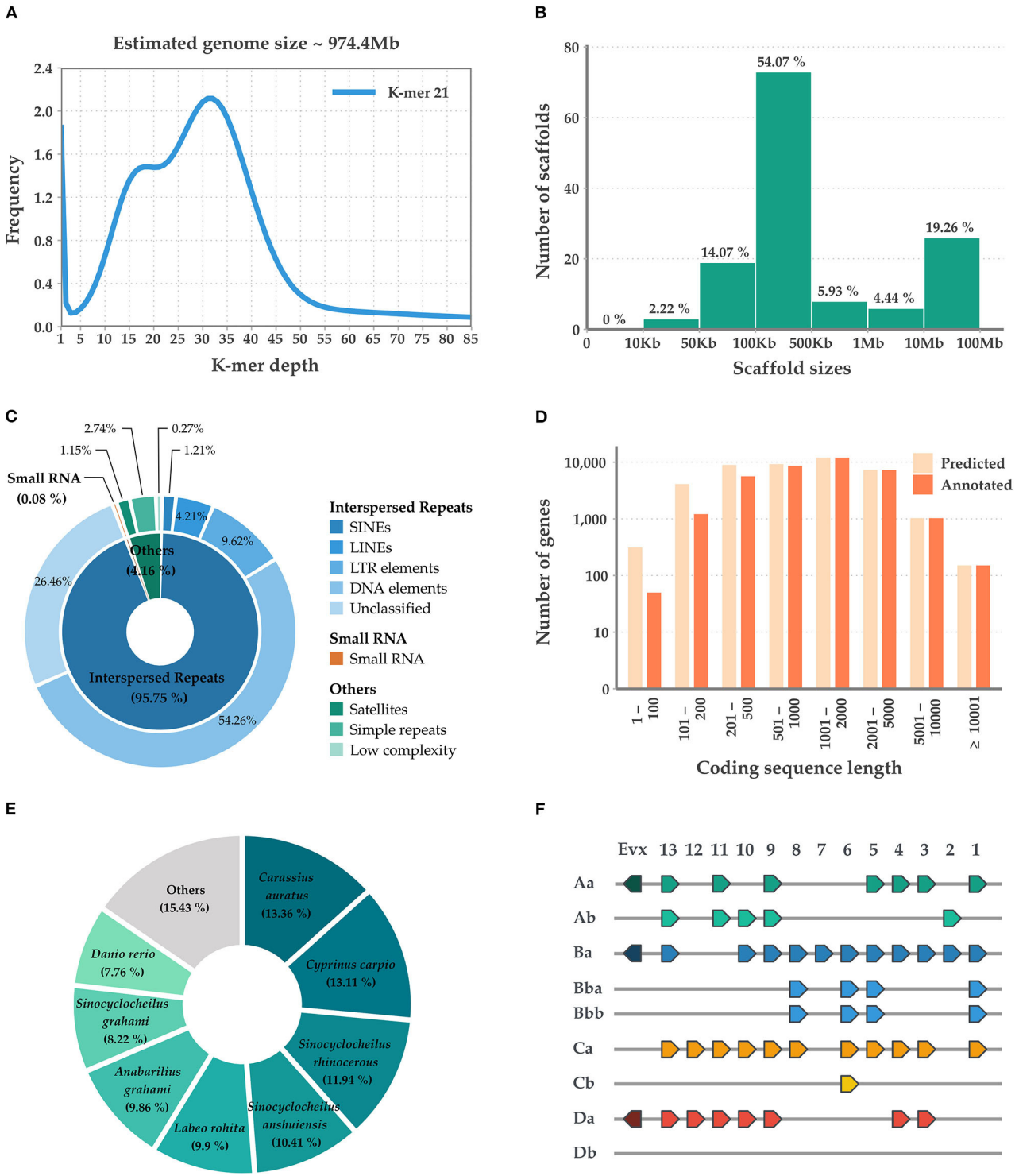

FIGURE 1 | Summary of the sequencing, assembly, and annotation of the *Misgurnus mizolepis* draft genome. **(A)** *K*-mer-based genome size estimation; **(B)** Length distribution of the assembled scaffolds; **(C)** *De-novo* repeat prediction for three classes (inner circle) and their sub-classes (outer circle); **(D)** Length distribution of predicted, functionally annotated genes; **(E)** Species distribution of the top NCBI-NR BLAST hits; and **(F)** Hox gene clusters.

mizolepis mitogenome sequence (NC_038151.1). The mitogenome annotated with MitoAnnotator from MitoFish database (Iwasaki et al., 2013).

Preliminary Analysis

Initially, the *M. mizolepis* genome was estimated to be 974.4 MB (**Figure 1A**), with 49.6 GB of short read sequences (**Table 1A**;

TABLE 1 | Summary of the sequencing for annotation of the *Misgurnus mizolepis* draft genome.

(A) Sequencing	
Illumina short-read yield	49,615,230,708 bp
Pre-processed short-read data	38,976,952,577 bp
PacBio long-read yield	105,939,920,101 bp
High-quality subread data	96,172,405,713 bp
(B) Assembly and scaffolding	
No. of scaffolds	135
Total bases	1,112,094,387 bp
Average length	8,237,736.20 bp
Minimum length	37,458 bp
Maximum length	77,600,393 bp
N50	41,826,286 bp
Ns	43,700 bp (0.00%)
GC ratio	38.07%
Repeats	574,403,339 bp (51.65%)
Complete BUSCO (Actinopterygii_odb10)	3,487 (95.8%)
(C) Gene prediction	
No. of genes	43,153
Average gene length	10,169.51 bp
Genome coverage	39.46%
Exon/gene	7.07
Average exon length	190.31 bp
Average intron length	1,454.05 bp
(D) Annotations	
NCBI nr BLAST hits	33,326
UniProt BLAST hits	29,212
Gene ontology hits	31,338
KEGG orthology hits	26,665
EggNOG hits	24,685
Pfam hits	26,036
SignalP hits	3,578
TmHMM hits	8,362
No annotation hits	7,287

Supplementary Tables 1, 2); 1.112 GB of the representative contigs were *de-novo* assembled from 96.2 GB of error-corrected long read sequences (**Supplementary Tables 2, 3**). Then, the *de-novo* assembled contigs were scaffolded into 135 scaffolds of the draft genome, with a scaffold length N50 of 41,826,286 bases and an average scaffold length of 8,237,736.20 bases (**Table 1B**; **Figure 1B**). In total, 574 MB (i.e., 51.65%) of the draft genome was covered by repeats, in which DNA elements dominated (i.e., 28.09%) (**Table 1B**; **Figure 1C**; **Supplementary Table 5**). First, 99.33% of the pre-processed whole-genome sequencing reads, and an average of 81.82% of the pre-processed RNA-Seq reads, were mapped on the draft genome (**Supplementary Table 1**; **Supplementary Figure 2**). In total, 43,153 genes predicted in the genome, with an average size of 10,169.51 bases and a 95.8% complete BUSCO score (**Tables 1B,C** and **Figure 1D**). Ultimately, 33,326 genes had homologous sequences in GenBank and 31,338 had Gene Ontology annotations (**Table 1D**). Of the 43,153 genes, 24,699 were found to be expressed and 13,385 were DEGs (**Supplementary Table 6**; **Supplementary Figure 3**). The mitogenome was assembled into a complete circular sequence of 16,570 bases, annotated with 13 protein-coding genes, 22 tRNA genes, and 2 rRNA genes (Greiner et al., 2019) (**Supplementary Table 7**; **Supplementary Figure 4**). The complete workflow used in this study is shown in **Supplementary Figure 1**. This is the first genome assembly for the family Cobitidae. Due to the lack of genomic knowledge of this lineage, most of the NCBI-NR BLAST annotations overlapped with the proteome of the closely related suborder Cyprinoidei, which have well-established genomic profiles (**Figure 1E**). Distinct HoxBb cluster duplication was inferred in the *M. mizolepis* genome, but was not found in most genomes of closely related teleosts, including zebra fish (*Danio rerio*) (Hoegg et al., 2007; Henkel et al., 2012) (**Figure 1F**). This first genome assembly for the family Cobitidae can be used to elucidate additional genomic features to better understand this lineage, and provides new insight for comparative genomic studies of teleosts.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

ETHICS STATEMENT

The animal study was reviewed and approved by National Institute Fisheries Science (2018-NIFS-IACUC-03). Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

YS and J-HJ: genome assembly and annotations. YS, G-HS, and B-HN: manuscript preparation. EN and HK: sampling and sequencing. B-HN: funding acquisition and modeling. EK and J-HJ: data curation. Y-OK: investigation. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2021.799148/full#supplementary-material

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An Old Pathogen in a New Environment–Implications of *Coxiella burnetii* in Australian Fur Seals (*Arctocephalus pusillus doriferus*)

Brett R. Gardner[1], John Stenos[2], Jasmin Hufschmid[1], John P. Y. Arnould[3], Rebecca R. McIntosh[4], Mythili Tadepalli[2], Anita Tolpinrud[1], Marc Marenda[1], Michael Lynch[5] and Andrew Stent[1]*

[1] Melbourne Veterinary School, The University of Melbourne, Werribee, VIC, Australia, [2] Australian Rickettsial Reference Laboratory, University Hospital Geelong, Geelong, VIC, Australia, [3] School of Life and Environmental Sciences, Deakin University, Burwood, VIC, Australia, [4] Conservation Department, Phillip Island Nature Parks, Cowes, VIC, Australia, [5] Zoos Victoria, Parkville, VIC, Australia

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***Correspondence:**
Brett R. Gardner
gardner.b@unimelb.edu.au

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The Australian fur seal (*Arctocephalus pusillus doriferus*) has experienced a slower than expected population recovery since the end of the commercial sealing era, with a high third trimester abortion rate. There is currently no known proximate cause. *Coxiella burnetii* (*Cb*) is a well-known cause of abortion in domestic and wildlife species and an important zoonotic pathogen. It has been recorded from a small number of northern hemisphere marine mammals and may be a potential contributory factor to decreasing populations of northern fur seals (*Callorhinus ursinus*) and Steller sealions (*Eumetopias jubatus*). It has not been recorded from marine mammals in the southern hemisphere but is well documented in ruminants and wildlife in Australia as a cause of reproductive failure. Third trimester aborted fetuses (n = 46) and full-term placentas (n = 66) from Australian fur seals, were collected on Kanowna Island and Seal Rocks in Bass Strait, south-eastern Australia. Utilizing routine hematoxylin and eosin histopathology, *Cb* immunohistochemistry and two different qPCR targets–htpAB and com1, *Cb* was identified. Routine histopathology and immunohistochemistry were insensitive for the detection of *Cb.* The detected *Cb* prevalence ranged from 10.6% for com1 up to 40.9% with htpAB. *Coxiella burnetti* was readily detected in full-term placentas but in aborted fetal material only in a single placenta associated with a still birth. The exact significance is currently unclear, but this highlights that *Cb* is present in Australian fur seals, breeding in Bass Strait. Bass Strait is in one of the world's fastest warming oceanic regions and marine mammals breeding in the area are likely to be key indicators of marine ecosystem stressors. This first description of *Cb* in a marine mammal from the southern hemisphere, highlights the need to further investigate the potential risks this pathogen poses to Australian fur seals and sympatric marine mammals. Additionally, it is important to determine the zoonotic risk of this pathogen to persons working with, and in proximity of, Australian fur seal breeding colonies.

Keywords: *Coxiella burnetii*, Australian fur seal, abortion, placenta, *Arctocephalus pusillus*, zoonotic risk

INTRODUCTION

There are many marine mammal populations that have recovered significantly since the shift toward conservation rather than exploitation. This has been most obvious in the pinnipeds. It appears that their near-shore habitat use and relatively shorter and faster life cycles compared to cetaceans, favor their recovery (Magera et al., 2013). Worldwide, most fur seal populations have displayed an annual recovery rate of 10–15% since bans on commercial harvesting were legislated (Wickens and York, 1997). However, this has not been the case for some species, such as the Australian fur seal (*Arctocephalus pusillus doriferus*, a benthic forager) (Arnould and Costa, 2006).

Originally, it was postulated that benthic foraging pinnipeds are faced with additional stressors (i.e., energetic expenditure and nutritional composition of benthic prey) that their pelagic foraging counterparts are not exposed to (Trites and Donnelly, 2003), potentially explaining the reduced recovery of certain pinnipeds (Rosen and Trites, 2005). More specifically, this "nutritional stress hypothesis" was considered as potentially the reason for lower fecundity in benthic foragers such as the Steller sea lion (*Eumetopias jubatus*), Australian sea lion (*Neophoca cinerea*), and the Australian fur seal (Costa and Gales, 2003; Trites and Donnelly, 2003; Gibbens et al., 2010). Recent studies of the critically endangered Galapagos sea lion (*Zalophus wollebaeki*) suggest, however, that benthic foraging modes are not necessarily less efficient than pelagic foraging (Blakeway et al., 2021). This then raises the question as to what other factors could be having a significant impact on the ability of the species such as the Australian fur seal to fully recover their population?

The breeding distribution of Australian fur seals is restricted to south-eastern Australia between 32°28′–43°52′ S and 142°00′–152°33′ E, including a limited number of islands within Bass Strait (McIntosh et al., 2018). They were locally extirpated from 17 of their 26 known historical colonies within Australia by the time they got full legislative protection in 1975 (Lancaster et al., 2010). Currently there are only nine regular breeding sites within Bass Strait (McIntosh et al., 2018). It was noted in certain colonies that the number of viable pups being produced each season declined after 2007 (McIntosh et al., 2018). This does not appear to be the case across all colonies but considering that Australian fur seals have shown much slower recovery than other fur seal species (Gibbens et al., 2010), it is possible that low recruitment from an unknown factor may be contributing to the slow recovery of this species (Pemberton and Gales, 2004).

Initial research has identified multiple possible causes for low recruitment, including environmental toxicants such as per-and polyfluoroalkyl substances (PFAS) (Taylor et al., 2021). However, the influence of infectious and toxicological agents, nutritional deficiencies, climate change and other factors has yet to be fully investigated. There has been a single study in which antibodies to *Brucella* sp. were detected in adult female Australian fur seals (Lynch et al., 2011a). In terrestrial mammals, brucellosis is a serious cause of reproductive failure and abortion but in marine mammals the literature on its pathogenicity is sparse. The organism has not definitively been detected in Australian fur seals and the seroprevalence indicated low levels of this abortifacient pathogen in circulation (Lynch et al., 2011a). One of the only other pathogens associated with reproductive failure that has been identified in Australian fur seals is *Mycoplasma* but, at present, there is no substantial evidence that it plays an important role in reduced fecundity (Lynch et al., 2011b).

Coxiella burnetii (*Cb*) was first described in Australia in 1937 as the causative agent of human disease, Query-fever or Q-fever (Eldin et al., 2017). This intracellular gram-negative bacterium has been associated with a broad range of hosts, including domestic animals and wildlife (González-Barrio and Ruiz-Fons, 2019). Although it is an obligate intracellular pathogen, it has two distinct growth forms. The intracellular form is known as the large cell variant (LCV), but once the organism is shed in the reproductive secretions, the organism transforms into a highly resistant condensed form known as the small cell variant (SCV), which is the most infective form of *Cb* (Abeykoon et al., 2021). In domestic ruminants, *Cb* has been noted to cause significant reproductive failure including abortion, still-birth, premature birth and weak full-term birth (Agerholm, 2013). It is considered an organism of significant zoonotic risk with the ability to be dispersed as an environmentally resistant aerosol that can remain infective for decades (Bond et al., 2018).

Although infection tends to be localized and with distinct patterns in various geographic regions, occasionally it can occur as large disease outbreaks (Eldin et al., 2017). In humans and domestic animals, the effects of a disease outbreak can be catastrophic, as was the case in Netherlands epidemic (2005–2009) with nearly 4000 human cases reported as a result of aerosols from infected goat herds (Roest et al., 2011). It is often less dramatic in humans, producing mostly influenza-like symptoms with a roughly 1% case fatality rate (Bond et al., 2018). *C. burnetii* is an important emerging disease in wildlife (González-Barrio and Ruiz-Fons, 2019), but despite *Cb* being well described in domestic animals and humans, data on its significance in wildlife are sparse in comparison, with very little literature available on the significance of infection in marine mammals. *C. burnetii* has been detected in the placental tissue of a few species of northern hemisphere marine mammals including harbor seals (*Phoca vitulina*), harbor porpoises (*Phocoena phocoena*), Steller sea lions and northern fur seals with the first report in the literature in 1999 (Lapointe et al., 1999; Duncan et al., 2012; Kersh et al., 2012). Whether it poses a significant risk as a pathogen of marine mammals is still uncertain, though it has been associated with declining populations of northern fur seal and Steller sea lion (Minor et al., 2013).

The aims of this study were to investigate whether *Cb,* a significant cause of reproductive failure in terrestrial mammals, could play a contributory role to low fecundity in Australian fur seals. Aborted fetuses, still births and full-term placentas from two of the largest Victorian breeding colonies were screened histologically and molecularly for the presence of *Cb* over a period of 3 years.

MATERIALS AND METHODS

Sample Collection

Spontaneously aborted third trimester Australian fur seal fetuses were purposely collected on Kanowna Island (39°15′48″S;

146°31′04″E) (n = 27) between July and October in 2019 and 2020 and opportunistically on Seal Rocks (38°31′34″S; 145°05′59″E) (n = 19) between August and October in 2018, 2019, and 2020. The Seal Rocks colony consists of approximately 18,400 individuals and is currently the largest for the species (McIntosh et al., 2018). Kanowna Island hosts a colony of approximately 13,000 individuals (Kirkwood et al., 2010) which is currently considered to be the third largest for the species (McIntosh et al., 2018). Fetuses were freshly sampled as per **Table 1** on Kanowna Island and those from Seal Rocks were stored frozen at −20°C and thawed at room temperature prior to processing.

Sixty-six fresh placentas from-full term Australian fur seal pups born on Kanowna Island were collected over a 3-week period during November and December 2020 and immediately processed *in situ*. The placental sections collected into formalin were cut full thickness from the maternal side of the placenta through to the fetal side of the placenta in the cleanest, grossly uncontaminated portions of the placenta. The incision and size of the tissue blocks ensured collection of all placental structures through from the fetal to maternal side of the placenta, including the trophoblast and hematophagous zones. The samples stored in RNAlater for DNA extraction were collected from the internal aspect of the opened placental sac-like unit, cutting on the fetal side of the placenta, to avoid environmental contamination of the samples.

A summary of the samples collected from each location is detailed in **Table 1**, indicating which were stored in 10% buffered formalin and in RNAlater (Thermo Fisher Scientific, Waltman, MA, United States). Some organs were absent due to scavenging activity of gulls.

Histopathology

Routine hematoxylin and eosin (HE) staining was performed on 5 μm thick sections of formalin fixed fetal tissues (heart, lung, trachea, esophagus, thymus, thyroid, lymph nodes, cerebrum, cerebellum, liver, spleen, kidney, adrenal gland, stomach, and umbilicus) and placenta embedded in paraffin wax. The sections were mounted on microscope slides and examined under light microscopy. Lesions across all representative fetal tissues were described. **Table 1** details all the fetal tissues collected and examined histologically. Two sections of representative placenta and placental vasculature were evaluated for each full-term placenta. The placental slides were evaluated for the presence of necrosis, mononuclear and neutrophilic infiltration, fibrin deposition, the accumulation of cellular debris and thrombosis as individual histological traits. Each histological trait was assigned a score between zero and three, to indicate the presence and degree of severity, with 0.5 increments. The total score for each slide was divided by five as five histological traits were examined for each slide to give an average score used to grade the inflammatory changes present.

DNA Extraction and Quantitative Polymerase Chain Reaction

Using a proprietary Invitrogen Purelink genomic DNA extraction kit (Thermo Fisher Scientific, Waltman, Massachusetts, United States), genomic DNA was extracted and purified from a total weight of 250–350 μg of RNAlater preserved tissue for each sample. For fetuses the tissues collected from one individual were combined and extracted together. For full-term placentas the total weight was comprised of solely placental tissue.

Coxiella burnetii was detected and semi-quantified using two real-time TaqMan polymerase chain reaction (qPCR) assays using the proprietary Invitrogen Platinum Quantitative PCR SuperMix-UDG (Thermo Fisher Scientific, Waltman, MA, United States), a two-color tracking dye system to prevent carryover contamination targeting the com1 and htpAB genes. The com1 is a single copy gene whereas the htpAB is a 20-copy gene, that codes for two heat shock proteins (htpA and htpB) and is more sensitive than the single gene com1 (Fournier and Raoult, 2003). The primers and probe concentrations are shown in **Table 2**. A total volume of 25 μl was used for each reaction, which included 5 μl of extracted DNA. A no template control was run with each reaction using 5 μl nuclease free water (Gibco, Mulgrave, Vic, Australia). A magnetic induction PCR cycler (Mic) was used to perform amplification and fluorescence

TABLE 1 | Tissue samples collected from aborted fetuses and full-term placentas from Australian fur seals (*Arctocephalus pusillus doriferus*) from Kanowna Island (KI), Seal Rocks (SR) in Victoria.

	Lung	Lymph node	Heart	Thymus	Thyroid	Spleen	Liver	Trachea	Kidney	Adrenal gland	Cerebellum and Cerebrum	Placenta
Kanowna Island												
Aborted fetuses (n = 28)												
10% F	28	28	24	27	11	21	22	28	21	21	21	7
RNA Later	28	28	0	27	0	21	22	0	6	0	0	7
Full-term placentas (n = 66)												
10% F												32
RNA Later												66
Seal Rocks												
Aborted fetuses (n = 19)												
	16	16	15	14	10	12	12	16	13	13	9	2
	18	15	0	13	0	12	12	0	0	0	0	2

Respective sample sizes (n) for preservation in 10% formalin (F) and RNA Later proprietary preservative are shown.

TABLE 2 | Primers and probes used for qPCR assays for the detection of *Coxiella burnetii* DNA in tissue samples from Australian fur seals (*Arctocephalus pusillus doriferus*).

Assay	Primer/ Probe	Sequence (5′–3′)	Final concentration (nm)	Amplicon size (bp)
Com1	Com1_F	AAAACCTCCG CGTTGTCTTCA	400	76
	Com1_R	GCTAATGATACTT TGGCAGCGTATTG	400	
	Com1_P	FAM[a]-AGAACTGCCCATT TTTGGCGGCCA-BHQ1[b]	200	
htpAB	htpAB_F	GTGGCTTCGC GTACATCAGA	400	114
	htpAB_R	CATGGGGTT CATTCCAGCA	400	
	htpAB_P	FAM-AGCCAGTACGGT CGCTGTTGTGGT-BHQ1	200	

All primers and probes were synthesized by Integrated DNA technologies (IDT).
The positive control, Nine Mile Phase II, Clone 4 (RSA439) was obtained after repeated passage in Vero cells and diluted to give Cq values between 26 and 30 per amplification.
Prepared single use aliquots and stored in −20°C.
[a] *6-Carboxyfluorescein.*
[b] *Black Hole Quencher-1.*

detection at 50°C for 3 min, thereby activating the uracil DNA glycosylase (UDG) in the SuperMix. Cycling then occurred at 95°C for 5 min to activate the platinum Taq DNA polymerase. For both the com1 and htpAB assays, 40 cycles of denaturation at 95°C for 20 s, with annealing and elongation occurring at 60°C for 40 s were used to amplify the *Cb* DNA. Data were analyzed using proprietary Mic PCR software version 2.8.13. A positive reaction for *Cb* DNA on either of the two qPCR was considered a positive result.

Immunohistochemistry

For immunohistochemistry (IHC), paraffin wax embedded placental sections were stained with anti-*Coxiella* antibodies (dilution 1:2,500) of only the PCR positive samples. PCR negative normal full-term goat placenta was used as the negative control tissue and the positive control was goat placenta with a known positive immunohistochemical staining for intracytoplasmic *Cb*. The assay was previously developed in-house at the University of Melbourne and internally validated; for details see Muleme et al. (2021).

Immunohistochemistry staining was applied to all placentas that produced a positive result on either of the two qPCRs and for which formalin fixed tissue was available (n = 11). Sections of formalin-fixed tissue of 5 μm thickness were mounted and deparaffinized on slides and then rehydrated with ethanol. Antigen retrieval was performed using a commercial kit (DAKO ARS S1700, Agilent, Santa Clara, CA, United States), the primary antibody was applied in 1:2500 and allowed to incubate for 30 min. Nova Red Chromogen Substrate (Vector Laboratories, Burlingame, CA, United States) was applied as per the manufacturer's instructions and the slides were then counterstained with hematoxylin.

RESULTS

Routine histopathology did not show any *Cb* organisms in any of the aborted fetal material including the associated aborted placentas. There were various non-specific lesions present throughout the fetuses, but none were positive for *Cb*. Amnion aspiration was commonly detectable through the presence of increased numbers of squamous epithelial cells present in the alveoli of the fetal lung and present in 29.8% of aborted fetuses. Nearly a fifth (23.4%) of the fetal lungs had what appeared to be histiocytic pneumonia with occasional neutrophilic infiltrates. There were non-specific cardiac lesions in 14.9% of the aborted fetuses including lymphohistiocytic epicarditis, regional myocarditis and myocardial necrosis. Of all the full-term placentas examined histologically on routine hematoxylin and eosin staining, intracytoplasmic bacteria were only detectable in one sample. The organisms were not uniformly distributed, but when present formed stippled cytoplasmic inclusions with characteristic foamy appearance within the placental trophoblasts (**Figure 1**). The *Cb* organisms did not appear to be associated with significant inflammatory reactions.

The grading of placental inflammatory lesions on HE, did not correlate with the presence of *Cb* as determined by qPCR. Placental inflammatory changes were quite focal and, even in more dramatic instances, were often interspersed with large amounts of normal appearing placenta. Many of the inflammatory changes appeared to be associated with the normal birthing process. These included focal areas of hemorrhage and thrombosis, mononuclear and neutrophilic white cell infiltration not associated with organisms or significant necrosis, mild fibrin deposition and the accumulation of surface cellular debris. Trophoblast necrosis was commonly seen as large strips of necrotic tissue surrounded by normal placental architecture. Occasionally the necrotic areas of trophoblast were associated with regions of fibrosis and mild focal calcification in 7.6% of placentas examined microscopically. The latter are not acute changes and not assumed to be associated with normal parturition.

A total of 45 third trimester abortions and a single full-term still birth collected from Kanowna Island and Seal Rocks were tested with both the htpAB and com1 qPCR. Due to scavenging by gulls, placental tissue was rarely available for the abortions and only nine (including the full-term still birth) were recovered and tested with the aborted fetuses. *Coxiella burnetti* DNA was not detected in any of these samples, except for the placental tissue of the full-term still birth. On the other hand, when the full-term placentas were evaluated using both qPCR, a substantial number of samples produced a positive result for the presence and amplification of *Cb* DNA. The htpAB produced 27 positive results from 66 samples (40.9%), whereas the com1 produced seven positive results from 66 full-term placental samples (10.6%). All samples that produced a positive com1 amplification were also positive for amplification on the htpAB (**Table 3**). The

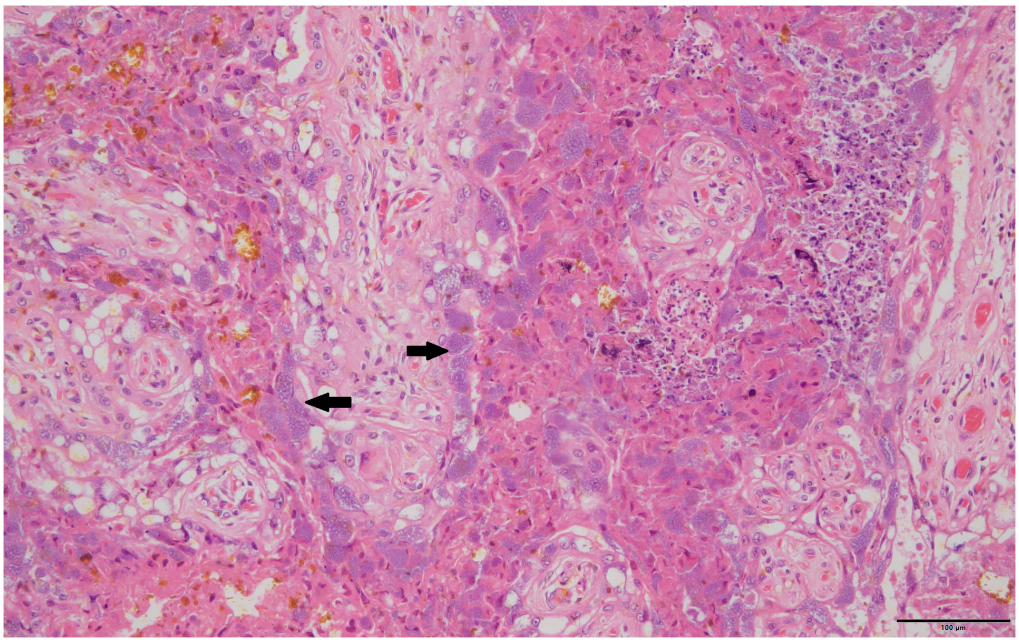

FIGURE 1 | Typical foamy *Coxiella burnetii* cytoplasmic inclusions within the placental trophoblasts are indicated by solid black arrows, 40×, hematoxylin and eosin stain.

quantification cycle (Cq) values ranged from 27.91 to 38.19 for the com1 and 24.58 to 39.64 for the htpAB. Using a Fisher's exact test, it appears that there is a statistically significant predicted difference using at a 95% confidence interval with a p value of 0.05 of 0.0238 between the presence of *Cb* in full-term placentas and the placental tissue of third trimester abortions.

Only full-term placentas that produced a positive result on the htpAB qPCR and for which formalin-fixed tissue was available were evaluated using IHC (n = 11). One of these placentas produced a positive result on IHC for immunoreactive coccobacilli (**Figure 2**), being the sample that was identified on routine histopathology. The IHC produced minimal non-specific staining of degenerate surface non-*Cb* material and was relatively easy to evaluate.

TABLE 3 | Summary of all test results for *Coxiella burnetii* in Australian fur seals (*Arctocephalus pusillus doriferus*) from Victoria, across all samples and testing modalities.

	HE	IHC	com1	htpAB
Aborted fetuses	0% (0.00; 0.08) (0/45)	0% (0.00; 1.79) (0/1)	0% (0.00; 0.08) (0/45)	0% (0.00; 0.08) (0/45)
Still births	0% (0.00; 1.79) (0/1)	0% (0.00; 1.79) (0/1)	100% (0.79; 2.00) (1/1)	100% (0.79; 2.00) (1/1)
Full-term placentas	3.3% (29.99; 30.17) (1/30)	10% (9.98; 10.40) (1/10)	10.6% (65.95; 66.20) (7/66)	40.9% (65.70; 66.53) (27/66)

Results shown as percentage (95% CI - lower; upper) and number of positive samples/total number of samples tested in brackets underneath.
HE, hematoxylin and eosin stain on histopathology; IHC, immunohistochemistry.

DISCUSSION

With a detected *Cb* prevalence ranging from 10.6 to 40.9%, this study brings to question what significance *Cb* holds for the reproductive ecology of Australian fur seals. *C. burnetii* is known to typically cause abortions, premature birth and other indirect negative impacts on reproduction (Agerholm, 2013). There is a growing body of evidence that *Cb* is associated with chronic infertility, especially in cattle that do not show the typical overt clinical signs of large-scale abortions as is seen in goats (De Biase et al., 2018). In 2018, it was first described that *Cb* can produce chronic endometritis in dairy cattle with resultant chronic sub-fertility (De Biase et al., 2018). The results of this study in full-term, previously presumed healthy births might indicate that Australian fur seals experience chronic sub-fertility in part from *Cb* infection.

Reduced pup abundance has been observed in Australian fur seals at various colonies (McIntosh et al., 2018). Increased pup mortality has also been noted in unpublished observations at Seal Rocks (McIntosh, unpublished data). Some of the colonies exhibit high rates of third trimester abortions (Gibbens et al., 2010). At present the exact cause of the decreased Australian fur seal pup numbers in certain colonies is unknown. *C. burnetii* is currently an important emerging disease in wildlife (González-Barrio and Ruiz-Fons, 2019) and is widely reported on the Australian mainland (Mathews et al., 2021). Considering the detected prevalence of *Cb* in full-term placentas, it brings

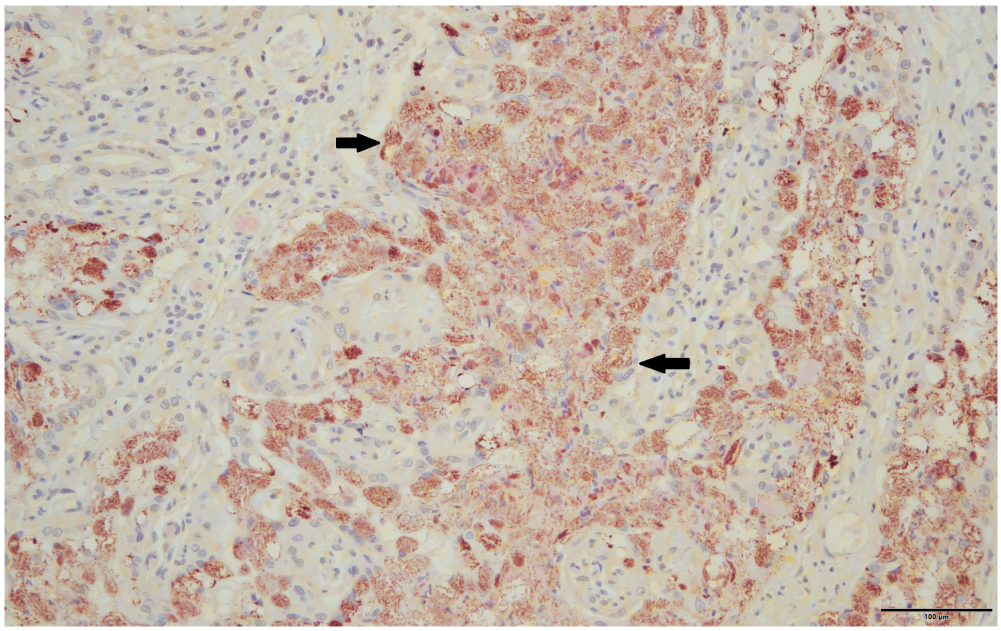

FIGURE 2 | Immunoreactive coccobacilli typical of *Coxiella burnetii* stain a brick red-orange on immunohistochemistry are indicated by solid black arrows, 40×.

to question if this may potentially have significant impacts on post-partum pup survival.

It has been noted that Australian fur seals tend to have higher rates of abortion in young females despite younger females having high rates of pregnancy (Gibbens et al., 2010). Potentially if younger females are naïve to *Cb* infection, this could contribute to higher rates of abortion, especially if combined with other environmental stressors. When *Cb* infection shows endemic circulation in a population, older animals often inadvertently develop more effective immunity (González-Barrio and Ruiz-Fons, 2019). These negative reproductive outcomes could be contributing to a direct lack of recruitment in the Australian fur seals colonies at both study sites through premature and full-term loss of pregnancy. Furthermore, if *Cb* can produce chronic sub-fertility in Australian fur seals, this would additionally decrease the fecundity of breeding colonies and could contribute to the observed slow population recovery rate.

Routine HE histopathology and IHC had poor sensitivity for the detection of infection with *Cb*. Full-term placentas regularly had minor histopathological changes that were presumed in most cases to be associated with normal parturition rather than distinct *Cb*-related pathology. The typical lesions described in this study, including surface debris accumulations associated with placental necrosis, inflammatory white cell infiltrations, thrombosis and occasional mineralization have been identified as non-specific changes associated with parturition and are not necessarily indicators of infectious placentitis in bovine placentas (Botta et al., 2019). In the routine histopathology and the immunohistochemical staining, the distribution of the presence of *Cb* in the placental tissue was patchy. These results are similar to findings published in a study of several marine mammals of the Pacific Northwest of the United States, which found that IHC is quite insensitive for detecting the presence of *Cb* in marine mammal placentas (Kersh et al., 2012).

The sensitivity of detection with IHC was very low at 9.1% for qPCR *Cb* positive samples in the study of marine mammals of the Pacific North-West (Kersh et al., 2012). In a study of northern fur seals, where qPCR was employed in conjunction with IHC, only 3% of placentas evaluated were positive on IHC, compared to qPCR that showed a 74.7% detection rate for *Cb* using the com1 gene (Duncan et al., 2012). Similarly reported low detection rates of 11% using IHC compared to 30% for the com1 qPCR have been recorded in marine mammals in the Pacific Northwest of the United States (Kersh et al., 2012).

Molecular testing was found to be a more sensitive modality for the determination of the presence of *Cb* in Australian fur seals. The authors believe the difference in detection of *Cb* between the abortions and full-term placentas is an inability to detect the organism due to a small sample size for placentas from the abortions. In the few reports of abortions associated with *Cb* in marine mammals, there were no fetal lesions and *Cb* organisms could only be detected in placental tissue (Lapointe et al., 1999; Kersh et al., 2010). The results presented here support this being the same in Australian fur seals. It is well documented that the placenta acts as a barrier to pathogens entering the fetus and is a far more common site of infection (Robbins and Bakardjiev, 2012). On the other hand, with abortions being sporadic and not synchronized as is pupping, the risk for cross contamination might also be lower, while the high prevalence in full-term placentas could be at least partially the result of such cross contamination.

Strict care was taken in the field sampling to avoid environmental and cross-contamination of samples. It is not

certain if some of the higher *Cq* (Quantification cycle) values of the qPCR indicate low levels of *Cb* across most samples or if those with higher *Cq* values potentially indicate a degree of environmental contamination at the time of maternal expulsion. During the pupping season there are dense congregations of females simultaneously giving birth, potentially increasing the risk of cross-contamination between infected and uninfected placentas.

The association of *Cb* with Australian fur seal placental tissue does not necessarily prove causality but highlights the need to determine whether this infectious organism is in part responsible for the decreased pup production of this species and its lower-than-expected recovery rate. The results of the present study are geographically restricted, with all samples collected from two colonies and all the positive samples from only one of these, Kanowna Island. The significance of *Cb* infection to Australian fur seals within this positive colony is not known and is even less so for the other colonies within the species' range. Indeed, very little is known of the importance of this pathogen in other marine mammals. Its origin in Australian fur seals is unknown and further genetic investigations are required to determine if it is related to spillover of terrestrial Australian genotypes or if it potentially an endemic marine adapted form of *Cb*. Such information is of importance as these two scenarios have completely different environmental implications.

There are significant zoonotic considerations to the findings presented here. Historically, persons working within the livestock industry, especially those working with small ruminants and in the abattoir industry have been associated with an increased risk of zoonotic exposure to *Cb* (Mathews et al., 2021). A highly effective national *Cb* vaccination strategy has been in place in Australia since 2002 for persons at high risk in the abattoir and farming sector (Gidding et al., 2009). It has become apparent, though, that wildlife rescuers and rehabilitators are twice as likely compared to the general Australian public to be exposed to *Cb* and seroconvert (Mathews et al., 2021). It is also well documented that Australia has one of the highest recorded rates of Q-fever infection in the world (Bond et al., 2018).

Coxiella burnetii can persist in the environment for many years as the SCV, which is extremely resistant to inactivation through environmental degradation (Eldin et al., 2017). This SCV is also highly infectious and even in dilute environmental numbers is still a considerable zoonotic risk (Eldin et al., 2017). Taking into consideration the sheer numbers of animals giving birth in Australian fur seal colonies and the often dry and windy weather conditions during the pupping season (November–December) (O'Grady and McInnes, 2010), there is a clear need to investigate if these conditions pose an occupational health and safety concern for human activities in and around Australian fur seal colonies and if there is a seasonal difference in these risks. In the interim it may well be important to ensure adequate vaccination of persons working in these situations as they should be considered high-risk for potential *Cb* exposure.

Australian fur seals have overlapping foraging areas, haul-outs and breeding colonies with other pinnipeds including New Zealand fur seals (*Arctocephalus forsteri*) and Australian sea lions (Shaughnessy et al., 2010; Hoskins et al., 2017). This could serve as a source of cross-species transmission and could have potentially serious risks to the already endangered Australian sea lion population. In South Australia (Kangaroo Island and Casuarina Island), New Zealand fur seals and Australian sea lions have in recent years had increasing contact with Australian fur seals as they expand their breeding range into South Australia (Shaughnessy et al., 2010). The Australian sea lion is Australia's only endemic pinniped, is classified as endangered and currently shows a decreasing population size facing a variety of population risks (Goldsworthy et al., 2021). If this population is naïve for *Cb*, there is the potential for introduction through expansion of Australian fur seal colonies that results in additional risk to an already unstable population. Unpublished observations from researchers working on Cape Bridgewater and Lady Julia Percy Islands have noted the presence of male Australian sea lions in Australian fur seal colonies. At present *Cb* has only been investigated in female animals and abortions. The risk that males pose to transmission of the organism is unknown. Alternatively, if *Cb* is already present in the population it would need to be explored as a possible contributing factor to the population decline of this endemic marine mammal.

To the authors' knowledge this is the first description of *Cb* in a marine mammal in the southern hemisphere despite the organism being first described on the Australian mainland nearly a century ago. It is currently unknown if the terrestrial and marine *Cb* organisms are of the same genotype. It is critical to understand the relationship between *Cb* in Australian fur seals and terrestrial genotypes of the organism. There is some preliminary evidence that genotypes might display a degree of host specificity (González-Barrio et al., 2016). Understanding the significance of this well-known zoonotic abortifacient organism is very important both to the ecology of Australian fur seals and other marine mammals. Additionally, also as a potential risk factor to researchers and ecotourism operators conducting work with and in the vicinity of Australian fur seal colonies.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Phillip Island Nature Parks Animal Ethics Committee and University of Melbourne Research Ethics and Integrity Committee.

AUTHOR CONTRIBUTIONS

BG and JH conceived the idea for the project. AS was the primary histologist with assistance by BG. Molecular work was done by AT, MT, and BG. BG led the data analysis with the assistance of JH, JS, JA, RM, and AT, led the writing of the manuscript with assistance from all authors, and collected and processed the

samples. All authors contributed critically to the drafts and gave final approval for the published manuscript.

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Evaluation of Spawning- and Natal-Site Fidelity of *Larimichthys polyactis* in the Southern Yellow Sea Using Otolith Microchemistry

Dade Song[1,2], Ying Xiong[1], Tao Jiang[3], Jian Yang[3], Xiaming Zhong[1]*, Jianhua Tang[1] and Zhongjie Kang[1,2]*

[1] Jiangsu Marine Fisheries Research Institute, Nantong, China, [2] College of Marine Sciences, Shanghai Ocean University, Shanghai, China, [3] Key Laboratory of Fishery Ecological Environment Assessment and Resource Conservation in Middle and Lower Reaches of the Yangtze River, Freshwater Fisheries Research Center, Chinese Academy of Fishery Sciences, Wuxi, China

Small yellow croaker *Larimichthys polyactis* is an important commercial fish species; however, industrial-scale fishing has largely contributed to the changes in its biological characteristics, such as individual miniaturization, faster growth, and younger average age. Robust understanding of the pivotal life history of *L. polyactis*, a typical oceanodromous species, is needed for its conservation and restoration. However, *L. polyactis* fidelity to natal or spawning sites is not well understood and, at present, there is no effective management strategy to guarantee the sustainable exploitation of *L. polyactis*. This study used laser ablation inductively coupled plasma-mass spectrometry to analyse the elemental composition of otoliths from 60 adult yellow croakers caught in the southern Yellow Sea, including two spawning groups with 1- and 2-year-old fish (S1 and S2, respectively) sampled close to China and one overwintering group including two-year-old fish (O2) sampled close to South Korea. The ratios of elements (Li, Na, Sr, and Ba) to Ca in the otolith core zones were significantly higher ($P < 0.05$) than in those of the year one (Y1) and year two (Y2) annual rings, but there were no significant differences in the elemental ratios between the Y1 and Y2 zones. Principal component analysis (PCA) of the elemental otolith signatures of the core, Y1, and Y2 zones in the three groups revealed two distinct clusters (cluster 1: S1-core, S2-core, and O2-core zones; cluster 2: S2–Y1, O2–Y1, S2–Y2, and O2–Y2 zones) and one zone (S1–Y1), suggesting spawning-site fidelity and natal-site fidelity uncertainty, especially considering the dispersal by current in prolonged period (50 h) from fertilized eggs to hatching and internal effect, such as yolk sac and maternal effect. Furthermore, these results indicated that the S2 and O2 groups could represent the same population, suggesting a stable migratory route for *L. polyactis* in Chinese and South Korean waters, whereas the S1 group could represent another population. This suggests the possibility a mixed *L. polyactis* population in the southern Yellow Sea. Characterization *L. polyactis* spawning-site fidelity is a crucial step toward linking spawning-site fidelity of this overexploited species with thorough conservation and management strategies.

Keywords: otolith microchemistry, *Larimichthys polyactis*, laser ablation inductively coupled plasma-mass spectrometry, spawning-site fidelity, connectivity

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Kit Yue Kwan,
Beibu Gulf University, China

Reviewed by:
Ying Xue,
Ocean University of China, China
Bilin Liu,
Shanghai Ocean University, China

***Correspondence:**
Ying Xiong
yxiongshfu@126.com
Xiaming Zhong
oceanxmzh@163.com

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INTRODUCTION

Migration is a fundamental behavioral characteristic of many animal species (Alo et al., 2020). All animals demonstrate some types of movement during their life span; among them, two have captivated the attention of scientists and non-experts: natal-site philopatry (i.e., return to birthplace) and spawning-site philopatry [i.e., return to a previous spawning site (Dingle and Drake, 2007; Chen et al., 2020)]. From an evolutionary point of view, natal philopatry enables isolation of specific breeding populations ("stocks") and provides potential for local adaptation in order to increase reproductive capacity, decrease the potential of recruitment failure, and stabilize the ability to adjust to environmental change and exploitation (Hilborn et al., 2003; Sadovy and Domeier, 2005). However, there are consequences to natal-site philopatry, including the risk of inbreeding depression (Miller et al., 2001) and increased competition (Sandercock, 1991; Di Franco et al., 2012). The phenomenon opposite to philopatry is dispersal, which is the migration away from the birthplace prior to reproduction (Mora and Sale, 2002). High rates of dispersal have been documented when the chance of inbreeding and population density are increased (Cote et al., 2010), suggesting that dispersal might counterbalance some of the negative consequences of natal-site philopatry for the population fitness. Therefore, reproductive fidelity in mobile marine species may regulate the spatial population structure and connectivity (Hastings and Botsford, 2006). The fidelity to natal or spawning sites can effectively be used to make informed decisions about conservation strategies, especially regarding future marine protected areas (MPAs), which, ultimately, should improve the management and conservation of marine fishery resources (Bardbury et al., 2008; Di Franco et al., 2012; Tripp et al., 2020).

Determination of natal-site fidelity is quite challenging, and requires the tracking of fish from eggs until breeding (Ovenden, 2013). During the past 30 years, a range of tagging techniques have been reported, which can be categorized as external (Florin and Franzén, 2010; Lowerre-Barbieri et al., 2013), implanted (Mellish et al., 2007), and natural (Reis-Santos et al., 2013; Chen et al., 2020; Stone et al., 2020). However, external and implanted tagging can have negative effects on health, reproduction, and survival; furthermore, they are costly and time-consuming, and may not be effective for small-sized and long-distance migratory fish because of the low recapture rate (Bishop et al., 2010; Humston et al., 2010). Recent technological advances have enabled the potential of distinct biological hard tissues as natural tags; among them, the elemental composition of otoliths has been used as an important marker for tracking movement and determining the connectivity patterns in fish species (Elsdon and Gillanders, 2003; Tanner et al., 2016; Honig et al., 2020; Martinho et al., 2020). Otoliths grow continuously over the lifespan in daily and annular rhythms and are not reabsorbed (Schulz-Mirbach et al., 2019); therefore, they have been widely used as indicators of fish age (Campana, 1999). Also, a substantial number of elements (e.g., calcium, Ca; strontium, Sr; barium, Ba; sodium, Na; magnesium, Mg; magnesium, Mn; potassium, K; lead, Pb; zinc, Zn), are co-precipitated in the otolith growth increments as the fish grows (Campana, 1999). Unfortunately, as otolith chemistry varies over time with respect to salinity, temperature, and ontogenetic stage, as well as ambient water occupied (Barnes and Gillanders, 2013; Taddese et al., 2019), single-element profiles may not be a reliable means of spatially tracking individuals (Kraus and Secor, 2004). Thus, the microchemical composition of equivalent otolith parts can serve as a natural marker to determine fish origin, reconstruct migration pathways, and discriminate among fish groups (Gillanders, 2002; Kraus and Secor, 2004; Svedäng et al., 2007; Munch and Clarke, 2008; Thorisson et al., 2010; Engstedt et al., 2013).

Small yellow croaker (*Larimichthys polyactis*) is an important commercial fish species endemic to the Northwest Pacific, particularly coastal waters of the East China, Yellow, and Bohai Sea between China and the Korean Peninsula (Lin, 1987). *L. polyactis* is an iteroparous species, which starts spawning at the age of 1 year, as evidenced by histological analyses of female individuals (Xie et al., 2021). For decades, *L.* polyactis has been an important source of seafood for local markets in China, Japan, and South Korea (Ma et al., 2020; Szuwalski et al., 2020); however, environmental deterioration and overfishing have led to a major decline in its population since the 1970s (Li et al., 2011). From 2000, the global capture production of *L. polyactis*, mainly by China and South Korea, has been increasing, reaching a maximum of 4.58×10^5 t in 2010 (Fishbase, 2019). Owing to intense fishing pressure, the current population of *L. polyactis* is characterized by individual miniaturization, decreased asymptotic length and weight, faster growth, and younger average age (Shan et al., 2017; Lee et al., 2019). Moreover, because of high fishing pressure *L. polyactis* has shifted its spawning grounds from nearshore to offshore waters (Lin et al., 2008). To avoid *L. polyactis* population collapse, such as happened to its sister species the large yellow croaker *Larimichthys crocea*, much stricter conservation measures based on scientific data must be implemented (Choi and Kim, 2020; Li et al., 2020).

Larimichthys polyactis is a typical oceanodromous fish that is born in estuaries or nearshore areas, grows and matures in nearby coastal waters, and migrates offshore to overwintering grounds (Xiong et al., 2017). Although progress has been made regarding the detailed migratory cycle of *L. polyactis* (Xu and Chen, 2009; Xiong et al., 2017; Song et al., 2022), understanding of its natal- or spawning-site fidelity is limited, hampering the development and implementation of appropriate conservation and resource management strategies for this overexploited and highly targeted species. Based on the morphological variations and migratory routes of *L. polyactis*, three wild populations have been suggested to exist in the China Seas: and the East China Sea population, the southern Yellow Sea population, and the northern Yellow/Bohai Sea population (Lin, 1987; Zhang et al., 2014). The southern Yellow Sea population has its overwintering grounds in the area defined by Sino-Korean fisheries agreements and in the exclusive South Korean economic zone (Lin, 1987; Baik et al., 2004; Han et al., 2020); however, its natal and spawning sites are uncertain. Therefore, it is critical to know whether *L. polyactis* returns to its natal and spawning sites to determine rational fishing quotas in China and South Korea (Xue, 2005).

Larimichthys polyactis analyzed in this study is an iteroparous species. Its spawning period in Chinese waters differs slightly from south to north, with the spawning peak of *L. polyactis* in the southern Yellow Sea being in April (Lim et al., 2010; Lin et al., 2018). Therefore, specimens of the spawning group were all sampled in April; the gonadosomatic indices (GSI) were all at stages V or VI, confirming the spawning period. The deposition of elements in the otoliths of *L. polyactis* exhibits annual periodicity (Li et al., 2013; Zhan et al., 2016; Zhang T. T. et al., 2019; Song et al., 2022). Accordingly, the otolith elemental composition in the equivalent parts of the otolith can be used as a biological tracer to examine natal- and spawning-site fidelity. Therefore, laser ablation inductively coupled plasma mass spectrometry (LA-ICPMS) was applied to the spawning group and overwintering group equivalent parts in sagittal otoliths of *L. polyactis*. The aim of this study was to examine the connectivity between *L. polyactis* spawning groups from China nearshore areas and overwintering groups from South Korea offshore areas based on otolith chemical signatures, which are metabolically stable, and thus to allow accurate stock classification. We also assessed the potential use of otolith element composition as a natural tag to examine natal- and spawning-site fidelity of *L. polyactis* in the southern Yellow Sea. This information should help to improve fishery management, including the development of appropriate fishing regulations and rational quotas for China and South Korea.

MATERIALS AND METHODS

Sampling and Laboratory Procedures

A total of 60 adult *L. polyactis* fish were collected from spawning grounds (close to China) and overwintering grounds (close to South Korea) in the Southern Yellow Sea (**Figure 1**); the samples included 20 1-year-old spawning individuals (S1) obtained in April 2017, 20 2-year-old spawning individuals (S2) obtained in April 2018, and 20 2-year-old overwintering individuals (O2) obtained in November 2018 (**Table 1**). Samples of S1/S2 and O2 fish were randomly collected from the catches of commercial vessels, which used anchored stow nets (cod-end mesh size, 25 mm) and gill nets (mesh size, 50 mm), respectively. The samples of fish were immediately refrigerated and transported to the laboratory. Fish body length, body weight, and sex were recorded in sequence. The nearest body length and weight are 0.1 mm and 0.1 g, respectively. Then, the gonadosomatic index (GSI) was determined by visual examination to confirm the assignment of *L. polyactis* to spawning groups (GSI: stage V and VI; Lim et al., 2010). Subsequently, sagittal otoliths were extracted, dried, weighed, and stored in plastic tubes; the numbers of seasonal growth rings (annulae) in the otoliths were counted to confirm fish age, and outlier specimens were excluded from further analysis. Detailed information on the samples used for otolith microchemistry analysis is presented in **Table 1**.

Otolith Elemental Analysis

Left sagittal otoliths were embedded in epoxy resin (Epofix; Struers, Copenhagen, Denmark), mounted on petrographic slides, sectioned by grinding in the sagittal plane on both surfaces with grit silicon carbide paper (to reveal the nucleus, daily growth increments), and annual growth increments, and further polished with 0.3 μm Al_2O_3-embedded lapping paper on an automated polishing wheel (Labopol-35, Struers, Copenhagen, Denmark) to remove major scratches. After final polishing, otoliths slides were sonicated in an ultrasonic cleaning bath for 5 min, rinsed with deionized water for 6 times, and dried at 38°C overnight for chemical analysis.

Elemental analysis of *L. polyactis* otoliths was performed using laser ablation inductively coupled plasma spectrometry (LA-ICP-MS; Jiang et al., 2016) in an NW213 laser ablation module (New Wave Research, Fremont, CA, United States) with an Agilent 7500ce inductively coupled plasma mass spectrometer (Agilent Technologies, Palo Alto, CA, United States). The laser was operated at a wavelength of 213 nm, pulse rate of 10 Hz, high voltage of 10 kV, and energy density of 12.58 J/cm^2. To assess natal- and spawning-site fidelity, ablated spots of 20 μm in diameter were located in the core and annual rings with a dwell time of 5 s (**Figure 2**). The numbers of ablated spots in the core and annual rings were 1 and 3, respectively, allowing for more reasonable and accurate elemental information representing the environment of natal and spawning site. And, the three spots on annuals were ablated on the front, middle, and back of the annual ring with 20 μm interval. The ablated material was transported from the laser unit to ICP mass spectrometer using a mixture of argon and helium gases. Two standard samples, MACS-3 (United States Geological Survey, Reston, VA, United States) and NIST612 (National Institute of Standards and Technology, Gaithersburg, MD, United States), were analyzed at the beginning and end of each sampling session (10 samples). Limits of detection (LOD) were determined based on 100 s background counts at the beginning and end, and the relative standard deviations (%) were calculated based on repeated measurements of a standard sample to determine the precision accuracy for each element. The results of elemental concentrations are expressed as the ratios of six elements (^{7}Li, ^{23}Na, ^{57}Fe, ^{59}Co, ^{88}Sr, and ^{138}Ba) to Ca.

Larimichthys polyactis can reach sexual maturity after 1 year (Lim et al., 2010; Xie et al., 2021), and fish of the S1 and S2 groups were believed to be in the spawning period. Therefore, the otoliths of the S1 group were analysed for the chemical composition of the core and the first annual ring (Y1) reflecting the local environment of the natal site and the first spawning site, respectively, and those of the S2 and O2 groups were in addition analysed for the composition of the second annual ring (Y2). The reliability of the described analysis could be illustrated by comparing the X-ray intensity map of Sr content and the light microscopy image of an *L. polyactis* otolith (**Figure 2**), which showed that low Sr (blue) regions, which correspond to the spawning period in coastal waters, matched the annual rings (Elsdon and Gillanders, 2003). These results indicat that combined with the sampling time and migratory patterns of *L. polyactis* (Lin et al., 2018), the ablation spots of the core and annual rings accurately represent the natal and spawning period environments, respectively.

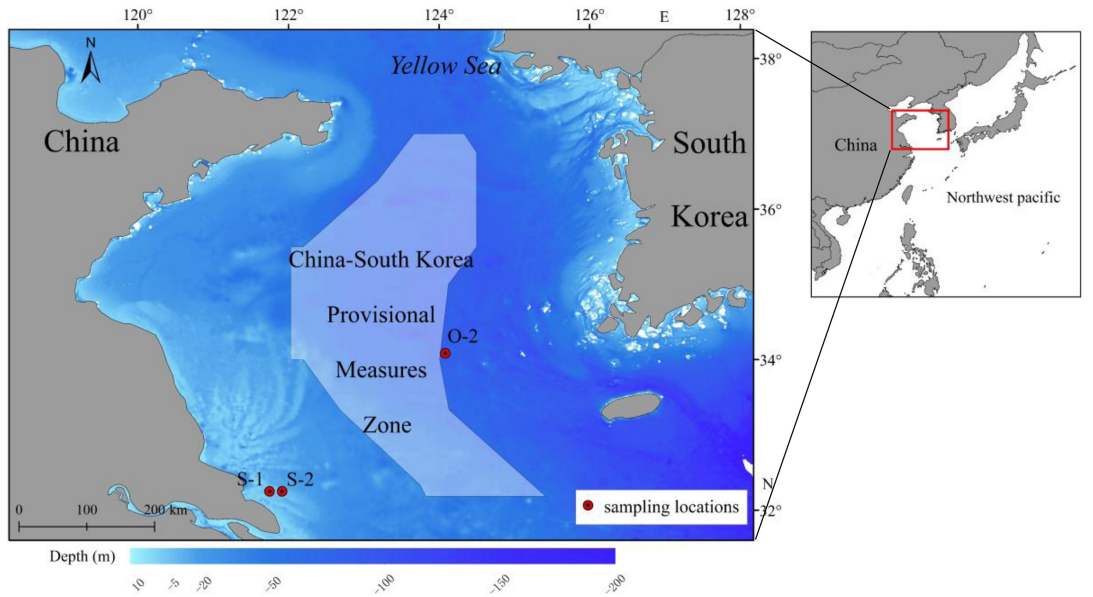

FIGURE 1 | Map showing *L. polyactis* sampling sites in the southern Yellow Sea and the provisional measures zone under the China-South Korea fishery agreement.

TABLE 1 | Characteristics of *L. polyactis* populations analysed for otolith microchemistry.

Sampling site	Site code	Sampling time	Sample size (*n*)	Body length, mm (mean ± SD)	Age (years)
South Yellow Sea nearshore zone	S1	April 2017	20	104.4 ± 6.0	1+
	S2	April 2018	20	129.2 ± 9.8	2+
South Yellow Sea offshore zone	O2	November 2018	20	145.5 ± 4.0	2+

Data Analysis

As the data did not fully meet homogeneity of variance/covariance and multivariate parametric assumptions of normality, we used methods that were based on relaxed assumptions or did not require them. Kruskal–Wallis tests was used to determine whether the elemental ratios of the core and Y1 ring differed among the S1, S2, and O2 groups. The Mann–Whitney *U*-test was used to determine the differences in Y2 ring composition between the S2 and O2 groups. Canonical discriminant analysis (CDA) with jackknife cross-validation (Amano et al., 2018) was carried out to assess the utility of element-to-Ca ratios in different ablation levels as a tag for *L. polyactis* stocks. Principal component analysis (PCA) was used to evaluate the potential of employing the element-to-Ca ratio in otoliths to discriminate between ablated zones in otoliths. The data were expressed as the mean ± standard deviation (SD) and $P < 0.05$ was considered to indicate statistical significance of difference. All statistical analyses were conducted using the IBM SPSS Statistics (V23.0) software.

RESULTS

Otolith Geochemistry

Otoliths were isolated from *L. polyactis* (S1, S2, and O2 groups) captured in the southern Yellow Sea. In total, 242 ablated spots were analyzed with a maximum of 7 per otolith. For the following elements, the detected concentrations in otoliths were above the LODs (mmol/mol): Li (2.53×10^{-3}), Na (3.33×10^{-1}), Fe (4.83×10^{-2}), Co (5.46×10^{-4}), Sr (1.63×10^{-4}), and Ba (1.63×10^{-4}). The analytical accuracy of the standards across all samples was high for the six elements with relative standard deviations ranging from 2.24 (Sr) to 9.27 (Co).

The 60 specimens of *L. polyactis* from the S1, S2, and O2 groups shared a common feature: the Li:Ca, Na:Ca, Sr:Ca, and Ba:Ca ratios in the core zone were significantly higher than those in the Y1 and Y2 zones ($P < 0.05$; **Figure 3**). No significant differences between/among the same ablated zone were observed among the elemental ratios of three sampling groups ($P > 0.05$) (**Figure 4**).

Canonical Discriminant Analysis of Element-to-Ca Ratios

Overall, natal- or spawning-site fidelity was reflected in the assignment to the actual zones of per otolith. According to CDA with jackknife cross-validation, the percentages of good assignment of different ablated zones to their actual zones were 95% for S1, 71.57% for S2, and 76.05% for O2 group (**Table 2**). The percentage of assignment of the core zone to its actual zone was high (95–100%) among the three sampling groups, but that of the Y1 and Y2 zones was

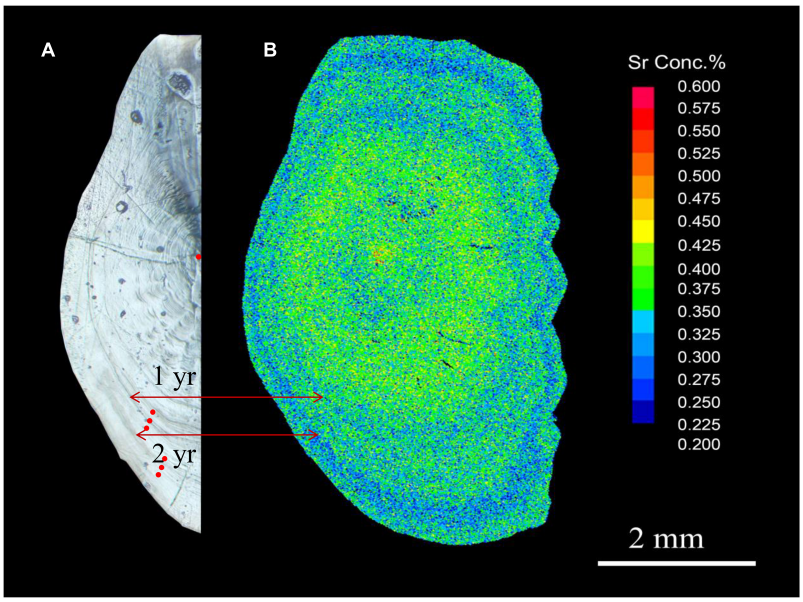

FIGURE 2 | Representative images of a sagittal section through the core of an *L. polyactis* otolith. **(A)** Otolith morphology (light microscopy). Red dots indicate the location of ablated spots in the core and annual rings; **(B)** Sr content (electron probe microanalysis).

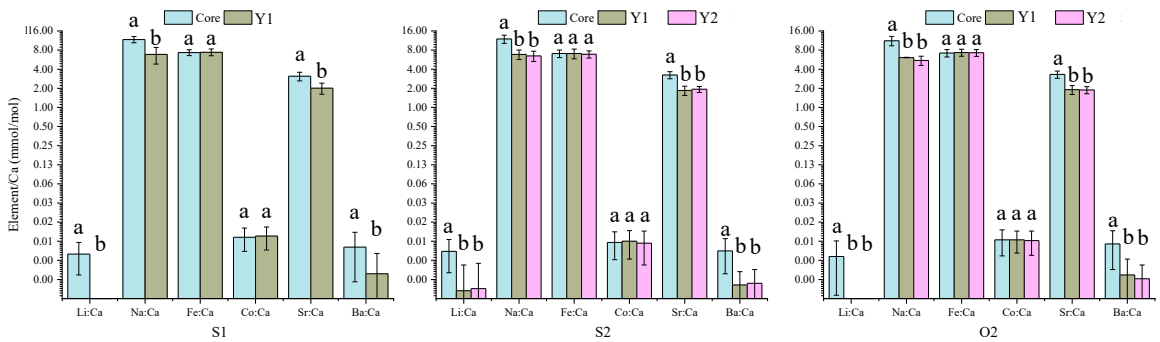

FIGURE 3 | Mean element-to-Ca ratios (mmol/mol) in the otolith's core zone and Y1 and Y2 rings of three *L. polyactis* age groups. Different low-case letters indicate significant differences between growth zones ($P < 0.05$).

rather low (50–70%), and one was frequently mistaken for the other (**Table 2**).

Canonical discriminant analysis provided clear separation between the core and Y1/Y2 zones in the S2 and O2 groups, which formed two distinct clusters: the natal environment cluster represented by the core zone and the spawning environment cluster represented by the Y1 and Y2 zones (**Figure 5**).

Group Discrimination

Principal component analysis conducted on the element-to-Ca ratios in different otolith zones explained 98.8% of the total variance (PC1:89.4% and PC2: 9.4%) and revealed spatial variations of the ablated zones within the sampling groups (**Figure 6**). Distinct geochemical signatures were identified for one zone (S1–Y1) and two clusters (cluster 1: S1-core, S2-core, and O2-core zones; cluster 2: S2–Y1, O2–Y1, S2–Y2, and O2–Y2 zones; **Figure 6**). The S1–Y1 zone was clearly separated from the other zones, whereas cluster 2 showed significant overlapping (**Figure 6**), suggesting that the S1 group represented a separate population, while the S2 and O2 groups represented the same population. Cluster 1 was isolated from S1–Y1 and cluster 2, suggesting that *L. polyactis* may have spawning-site fidelity but not natal-site fidelity.

DISCUSSION

Natal- and Spawning-Site Fidelity

The results of this study indicate that the natal sites of the S1, S2, and O2 *L. polyactis* groups can be discriminated with high accuracy using element-to-Ca ratios (**Table 2** and **Figure 5**), among which Na:Ca, Li:Ca, Ba:Ca, and Sr:Ca ratios

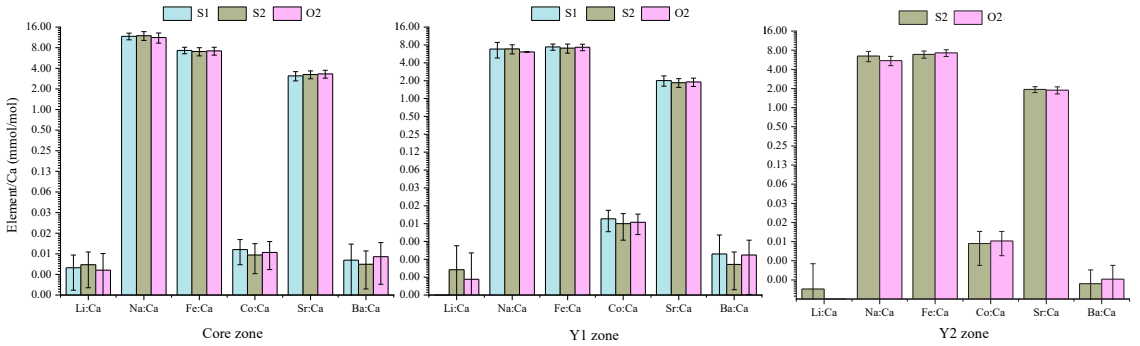

FIGURE 4 | Comparison of mean element-to-Ca ratios (mmol/mol) in otolith growth zones of *L. polyactis* from different groups.

TABLE 2 | Re-classification matrix constructed based on CDA with jackknife cross-validation of element-to-Ca ratios in otolith zones of the S1, S2, and O2 *L. polyactis* groups.

Sampling group	Original ablated zone	Predicted ablated zone			Sample number	Correct prediction	Total correct prediction
		Core	Y1	Y2			
S1	Core	**19**	1		20	95.00%	95.00%
	Y1	1	**19**		20	95.00%	
S2	Core	**20**	0	0	20	100%	71.57%
	Y1	0	**10**	10	20	50.00%	
	Y2	0	6	**11**	17	64.71%	
O2	Core	**19**	1	0	20	95.00%	76.05%
	Y1	0	**14**	6	20	70.00%	
	Y2	0	7	**12**	19	63.16%	

Bold numbers indicate individual assignment to capturing locations.

are particularly useful natal signatures. The ratios of these four elements to Ca in the core zone were generally higher than those in the Y1 and Y2 zones ($P < 0.05$). Element Sr is a key element involved in the formation of otoliths (Walther, 2019) and higher Sr:Ca ratios in the core zone have also been observed in *Urophycis brasiliensis* (Biolé et al., 2019), *Anguilla japonica* (Tzeng, 1996; Tsukamoto and Arai, 2001), *Collichthys lucidus* (Liu et al., 2015), and *Miichthys miiuy* (Xiong et al., 2015). The potential reasons for the higher Sr:Ca ratios in the core zone of *L. polyactis* otoliths have been summarized by Song et al. (2022): (1) before hatching, *L. polyactis* feeds on the yolk sac and not on exogenous sources (Zhan et al., 2016); (2) Sr:Ca ratios are mainly influenced by the habitat environment (Elsdon and Gillanders, 2003); (3) during the embryonic stage of fish species, higher concentrations of protein and lower Ca could lead to an increase in the levels of other alkaline earth metals from the Ca group, such as Sr and Ba (Campana, 1999; Murayama et al., 2004). Indeed, Ba:Ca ratios, similar to Sr:Ca ratios, were also higher in the otolith core zone, which is consistent with the observation that high Sr concentrations facilitate Ba uptake into fish otoliths (De Vries et al., 2005). Although the underlying mechanisms are not fully understood, the differences in Ba:Ca ratios are thought to reflect the levels of Ba dissolved in ambient water (Campana, 1999; Ashford et al., 2005). Ba has a nutrient-like vertical distribution with a much higher concentration in deeper waters, and its surface concentration is significantly increased during upwelling (Arkhipkin et al., 2004), as observed along the Lvsi coast on the north and west sides of the submarine canyon off the Yangtze River estuary (Lv et al., 2006; Hu and Wang, 2016), where the individuals of the S1 and S2 groups were sampled. These sampling areas are characterized by large-scale coastal upwelling centered on 31°30′N and 122°40′E, which is influenced significantly by the massive coastal currents (e.g., Yellow Sea and Zhejiang–Fujian coastal currents) and riverine inputs (Changjiang River Diluted Water) (Lv et al., 2006; Hu and Wang, 2016). Therefore, these areas tend to be more Ba-enriched and productive because of supplementation by terrestrial runoff, nutrient-rich bottom waters, and dissolution of Ba in submarine sediments (Hu and Wang, 2016). Among the four elements that were significantly higher in the core zone, Ba and Li concentration mostly reflect the physico-chemical status of the water environment (Sturrock et al., 2012; Grammer et al., 2017). Element Li shows strong positive correlation with the ambient chlorophyll level and could be a potential indicator of marine productivity (Grammer et al., 2017). However, Na should be mentioned as a necessary physiological element required for metabolic reactions and processes associated with growth and reproduction (Loewen et al., 2016). Moreover, Na deposition in otoliths appears to be affected by ontogenetic stages and, to a lesser extent, by stress (Mohan et al., 2014; Grammer et al., 2017).

As elements incorporated into fish otoliths are mainly derived from the environment (Elsdon and Gillanders, 2003), the

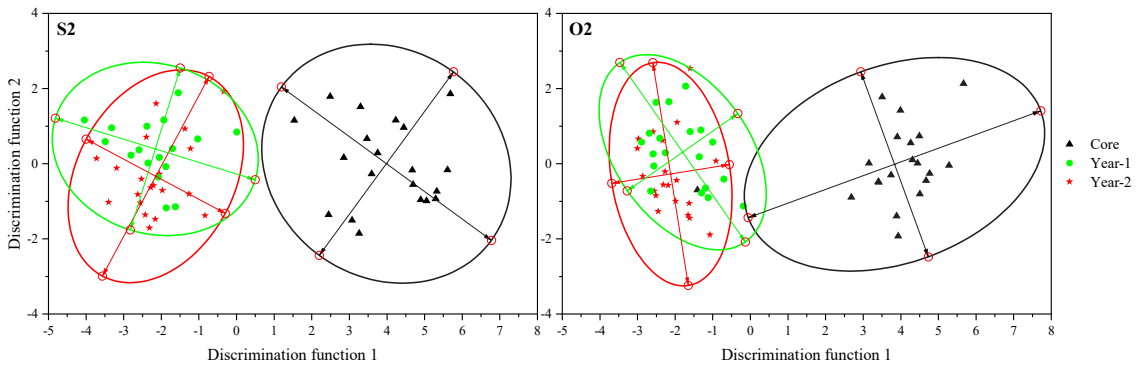

FIGURE 5 | Scatter plots showing the separation of the core, Y1, and Y2 otolith zones in the S2 and O2 groups (based on CDA). Ellipses indicate the mean 95% confidence level contours for each zone.

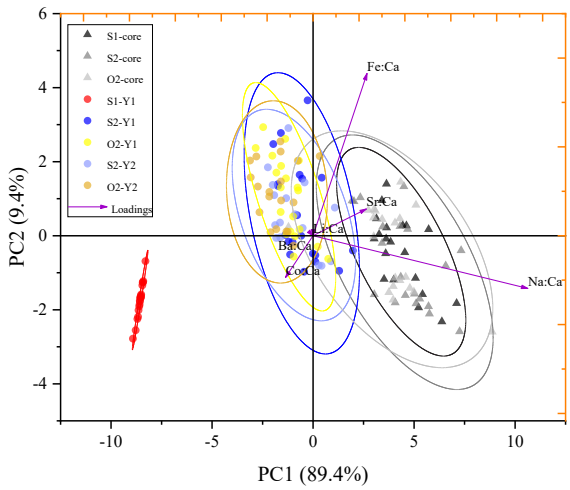

FIGURE 6 | PCA for element-to-Ca ratios in ablated zones of otoliths from S1, S2, and O2 *L. polyactis* groups. Ellipses indicate the mean 95% confidence level contours for each zone; triangles and circles indicate the natal and spawning sites, respectively.

significant difference in the elemental composition between the core zone and Y1/Y2 zones suggests that the hatching and spawning areas do not completely coincide. The pelagic eggs of *L. polyactis* are likely to be carried by ocean currents for nearly 2 days. The hatching of fertilized eggs takes ∼ 50 h in seawater (salinity 28) at 18°C (Zhan et al., 2016). Li and Ba concentrations are increased in the nutrient-rich environment, and these important elements contribute to the hatching and forage of *L. polyactis*, which is reflected by their higher ratios in the core zone compared with the Y1 and Y2 zones.

In addition to the effect of the water environment on the elemental incorporation in otoliths, the contribution of the internal factors was also considered. The otolith core is the area mostly formed during embryogenesis (i.e., the primordium and yolk sac stages; Zhan et al., 2016). Previous studies showed that the 4th increment appears after the first-feeding check with a distance of 20.67 ± 2.28 μm from the central nucleus; it is wide, dark, and well-defined (Li et al., 2013; Zhang T. T. et al., 2019; Song et al., 2022). The 20 μm ablation spots of the core zone were fully within the boundaries of the embryonic stage, indicating that the deposition of elements in the core zone was influenced by endogenous factors. The otolith Sr:Ca ratio, which is widely used to reveal the migration of fish species, in the core zone should reflect maternal rather than environmental effects (Campbell et al., 2002; Shippentower et al., 2011). It can be inferred that the elemental chemistry of the otolith core zone in *L. polyactis* depends on the internal conditions at the primordium and yolk sac stages and not on the surrounding water. Still, regardless of the factors behind the specificity of the elemental composition in the core zone, the natal-site fidelity of *L. polyactis* remains uncertain at present.

In contrast, it was difficult to discriminate between the Y1 and Y2 zones representing the spawning site, since the CDA and PCA scatter plots for the S2 and O2 groups notably overlapped (**Figure 5** and **Figure 6**). There were no significant differences in the element-to-Ca ratios between the Y1 and Y2 zones of the S2 and O2 fish sampled in the southern Yellow Sea, indicating that the spawning-site environments for these groups were very similar and suggesting the existence of spawning-site fidelity in *L. polyactis*. To fully understand the philopatry in *L. polyactis* populations, natal- and spawning-site fidelities must be considered separately because the latter without the former does not lead to genetic isolation and local adaptation among spawning stocks (Miller et al., 2001). Previous population genetic studies of *L. polyactis* using diverse types of genetic markers have shown persistently high levels of genetic connectivity and a lack of population structure in *L. polyactis* (Wang et al., 2013; Zhang et al., 2020). Fish must first spawn at the previous grounds to avoid intermating and genetic homogenization within populations (Miller et al., 2001). Therefore, at this moment, we suggest that *L. polyactis* exhibits spawning-site fidelity.

Connectivity Between Overwintering and Spawning Grounds

The PCA scatter plot indicated that the Y1 zone of the S1 group was clearly isolated from the other zones, whereas the Y1

and Y2 zones of the S2 and O2 groups significantly overlapped (**Figure 6**). These results, together with previous data on the *L. polyactis* population division (Lin, 1987; Zhang et al., 2014), indicate that the S2 and O2 groups represent the same southern Yellow Sea population, whereas the S1 group represents another population. Some individuals of the southern Yellow Sea and East China Sea populations are mixed in the southern Yellow Sea (Lin et al., 2010; Xu and Chen, 2009), suggesting that the S1 group may be the East China Sea population. The fact that the S2 and O2 groups are the same population indicates that *L. polyactis* has a stable migratory route in Chinese and South Korean waters. *L. polyactis* spawns in the coastal region of China but afterward migrates to regions of the Yellow Sea, where it can also be caught by South Korean fishing vessels (Xue, 2005). The discovery of the connectivity between overwintering and spawning groups and the existence of mixed populations in the southern Yellow Sea could contribute to the implementation of fishery management strategies for *L. polyactis* in China. Although spatial management, including MPAs, has been promoted as a method to conserve biodiversity and manage fishery resource (Agardy, 2017; Li et al., 2020), its design faces many practical issues and requires sound knowledge of ecological processes and population dynamics (Li et al., 2020). Continuing debates on the efficacy of MPAs have determined the need for analytical models that capture the spatial dynamics of marine species, especially in relation to natal- or spawning-site fidelity and larval dispersal (Jones et al., 2009; Krueck et al., 2017). The finding of spawning-site fidelity of *L. polyactis* has important consequence for the design of MPAs, which should be based on scientific data in order to be effective in the conservation of marine resources, because ultimately, the credibility of agencies that promote and administer MPAs will be evaluated based on the choice of suitable geographical location for protection.

According to previous studies on fish morphology and spawning migration routes, three populations are distinguished: the East China Sea, the southern Yellow Sea, and the Bohai/northern Yellow Sea populations (Lin, 1987; Liu, 1990). With the introduction of various methods of population division analysis, such as evaluation of otolith morphology and microchemistry (Zhang et al., 2014; Wang et al., 2016), morphometric characteristics (Zhang et al., 2015), and population genomics (Zhang B. D. et al., 2019), an increasing number of cryptic populations have been reported. In addition to an unrecognized *L. polyactis* cryptic population, its spawning grounds also should also be considered in the design of management strategies. Previous studies have shown that spawning grounds have expanded from nearshore to offshore waters (Ding et al., 2007; Lin et al., 2008; Song et al., 2022). Despite new data on cryptic populations, biological characteristics, and the range of spawning grounds, spatial management measures, especially MPAs, have not been improved since the implementation of the national aquatic germplasm resources reserve of *L. polyactis* in the Lvsi fishing grounds in December 2009 (Ministry of Agriculture [MOA], 2021), and the monitoring and enforcement of protected areas have been unsatisfactory (Cao et al., 2017). The unprecedented changes in the biological characteristics and population structure of *L. polyactis* call for new management approaches to counteract anthropogenic impacts (Li et al., 2020). Moreover, regarding stocks that occur within the exclusive economic zones of two or more states, Article 63 of the Law of the Sea Convention requires relevant countries to collaborate and ensure the rational utilization of stocks, because for shared species, protective measures implemented only by one country may have a weak effect unless they are also observed by the others. Cooperation between China and South Korea along the same migratory route of *L. polyactis* is crucial for protecting this overexploited and highly targeted species.

In conclusion, in this study we analysed the natal- and spawning-site fidelity and connectivity among three groups of *L. polyactis* sampled in different locations based on the elemental signatures of otolith zones. The results indicate that (1) *L. polyactis* has spawning-site but not natal-site fidelity and that (2) the three groups represent two *L. polyactis* populations, namely, the southern Yellow Sea population (S2 and O2) and the East China Sea population (S1), indicating a stable migratory route of *L. polyactis* between Chinese and South Korean waters. The identification of *L. polyactis* natal- and spawning-site fidelity is critical for marine spatial planning and management of this highly targeted fish species through cooperation between China and South Korea. With the development of advanced analytical instruments for fine scale elemental analysis, in future study, we intend to investigate the oxygen and carbon stable isotopic ratios ($\delta^{18}O$ and $\delta^{13}C$) of otoliths from the core to the edge for comparison with the locations of annual rings, reaching a robust and reliable conclusion on natal-site fidelity. Of course, multidisciplinary techniques are often required in population studies; owing to complexity of the stock, the results from different methods should therefore be integrated to reach a consensus. In future study on population division, we will use various methods, including otolith shape, body morphology, and genetics to reach a robust conclusion.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The animal study was reviewed and approved by institutional animal care and use Committee of Shanghai Ocean University.

AUTHOR CONTRIBUTIONS

DS, ZK, and YX conceived and designed the experiments. DS performed the experiments, wrote the manuscript, analyzed the data, and applied the statistical analyses with the help of YX. All authors provided editorial advice and agreed this version of the manuscript for submission.

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Microbiomes of Healthy and Bleached Corals During a 2016 Thermal Bleaching Event in the Andaman Sea of Thailand

Suchana Chavanich[1,2], Heru Kusdianto[1], Chitrasak Kullapanich[3,4], Suppakarn Jandang[1], Doonyapong Wongsawaeng[5], Jamal Ouazzani[6], Voranop Viyakarn[1,2] and Naraporn Somboonna[3,4]**

[1] Reef Biology Research Group, Department of Marine Science, Faculty of Science, Chulalongkorn University, Pathumwan, Thailand, [2] Aquatic Resources Research Institute, Chulalongkorn University, Pathumwan, Thailand, [3] Department of Microbiology, Faculty of Science, Chulalongkorn University, Pathumwan, Thailand, [4] Microbiome Research Unit for Probiotics in Food and Cosmetics, Chulalongkorn University, Pathumwan, Thailand, [5] Department of Nuclear Engineering, Faculty of Engineering, Chulalongkorn University, Pathumwan, Thailand, [6] Centre National de la Recherche Scientifique, Institut de Chimie des Substances Naturelles ICSN, Gif-sur-Yvette, France

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***Correspondence:**
Voranop Viyakarn
Voranop.V@chula.ac.th
Naraporn Somboonna
Naraporn.S@chula.ac.th

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As seawater temperature rises, repeated thermal bleaching events have negatively affected the reefs of the Andaman Sea for over decades. Studies on the coral-associated microbial diversity of prokaryotes and microbial eukaryotes (microbiome) in healthy and bleached corals are important to better understand the coral holobionts that involved augmented resistance to stresses, and this information remains limited in the Andaman Sea of Thailand. The present study thereby described the microbiomes of healthy (unbleached) and bleached colonies of four prevalent corals, *Acropora humilis*, *Platygyra* sp., *Pocillopora damicornis*, and *Porites lutea*, along with the surrounding seawater and sediments, that were collected during a 2016 thermal bleaching event, using 16S and 18S rRNA genes next-generation sequencing (NGS). Both prokaryotic and eukaryotic microbes showed isolated community profiles among sample types (corals, sediment, and seawater) [analysis of similarities (ANOSIM): $p = 0.038$ for prokaryotes, $p < 0.001$ for microbial eukaryotes] and among coral genera (ANOSIM: $p < 0.001$ for prokaryotes and microbial eukaryotes). In bleached state corals, we found differences in microbial compositions from the healthy state corals. Prevalent differences shared among bleached coral genera (shared in at least three coral genera) included a loss of reported coral-beneficial microbes, such as Pseudomonadales, Alteromonadales, and *Symbiodinium*; meanwhile an increase of putative coral-pathogenic *Malassezia* and *Aspergillus*. This difference could affect carbon and nitrogen availability for coral growth, reflective of a healthy or bleached state. Our findings in part supported previously microbial dysbiosis knowledge of thermal bleaching coral microbiomes around South East Asia marine geography, and together ongoing efforts are to support the understanding and management of microbial diversity to reduce the negative impacts to corals in massive thermal bleaching events.

Keywords: coral bleaching, coral reefs, microbiome, bacteria, fungi, next generation sequencing, Andaman Sea

INTRODUCTION

Coral bleaching is defined as a breakdown of coral-algae symbiosis, when the corals are stressed by a variety of factors, in which the increased atmosphere temperature (thus seawater temperature) is the major triggering cause, and the endosymbiotic algae (in particular photosynthetic dinoflagellate *Symbiodinium*) expel (Hoegh-Guldberg and Smith, 1989; Bruno et al., 2001; Eakin et al., 2010). This results in the loss of coral color (become white) and of food that is supplied through photosynthesis by the dinoflagellates for coral growth, reproduction, resistance to diseases, and survivability (Baker et al., 2008). Nowadays, thermal bleaching events are considered as the most problematic coral situation worldwide, including in the Andaman Sea coast of Thailand where the first widespread thermal bleaching event was recorded in 1991 and subsequently recurred in 1995, 1998, 2003, 2010 (severe event), and 2016, respectively (Phongsuwan and Chansang, 2012). The 2010 bleaching event caused 44.2–99.0% coral mortality (Brown et al., 1996; Phongsuwan and Chansang, 2012). In the 2016 bleaching event, approximately 50% of the corals were affected; this was considered moderate bleaching-induced mortality (Pootakham et al., 2018). The mortality of corals affects the nursing habitats of diverse marine fish and invertebrates (Boilard et al., 2020).

Other factors to cause coral bleaching include water salinity increase (Berkelmans et al., 2012; Kuanui et al., 2015), water pH decrease (ocean acidification) (Anthony et al., 2011; Burke et al., 2012), increased level of sediment till become covering corals and blocking sunlight to coral (affect photosynthesis of coral-photobionts) (Peters, 1984), and dysbiosis of the coral-associated microbiome (Ritchie, 2006; Bourne D. G. et al., 2008). This symbiotic relationship involves algal *Symbiodinium* (also known as zooxanthellae), prokaryotes (bacteria and archaea), eukaryotic microbes, and viruses and was reported dynamic and differentially associated in healthy vs. bleaching state, and bleaching-sensitive vs. -tolerant corals (Rohwer et al., 2002; Rosenberg et al., 2007; Boilard et al., 2020). Corals provide habitat to the coral symbionts (algae and microbes), while the microbial symbionts provide corals with nutrients through photosynthesis and/or decomposition of organic and inorganic substances, competition for space and nutrients against coral pathogens, and antibiotics or antioxidants to promote coral resistance against pathogens and coral bleaching (Lesser et al., 2004; Rosenberg et al., 2007; Lema et al., 2012; Webster and Reusch, 2017). The compositions of these beneficial microbes and negatively impacted microbes affect how well different coral hosts (i.e., coral species) sensitive to thermal bleaching (Csaszar et al., 2010; Leggat et al., 2011; Gardner et al., 2019). Among the coral genera affected by bleaching, some genera were more bleaching tolerant than the others (e.g., *Porites* > *Acropora*), likely due to the differences in host genetic and their symbiont compositions (Phongsuwan and Chansang, 2012; Gardner et al., 2019). The severe 2010 and 2016 bleaching events found that *Acropora* spp. is more prone to bleaching-induced mortality (75–100%) than *Porites* spp. (25–49%), in which scientists had suggested to be associated with the different *Symbiodinium* clades between *Acropora* and *Porites* (C3 and C15, respectively) (Fisher et al., 2012; Phongsuwan and Chansang, 2012; Gardner et al., 2019). Additionally, a shift of *Symbiodinium* clades from type C in healthy *Acropora millepora* to type D in bleached *A. millepora* (Berkelmans and van Oppen, 2006; Oliver and Palumbi, 2011) and the higher abundance of coral pathogens had been reported in bleached *Acropora* (Roder et al., 2014; Kusdianto et al., 2021).

Besides, studies reported different coral-associated microbiome patterns among coral genera and sites (Gardner et al., 2019). For instance, Pogoreutz et al. (2018) discovered the consistent pattern of, in particular, the dominance of order Oceanospirillales in coral *Pocillopora verrucosa* in Saudi Arabia coastal and other marine resources albeit healthy to bleaching and mortality state corals and suggested that the range in microbial flexibility may correlate an ability of corals to serve as hosts. This microbiome specificity to coral genera (or coral species) might affect the corals in adaptive to environmental changes. Many scientists, including Morrow et al. (2018) described the diversity of microbiomes associated with coral abilities to respond to thermal bleaching events and other environmental stresses (e.g., nutrient limitation). For sites, different marine geographies in the world affect reef types and climates, and thus, the microbiome diversity (Somboonna et al., 2017). Further, van Oppen and Blackall (2019) also pointed the temporal factor on microbiome changes, and specific microbiomes could support acclimatization of the corals to climate warming, heightening the coral insensitivity to bleaching (Gardner et al., 2019). Supportively, scientists found that microbiome transplantation using homogenized coral tissues of heat-tolerant donors *Porites* and *Pocillopora* in the wild could allow the recipient corals to bleach at lower rates (Doering et al., 2021). In vice versa, certain coral-associated microbiomes might cause coral to bleach at normal to high rates. Nevertheless, specifically preferred microbiomes for bleaching resilience remained none classified to date in the Andaman Sea of Thailand.

Following the aforementioned reports that successfully utilized universal 16S and 18S rRNA genes to analyze coral associated prokaryote and eukaryote diversities (Hadziavdic et al., 2014; Wang et al., 2014; Banos et al., 2018; Kusdianto et al., 2021), this report, therefore, utilized similar methods [16S and 18S rRNA genes next-generation sequencing (NGS)] to describe prokaryotic and eukaryotic microbiomes in healthy and bleached corals *Acropora humilis*, *Platygyra* sp., *Pocillopora damicornis,* and *Porites lutea*, in the Andaman Sea around Racha Yai Island, Phuket province, along with the microbiomes of the surrounding seawater and sediments, during a thermal bleaching event in 2016. Previously, the coral-associated microbiome of prokaryotes during the 2016 bleaching event in the Andaman Sea had been investigated (Pootakham et al., 2018); however, that study only reported on *P. lutea* leaving other three coral genera that are prevalent in the studied locations and also did not include analyses of the eukaryote microbiomes. Therefore, the present study included alpha and beta biodiversity analyses of both prokaryotic and eukaryotic microbiota of healthy and bleached colonies of four prevalent coral species. Our and other data will be crucial to allow characterization of the coral-beneficial microbes (or microbiome) against thermal bleaching events in

the Andaman Sea, supporting the microbe-mediated strategies to protect and recover corals from thermal bleaching events (van Oppen and Blackall, 2019).

MATERIALS AND METHODS

Sample Collections

Corals of species *A. humilis* (abbreviated as CA), *Platygyra* sp. (CT), *P. damicornis* (CC), and *P. lutea* (CR), seawater (W), and sediment (S) samples were collected from reef around Racha Yai Island (7°36′27.9″N 98°22′40.9″E), in Phuket province, Thailand (**Figure 1A**). Three independent replicates were collected for each sample. Healthy (H) and bleached (B) coral colonies were determined *via* their appearance [healthy coral colony appeared light or dark brown color (natural color, 0% sign of bleaching), and bleached coral colony appeared white color by on-site marine scientist divers (S. Chavanich, S. Jandang, and V. Viyakarn) (Bulan et al., 2018a,b; Kusdianto et al., 2021)]. The four coral genera had an identical bleached appearance, and the bleached sample was collected in the bleached area (completely white area) of coral color that bleached more than 50%. Of each coral sample, a fragment approximately 5 cm in length and 5 cm in diameter (for branching corals *Acropora* and *Pocillopora,* the samples were approximately 5 cm in length, while the compound colony *Porites,* approximately 5 cm in diameter of each sample was collected), at approximately 8–10 m depth from the sea surface, was collected haphazardly using hands in gloves, and the distance between each sampled colony was approximately 5 m. Seawater (6 L) was collected directly above each of the sampled coral colonies, and sediment (approximately 50 g) was collected directly below each of the sampled coral colonies. All samples were collected at the same day and time (around noon) during the global thermal bleaching event in May 2016, and at the same site and thus, the environmental factors were the same. All samples were transported immediately within the same day to the laboratory and stored in −20°C. The abbreviations for samples are sample (CA, CT, CC, CR, W, or S) followed by coral condition (H or B) (for coral) and independent replicate number (1, 2, or 3). For example, CAH1 represents healthy *Acropora* coral of independent replicate number 1.

Metagenomic Extraction

Coral samples (containing surface mucus layer, tissue, and skeleton) were ground using a sterile mortar and pestle, and 1 g of coral fragment and 1 g of sediment were used. Noted several sampling techniques to profiling the coral microbiome, including grinding entire coral like ours (Bulan et al., 2018a,b; Hernandez-Agreda et al., 2018; Pootakham et al., 2018; Rosales et al., 2019), collecting only surface mucus layer (Badhai et al., 2016), or scraping some tissue (Glasl et al., 2017), were reported. The surface mucus layer and tissue are important as an interface habitat between the coral and the surrounding (Glasl et al., 2016; Hadaidi et al., 2017), while the skeleton showed roles in physico-chemical nutritional exchanges and decalcification, which coral bleaching also affected alteration in skeleton microbiome and scientists discussed the skeleton may function as a microbial reservoir for tissue microbiome recolonization, i.e., following bleaching dysbiosis to support coral microbiome return to homeostasis (Ainsworth et al., 2015; Ricci et al., 2019). For seawater, 2.5 L was filtered through a sterile 0.22-μm filter membrane (Merck Millipore, MA, United States) and the filtered membrane was used. Metagenomic DNA was extracted using Power Soil DNA Isolation Kit for coral and sediment samples, and Power Water DNA Isolation Kit for seawater samples (MoBio, Carlsbad, CA, United States), following the instructions

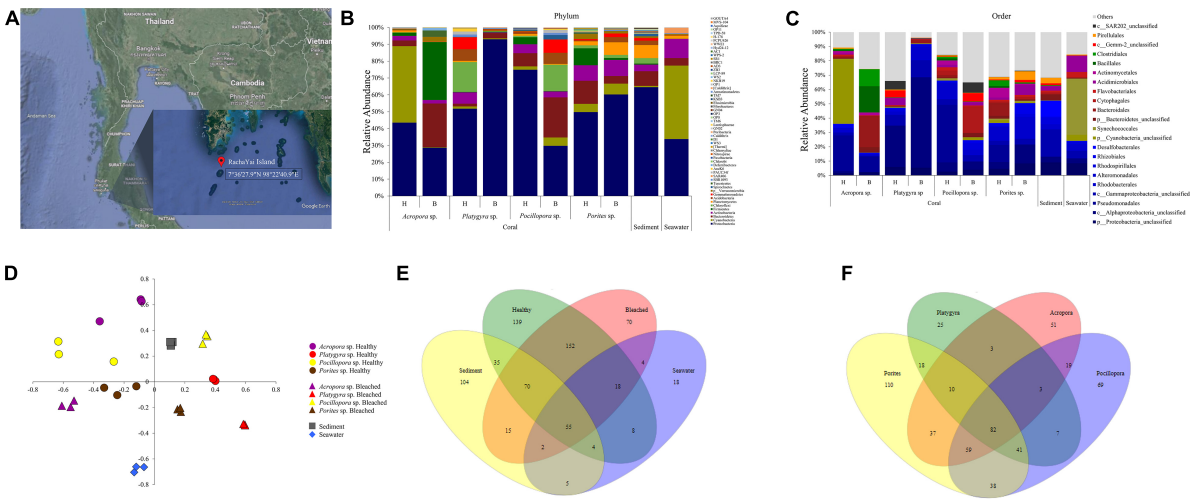

FIGURE 1 | (A) Map representing sampling site of Racha Yai Island in the Andaman Sea; average bacterial compositions in coral, sediment, and seawater samples at **(B)** phylum level and **(C)** order level; **(D)** their non-metric multidimensional scaling analyses based on ThetaYC index at genus level; and Venn diagrams showing the numbers of shared and unique OTUs **(E)** among healthy corals, bleached corals, and surrounding sediment and seawater; and **(F)** among four coral species *Acropora humilis*, *Platygyra* sp., *Pocillopora damicornis*, and *Porites lutea*. In panel **C**, "Others" represent order OTUs with <1% abundance. For OTU classification where order could not be identified, the deepest classification level was given (abbreviated p for phylum and c for class).

of the manufacturer and previous works of literature (Bulan et al., 2018a,b; Kusdianto et al., 2021). The quantity and quality of the extracted DNA were analyzed by NanoDrop™ 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, United States) and agarose gel electrophoresis (0.55% agarose gel w/v). The metagenomic DNA was stored at −20°C until further analyses.

16S and 18S rRNA Gene Library Preparation and Next-Generation Sequencing

The 16S rRNA gene V4 region and 18S rRNA gene V9 region were amplified by PCR using universal prokaryotic primers 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′), and universal eukaryotic primers Illumina_Euk_1391F (5′-GTACACACCGCCCGTC-3′) and lllumina_EukBr (5′-TGATCCTTCTGCAGGTTCACCTAC-3′), to create libraries of prokaryotes and microbial eukaryotes (fungi and small eukaryotes), respectively, according to Caporaso et al. (2012). Noted the 18S rRNA gene V9 is used elsewhere as adequate for analyzing microbial eukaryotic lineages (Vestheim and Jarman, 2008; Amaral-Zettler et al., 2009; Stoeck et al., 2010; Hadziavdic et al., 2014; Wang et al., 2014; Gong and Marchetti, 2019) and including the standard Earth Microbiome Project protocols (earthmicrobiome.org); and the 18S rRNA gene sequence database is increasingly large to support operational taxonomic units (OTUs) classification for microbial eukaryotes (Banos et al., 2018). For each sample, a 25-μl PCR reaction comprised 1 × EmeraldAmp® GT PCR Master Mix (TaKaRa, Shiga, Japan), 0.3 μM of each primer, and 75 ng of the metagenome DNA. The 18S rRNA gene PCR also included 10 μM of a mammal-blocking primer (5′-GCCCGTCGCTACTACCGATTGGIIIIITTAGTGAGGCCCT3S pC3-3′) (Caporaso et al., 2012). The 16S rRNA gene PCR conditions were 94°C for 3 min, and 30 cycles of 94°C for 45 s, 50°C for 60 s, and 72°C for 1 min 30 s, followed by 72°C for 10 min; and the 18S rRNA gene PCR conditions were 94°C for 3 min, and 30 cycles of 94°C for 45 s, 65°C for 15 s, 57°C for 30 s, 72°C for 90 s, followed by 72°C for 10 min. Of each sample, independent triplicate PCRs were performed and pooled to prevent stochastic bias. PCR products of ≈381 bp (16S rDNA) and ≈260 bp (18S rDNA) in length were excised from agarose gels and purified using GF-1 Gel Extraction Kit (Vivantis Technologies Sdn Bhd, Selangor, Malaysia). Qubit™ dsDNA HS assay kit (Invitrogen, Waltham, MA, United States) and Qubit 3.0 Fluorometer (Thermo Fisher Scientific) were used to quantify the dsDNA of the PCR products, and 200 ng of each sample was sequenced on Miseq 300 NGS platform (Illumina, San Diego, CA, United States) at OMICS Sciences and Bioinformatics Center, Faculty of Science, Chulalongkorn University (Bangkok, Thailand).

Bioinformatics and Statistical Analyses

Sequences were processed according to Mothur's standard operating procedure (SOP) (Schloss et al., 2009). For quality screening, reads containing (i) ambiguous bases; (ii) >1 mismatch base in the reverse primer region, (iii) >10 homopolymer, (iv) sequence length <100 bp, and (v) chimera sequence, were removed. Silva databases (version 1.32) were used to align the sequences and remove contaminated sequences, such as mitochondria and chloroplast sequences. For taxonomic classification into OTUs, the quality 16S and 18S rDNA sequences were determined using Mothur (Schloss et al., 2009) and Greengenes (version 13.8); and Good's coverage index was computed to estimate a sequencing coverage (sequencing depth) of each sample. Then, these data samples for 16S and 18S rRNA genes sequences were normalized at 9,045 and 38,429 sequences per sample to prevent a bias from various sequencing depth across samples when comparisons among samples and groups were performed; noted a data sample with fewer sequences than the normalization (samples CAH2-3, CCH1-3, and CRH1-3) was pooled with other independently replicate sample followed by subsampling to normalization or using all those sequences. Venn diagram, alpha diversity (Chao1 richness, and Shannon diversity indices), and beta diversity [thetaYC dissimilarity index and non-metric multidimensional scaling (NMDS)], with default parameters, were determined using Mothur (Schloss et al., 2009; Kusdianto et al., 2021).

Mean and SD were computed. For statistical analysis, the Student's *t*-test and analysis of similarities (ANOSIM) were used to test for significance between and among groups ($p < 0.05$).

Availability of Supporting Data

Nucleic acid sequences of 16S and 18S rDNA were deposited in an NCBI open access Sequence Read Archive database, accession number PRJNA700134.

RESULTS

16S and 18S rRNA Gene Sequencings and Alpha Diversity

From the sequencing results, a total of 766,434 quality reads for 16S rRNA gene sequences and 6,103,914 quality reads for 18S rDNA sequences were obtained. The average quality reads per sample were 25,548 and 203,464 for 16S and 18S rRNA genes sequences, respectively; and hence, the Good's coverage indices demonstrated relatively sufficient sequencing depth: genus-level OTUs were averaged at 98.89 and 99.99% in 16S and 18S rRNA genes, respectively (**Supplementary Tables 1, 2**).

The alpha diversity (a measurement of biodiversity to a single sample) of corals and sediments demonstrated the greater genus richness (represented by OTUs and Chao index) for prokaryotes than microbial eukaryotes, meanwhile in seawater demonstrated similar genus richness between prokaryotes and microbial eukaryotes (**Supplementary Tables 1, 2** and **Supplementary Figures 1A,C**). In corals and at the genus level, prokaryotes had an average (avg.) of 252.71 OTUs, microbial eukaryotes avg. 60.42 OTUs; in sediments, prokaryotes had avg. 504.67 OTUs, microbial eukaryotes avg. 143.67 OTUs; and in seawater, prokaryotes had avg. 189.00 OTUs, and microbial eukaryotes avg. 227.67 OTUs. Hence, the highest richness was observed for prokaryotes in sediment samples

(avg. Chao 581.05) followed by corals (avg. Chao 318.48) and seawater (avg. Chao 254.00; **Supplementary Table 1**). Analyzing together with the genus evenness (represented by Shannon index) found a similar trend: corals and sediments had the higher biodiversity for prokaryotes than microbial eukaryotes (with the highest in coral *Porites* (avg. Shannon 4.06) than the other three coral genera (avg. Chao 2.87)), meanwhile, seawater demonstrated similar biodiversity between prokaryotes and microbial eukaryotes (**Supplementary Tables 1, 2** and **Supplementary Figures 1B,D**). For microbial eukaryotes, both genus richness and evenness were observed the highest in seawater (avg. Chao 245.89, avg. Shannon 2.92), followed by sediment (avg. Chao 164.73, avg. Shannon 1.96), and corals (avg. Chao 74.19, avg. Shannon 1.00) (**Supplementary Figures 1C,D**).

Interestingly, comparing between healthy and bleached coral conditions showed an alteration in their biodiversity, both in genus richness and evenness (**Supplementary Figures 1, 2**). For prokaryotes, bleached corals showed less biodiversity in *Platygyra* and *Porites*. For microbial eukaryotes, bleached corals showed less biodiversity in *Acropora*, *Platygyra,* and *Pocillopora.*

Beta Diversity Analyses for Prokaryotic Communities

While the relatively close biodiversity compositions within independently triplicate samples ($p > 0.01$) are shown in **Supplementary Figure 2A**, the diverse biodiversity compositions were observed for corals of different genera and sample types (corals vs. sediment vs. seawater). Analyzing the relative abundance at the phylum level revealed that Proteobacteria (avg. 53%) was the dominant phylum across all sample types and coral genera (**Figure 1B**). For the other dominant phyla, each sample group displayed quite a distinct prokaryotic composition; for example, the high abundance of Cyanobacteria was found only in seawater and healthy *Acropora*. Moreover, a dysbiosis of prokaryotic compositions was found specific to each bleached coral genus; and the dysbiosis between the healthy and bleached conditions was found more distinct in coral *Acropora*, *Pocillopora*, and *Platygyra* than *Porites*, in orderly (**Figures 1B,D**). For instance, the dominant Cyanobacteria (**Figure 1B**: avg. 45.34%) in healthy *Acropora* was changed to Bacteroidetes (avg. 25.93%) and Firmicutes (avg. 34.41%) in bleached *Acropora*, and the bacterial alteration was found in bleached *Porites* where the abundances of Firmicutes, Bacteroidales, Pseudomonadales, and Actinomycetales were reduced and the abundances of Planctomycetes, Rhodobacterales, Acidimicrobales, Rhizobiales, and Pirellules increased in proportion. Surprisingly, a contradictory trend was observed for Gemmatimonadetes, Acidobacteria, and Chloroflexi, which were present in relatively high abundances in healthy than in bleached *Platygyra*. It was noted that at phylum level analyses, the prokaryotic composition in bleached *Porites* to that in the sediment and the prokaryotic composition in CAH2 and CAH3 to that in seawater were quite similar (**Figure 1B** and **Supplementary Figure 2A**).

When analyzing the relative abundance at the order level, the prokaryotic compositions showed the clearer distinct in all sample types, and the similarity of bleached *Porites* to sediment and healthy *Acropora* to seawater was no longer observed (**Figure 1C**). The results also showed a specific dysbiosis of prokaryotic composition in bleached vs. healthy conditions of every coral genus; for example, in *Acropora*, an unclassified order of phylum Cyanobacteria was decreased in abundance while Bacteroidales, Bacillales, and Clostridiales were increased when undergoing bleaching. For *Platygyra*, an unclassified order of class Alphaproteobacteria and Gammaproteobacteria was predominantly present in healthy *Platygyra*; however, in bleached conditions, these bacterial taxa were increased even further along with Bacteroidales and Alteromonadales. For *Pocillopora*, healthy coral exhibited a greater proportion of Pseudomonadales and Rhodobacterales while bleached coral showed a higher proportion of Cytophagales, Rhodospirillales, and an unclassified order of class Gemm-2 and SAR202. Interestingly, a common bacterial alteration was observed among the coral genera, which is the reduction of order Pseudomonadales shared across three bleached coral genera (*Acropora*, *Pocillopora*, and *Porites*) (**Figure 1C**), which may suggest its importance in healthy corals.

The beta diversity (a measurement of biodiversity among samples) *via* NMDS analysis further demonstrated the community separation among coral genera. All bleached communities demonstrated separation from their healthy communities with the most separation being *Acropora* (**Figure 1D**), which is likely due to the aforementioned alteration seen in **Figure 1C** for each coral genus.

These results indicated that the prokaryotic profile of each coral genus is unique. Moreover, the results demonstrated that bleaching in coral may be associated with prokaryotic dysbiosis; however, the degree of alteration and species of prokaryotic changes may differ per coral species.

Beta Diversity Analyses for Microbial Eukaryotic Communities

The eukaryotic profiles obtained from 18S rRNA gene sequencing revealed multiple kingdoms of eukaryotes both unicellular and multicellular organisms, with the top abundance being Chromista (**Supplementary Figure 2B**: avg. 88.23%) followed by Animalia (9.10%), Plantae (0.86%), Fungi (0.23%), and then Protists (0.12%). At the phylum level, Dinoflagellata (avg. 73.49%) dominated the eukaryotic composition of all coral genera, while Ochrophyta (13.92%) dominated that of sediment and seawater. In addition to Ochrophyta, phyla Arthropoda, Protalveolata, and Dinoflagellata were also found to be dominant in seawater samples of Racha Yai Island. In certain coral genera (*Platygyra*, *Pocillopora*, and *Porites*), a decent abundance of Porifera (6.08%) was also observed as part of the dominant phyla; however, this is not the case for both coral states, such as bleached *Pocillopora* (0.0016%) and healthy *Porites* (0.007%) (**Figure 2A**). This result indicated the different eukaryotic profiles of coral, sediment, and seawater samples.

The composition of microbial eukaryotes Dinoflagellata (a single-cell protist and usually considered algae) and Fungi at a genus level was specifically analyzed to characterize the profiles at the genus level (**Figures 2B,C**, respectively). The

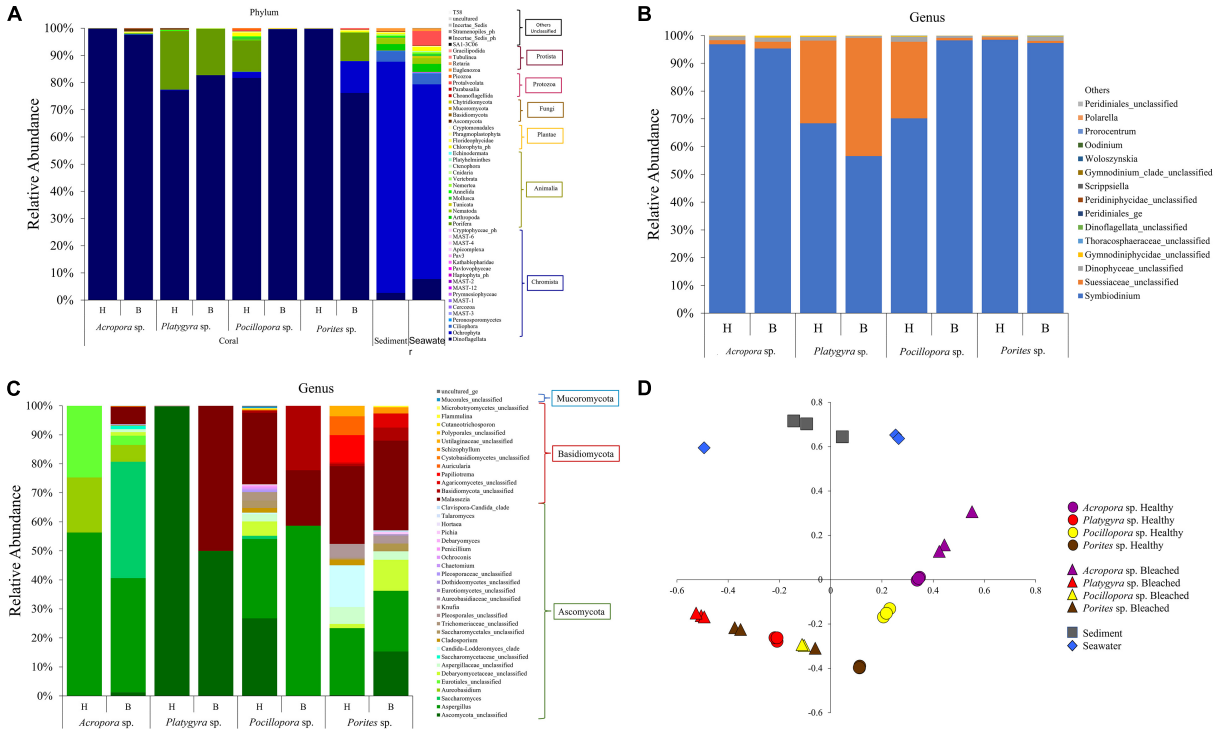

FIGURE 2 | Average microbial eukaryotic compositions in coral, sediment, and seawater samples at **(A)** phylum level and **(B**,**C)** genus level (genus compositions of **(B)** Dinoflagellata and **(C)** fungi); and **(D)** their non-metric multidimensional scaling analyses based on ThetaYC index at the genus level. In panels **(B,C)**, "Others" represent genus OTUs with <1% abundance. For OTU classification where genus could not be identified, the deepest classification level was given.

result showed that most of the Dinoflagellata found were classified as *Symbiodinium* and that it is highly abundant in all coral genera. An unclassified genus of the family *Suessiaceae* was also observed to be highly abundant in healthy corals specifically in *Platygyra* and *Pocillopora*. Comparing healthy and bleached corals, the abundance of *Symbiodinium* was found to be reduced in all bleached coral genera except for bleached *Pocillopora* (**Figure 2B** and **Supplementary Figure 2B**). The finding supported previous knowledge of the symbiotic relationship of *Symbiodinium* to healthy state coral. However, for the *Pocillopora* genus, the replacement of *Symbiodinium* by an unclassified genus of the family *Suessiaceae* (from avg. 22.54% to avg. 0.87%), which was opposite to that in bleached *Platygyra*, might highlight one coral-holobiont advantageous for thermal-resistant *Pocillopora*.

Investigation into the fungal communities showed that each coral genus comprised quite a unique composition of fungal genera in mainly *Ascomycota* and *Basidiomycota* (**Figure 2C**). Overall, the result revealed that *Pocillopora* and *Porites* were more associated with diverse fungal communities than *Acropora* and *Platygyra*. For example, healthy *Platygyra* was revealed to compose solely of an unclassified genus of phylum Ascomycota while other coral genera were associated with a more diverse fungal community. Nevertheless, a shared fungal genus was observed for *Aspergillus* abundance in three coral genera (*Acropora*, *Pocillopora,* and *Porites*), which may suggest their association in the coral. When compared between healthy and bleached corals, the abundance of *Malassezia* was found to be commonly increased in bleached conditions of three coral genera (*Acropora*, *Platygyra,* and *Porites*) apart from *Pocillopora*, which suggested their pathogenicity in the coral (Amend, 2014; Kusdianto et al., 2021). Other observations include abundance shifts of Eurotiales (**Supplementary Figure 2C**) to Saccharomyces in bleached *Acropora*, declining fungal diversity in bleached *Pocillopora* (remaining genera: *Aspergillus*, *Malassezia,* and an unclassified genus of phylum Basidiomycota), and the fungal dysbiosis in bleached *Porites* where an unclassified genus of phylum Ascomycota and an unclassified genus of family Debaryomycetaceae both increased in their abundance while Candida-Lodderomyces, an unclassified genus of family Pleosporaceae and Chaetomium, diminished.

Non-metric multidimensional scaling analysis in **Figure 2D** showed that the microbial eukaryotic diversity between sample types (corals vs. sediment, seawater) was separate; and *Platygyra*, *Pocillopora,* and *Porites* shared some overlapping community positions, while *Acropora* communities were more disparate. Of each coral genus, slight separation between healthy and bleached profiles was observed. The results also indicated that the microbial eukaryotic composition obtained by 18S rRNA gene sequences in each coral genus is quite similar due to the dominant abundance of Dinoflagellata composition (**Figure 2A**). However, the dysbiosis caused by the bleaching event was observable and the compositions of fungi were revealed to be unique in each coral genus.

Identification of Important Bacteria and Fungi Operational Taxonomic Units in Healthy and Bleached Corals

Specific analyses were performed on the microbial taxa that were known to be associated with coral bleaching which include bacterial orders Vibrionales, Oceanospirillales, Alteromonadales, Rhodospirillales, Rhizobiales, and fungal genus *Aspergillus*, for example (**Supplementary Figure 3**). Vibrionales were reported to be associated with bleached corals and were found to increase during heat stress (Kushmaro et al., 1998; Bourne D. et al., 2008; Tout et al., 2015); however, in the present study, the Vibrionales abundance was relatively low in all coral genera and was almost absent in bleached corals. Oceanospirillales were shown to be involved in dimethylsulfoniopropionate (DMSP) degradation and antimicrobial compounds production, which may provide nutrients and resistance against coral diseases (Kirkwood et al., 2010; Raina et al., 2016). Apparently, this is likely the case for *Pocillopora* (from 0.49 to 0.09%) and *Porites* (from 0.84 to 0.13%) genera, which showed a significant reduction in Oceanospirillales in bleached corals; however, in bleached *Acropora* (from 0.40 to 0.72%) and bleached *Platygyra* (from 0.93 to 1.69%), Oceanospirillales were found to be increased instead. Alteromonadales were reported to be beneficial as nitrogen fixers and the nitrogen availability serves as one key for *Symbiodinium* growth, so they were generally associated with healthy state corals (Ceh et al., 2013; Rädecker et al., 2015); however, they were sometimes reported to be increased when corals are stressed or developing diseases, perhaps might mediate coral or algal parasitism growths (Sunagawa et al., 2009; Morris et al., 2019). In the present result, *Acropora*, *Pocillopora*, and *Porites* all exhibited a reduction of Alteromonadales in bleached corals; however, in *Platygyra*, Alteromonadales were significantly increased when bleached. Finally, Rhodospirillales and Rhizobiales were known for their role in nitrogen fixation (Lema et al., 2014; Tsoy et al., 2016). Interestingly, these bacterial orders (Rhodospirillales and Rhizobiales) were found to be increased in bleached *Pocillopora* and bleached *Porites*, suggesting their role in adapting to thermal stresses in these coral genera.

To date, the role of fungi in coral bleaching is very limited. *Aspergillus sydowii* was reported to be causing diseases in corals (Geiser et al., 1998; Smith and Weil, 2004). In analyzing the abundance of *Aspergillus*, the present result obtained a relatively low abundance of this fungal genus. However, the *Aspergillus* abundance was found to be increased in bleached *Acropora*, which could play its pathogenic role during a thermal bleaching event (Alker et al., 2001; Kusdianto et al., 2021). Additionally, increased temperature affects coral immune vulnerability and thus decreasing coral resistance and increasing coral pathogen diseases (Ward et al., 2007).

DISCUSSION

Global warming has negatively impacted massive corals including those in the Andaman Sea for over decades since 1991 (Phongsuwan and Chansang, 2012). The importance of coral-associated microbiomes is recently being recognized as they function fundamentals to the coral developmental cycle, ranging from providing nutrients, antimicrobial substances, and microbial metabolites, to competition against pathogens, which together could support the ability of corals to resist or adapt to harsh environments, including thermal bleaching event (Lesser et al., 2004; Kelman et al., 2006; Rosenberg et al., 2007; Lema et al., 2012; Webster and Reusch, 2017). Phuket province is surrounded by coral reefs of four prevalent coral genera; however, their coral-associated microbiomes of prokaryotes and microbial eukaryotes have not been determined with respect to the thermal bleaching event. Subsequently, this study aimed to provide the prokaryotic and eukaryotic microbial profiles of these four prevalent coral genera (*Acropora*, *Platygyra*, *Pocillopora*, and *Porites*) in healthy and bleached status in Phuket province, Andaman Sea.

Revealing the microbial diversity at the genus level, a similar pattern to our previous study conducted in the upper Gulf of Thailand in the year 2016 was obtained where the sediment was evaluated to have the highest prokaryotic richness among all sample types and where seawater was shown to have the highest microbial eukaryote richness (**Supplementary Figures 1A,C**; Hoshino et al., 2020; Kusdianto et al., 2021). Moreover, a similar result to Kusdianto et al. (2021) was observed in microbial diversity in coral samples, which comprised a higher richness of bacteria than that of microbial eukaryotes (**Supplementary Figures 1B,D**). Compared to the coral genera, *Porites* displayed relatively high biodiversity in both prokaryotic and microbial eukaryotic profiles (**Figure 1**). This result is in good agreement with a previous study from Gardner et al. (2019) who revealed a higher microbial diversity in heat-tolerant corals *Porites* and *Coelastrea*, than in a heat-sensitive coral, *Acropora*. Interestingly, when bleached these coral genera, they demonstrated the unique dysbiosis to their prokaryotic and microbial eukaryotic biodiversity (**Figure 1** and **Supplementary Figure 1**). This species alteration could be due to the specific microbial composition and unique coral genus host-bacterial interaction (Bourne D. et al., 2008; Carlos et al., 2013; McDevitt-Irwin et al., 2017; Osman et al., 2020; Sun et al., 2020). Venn diagrams showed that bleached corals had relatively fewer number of genus OTUs than healthy corals in approximately half the number (**Figure 1E**; Pollock et al., 2019); nonetheless, this lower alpha diversity was not held true to every coral species, in part highlighting the report that the alpha diversity of the stressed corals, regardless of the stressor, could carry the more alpha diversity (McDevitt-Irwin et al., 2017), and these differences might be associated with coral species and their associated microbiome (Kusdianto et al., 2021). Further, among four coral genera, *Pocillopora* and *Porites* had the highest number of OTU diversity (**Figure 1F**), which supported the previous reports of heat-tolerant *Porites* and *Pocillopora* in the wild and that the microbiota from the heat-tolerant *Porites* and *Pocillopora* in the wild could help the recipient corals to bleach at lower rates (Doering et al., 2021).

The symbiotic relationship between corals and photosynthetic dinoflagellates (*Symbiodinium*) is well recognized for the symbionts' provision of nutrients and energy required by the corals (Falkowski et al., 1984; Mieog et al., 2009). Unfortunately,

this positive interaction between corals and *Symbiodinium* can be easily broken with the slight increase of seawater temperature, leading to the bleaching phenomenon (Salih et al., 1997; Gardner et al., 2019). In profiling, the coral-associated microbiome during the 2016 thermal event in Racha Yai Island, a consistent reduction of *Symbiodinium* was found in three bleached corals genera (*Acropora*, *Platygyra*, and *Porites*) (**Figure 2B** and **Supplementary Figure 2B**). Even though the reduction of *Symbiodinium* was relatively low, this might make insufficient to support the healthy state (Sebastian et al., 2009; Cunning et al., 2017). The remaining *Symbiodinium* might also comprise of different clades/types and confer a different degree to support the healthy coral phenotype (Berkelmans and van Oppen, 2006; Stat et al., 2008; Gardner et al., 2019). This was previously reported in the case of *Pocillopora* that exhibited an increased proportion of putative thermal-resistant clade D *Symbiodinium* in a bleached state (Berkelmans and van Oppen, 2006; Panithanarak, 2015) and supported our finding of relative abundant *Symbiodinium* in bleached *Pocillopora*. Meanwhile, *Pocillopora* was previously reported to exhibit variable zooxanthellae species composition, concordant with inferred thermal stress, and thus, the smaller percentage of *Symbiodinium* in our reported healthy *Pocillopora* might merely be due to the great diversity of zooxanthellae species composition during its healthy state (Torres et al., 2021). Moreover, comparing among coral genera, it was observed that *Platygyra* and *Pocillopora* did not depend solely on *Symbiodinium* but that they also relied on an ancient photosynthetic dinoflagellate, Suessiaceae, which was suggested to be symbionts of the earliest Scleractinian corals, evolving through the Triassic period (Palliani and Riding, 2000; Boulotte et al., 2016; Janouškovec et al., 2017) and was present in a high proportion in healthy *Platygyra* and *Pocillopora*. Thus, in terms of supporting healthy state corals for *Pocillopora* could be explained by the abundance of Suessiaceae, while in *Platygyra* the proportion is relatively high yet in bleaching state (**Figure 2B**). However, our reported numbers are all in relative abundance percentage, and thus in future studies, we plan to include the copy numbers of each species (as known as quantitative microbiota) to reveal the microbial abundance in explicit numbers as copies (Steinert et al., 2020) and also may include the analyses of ITS and/or 18S rRNA gene V4 region together with the 18S rRNA gene V9 region to maximize the characterization for phototrophic communities (Bradley et al., 2016).

Differences in bleaching susceptibility among coral genera were not entirely resolved by *Symbiodinium* abundance and composition but were complemented by the associated bacterial communities (e.g., bacteria of carbon synthesis function to replace *Symbiodinium*'s photosynthesis function and bacteria of nitrogen synthesis function, for coral growth) (Rädecker et al., 2015; Cunning et al., 2017; Sweet and Bulling, 2017; Morrow et al., 2018). Compared to the microbial eukaryotic profiles, the prokaryotic community shifts between healthy and bleached corals are more apparent in all coral genera (**Figure 1**). This is likely due to the wide metabolisms and rapid generation times of the prokaryotes making them more rapid in responding to stresses than *Symbiodinium* (Pogoreutz et al., 2018). In examining the coral genera, each coral genus exhibited a unique bacterial composition as evidenced in relative abundance and NMDS analyses (**Figure 1**). This is likely due to the selection of host-specific microbiome, which finetuned a distinct microbial assemblage for each coral genus (Bourne D. et al., 2008; Bernasconi et al., 2019; Damjanovic et al., 2020; Kusdianto et al., 2021). Nevertheless, common bacterial community shifts of order Pseudomonadales and Alteromonadales were observed, which were present in more abundance across three healthy coral genera (*Acropora*, *Pocillopora*, and *Porites*) but declined in bleached corals (**Figure 1** and **Supplementary Figures 2A, 3**). This is similar to the previous report of Pseudomonadales where its abundance was replaced by other pathogenic bacteria when undergoing a bleaching event (Rosenberg et al., 2007) and is also in good agreement with its role in inhibiting coral pathogens in soft coral *Sarcophyton glaucum* (ElAhwany et al., 2015). As Alteromonadales was reported to be able to degrade toxic DMSP to a carbon source of food (Reisch et al., 2011; Raina et al., 2013), this further supported the abundance of these taxa in healthy corals. Although relatively low in their abundance, Vibrionales was surprisingly found in healthy corals across all coral genera and was found to decline in bleached coral samples (**Supplementary Figure 3**). This was unexpected as Vibrionaceae were reported to be opportunistic and potentially pathogenic bacteria associated with coral diseases and coral bleaching (Bourne D. et al., 2008; Tout et al., 2015). However, this pattern was similar to that of our previous study in corals around the upper Gulf of Thailand (Kusdianto et al., 2021), which could be due to the nitrogen-fixing ability in certain *Vibrio* spp. (Criminger et al., 2007; Chimetto et al., 2008). Reshef and colleagues (Reshef et al., 2006; Rädecker et al., 2015) proposed "coral probiotic hypothesis" that the relationship between symbiotic microbes and their coral host was dynamic and might evolutionarily select toward the advantageous compositions of the coral holobionts under varying environmental conditions in a year. Other changes were found to be specific to coral genera, where *Acropora* and *Platygyra* shared a similar alteration while *Pocillopora* and *Porites* shared the same fate of bacterial dysbiosis when bleached to orders Oceanospirillales, Rhizobiales, and Rhodospirillales (**Figure 1C** and **Supplementary Figure 3**). It is still unclear how these bacterial taxa that were reported to play roles in carbon and sulfur recycling processes (Oceanospirillales) (Raina et al., 2016), and nitrogen fixation (Rhizobiales and Rhodospirillales) (Lema et al., 2014; Tsoy et al., 2016) were more abundant in a healthy state of certain coral genera but were also more abundant in a bleached state of other coral species. Nevertheless, it is hypothesized that the specific interaction put by these bacterial species and their host which was unique for each coral genus could be the underlining reason. Moreover, these specific community shifts for each coral genus were also evidenced in previous studies where specific bacterial taxa (e.g., Vibrionales, Rhodobacterales, Alteromonadales, Rhizobiales, Rhodospirillales, and Oceanospirillales) were found to be dominant in a healthy state of certain coral species but were also more abundant in a bleached state of other coral species (Bourne and Munn, 2005; McDevitt-Irwin et al., 2017; Sun et al., 2020).

For the fungal communities, although unique patterns were observed against coral genera, common increases of

genus *Malassezia* for bleached *Acropora*, *Platygyra*, and *Porites* were observed. Another alteration in the fungal community was the decrease of genus *Aspergillus* for bleached *Acropora* and unclassified genus in family Aspergillaceae for bleached *Pocillopora* and *Porites* (**Figure 2C**). The association of *Malassezia* to coral bleaching is yet unknown; however, its pathogenic role had been reported in association with skin disorders in the human for *Malassezia* (McGinley et al., 1975; Velegraki et al., 2015). For *Aspergillus*, its role as an aspergillosis disease in coral communities had been reported (Geiser et al., 1998; Kim and Rypien, 2015). Moreover, the increase of these fungal taxa in bleached corals is similar to our previous study in 2016 thermal bleaching event in the upper Gulf of Thailand (Kusdianto et al., 2021).

CONCLUSION

In summary, as Thailand is bounded by the coastlines of the Andaman Sea on one side and the Gulf of Thailand on the other side (**Figure 1A**), this presents a slight difference in marine geography (e.g., the corals in the Andaman Sea have more hard corals than in the Gulf of Thailand). Following our first report of both prokaryotic and microbial eukaryotic profiles associated with coral genera and their sediment and seawater surroundings in the upper Gulf of Thailand (Kusdianto et al., 2021), the present study continued firstly characterizing the microbiome compositions (prokaryotes and microbial eukaryotes) of four coral genera exhibited in healthy and bleached corals collected from the Andaman Sea of Thailand during the 2016 thermal bleaching event to better understand the healthy and bleached coral-associated microbiome and in the slight different Thailand marine geography. This study found distinct prokaryotic and microbial eukaryotic profiles between *Acropora, Platygyra, Pocillopora*, and *Porites* and suggested microbiome composition that might support coral bleaching susceptibility or tolerance based on previous works of literature. Potential coral-beneficial microbes from the previous reports as aforementioned were found to be reduced in the bleaching state corals (e.g., Pseudomonadales and Alteromonadales) but the previously reported coral-pathogenic microbes were found to increase (e.g., *Malassezia* and *Aspergillus*). Overall, while similar findings across bleach vs. healthy microbiota were found between our studied locations in this study (the Andaman Sea) and the Gulf of Thailand by Kusdianto et al. (2021), new findings that were highlighted in this study were Pseudomonadales (no reduction in bleached state corals *Acropora* and *Platygyra* in the Gulf of Thailand), Alteromonadales (no reduction in bleached state *Porites* in the Gulf of Thailand), and Vibrionales (higher in bleached state *Porites* in the Gulf of Thailand). However, the present analysis is a time point analysis of coral microbiome in 2016 in the studied location, further studies of upcoming bleaching events and diverse locations in the Andaman Sea of Thailand should be performed to allow complete understanding and identification of microbiomes representing the healthy vs. bleaching corals and to interpret the associated microbiomes to support against thermal bleaching stress. In addition, future studies should separate coral tissue and coral skeleton microbiomes analyses; coral tissue represents a direct interface and coral skeleton represents a reservoir for inner microbes and physico-chemical nutrients *via* decalcification (Ainsworth et al., 2015; Glasl et al., 2016; Ricci et al., 2019). Eventually, understanding these coral-associated microbiomes would help in restoring sustainable reefs (i.e., understanding which core microorganisms support bleaching resistance and coral reef restoration in the Andaman Sea of Thailand).

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/, PRJNA700134.

AUTHOR CONTRIBUTIONS

SC collected the samples, helped conceive of the study, and helped revise the manuscript. HK and CK did molecular biology experiments and data analysis. CK helped draft the manuscript. DW commented. SJ and VV collected the samples. JO and VV conceived of the study. VV helped revise the manuscript. NS conceived of the study, coordinated the experiments and data analysis, and wrote and revised the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2022.763421/full#supplementary-material

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Selection Signatures of Pacific White Shrimp *Litopenaeus vannamei* Revealed by Whole-Genome Resequencing Analysis

Hao Wang[1†], Mingxuan Teng[1†], Pingping Liu[1,2], Mingyang Zhao[2], Shi Wang[1,2,3], Jingjie Hu[1,2], Zhenmin Bao[1,2,4] and Qifan Zeng[1,2,4]*

[1] MOE Key Laboratory of Marine Genetics and Breeding, College of Marine Life Sciences, Ocean University of China, Qingdao, China, [2] Key Laboratory of Tropical Aquatic Germplasm of Hainan Province, Sanya Ocean Institute, Ocean University of China, Sanya, China, [3] Laboratory for Marine Biology and Biotechnology, Qingdao National Laboratory for Marine Science and Technology, Qingdao, China, [4] Laboratory for Marine Fisheries Science and Food Production Processes, Qingdao National Laboratory for Marine Science and Technology, Qingdao, China

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Anthony Lee Dellinger, Kepley BioSystems Inc., United States

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Zhongkai Wang, Qingdao Agricultural University, China
Quanchao Wang, Yantai Institute of Coastal Zone Research (CAS), China
Chaozheng Li, Sun Yat-sen University, China

***Correspondence:**
Qifan Zeng
zengqifan@ouc.edu.cn

[†] These authors have contributed equally to this work

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The Pacific white shrimp *Litopenaeus vannamei* is among the top aquatic species of commercial importance around the world. Over the last four decades, the breeding works of *L. vannamei* have been carried out intensively and have generated multiple strains with improved production and performance traits. However, signatures of domestication and artificial selection across the *L. vannamei* genome remain largely unexplored. In the present study, we conducted whole genomic resequencing of 180 Pacific white shrimps from two artificially selective breeds and four market-leading companies. A total of 37 million single nucleotide polymorphisms (SNPs) were identified with an average density of 22.5 SNPs/Kb across the genome. Ancestry estimation, principal component analysis, and phylogenetic inference have all revealed the obvious stratifications among the six breeds. We evaluated the linkage disequilibrium (LD) decay in each breed and identified the genetic variations driven by selection. Pairwise comparison of the fixation index (F_{st}) and nucleotide diversity (θ_π) has allowed for mining the genomic regions under selective sweep in each breed. The functional enrichment analysis revealed that genes within these regions are mainly involved in the cellular macromolecule metabolic process, proteolysis, structural molecule activity, structure of the constituent ribosome, and responses to stimulus. The genome-wide SNP datasets provide valuable information for germplasm resources assessment and genome-assisted breeding of Pacific white shrimps, and also shed light on the genetic effects and genomic signatures of selective breeding.

Keywords: Pacific white shrimp, SNP, population structure, linkage disequilibrium, selective sweep

INTRODUCTION

Aquaculture produces almost half of the seafood consumed by humans and is becoming one of the fastest-growing food production sectors in the world (FAO, 2020). To match the ever-increasing food demands of the growing population, aquaculture production should increase fivefold in the next three decades (Costello et al., 2020). The recent annual global production of farmed shrimps

reached more than 7.7 million tons, representing a value of over 33 billion US dollars (FAO, 2020). Benefitting from technological innovation and policy reforms, shrimp farming has developed steeply from traditional and small-scale activities into a global industry, holding a great promise for enhancing the contribution of aquaculture production to food supply (Lotz, 1992; Briggs et al., 2005).

Pacific white shrimp (*Litopenaeus vannamei*) is the top shrimp species of commercial importance. The annual global yield of *L. vannamei* reached 4.4 million tons with a production value of about 26.7 billion USD, accounting for 80% of the total cultured shrimp production (FAO, 2020). The success of Pacific white shrimp aquaculture is largely attributed to a series of breeding programs since the 1970s (Lotz, 1992; FAO, 2011). Genetic improvements in performance traits and disease resistance have achieved remarkable progress over the last decade (Argue et al., 2002; Campos-Montes et al., 2012; Montaldo et al., 2013; Lillehammer et al., 2020). Several specific pathogen-free (SPF) *L. vannamei* breeds, with superior health and efficiency adapted to distinct farming conditions, have been cultivated and shipped worldwide (Briggs et al., 2005; Fletcher, 2020; Ren, 2020). Benefitting from the rapid development of high-throughput sequencing and genotyping technologies, several causative genes responsible for phenotypic variations of *L. vannamei* have been reported (Wang et al., 2019; Zhang X. et al., 2019; Lyu et al., 2021). For instance, genes encoding class C scavenger receptor (*SRC*), deoxycytidylate deaminase (*dCMPD*), and non-receptor protein tyrosine kinase (*NPTK*) were identified as potential genes related to the growth rate (Wang et al., 2019; Lyu et al., 2021). Whereas, genomic signatures underlying artificial selection remain largely unexplored. Dissection of the high-resolution genomic variation map is essential for a better understanding of genetic diversity, population structure, and genomic features during generations of selection.

In the present study, to profile the genome signatures of selection in *L. vannamei*, we performed a whole genomic resequencing analysis of shrimps from two artificially selective breeds (Renhai No. 1 and Kehai No. 1) and broodstocks of four market-leading companies (Benchmark Genetics, Charoen Pokphand, Shrimp Improvement Systems, and Top Aquaculture Technology). The genome-wide single nucleotide polymorphisms (SNP) datasets revealed the population structure and genetic effects during the breeding process, providing valuable information for germplasm resource assessment and genome-assisted breeding of Pacific white shrimps.

MATERIALS AND METHODS

Sampling and DNA Extraction

A total of 180 samples from the *L. vannamei* broodstock of Renhai No. 1 (RH), Kehai No. 1 (KH), Benchmark Genetics (BMK), Charoen Pokphand (CP), Shrimp Improvement Systems (SIS), and Top Aquaculture Technology (TA) were collected from Hairen Aquatic Seed Industry Technology Co., Ltd (Hebei, China) (**Supplementary Table 1**). The muscle samples of each individual were collected for DNA extraction and genome resequencing. Genomic DNA was extracted using the TIANamp Marine Animal DNA Kits (TIANGEN Biotech Co., Ltd. Beijing, China). Paired-end sequencing libraries, with an insert size of 250 to 350 bp, were constructed by the VAHTS Universal Plus DNA Library Prep Kit for MGI (Vazyme Biotech Co., Ltd., Nanjing, China) in their lab, and sequenced on an MGI DNBSEQ-T7 system (BGI Genomics Co., Ltd., Shenzhen, China). All raw sequencing data were subject to quality control[1]. Low-quality bases and reads were trimmed by *Trimmomatic* with parameters "LEADING:3 TRAILING:3 SLIDINGWINDOW:4:15 MINLEN:36" (Bolger et al., 2014).

Sequencing and Genotyping

The reference genome was downloaded from the National Center for Biotechnology Information (NCBI) with the accession of GCA_003789085.1 (Zhang X. et al., 2019). We anchored the contigs into scaffolds by the guidance of a genetic linkage maps (Yu et al., 2015; Jones et al., 2017), and all trimmed reads were aligned to this genomic assembly using the *BWA* (version 2.3.4.1), with the default parameters (Li and Durbin, 2009). The Binary Alignment Map (BAM) files were imported to the *samtools* (v0.1.19) for reference index building, format conversion, and reads sorting (Li et al., 2009). The *Picard*[2] (v1.92) and the *sambamba* (v0.8.0) were used to assign read group information (Tarasov et al., 2015). PCR and optical duplicates were marked and filtered using *GATK* (v4.1). Variants and haplotypes were identified using the *HaplotypeCaller* algorithm in Genomic Varient Call Format (GVCF) mode (Mckenna et al., 2010). The *GenotypeGVCFs* were used subsequently for joint genotyping.

To keep the most reliable SNPs for subsequent analysis, variant sites were marked and filtered by the *vcffilter* with the following criteria: low quality score (GQ $<$ 20); low quality by depth score (QD $<$ 10); high Fisher strand score (FS $>$ 10); low mapping quality (MQ $<$ 40); low read position rank sum score (ReadPosRankSum $<$ 8); high strand odds ratio (SOR $>$ 4); and low mapping quality rank-sum score (MQRankSum $<$ 12.5). The SNP loci with multiple alleles, low minor allele frequency (MAF $<$ 0.05), and missing genotypes (max-missing $<$ 1) were also removed using the *VCFtools* (v0.1.16) (Danecek et al., 2011). The potential effect of each SNP was annotated using the *SNPEff* (Cingolani et al., 2012).

Population Structure Analysis

Principal component analysis (PCA) was performed using the *PLINK* (Purcell et al., 2007). Ancestry estimation and population structure were analyzed using the *ADMIXTURE* (Alexander et al., 2009). Pairwise fixation index (F_{st}) was calculated by *VCFtools* with parameters –fst-window-size 50,000 and –fst-window-step 10,000 (Danecek et al., 2011). The genomic observed heterozygosity, expected heterozygosity, and inbreeding coefficient were evaluated by the *VCFtools* with parameter –het (Danecek et al., 2011). For the phylogenetic

[1]https://github.com/s-andrews/FastQC

[2]http://broadinstitute.github.io/picard/

analysis, representative SNP markers were extracted using the *VCFtools* with parameter –thin 10,000 to mitigate the effects of LD, homozygous sites were removed from the alignment file. Maximum likelihood phylogenetic inference was carried out by *IQ-TREE* (Nguyen et al., 2015).

The SNPs with specific alleles in one population were defined as population-specific SNPs (ps-SNPs). If the frequency of the population-specific allele was greater than 50% in a population, the SNPs were defined as common population-specific SNPs (cps-SNPs). The LD decay was estimated for each population using the *PopLDdecay* (Zhang C. et al., 2019). Genomic inbreeding coefficients F_{ROH} were calculated according to the following equation, with a minimum window of 100 kb (McQuillan et al., 2008):

$$F_{ROH} = \Sigma \frac{L_{ROH}}{L_{Auto}}$$

Selective Sweep Analysis

The pairwise F_{st} and the ratio of genetic polymorphisms (θ_π) were calculated using a 50 kb window and a 10 kb step between each breed. Genomic regions with signals of differences in the spectrum of genetic polymorphisms were identified as regions that are potentially under selection. Empirical cut-offs for F_{st} and θ_π ratio were set as the top 2% largest and the top 5% largest or smallest, respectively (Li L. et al., 2018; Wang et al., 2021). Candidate genes under selection were defined as within or overlapping with the regions showing signals of selection. Candidate genes were characterized with Gene Ontology (GO) enrichment analysis implemented in the *EnrichPipeline* based on Fisher's exact test (Huang et al., 2009). The GO terms with a *P-value* < 0.05 were considered significantly enriched.

RESULTS

SNP Identification and Annotation

An average of 164 million 300 bp paired-end reads were generated for each library, representing ∼18.7 × coverage. After quality control, approximately 80% of reads could be aligned to the reference genome (∼12.34 × mean unique coverage) and used for variant calling. A total of 37,308,098 SNPs were identified in the six populations. The number of SNPs in the chromosomes ranged from 182,599 to 1,274,977, and the density was between 20.60 SNPs/Kb and 24.58 SNPs/kb. Of these, 4,509,526 reliable SNPs were kept after the removal of low-quality samples and those within repetitive regions of missing genotypes. The transitions/transversions ratio in this biallelic SNPs set was found to be 1.63, with 1,117,185,030 transitions and 72,199,828 transversions, respectively. The SNPs were unevenly distributed across the genome, with over 10.56% (1,460,366) located in the intergenic regions, about 55.43% (7,662,564) located in the introns, and only 2.283% (238,482) located in the exons (**Table 1**). More than 73.89% of the SNPs (221,021) were silent mutations, the remaining 25.82 (77,219) and.30% of SNPs (886) could result in missense and non-sense mutations, respectively (**Table 1**).

TABLE 1 | SNP annotation by genomic region, impact and function class.

Category	Type (alphabetical order)	Count	Percent (%)
Region	Downstream	1,237,840	8.954
	Exon	315,610	2.283
	Intergenic	1,460,366	10.563
	Intron	7,662,564	55.427
	Splice site acceptor	272	0.002
	Splice site donor	367	0.003
	Splice site region	26,386	0.191
	Transcript	1,811,097	13.100
	Upstream	1,132,578	8.192
	UTR_3_prime	138,137	0.999
	UTR_5_prime	39,435	0.285
Impact	High	1,825	0.013
	Low	249,177	1.802
	Moderate	76,897	0.556
	Modifier	13,496,753	97.628
Function class	Missense	77,219	25.815
	Non-sense	886	0.296
	Silent	221,021	73.889

Population Structure

The population structure was inferred based on the genome-wide biallelic SNPs. As shown by the PCA, the first three components cumulatively explained 24.56% of the variance (**Figure 1A** and **Supplementary Figures 1, 2**). Samples from the six populations were grouped into three major clusters, which largely restored the sampling sources. Obvious stratifications were observed among BMK, SIS, and the Asian breeds (RH, KH, TA, and CP). Although RH, KH, TA, and CP were distributed closely on the first two components, they possessed distinct population structures (**Figure 1** and **Supplementary Figure 2**). The pairwise comparison revealed that the F_{st} values among the six populations ranged from 0.0005 to 0.13415, and that BMK had the largest genetic distances from the others. The KH and TA share a relatively higher similarity in genetic structure (**Figure 1B**). Populations defined by estimated genetic ancestries also confirmed the distribution of samples, where six ancestral populations were inferred to be the optimal scenario by cross-validation (**Figure 1C** and **Supplementary Figure 3**). The phylogenetic relationships were reconstructed *via* the maximum likelihood inference. Except for two samples of TA clustered together with CP, samples from each population formed monophyletic clades, which is consistent with the population structure inferred by PCA and ancestral estimation (**Figure 1D**).

Genome Selection Signatures

As shown in **Figure 2A**, population-specific SNPs were identified in each breed. The BMK and SIS possessed 83,273 and 15,980 cps-SNPs, respectively, which are much larger than that of the other four breeds. The RH, KH, TA, and CP only had 3,153, 1,889, 485, and 836 cps-SNPs, respectively (**Table 2**). We evaluated the genetic diversity by the observed heterozygosity, expected heterozygosity, and inbreeding coefficient (**Supplementary Data Sheet 1**). These six populations have an average genomic

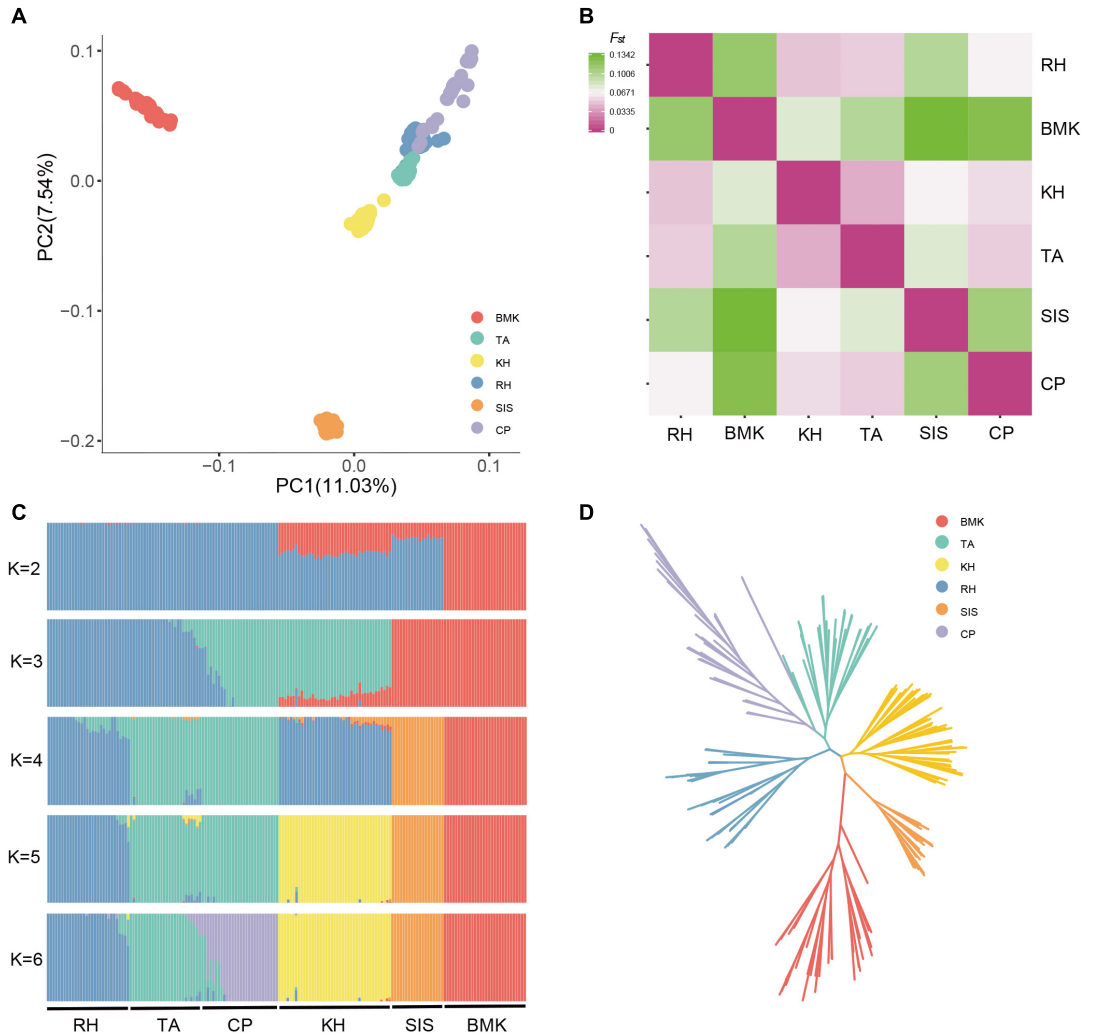

FIGURE 1 | Population structure analysis of the six populations by principal component analysis (PCA) **(A)**, Pairwise F_{st} distance **(B)**, ancestral estimation **(C)**, and phylogenetic inference **(D)**.

observed heterozygosity of 0.122, 0.137, 0.144, 0.147, 0.149; and 0.156 in BMK, CP, TA, RH, KH, and SIS. The extent of genome-wide LD decay was measured against the physical distance in each population (**Figure 2B**). Asian breeds (RH, KH, TA, and CP) typically exhibited an overall lower level of LD than the others. Their LD levels (r^2) fell below 0.1 at less than 15Kb, whereas, the physical distances reached over 70 kb in BMK and SIS. Analysis of runs of homozygosity (ROH) revealed that BMK and CP reserved higher levels of inbreeding than the others (**Figure 2C** and **Supplementary Figure 4**). Homozygous segments larger than 1Mb were mostly identified in these two populations. Despite that the genome-wide distribution of ROH is distinct in each population, peaks in specific regions of chromosomes 10, 15, and 42 were identified in each population (**Figure 2D**).

Genes Under the Selective Sweep

The pairwise F_{st} and θ_π ratio were calculated to scan the genomic regions with genetic signals of divergence. The top 5% of the windows, with high values of F_{st} and differentiation of polymorphism frequency spectrum, were identified as regions that are potentially under selection (**Figure 3A** and **Supplementary Figure 5**). Genome-wide selective sweeps in BMK were illustrated in **Figure 3A**. We detected 271, 440, 257, 269, 431, and 429 candidate genes under a strong selection from 166,258 genomic windows in RH, BMK, KH, TA, SIS, and CP, respectively (**Supplementary Data Sheet 2**). Interestingly, 206 genes were under selection in more than one population (**Supplementary Data Sheet 2**). Function enrichment analysis of these candidate genes provided 631 significant enriched categories (**Supplementary Data Sheet 3**). For example, genes under selection in BMK were enriched in response to stimulus, carbohydrate transport, and several biological processes (**Figure 3B**). Selective genes in RH were mainly enriched in trialkyl sulfonium hydrolase activity, protein modification by a small protein conjugation, and phospholipid binding. Selective genes in KH were mainly

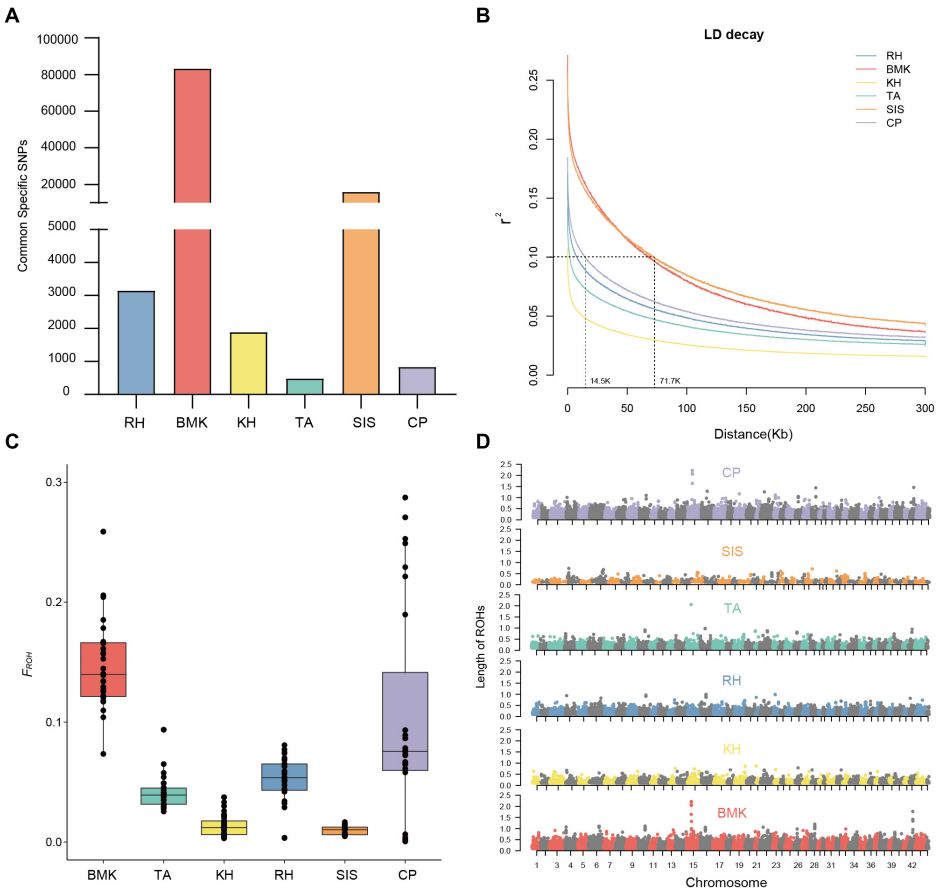

FIGURE 2 | (A) cps-SNPs among the six populations. **(B)** linkage disequilibrium (LD) decay at a distance of 0–300Kb. **(C)** Box plot of F_{ROH} statistic. **(D)** Genome-wide distribution of ROHs in the six populations.

enriched in the structural constituent of ribosome, structure molecule activity, and cellular macromolecule metabolic process. Selective genes in TA were enriched in the pyruvate kinase activity, peptidase inhibitor activity, and ATP metabolic process. Selective genes in SIS were enriched in the ribonuclease H2 complex, GTP cyclohydrolase I activity, and cellular macromolecule metabolic process. Selective genes in CP were mainly enriched in the structural molecule activity, regulation of ATPase activity, and 5′-deoxynucleotidase activity (please check **Supplementary Data Sheet 3** for detailed in section "Results"). Notably, *B3GT5*, *CDC42, PXDN,* and several genes that play important roles in cellular macromolecule metabolic process, proteolysis, and responses to stimulus were identified under selection in more than one population (**Figure 4**).

DISCUSSION

Genetic breeding of *L. vannamei* has achieved remarkable progress and cultivated multiple novel breeds with significant improvement in growth and adaptation to modern farming conditions after generations of artificial selection (Argue et al., 2002; Campos-Montes et al., 2012; Montaldo et al., 2013; Lillehammer et al., 2020). As exploring the full range of genetic diversity has practical implications for the management of germplasm resources and breeding programs, several studies have been carried out to decipher the spectrum of genetic polymorphism (Castillo-Juarez et al., 2015; Garcia et al., 2021). Whereas, these studies were mainly based on a limited number of markers by targeted or by reduced-representation genotyping (Li and Wu, 2003; Perez-Enriquez et al., 2009, 2018b;

TABLE 2 | Genetic statistical summary of the six population.

Population	Common specific SNPs	Rare specfic SNPs	Total number of ROH	Mean length of ROHs	Froh
RH	3,153	900,321	487	175.78	0.052
BMK	83,273	1,015,660	1282	188.64	0.147
KH	1,889	3,979,378	133	162.41	0.013
TA	485	1,293,688	392	174.64	0.042
SIS	15,980	850,211	97	163.86	0.01
CP	836	606,685	956	175.95	0.111

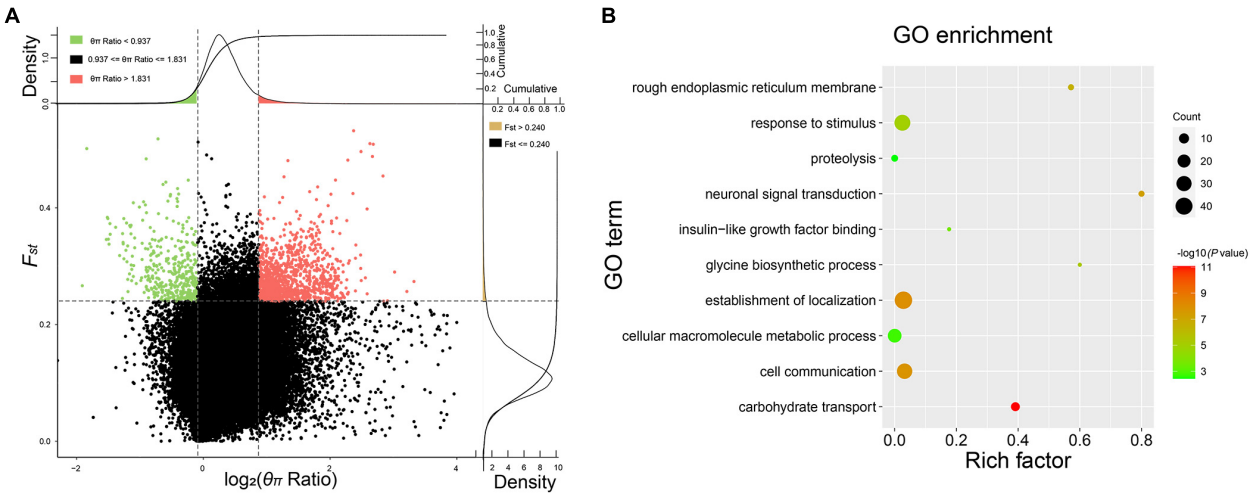

FIGURE 3 | Selective sweep and Gene Ontology (GO) enrichment analysis of the BMK samples. **(A)** Distribution of θ_π ratios (θ_π reference population/θ_π selective breed) and F_{st} values calculated in 50 kb window with a 10 kb step. Windows with the 5% largest or smallest of θ_π ratio, and the top 2% largest F_{st} were highlighted as regions under selection. **(B)** Enriched GO terms of genes under selection.

GO term	TA	RH	BMK	KH	SIS	CP
cellular macromolecule metabolic process	*DNM3A, EDC4, HNK2, ILPR, IRAK4, MED15, PAX9, PGTB2, PMGT1, POL, RM32, RT16*	*GATAC, MOS, PGTB2, PIM3, PMGT1, POL3, R144A, RS11, RT16, SCAP, UBP8*	*C1GLT, GSBN, HIRA, MOS, NHR48, PHKG2, POL3, RL9, ROR1, STT3B, TICRR*	*ATM, AURAB, B3GT5, NU301, PAX7, PHKG2, POL, PSA7, RL21, ROR1, RS21, RT18A, UBP12*	*ANM3, ATM, AURAB, B3GT5, C1GLT, CUL5, FANCL, GATAC, PAX7, PHKG2, PMGT1, POL, RL21, RPA1, UBP12*	*ACH92, B3GT5, C1GLT, CC50A, CGAT2, GRM, MRP4, OPSC1, PTHD3, QCR8, TSN31, TSPO, VAMP4, ZNT6*
structural molecule activity	*RM32, RT16*	*NU133, RS11, RT16, SPTCB*	*RL9, SNTG1, TBA1*	*NU133, RL21, RS21, RT18A, RT25*	*NU133, RL21, RLA2, RT25, SNTG1*	*NU155, NUP98, RL34, RL37A, RS9*
structural constituent of ribosome	*RM32, RT16*	*RS11, RT16*	*RL9*	*RL21, RS21, RT18A, RT25*	*RL21, RLA2, RT25*	*RL34, RL37A, RS9*
response to stimulus	*CALCR, FSHR, IRAK4, KCTD9, PXDN2, RAS, RIT1*	*GRM, GUTR1, RXFP1, SCAP*	*ASPC1, CDC42, CHIN, IQEC1, PXDN, RAC1, RGF1B, RHG26, RHO1, ROR1, WDR24*	*ATM, RASF4, RGPS1, ROR1, RXFP1, SOCS7*	*ATM, DNLI3, FANCL, FZD10, PDH1, PERC, RASF4, RGPS1, SOCS7*	*ADT1, AGRD1, BMR1B, GKAP1, GRM, OPSC1, PXDN, PXDN2, THOC1, TITIN, TM198, TOLL8, ZFY26*
proteolysis	*POL2*	*ASPP, RMD5A, UBP8*	*CTR2, NICA, POL3, POL4, PSMD8*	*ACE, ECE1, MMEL1, PSA7, SPCS3, TMPS9, UBP12, YCF2E*	*AFG31, CFB, CTRB2, CUL5, MBTP1, OTUBL, SPCS3, STUB, TMPS6, TMPS9, UBP12*	*ADA10, DPEP1, DPP10, NUDEL, TRY1, UBP14, UBP4*

Note: *P* value < 0.01; 0.01 ≤ *P* value < 0.05; *P* value > 0.05.

FIGURE 4 | Shared enriched GO terms of selective genes among each breed.

Garcia et al., 2021; Prithvisagar et al., 2021). The genome-wide landscape of variations shaped by artificial selection remains largely unexplored. In this study, we conducted whole genomic resequencing of *L. vannamei* and investigated the distribution of SNP sites among six populations. In total, 4.5 million high confidence SNPs were identified across the genome, reaching an average density of 22.5 SNPs/kb across the genome. Interestingly, population structures inferred by PCA, ancestry estimation, and phylogenetic analysis provided strong evidence for interpopulation stratifications. Considering that the founders of *L. vannamei* breeds were originally introduced from native populations in the Pacific coast from Mexico to Northern Peru in the late 1970s and 1980s (Wyban and Sweeney, 1991; Rosenberry, 2000), it was suggested that genetic drift and artificial selection could potentially contribute to the differentiation. The genomic heterozygosity of the six populations ranges from 0.122 to 0.156. The BMK and CP exhibit the lowest level of heterozygosity and high inbreeding coefficient, which is consistent with the ROH

analysis. The observed heterozygosity levels are relatively lower than previous estimations (De Freitas and Galetti, 2005; Artiles et al., 2011; Rezaee et al., 2016; Perez-Enriquez et al., 2018a). The decay of LD provides important information about the historical recombination of population and is widely used to understand the evolutionary and demographic processes (Slatkin, 2008). Our results revealed that the overall LD levels of the four Asian populations are comparable to the breeding population of Ecuador (Garcia et al., 2021), while LD at long-range was stronger in BMK and SIS. Despite the biases in sampling size and the relatedness among individuals, LD extension could be attributed to demographic and biological factors, such as the admixture, mutation, and fluctuation of the effective population size (Qanbari, 2019). As the natural distribution of *L. vannamei* is restricted in America, we presumed that the observed long-range LD in American strains may be introduced by recurrent inclusion of wild strains, which was absent in the Asian populations (Moss et al., 2012). This phenomenon was also reported in salmonids, where admixture is the major factor contributing to long-range LD (Odegard et al., 2014; Barria et al., 2018; Vallejo et al., 2018).

The F_{st} values and θ_π ratios have been broadly used to identify genetic differentiation and genome-wide selective sweeps (Barreiro et al., 2008; Li L. et al., 2018). In the present study, we revealed numerous breeding signatures reflecting the complex genomic architectures from six breeds with distinct genetic backgrounds. A series of positive selective genes, such as *B1GT3, C1GLT, NU133, PXDN, SOCS7*, and *UBP8*, etc., have been identified in more than one breed. Previous studies in arthropods have revealed that many of these genes are involved in important physiological and biochemical functions. For example, *PXDN, SCAP (SREPF* Chaperone*), SOCS7, and TOLL8* are related to antiviral or antibacterial immunity in Pacific white shrimps and other Crustaceans (Du et al., 2013; Wang et al., 2016; Li H. et al., 2018; Aweya et al., 2020). The *CDC42* plays an important role in Reactive oxygen species (ROS) production and apoptosis by Mitogen-activated protein kinases (MAPK) pathway in *L. vannamei* (Peng et al., 2015). As selective breeding of these populations has been carried out for generations, the identified selective genes seem to coincide with the improved performance traits. Previous studies revealed that target genotyping of trait-linked markers can improve the accuracy of breeding value estimation (Li et al., 2017). Thus, we expect that these loci could serve as important markers for genetic improvement *via* targeted genotyping.

CONCLUSION

We conducted a population genetic study on six *L. vannamei* breeds by whole genomic resequencing and identified over 37 million SNPs. Population structure revealed by PCA, ancestral estimation, and phylogenetic analysis confirmed the stratification among these populations. Selective sweep analysis detected that 206 genes were under selection in more than one population. Among them, several candidate genes act as key regulators in cellular macromolecule metabolic process, proteolysis, and responses to stimulus, which could be responsible for the improved economic traits and adaptation to modern aquaculture. This study not only provides valuable information for germplasm resources assessment and genome-assisted breeding of *L. vannamei*, but also sheds light on the genetic effects and genomic signatures of selective breeding.

DATA AVAILABILITY STATEMENT

The data presented in the study are deposited in the repository of Aquaculture Molecular Breeding Platform (AMBP, http://mgb.qnlm.ac).

AUTHOR CONTRIBUTIONS

ZB, QZ, JH, and SW designed the experiments. MT, MZ, and PL collected the samples. HW, MT, and PL performed the experiments. HW, MT, and MZ analyzed the data. HW and QZ wrote the manuscript. All authors have read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2022.844597/full#supplementary-material

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