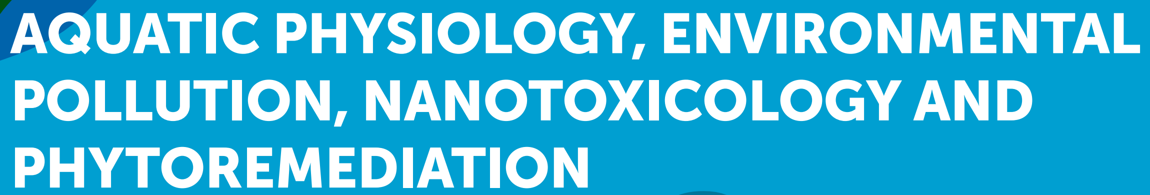

AQUATIC PHYSIOLOGY, ENVIRONMENTAL POLLUTION, NANOTOXICOLOGY AND PHYTOREMEDIATION

EDITED BY: Alaa El-Din Hamid Sayed, Francesco Fazio, Tomomi Watanabe-Asaka and Vincenzo Parrino

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frontiers Research Topics

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AQUATIC PHYSIOLOGY, ENVIRONMENTAL POLLUTION, NANOTOXICOLOGY AND PHYTOREMEDIATION

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This Research Topic is part of the Aquatic Physiology, Environmental Pollution, Nanotoxicology and Phytoremediation series:

Aquatic Physiology, Environmental Pollution, Nanotoxicology and Phytoremediation, Volume II

Environmental pollution as a result of increasing industrialization is a major problem worldwide. The toxicity of the chemicals, hazards, radiation, and environmental stressor to the aquatic fauna was studied. Although, recently, the excess levels of wastes discharged in water caused severe toxicity in aquatic environments and their fauna, still there is some shortage in the nanotoxicology and phytoremediation studies. So, the aim of this Research Topic is to create some knowledge about the environmental pollution and remediation in aquatic environment in collaboration with experts in physiology, biochemistry, endocrinology, morpho-histology of aquatic fauna.

The relation between physiology and other research fields is strong enough as all researchers in biology field use some extent physiological parameters to evaluate the organisms' health status in normal and stressful conditions. In addition, physiology with endocrinology and neurology can provide a contribution on the endocrine stress response of aquatic vertebrates and regulate the responses of vertebrates to stressors. Whilst the physiology of most aquatic animals has been well studied, not many articles provide sufficient data that helps understanding the common bases of the stress response after exposure to environmental pollutants and mechanisms of action. Such approach needs to be taken both in terms of comparative responses among vertebrates but also among classes or orders within groups of vertebrates. Another aspect that has not been sufficiently approached so far is physiological stress response in relation to immunity, growth, reproduction or behavior and embryology of the aquatic organisms, which expands the knowledge on the interactions between physiological systems to build an overall stress response.

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Table of Contents

05 ***The Autophagic Flux Inhibitor Bafilomycine A1 Affects the Expression of Intermediary Metabolism-Related Genes in Trout Hepatocytes***
Sarah Séité, Tracy Pioche, Nicolas Ory, Elisabeth Plagnes-Juan, Stéphane Panserat and Iban Seiliez

16 ***Intestinal Function of the Stomachless Fish, Ballan Wrasse (*Labrus bergylta*)***
Hoang T. M. D. Le, Xinting Shao, Åshild Krogdahl, Trond M. Kortner, Ingrid Lein, Katerina Kousoulaki, Kai K. Lie and Øystein Sæle

31 ***Regulation of Dietary Lipid Sources on Tissue Lipid Classes and Mitochondrial Energy Metabolism of Juvenile Swimming Crab,* Portunus trituberculatus**
Ye Yuan, Peng Sun, Min Jin, Xuexi Wang and Qicun Zhou

44 ***Integrated Remediation Processes Toward Heavy Metal Removal/Recovery From Various Environments-A Review***
Adikesavan Selvi, Aruliah Rajasekar, Jayaraman Theerthagiri, Azhagesan Ananthaselvam, Kuppusamy Sathishkumar, Jagannathan Madhavan and Pattanathu K. S. M. Rahman

59 ***Offshore Crude Oil Disrupts Retinoid Signaling and Eye Development in Larval Atlantic Haddock***
Kai K. Lie, Sonnich Meier, Elin Sørhus, Rolf B. Edvardsen, Ørjan Karlsen and Pål A. Olsvik

73 ***Effects of Dietary Inclusion of Shrimp Paste on Growth Performance, Digestive Enzymes Activities, Antioxidant and Immunological Status and Intestinal Morphology of Hybrid Snakehead (*Channa maculata ♀ × Channa argus ♂*)***
Haohang Fang, Jiajun Xie, Shiyu Liao, Tianyu Guo, Shiwei Xie, Yongjian Liu, Lixia Tian and Jin Niu

81 ***Diet Supplemented With Synthetic Carotenoids: Effects on Growth Performance and Biochemical and Immunological Parameters of Yellow Perch (*Perca flavescens*)***
Eman A. Abd El-Gawad, Han-Ping Wang and Hong Yao

94 ***Transcriptome Analysis Reveals That Naphthenic Acids Perturb Gene Networks Related to Metabolic Processes, Membrane Integrity, and Gut Function in* Silurana (Xenopus) tropicalis *Embryos***
Juan Manuel Gutierrez-Villagomez, Christopher J. Martyniuk, Lei Xing, Valerie S. Langlois, Bruce D. Pauli, Jules M. Blais and Vance L. Trudeau

111 ***Gonadal Histopathological Disorders in* Mytilus galloprovincialis *Male Exposed to Tars Used in Mussel Farms***
Ana Alonso, Pilar Suárez, Yolanda Ruiz, Vladimir Dobal and Fuencisla San Juan

131 ***The Potential Use of Invasive Ascidians for Biomonitoring Heavy Metal Pollution***
Roni Tzafriri-Milo, Tal Benaltabet, Adi Torfstein and Noa Shenkar

148 ***Multiple Stressors in the Environment: The Effects of Exposure to an Antidepressant (Venlafaxine) and Increased Temperature on Zebrafish Metabolism***

Hossein Mehdi, Leslie M. Bragg, Mark R. Servos and Paul M. Craig

158 ***Continuous Inking Affects the Biological and Biochemical Responses of Cuttlefish*** **Sepia pharaonis**

Maowang Jiang, Chenxi Zhao, Runxuan Yan, Jianping Li, Weiwei Song, Ruibing Peng, Qingxi Han and Xiamin Jiang

170 ***Significance Assessment of*** **Amphora coffeaeformis** ***in Arsenic-Induced Hemato- Biochemical Alterations of African Catfish (*****Clarias gariepinus*****)***

Imam A. Mekkawy, Usama M. Mahmoud, Rehab H. Moneeb and Alaa El-Din H. Sayed

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The Autophagic Flux Inhibitor Bafilomycine A1 Affects the Expression of Intermediary Metabolism-Related Genes in Trout Hepatocytes

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Autophagy is an evolutionarily conserved process of cellular self-eating which emerged these last years as a major adaptive metabolic response to various stresses such as fasting, hypoxia, or environmental pollutants. However, surprisingly very few data is currently available on its role in fish species which are directly exposed to frequent environmental perturbations. Here, we report that the treatment of fasted trout hepatocytes with the autophagy inhibitor Bafilomycine A1 lowered the mRNA levels of many of the gluconeogenesis-related genes and increased those of genes involved in intracellular lipid stores. Concurrently, intracellular free amino acid levels dropped and the expression of the main genes involved in the endoplasmic reticulum (ER) stress exhibited a sharp increase in autophagy inhibited cells. Together these results highlight the strong complexity of the crosstalk between ER, autophagy and metabolism and support the importance of considering this function in future studies on metabolic adaptation of fish to environmental stresses.

Keywords: fish, hepatocyte, autophagy, intermediary metabolism, ER stress, gene expression

INTRODUCTION

Macroautophagy (autophagy hereafter) is a cellular function conserved in eukaryotes that allows the recruitment of substrates into lysosomes for their degradation (Bento et al., 2016). In addition to its role as a "cell cleaner," autophagy allows providing energy during fasting or other cellular stress in order to promote survival (Yang and Klionsky, 2010). In mammals, several studies demonstrated that autophagy maintains cellular, and energy homeostasis by degrading and recycling the main

Abbreviations: Acc2, acetyl CoA carboxylase 2; ATF6, activating transcription factor 6; Baf A1, Bafilomycine A1; chop, C/EBP homologous protein; CoA, stearyl coenzyme A; Dgat2, diacylglycerol acetyltransferase 2; edem1, ER degradation enhancing alpha-mannosidase like protein 1; eef1a1, eukaryotic elongation factor 1 α 1; eIF2α, eukaryotic initiation factor 2α; fas, fatty acid synthase; fbp, fructose 1,6-bisphosphatase; g6pc, glucose 6-phosphatase; IRE1, inositol requiring 1; pck1, phosphoenol pyruvate carboxykinase 1 (cytosolic); pck2, phosphoenol pyruvate carboxykinase 2 (mitochondrial); PERK, PKR-like ER kinase; plin2, perilipin 2; plin3, perilipin 3; Scd1, stearyl desaturase 1; SIDT2, SID-1 transmembrane family member 2; SREBP, sterol regulatory element–binding proteins; TUBB, β-tubulin; UPR, unfolded protein response; xbp1, X-box binding protein 1.

energy sources (proteins, lipids or glycogen) on exposure to various stresses (Madrigal-Matute and Cuervo, 2016). One of the first metabolic functions attributed to autophagy has been the release of amino acids through protein degradation during starvation. The released amino acids not only sustain protein synthesis under fasting condition, but also feed the tricarboxylic acid cycle for ATP production (Lum et al., 2005; Rabinowitz and White, 2010; Ezaki et al., 2011; Thomas et al., 2018). Furthermore, autophagic proteolysis in liver has been shown to makes a significant contribution to the maintenance of glycaemia during fasting by releasing amino acids for glucose production via gluconeogenesis (Ezaki et al., 2011). In addition to its role in protein breakdown, autophagy has also been shown to play an important role in the degradation of hepatic lipid stores through a selective form of autophagy termed lipophagy (Singh et al., 2009). During this process, autophagy-dependent breakdown of lipid droplets supplies free fatty acids, which undergo β-oxidation in the mitochondria to support ATP production (Singh et al., 2009; Rambold et al., 2015; Welte, 2015). Although less studied than the two former autophagic processes, lysosomal breakdown of hepatic glycogen might also contribute to glucose homeostasis during some critical periods. In mice, this process known as glycophagy has thus been shown to be necessary to sustain life during the period of postnatal hypoglycemia (Kotoulas et al., 2006). Collectively, these data highlight the critical importance of autophagy for the adaptation of intermediary metabolism to environmental changes.

In fish, this cellular function is attracting growing interest and the number of studies in this field is constantly increasing. As such, induction of autophagy has been demonstrated upon different biotic or abiotic stress situations including pollution (Khangarot, 1992; Chen et al., 2015; Xing et al., 2015), hypoxia (Beck et al., 2016), viral contamination (Liu et al., 2015), fasting, or nutritional imbalance (Seiliez et al., 2012; Yabu et al., 2012; Wei et al., 2017, 2018; Séité et al., 2018; Wang et al., 2018). In these last few years, increasing research also focused on the mechanisms involved in the control of this cellular function in fish, particularly in zebrafish (He et al., 2009; Dowling et al., 2010; He and Klionsky, 2010; Mathai et al., 2017). In contrast, surprisingly, its metabolic role remains poorly explored in these species and very little data is currently available on this subject.

However, we previously reported that rainbow trout treated with the autophagy flux inhibitor agent Colchicine exhibited severe alterations in hepatic carbohydrate and fat metabolisms, as revealed by a significant decrease in plasma glucose levels associated with a decrease of the concentration of some glucogenic amino acids in the liver, but also an increase in hepatic triglyceride and lipid droplet contents (Seiliez et al., 2016). Similarly, recent works provided the evidence for the degradation of lipid stores through lipophagy in the liver of fasted zebrafish (Wang et al., 2018) and yellow catfish (*pelteobagrus fulvidraco*) fed zinc supplemented diet (Wei et al., 2018). Together, these data are in close agreement with the aforementioned metabolic role of autophagy demonstrated in mammals (Madrigal-Matute and Cuervo, 2016). However, they also suggest that in addition to this previously reported role of autophagy in providing substrates for glucose production, energy furniture, or the synthesis of specific proteins, it could also play a major role in the regulation of the expression of some key metabolic genes. Indeed, these studies pointed out that autophagy inhibited fish exhibited strong perturbations in the mRNA levels of genes involved in hepatic carbohydrate and fat metabolisms (Seiliez et al., 2016; Wang et al., 2018). This would be an unknown function for autophagy. However, they show conflicting outcomes on specific gene expression regulations, precluding a clear picture of the role of autophagy in this process. Such differences could be explained by the divergence of experimental protocols such as the use of (1) different fish models (zebrafish vs. rainbow trout) and (2) different autophagy flux inhibitors (chloroquine vs. colchicine) and deserved further investigations.

In the present study, we treated primary cultures of trout hepatocytes with another autophagy flux inhibitor, the Baf A1, to assess the specificity of the previously reported *in vivo* effect of colchicine-mediated autophagy inhibition on the expression of several metabolism-related genes in this species. Baf A1 is widely used *in vitro* as an autophagic flux inhibitor. This drug inhibits the lysosomal V-ATPase to prevent its acidificationas well as the Ca2+ pump SERCA to disrupt autophagosome-lysosome fusion, together resulting in a strong block of autophagic flux (Mauvezin and Neufeld, 2015). The use of primary cultures of trout hepatocytes is an additional asset for our study, as they allow testing the response of the studied factors to specific stimuli independently of their systemic effects. This model is now widely used to improve understanding of intermediary metabolism in fish (Moon et al., 1985).

MATERIALS AND METHODS

Animals

Sexually immature rainbow trout having a mean initial weight of 200 g were obtained from the INRA experimental facilities at Donzacq (Landes, France). Fish were maintained in tank kept in open circuits at a constant water temperature of 17°C, under natural photoperiod. They were fed to satiety every 2 days with a commercial diet (T-3P classic, Trouw, France). The experiments performed in the present study comply with the EUdirective 2010/63/EU on the protection of animals used for research as well as the decree No 2013-118, 1 February 2013 of the French legislation on the ethical treatment of animals.

Hepatocyte Cell Culture

Rainbow trout liver cells were isolated from 3 days feed-deprived fish according to the previously detailed protocol (Lansard et al., 2010). We measured the cell viability (>98%) with trypan blue exclusion method (0.04% in 0.15 mol/L NaCl) and cells were counted using Neubauer chamber. They were then plated in a 6-well Primaria culture dish (BD) at a density of 3.106 cells/well and incubated at 18°C, the optimal temperature for cell cultures of trout origin, with complete medium containing modified Hanks' medium (136.9 mmol/L NaCl, 5.4 mmol/L KCl, 0.8 mmol/L $MgSO_4$, 0.44 mmol/L KH_2PO_4, 0.33 mmol/L Na_2HPO_4, 5 mmol/L $NaHCO_3$, and 10 mmol/L HEPES) supplemented with 1% defatted BSA, 3 mmol/L glucose, 2%

MEM essential amino acid mixture, 1% MEM non-essential amino acid mixture and 1% antibiotic antimycotic solution (1X) (sigma). The incubation medium was replaced every 24 h over the 48 h of primary cell culture. Microscopic examination ensured that hepatocytes progressively re-associated throughout culture to form cell heap. After 2 days of culture, the cells were incubated in a minimal medium deprived of serum and amino acids (a condition known to activate autophagy) in presence or absence of 100 nM of Baf A1 a concentration commonly used to block autophagosome-lysosome fusion *in vitro* (Klionsky et al., 2016). Cells were then sampled 4, 8, 16, and 24 h after the treatment and were prepared for western blot analysis or resuspended in TRIZOL reagent (Invitrogen, Carlsbad, CA, United States) and stored at −80°C for subsequent analyses. Each experiment was repeated 2 times.

Protein Extraction and Western Blot Analyses

Cells were prepared for western blot analyses according to the previously detailed protocol (Lansard et al., 2010). LC3-II levels were measured by western blot as described previously in Belghit et al. (2014) and using the following antibodies: anti-LC3b (#2775 Cell Signaling Technology) and anti-TUBB (#2146, Cell Signaling Technology). These antibodies have already been validated in rainbow trout (Belghit et al., 2014).

Quantitative RT-PCR Analyses

The protocol conditions for sample preparation and quantitative RT-PCR have been previously published (Lansard et al., 2010). The primers used for real time RT-PCR assays are listed in **Table 1**. Primer of *edem1* and *xbp1* were newly designed using Primer3 software. The primers that amplified glucose and lipid metabolism-related genes have already been described in previous studies (Plagnes-Juan et al., 2008; Marandel et al., 2015; Seiliez et al., 2016). For the expression analysis, relative quantification of target gene expression was done using the ΔCT method described by Pfaffl et al. (2002). The relative gene expression value of *eef1a1* was used for the normalization of the measured expression values of the target mRNA, and was found to not change significantly over sampling time or among treatments (data not shown).

TABLE 1 | Sequences of the primer pairs used in the quantitative real-time RT-PCR assays.

Genes	Forward primer	Reverse primer
Gluconeogenesis related genes		
pck1	ACAGGGTGAGGCAGATGTAGG	CTAGTCTGTGGAGGTCTAAGGGC
pck2	ACAATGAGATGATGTGACTGCA	TGCTCCATCACCTACAACCT
fbp1b1	CTCTCAAGAACCTCTACAGCCT	TCAGTTCTCCCGTTCCCTTC
g6pca	GATGGCTTGACGTTCTCCT	AGATCCAGGAGAGTCCTCC
g6pcb1	AGGGACAGTTCGAAAATGGAG	CCAGAGAGGGAAGAAGATGAAGA
g6pcb2	CCTGCGGAACACCTTCTTTG	TCAATTTGTGGCGCTGATGAG
Lipid metabolism related genes		
fas	TGATCTGAAGGCCCGTGTCA	GGGTGACGTTGCCGTGGTAT
plin2	CATGGAGTCAGTTGAAGTCGTC	AATTTGTGGCTCCAGCTTGCC
plin3	GATGTCCAACACCGTCACAG	TCGATTTCCAACTCGTCCTC
ER stress related genes		
chop	CTGCACACGGTCTGGAGCTG	GGATCTCGTCTGGGATCAGGT
edem1	GAACATCCAAACGGGACAGT	TGAGAAGAGGGAGGGAGTCA
xbp1	CAACCCCGAGAACACAGTTT	AAGTGACACACGCTGTGGTC
Reference gene		
eef1a1	TCCTCTTGGTCGTTTCGCT	ACCCGAGGGACATCCTGTG

Free Amino Acid Analyses

Free amino acid concentrations in hepatocytes were determined by ion exchange chromatography with a ninhydrin post-column reaction (L-8900 Amino Acid Analyzer, Hitachi High-Technologies Corporation, Tokyo, Japan).

Statistical Analyses

Data are expressed as means ± SD. Normality was assessed using the Shaprio–test, while the equality of variances was determined using Levene's test. When the normality and/or equal variances of data were respected, two-way ANOVA was used to detect significant differences. Following two-way ANOVA analysis, the Tukey test was used for *post hoc* analysis. For all statistical analyses, the level of significance was set at $P < 0.05$.

RESULTS

Baf A1 Inhibits Autophagy in Trout Hepatocytes

We first tested the ability of Baf A1 to block autophagy in our cell culture model. For this purpose, we analyzed by western blot the well-established autophagy marker LC3II in cells incubated in a serum- and amino acid-deprived medium (a condition known to activate autophagy) and treated or not with Baf A1 for 4, 8, 16, and 24 h. During autophagy, LC3 is converted from a non-lipidated cytosolic form (LC3-I) to a phosphatidylethanolamine-conjugated form (LC3II) on the autophagosomal membrane (Klionsky et al., 2016). However, LC3-II is also degraded during the late stage of autophagy, and it is now well accepted that the exposure of cells to lysosomal inhibitors, protease inhibitors or agent that block fusion of autophagosome with lysosomes, leads to LC3-II accumulation (Klionsky et al., 2016). As shown in **Figure 1**, the ratio of LC3-II to TUBB reached significantly higher levels in Baf A1 treated cells compared to non-treated cells. These results indicated that Baf A1 treated cells displayed a loss of autophagy function and that this drug is useful in our cell culture model.

Baf A1 Treatment Affects the Expression of Key Genes of the Intermediary Metabolism in Trout Hepatocytes

We next addressed the consequences of Baf A1 treatment on the expression of several metabolism-related genes. We first monitored the expression of several genes of the gluconeogenesis in cells incubated in the same conditions described above with or without Baf A1. The obtained results showed that the addition of

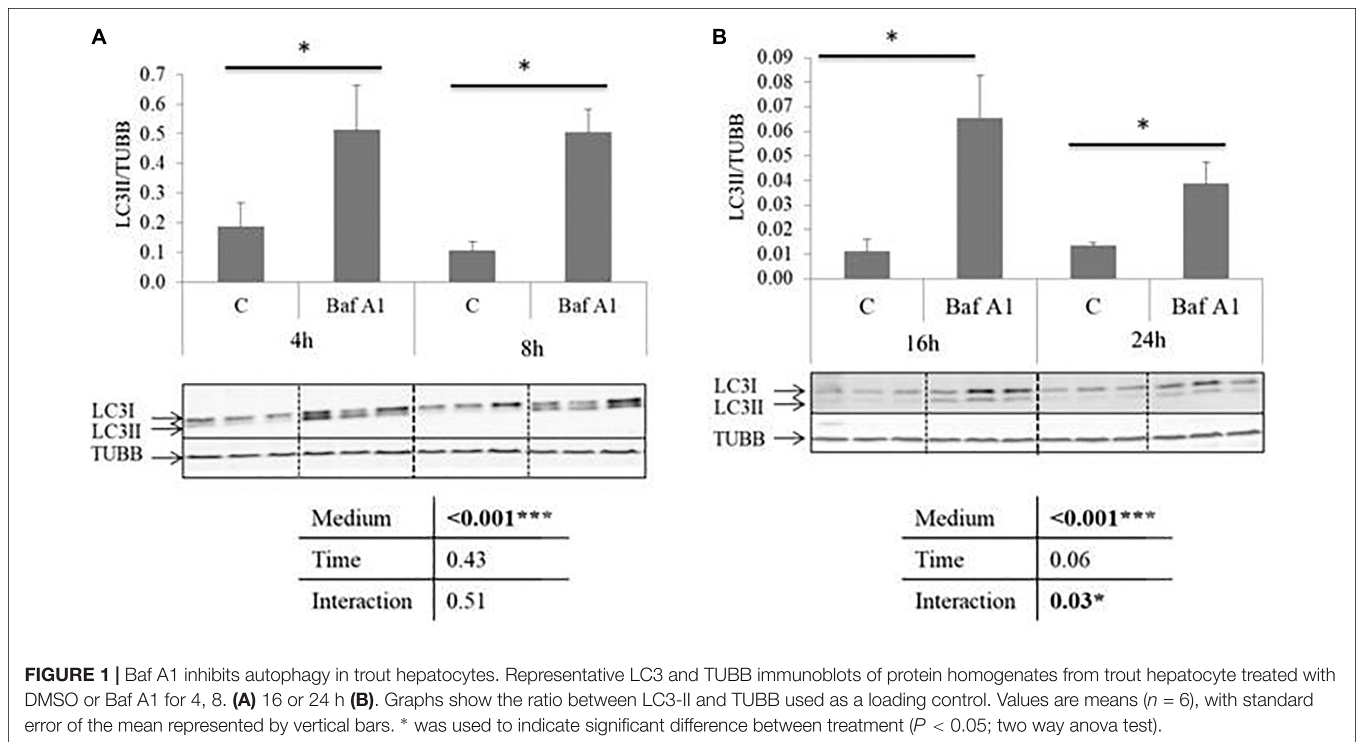

FIGURE 1 | Baf A1 inhibits autophagy in trout hepatocytes. Representative LC3 and TUBB immunoblots of protein homogenates from trout hepatocyte treated with DMSO or Baf A1 for 4, 8. **(A)** 16 or 24 h **(B)**. Graphs show the ratio between LC3-II and TUBB used as a loading control. Values are means (n = 6), with standard error of the mean represented by vertical bars. * was used to indicate significant difference between treatment (P < 0.05; two way anova test).

Baf A1 to the media led to a significant decrease of mRNA levels of gluconeogenesis-related genes *g6pcb1* and *pck1* regardless of the time of treatment (**Figures 2A,E**). Similar results were obtained for *g6pca* and *fbp1b1* at 24 h after the treatment (**Figures 2C,D**) and for *pck2* at 16 and 24 h after the treatment (**Figure 2F**). In contrast, we observed an increase of mRNA levels of one of the *g6pc* paralogs, the *g6pcb2,* in trout hepatocyte treated with Baf A1 (**Figure 2B**).

We then analyzed the expression of several genes involved in lipid metabolism. The obtained results showed that mRNA levels of *fas* increased in cells treated with Baf A1 (**Figure 3A**). Similar results were obtained for *plin2 and plin3,* two critical regulators of hepatic neutral lipid storage (**Figures 3B,C**).

Overall, these data confirmed our previous *in vivo* results obtained with Colchicine and established a tight link between the activity of autophagy and the expression of several glucose and lipid metabolism-related genes.

Baf A1 Treatment Lowers the Level of Free Amino Acids in Trout Hepatocytes

It is now well established that the expression of many metabolism-related genes is under the tight control of amino acid availability (Lansard et al., 2010, 2011). Autophagy being one of the main systems for the release of free amino acids during fasting, we wondered whether the effects of Baf A1 on metabolic gene expression could be related to a decrease in free amino acid levels in hepatocytes whose autophagy has been inhibited. We therefore, monitored the concentration of the main amino acids in fasted cells treated or not with Baf A1. As shown in **Figure 4**, hepatocytes treated with Baf A1 exhibited lower levels of most of the analyzed amino acids, in accordance with the reported role of liver autophagy on amino acid release during starvation. This global decrease in amino acid release in hepatocytes treated with BafA1 could therefore contribute to perturb the expression of the studied genes.

Baf A1 Treatment Leads to ER Stress

Another hypothesis to explain the effect of Baf A1 on the expression of the studied genes concerns the endoplasmic reticulum (ER) stress. Accumulating evidences demonstrated that autophagy dysregulation causes ER stress (Yang et al., 2010), which has been shown to strongly impact the expression of intermediary metabolism-related genes (Lee et al., 2012; Wang and Kaufman, 2014; Zhou and Liu, 2014). However, to our knowledge, few if no data is available on the effect of autophagy dysregulation-mediated ER stress on the expression of intermediary metabolism-related genes. In the present study, we therefore sought to determine whether Baf A1 caused ER stress in our cells. To this end, we analyzed, in fasted hepatocytes treated with or without Baf A1, the expression of three target genes *chop, xbp1,* and *edem1* of the main ER-stress sensing pathways PERK, ATF6, and IRE1 pathways, respectively. As shown in **Figure 5A**, the mRNA levels of *chop* significantly increased in Baf A1 treated cells in comparison to control cells 4, 8 and 16 h after the treatment. Similar results were obtained for *xbp1*, with an increase at 4 and 8 h after the treatment (**Figure 5B**). Likewise, the mRNA levels of *edem1* increased 24 h after the treatment (**Figure 5C**), in line with previous findings demonstrating that *edem1* is a late ER-stress marker. Overall, the results obtained clearly show that hepatocytes treated with Baf A1 display sign of ER stress, which in turn could affect the expression of the studied genes.

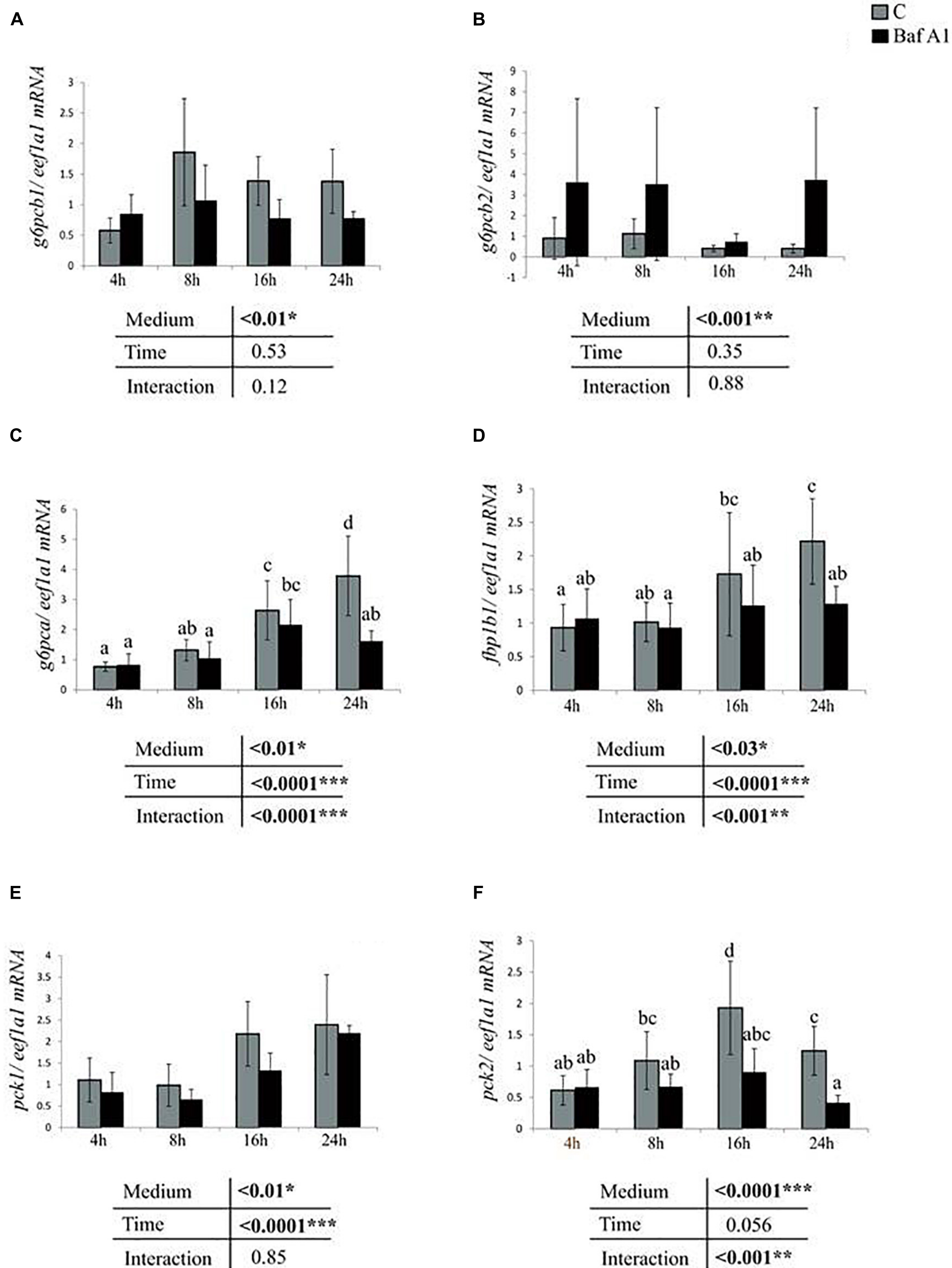

FIGURE 2 | Baf A1 treatment affects mRNA levels of gluconeogenic genes. Hepatocytes were treated with DMSO or Baf A1 for 4, 8, 16, or 24 h. Hepatocyte mRNA levels of **(A)** *g6pcb1*, **(B)** *g6pcb2,* **(C)** *G6pca,* **(D)** *fbp1b,* **(E)** *pck1*, and **(F)** *pck2* were measured using quantitative real time RT-PCR assays. Expression values are normalized with the *eukaryotic translation elongation factor 1 α 1 (eef1a1)* mRNA. Value are means (n = 6) with standard error represented by vertical bars and were analyzed using two-way ANOVA ($P < 0.05$), followed by Tukey's *post hoc* test for multiple comparisons. When interaction between sampling time and treatment is significant, lowercases letters (a, b, c, and d) represent statistically significant differences ($P < 0.05$, Tukey's HSD).

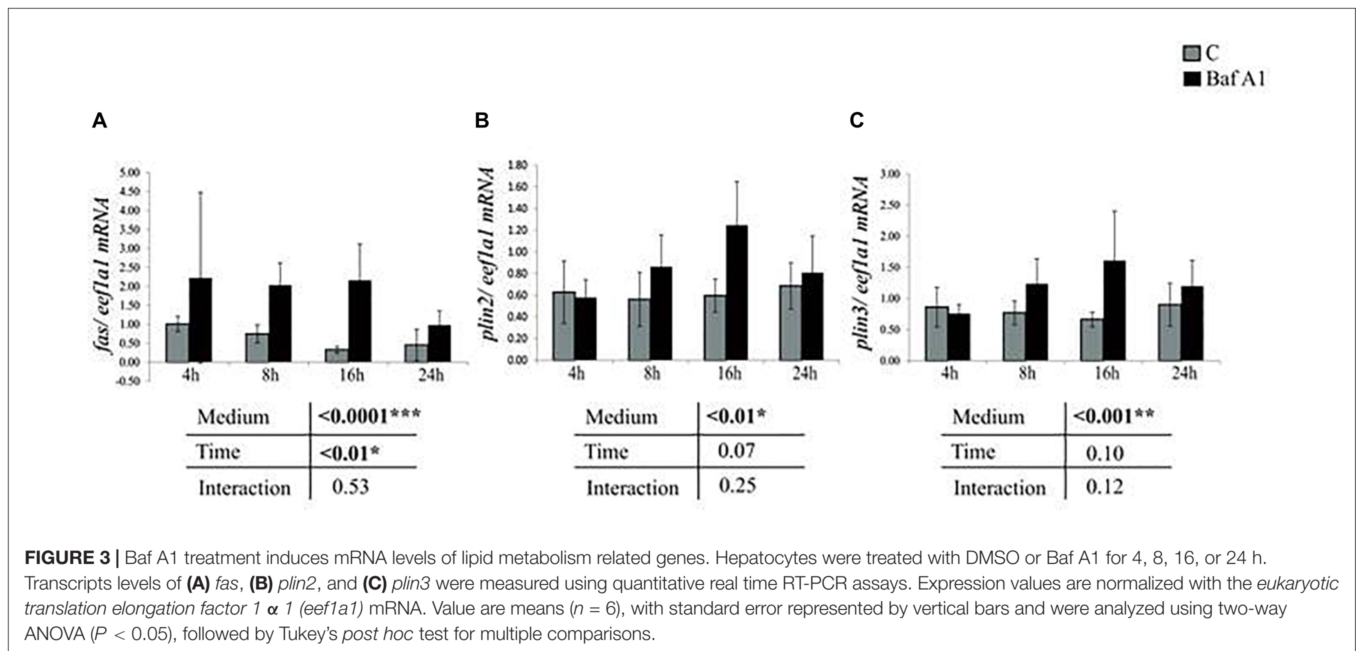

FIGURE 3 | Baf A1 treatment induces mRNA levels of lipid metabolism related genes. Hepatocytes were treated with DMSO or Baf A1 for 4, 8, 16, or 24 h. Transcripts levels of **(A)** *fas*, **(B)** *plin2*, and **(C)** *plin3* were measured using quantitative real time RT-PCR assays. Expression values are normalized with the *eukaryotic translation elongation factor 1* α *1 (eef1a1)* mRNA. Value are means (n = 6), with standard error represented by vertical bars and were analyzed using two-way ANOVA ($P < 0.05$), followed by Tukey's *post hoc* test for multiple comparisons.

DISCUSSION

Autophagy has long been considered merely as a cellular waste disposal and recycling mechanism. However, studies in recent years have highlighted its major role for the adaptation of metabolism to environmental changes (Kroemer et al., 2010; Chen et al., 2015; Chiarelli et al., 2016; Tang, 2016). In this regard, we and others showed that treatment of fasted fish (rainbow trout or zebrafish) with autophagy flux inhibitor agents (colchicine or chloroquine) led to strong defaults in intracellular substrates delivery for glucose production or energy furniture (Seiliez et al., 2016; Wang et al., 2018). Interestingly, these studies also pointed out sever perturbations in the mRNA levels of several intermediary metabolism-related genes in these fish, establishing a new potential link between autophagy and intermediary metabolism. However, probably due to divergences of experimental protocols (including the species investigated and the used autophagy flux inhibitors), these studies led to conflicting results with respect to the regulations of specific gene expression, precluding a clear picture of the role of autophagy in this process.

In the present study, we demonstrated that Baf A1 treatment of trout hepatocytes decreased the mRNA levels of genes involved in gluconeogenesis and conversely, increased those of genes involved in lipogenesis and lipid storage. Although it is well accepted that Baf A1 is an autophagy inhibitor, it may also have other side effects. For instance, some data reported that it has some effects on mitochondria quality (Yuan et al., 2015; Redmann et al., 2017), making it difficult to determine which effects on metabolism-related mRNAs could be a consequence of inhibiting autophagy or of direct effects on mitochondria independently of autophagy. However, our results are in close agreement with those previously reported in trout showing that *in vivo* treatment with colchicine (which act on autophagy by inducing microtubule disassembly) led to a similar lowering effect on the mRNA levels of gluconeogenesis-related genes and an increasing effect on both *plin2* and *plin3* (Seiliez et al., 2016), suggesting that the observed effects are specific to autophagy inhibition. Interestingly, previous findings in mammals also evidenced a tight link between the activity of autophagy and mRNA levels of some enzymes involved in glucose metabolism (Wang et al., 2015). Acute suppression of autophagy with lysosome inhibitors (Chloroquine or Bafilomycin A1) in statin treated human liver cancer cell line (HepG2 cells) has thus been shown to reduce mRNA levels of the two gluconeogenic enzymes *g6pc* and *pck1* (Wang et al., 2015). Similarly, the statin-induced increase in expression of *g6pc* and *pck1* was blocked in Atg7-deficient hepatocytes, providing a genetic confirmation of these results (Wang et al., 2015). However, another study suggests the opposite role of autophagy in gluconeogenesis with the finding that overexpression of Atg7 reduces mRNA levels of *g6pc* and *pck1* in the livers of mice (Yang et al., 2010); But the induction of autophagy by Atg7 overexpression was not verified in this study, preventing to conclude on the specific role of this function in the observed effects. More recently, Wang et al. (2018) showed that chloroquine treatment of fasted zebrafish inhibited the hepatic expression of most genes related to lipid metabolism and conversely upregulated those of carbohydrates metabolism, making possible the existence of species-dependent effects of autophagy inhibition. Overall, these data support a close link between autophagy and the mRNA levels of metabolic genes, although the exact nature of this relationship, which likely depends on many factors (including the species studied and/or the protocol used to monitor this link), remains to be clarified.

It is now clearly established that the expression of a wide range of hepatic genes involved in the intermediary metabolism is under the control of amino acid availability. In trout hepatocytes, free amino acid addition to an amino acid-deprived medium

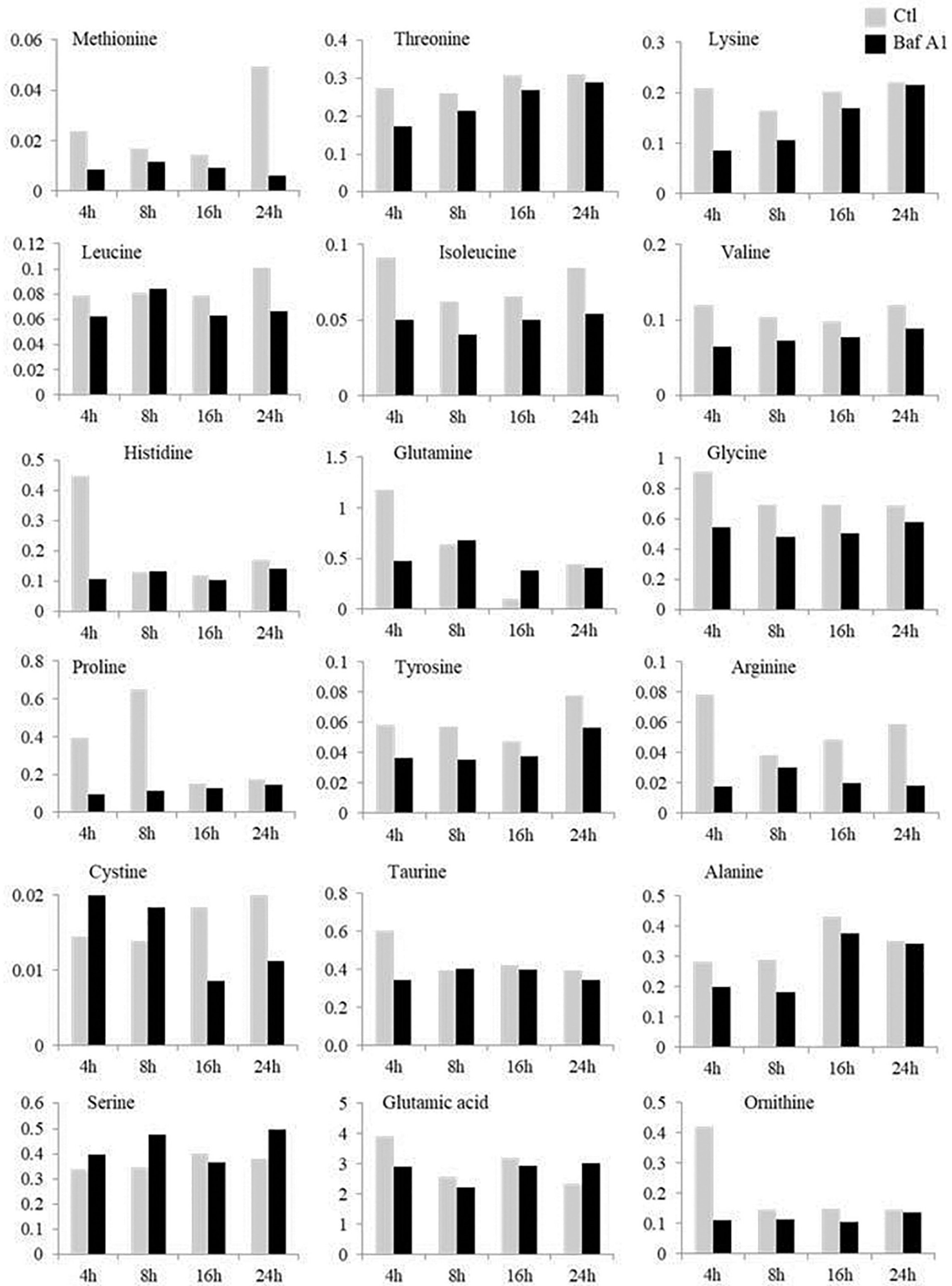

FIGURE 4 | Time courses of the changes in free amino acids in fasted hepatocytes treated or not with Baf A1. Fasted trout hepatocytes were treated with DMSO or Baf A1 for 4, 8, 16, or 24 h. The concentration of each amino acid is expressed as μmol/l cell homogenate and was determined on a pool of samples from two independent experiments.

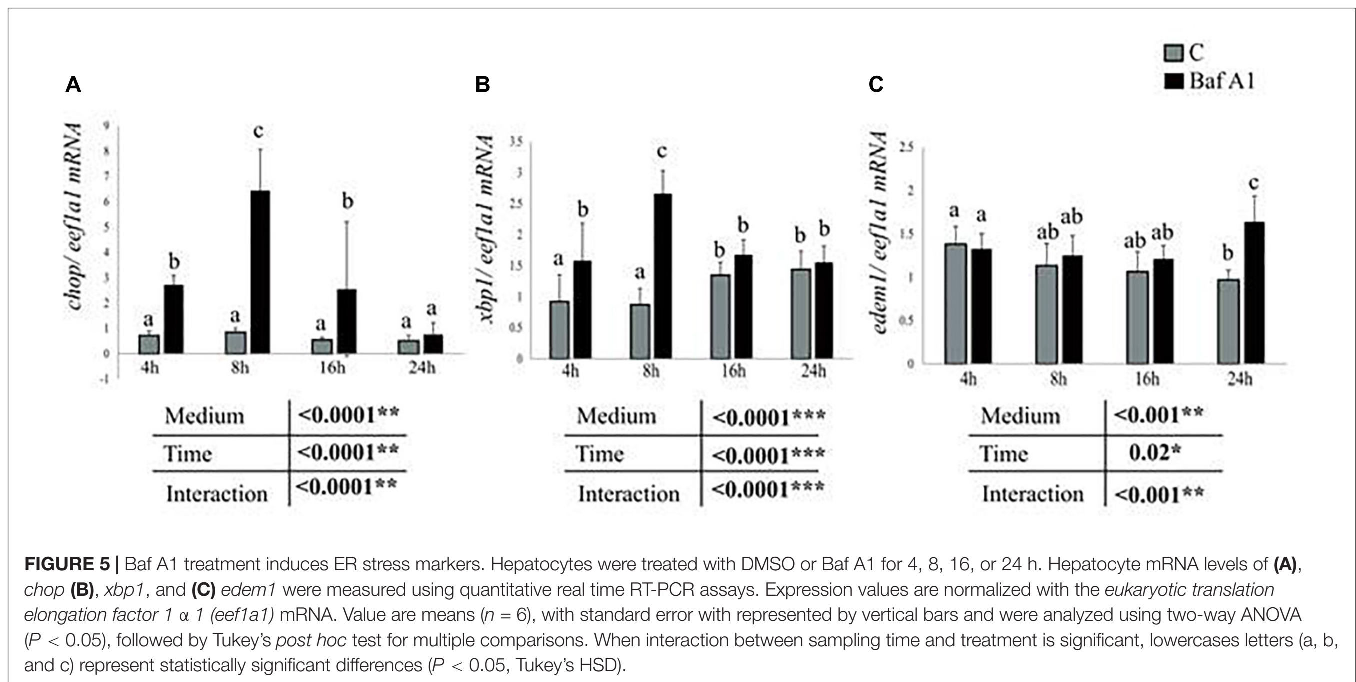

FIGURE 5 | Baf A1 treatment induces ER stress markers. Hepatocytes were treated with DMSO or Baf A1 for 4, 8, 16, or 24 h. Hepatocyte mRNA levels of **(A)**, *chop* **(B)**, *xbp1*, and **(C)** *edem1* were measured using quantitative real time RT-PCR assays. Expression values are normalized with the *eukaryotic translation elongation factor 1 α 1 (eef1a1)* mRNA. Value are means ($n = 6$), with standard error with represented by vertical bars and were analyzed using two-way ANOVA ($P < 0.05$), followed by Tukey's *post hoc* test for multiple comparisons. When interaction between sampling time and treatment is significant, lowercases letters (a, b, and c) represent statistically significant differences ($P < 0.05$, Tukey's HSD).

has thus been shown to up-regulate the mRNA levels of gluconeogenesis-related enzymes (Lansard et al., 2010, 2011). As autophagy is described as one of the main amino acid provider during fasting (Kuma et al., 2004; Ezaki et al., 2011), we therefore hypothesized that the observed effect of Baf A1 treatment on the mRNA levels of metabolic genes could be due to a default in free amino acid release in autophagy-inhibited hepatocytes. Accordingly, Baf A1 treated cells exhibited lower levels of most of the analyzed amino acids compared to control cells. Such an effect of autophagy in providing amino acids endogenously to sustain mechanistic target of rapamycin complex 1 (mTORC1) signaling when extracellular amino acids are limited has previously been reported in C2C12 murine myotubes (Yu and Long, 2014), and could therefore be also at play in the observed effect of Baf A1 on the studied genes. Interestingly, the expression of *g6pcb2* which, in contrast to the other analyzed gluconeogenesis-related genes increased in Baf A1 treated hepatocytes, was previously shown to exhibit an opposite regulation by feeding different levels of proteins and by amino acids levels in hepatocytes compared to other *g6pc* paralogs (Marandel et al., 2015; Lucie et al., 2016), tipping the scale in favor of a default of autophagy-dependent release of amino acids in the observed Baf A1 effect. However, not all studied genes are known to be under the control of amino acids *per se*. This is particularly the case for the gene *fas*, whose expression has already been shown to be not directly affected by the addition of amino acids to an amino acid-free medium in trout hepatocytes (Lansard et al., 2010, 2011). Instead, it is possible that the induction of ER stress observed in autophagy-inhibited hepatocytes plays an important role in the observed effect of Baf A1 treatment on these genes. Indeed, previous studies have shown that ER stress plays a critical role in regulation of lipid metabolism (Sriburi et al., 2004; Bobrovnikova-Marjon et al., 2008; Oyadomari et al., 2008; Rutkowski et al., 2008; Kammoun et al., 2009; Zhang et al., 2014). According to these studies, ER stress leads to activation of the evolutionarily conserved UPR signaling system in order to restore ER homeostasis (Shen et al., 2004). Accumulating evidence shows that activation of the UPR pathways can modulate lipid metabolism by controlling the transcriptional regulation of lipogenesis and triglyceride storage (Basseri and Austin, 2012; Han and Kaufman, 2016). For example, PERK and eIF2α phosphorylation are induced by antipsychotic drugs, resulting in increased lipid accumulation in hepatocytes through activation of sterol regulatory element–binding proteins SREBP-1c and SREBP-2, two transcription factors that regulate the expression of critical enzymes involved in lipogenic pathways including *fas* (Gosmain et al., 2005; Lauressergues et al., 2012). XBP1 also seems to be involved in the lipid metabolism through both direct and indirect activation of the transcription of key lipogenic genes in the liver, including *fas*, *plin2* as well as *CoA*, *desaturase 1* (*Scd1*), *Dgat2*, and *Acc2* (Lee et al., 2008, 2012). Together, these data support a possible role of ER stress in the observed effect of Baf A1 on the mRNA levels of enzymes involved in lipid metabolism. Noteworthy, UPR signaling has also been shown to affect the expression of genes involved in glucose metabolism and more particularly those of the gluconeogenesis pathway (Wagner and Moore, 2011).

Finally, recent findings in mammals reported a novel RNA degradation system called RNautophagy, during which direct import of RNA into lysosomes followed by degradation takes place (Fujiwara et al., 2013). During this process, the putative nucleic acid transporter SIDT2 predominantly localizes to lysosomes and mediates the translocation of RNA into lysosomes (Aizawa et al., 2016; Contu et al., 2017). Interestingly, the authors found that treatment of cells with lysosome inhibitors (chloroquine or Bafilomycin A1) hindered the SIDT2 overexpression-mediated increase in intracellular

RNA degradation. These data make therefore the impairment of RNautophagy a possible mechanism of the observed inducing effect of Baf A1 in the level of some mRNAs. However, it remains to be established whether or not RNautophagy or a RNautophagy-like process exists in fish.

Wrapping-Up

In the present study, we report that the treatment of fasted trout hepatocytes with Baf A1 strongly perturb the mRNA expression of several genes involved in glucose and lipid metabolisms. These results are in close agreement with those already reported with other autophagy inhibitors both in mammals and fish, and support a tight link between autophagy activity and the mRNA levels of metabolic genes. The underlying mechanisms are likely multiple and highlight the complexity of the crosstalk between ER, autophagy and metabolism.

Interestingly, the observed decrease in mRNA levels of gluconeogenic genes in cells treated with Baf A1 is also consistent with the reported role of autophagy in the maintenance of blood glucose during fasting by releasing amino acids for glucose production via gluconeogenesis (Ezaki et al., 2011). Similarly, we observed an increase in mRNA levels of FAS and the two LD-associated proteins PLIN2 and PLIN3 in Baf A1 treated cells in agreement with the well-established role of autophagy in the control of lipid stores during fasting (Singh et al., 2009; Wang et al., 2018; Wei et al., 2018). Autophagy could thus combine its role as a supplier of substrates for the production of glucose or energy furniture with the molecular regulation of several related metabolic enzymes.

In the future, important issues will be to confirm these observations by establishing fish cell lines whose autophagy is genetically invalidated, which is now possible with the CRISPR-Cas9 technology. Gaining knowledge in the relationships between ER, autophagy and metabolism is of paramount for a better understanding of the mechanisms involved in metabolic adaptation of fish to environmental stresses.

AUTHOR CONTRIBUTIONS

SS, SP, and IS designed the research. TP, NO, and EP-J conducted the analyses. All the data were obtained and analyzed by TP, NO, and SS under the supervision of SP and IS. The manuscript was written by SS and critically revised by IS and SP. All authors have read and approved the final manuscript. IS had primary responsibility for final content.

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Intestinal Function of the Stomachless Fish, Ballan Wrasse (*Labrus bergylta*)

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Little is known about the digestion of nutrients in stomachless (agastric) fishes with short intestines, such as wrasse. This study describes the digestion, absorption and evacuation rates in ballan wrasse (*Labrus bergylta*) fed either dry or pre-soaked diets. Ballan wrasse juveniles received either dry or pre-soaked diets containing inert markers to determine intestinal evacuation rates over a 4–14 h period after ingestion. The digestive tract evacuation was completed in 12–14 h, and was not affected by dietary moisture level. Within the different segments of the intestine, 90% of the digesta moved from the foregut to the midgut after 4–8 h, and over 7% of the digesta had arrived in the hindgut 4 h after feeding. The foregut was a major site of digestion, where 86% of carbohydrates, 74% of proteins, and 50% of lipids, were absorbed from the diet. The absorption of carbohydrates and lipids increased until the hindgut (98 and 80%, respectively). Protein absorption continued along the length of the intestine reaching 90% absorption at the hindgut. Protein and carbohydrate absorption was slightly higher in the foregut for fish fed dry versus moist diets, but this difference disappeared in the midgut. The moisture levels of diet had no effect on the digesta moisture levels, expression of metabolic genes, the levels of nutrients in plasma, or intestinal health. Nine aquaporins were found to be expressed in the wrasse intestinal tissue. Vertebrate aquaporins are involved in water transport from the intestinal lumen into the body and may play a role in maintaining stable moisture level of the digesta in the intestine of ballan wrasse regardless of the initial dietary moisture levels.

Keywords: intestine, digestibility, evacuation rate, stomachless fish, aquaporin, wrasse, digestive physiology, transcriptome

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INTRODUCTION

The stomach is a specialized organ located in the anterior gut in many vertebrates, characterized by the occurrence of acid (HCl) and pepsin-producing gastric glands (Koelz, 1992). The stomach has multiple functions which include storage of food, initiation of protein digestion, inactivation of pathogens and controlling chyme release to the intestine (Wilson and Castro, 2010). The storage function of the stomach allows an animal to ingest larger meals, which is particularly important for

animals that swallow whole large prey, as it occurs in many fish species. However, the stomach has been lost at least 15 times in the evolution of fishes (Castro et al., 2014), including in the ancestor of extant wrasses.

During digestion, the ingested feed is degraded by a combination of mechanical and enzymatic processes. Retention and transferring of digesta to different compartments within the gastrointestinal tract are tightly controlled processes that have evolved to optimize the digestion and absorption of nutrients from food. In gastric fishes, food is temporarily stored in the stomach, allowing time for lowering pH as well as secretion and activation of pepsinogen. The active pepsin hydrolyses specific peptide bonds and initiate protein hydrolysis. For instance, in juvenile Korean rockfish (*Sebastes schlegeli*), the stomach has been shown to remain full at 4 h post feeding and was gradually evacuated until empty by 20 h post feeding (Lee et al., 2000). Many gastric fish also have pyloric caeca, i.e., finger-like blind end tubes projecting off the anterior intestine. The pyloric caeca increase the overall intestinal absorptive surface area, and thus the absorption efficiency of nutrients of the gastrointestinal tract. While lacking a stomach, agastric fish may possess an intestinal bulb or swelling in the anterior intestine instead, which has been reported to function as a food storage area (Barrington, 1942; Kapoor et al., 1976; Guillaume and Choubert, 2001). Furthermore while also lacking pyloric caeca, agastric fish may have a relatively long intestine measuring several times the animal body length (e.g., bighead carp *Aristichthys nobilis*) (Opuszynski and Shireman, 1991), which increases the overall surface area of the intestine. However, it remains unknown how efficiently nutrients are digested and absorbed in agastric fish with short digestive tracts such as the ballan wrasse (**Figure 1**) (Deady and Fives, 1995; Figueiredo et al., 2005; Skiftesvik et al., 2014; Lie et al., 2018; Artüz, 2019). It is also unknown if the wider anterior segment of ballan wrasse functions as a food storage area and thus if the intestinal bulb should be considered a pseudogaster.

Physical characteristics and chemical composition of the diet fed to an animal affect gastrointestinal evacuation rates, which in turn is an important factor in determining the utilization of ingested dietary nutrients. Prolonging the passage time of feed has the potential to increase enzymatic digestion and mucosal absorption of nutrients and may also allow beneficial microorganisms to assist in digestion (Olsen and Ringø, 1997; Venero et al., 2015). As such, a reduced gut transit time has been found to diminish the efficiency of digestion and absorption of ingested feed in several fish species (Grove et al., 1978; Flowerdew and Grove, 1979; Jobling, 1981, 1987; Garber, 1983; Lee et al., 2000). In gastric species moist diets can have shorter passage rates than dry diets, as found for turbot *Scophtalamus maximus* (Grove et al., 2001). In several studies, faster gastrointestinal transit has been shown to enhance feed intake rates, e.g., in greater amberjack *Seriola dumerili* (Papadakis et al., 2008) and Atlantic salmon *Salmo salar* (Aas et al., 2013), but reduce nutrient digestibility, e.g., in European seabass *Dicentrarchus labrax* (Adamidou et al., 2009) and gilthead sea bream *Sparus aurata* (Venou et al., 2009). However, other studies found that the dietary moisture level did not affect the rate of gastrointestinal evacuation in Korean rockfish (Lee et al., 2000) and olive flounder *Paralichthys olivaceus* (Kim et al., 2011); or the digestibility of nutrients in rainbow trout *Oncorhynchus mykiss* (Aas et al., 2011) and Atlantic salmon (Oehme et al., 2014).

In this study, we investigated the effect of dietary moisture level on gut evacuation rates in ballan wrasse. Ballan wrasse were fed either dry or pre-soaked (moist) diets, and then the rate of evacuation and absorption of macronutrients from the diets in individual segments, as well as overall, were measured. Furthermore, gene expression and histology of the intestine was evaluated to identify any transcriptional or histomorphological changes due to dietary moisture level. We found that the food is evacuated from the ballan wrasse gastrointestinal tract within 12–14 h, and that the first segment of the intestine plays a major role in nutrient digestion and absorption. Dietary moisture level had no effect on the overall gastrointestinal evacuation rate or the moisture level of digesta, histomorphology and had a limited effect on gene expression. The results demonstrate that in spite of possessing a rather basic gastrointestinal tract, ballan wrasse can efficiently digest and absorb nutrients in a similar manner to species with stomach, pyloric caeca and/or long intestinal tract.

MATERIALS AND METHODS

Diets

The experimental diets were produced at Nofima's Feed Technology Centre (Bergen, Norway) in a co-rotating twin screw extruder (TX52, Wenger Manufacturing Inc., Sabetha, KS, United States) and dried in a dual layer carousel dryer (Model 200.2; Paul Klockner GmbH, Nistertal, Germany). The pellets were 2 mm in diameter and sank slowly in seawater (density >1.024 g/cm^3). The diets were formulated based on information developed through a series of feeding studies (e.g., Kousoulaki et al., 2014, 2015; Krogdahl et al., 2014; Bogevik et al., 2016), and were based on high quality marine raw materials (see **Table 1**). Two batches were made, one with yttrium the other with ytterbium as inert markers. Moist diets were prepared by soaking dry pellets in seawater for 4–5 min prior to feeding and the proximate composition of nutrients in the dry and pre-soaked (moist) diets are showed in **Table 2**.

Experimental Design and Sampling

The fish used for these experiments had been reared for 1 year in a commercial farm (Marine Harvest, Bergen, Norway) before transfer to the Nofima AS land based research facility in Sunndalsøra, Norway. Before arrival, the fish had been fed the yttrium labeled dry diet for 6 weeks. The fish were transported on September the 2nd, 2015, by truck for 4 h and distributed to 12 cylindrical flat-bottomed black tanks with a volume of 350 l, equipped with separate light sources. The tank water temperature during the experiments was 14.5–17°C and the photoperiod was of 24-h daylight. Four days after arrival all fish were individually tagged [passive integrated transponder (PIT), weighed (average 89 ± 2 g, mean ± SEM) and length measured (average 17.1 ± 1.0 cm, mean ± SEM)]. Fish densities were adjusted to 20 kg/m^3. Feed was distributed automatically every 10th min. using automatic belt feeders for the duration of the

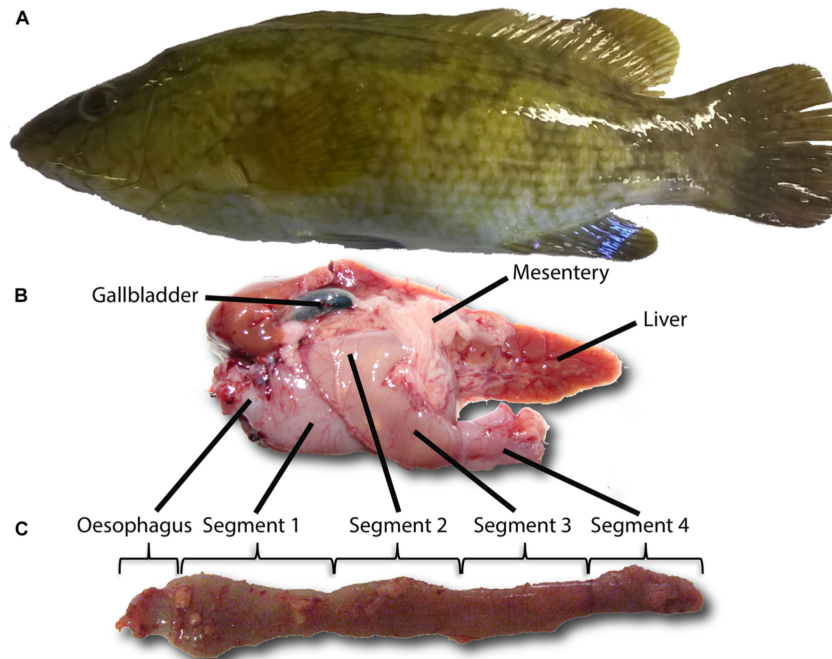

FIGURE 1 | Digestive system of ballan wrasse. Ballan wrasse (*Labrus bergylta*) **(A)**; digestive system as found *in vivo* **(B)**; anatomy of the intestine and the division of the intestinal tract into 4 segments **(C)**, based on Lie et al. (2018).

experiment. Each belt feeder (one per tank) was loaded with feed equivalent to 12 g feed/kg fish per day. The moist diet was prepared by weighing the daily feed amount for each tank before adding enough water to just cover the dry diet, all of the water was absorbed by the pellets. Four minutes later the moist diet was distributed to each automatic belt feeder and leveled out using a rubber spatula. Dry diet was also added and levelled out on automatic belt feeders daily.

The two feeding experiments followed the Norwegian animal welfare act guidelines, in accordance with the Animal Welfare Act of 20th December 1974, amended 19th of June, 2009. The facility at Sunndalsøra, Nofima (division 60) got a permission granted by the Norwegian Food Safety Authority, (FOTS ID 8060) to run these experiments. The decision was made on the basis of Regulations 18. June 2015 on the use of animals in experiments, §§6, 7, 9, 10, and 11.

Experiment 1 (Passage Rate)

To evaluate the passage rate of feed, the replacement of yttrium labeled feed by ytterbium labeled feed in the intestinal lumen was analyzed. At the start of the experiment, diets containing yttrium (Y-dry and Y-moist) were replaced by diets with ytterbium (Yb-dry and Yb-moist). Gut contents were collected at 4, 6, 8, 10, 12, and 14 h after changing to diets containing ytterbium. At each time point, 6 fish from 2 tanks per treatment were euthanized with an overdose of MS-222 before dissection. The whole intestine was dissected out and divided into 4 segments as shown in **Figure 1C**. Gut contents were collected from each intestinal segment and used for yttrium and ytterbium analysis.

Experiment 2 (Digestibility)

To evaluate macronutrient digestibility, gut transcriptomes and plasma indicators of metabolism, fish in two tanks were fed Y-labeled dry diet and two tanks fed Y-labeled moist diet for 5 weeks. At the termination of the feeding trial, fish were randomly taken from the tanks and euthanized with an overdose of MS-222 before tissue sampling. Firstly, blood samples were collected to analyze circulating levels of nutrients. Then the intestine was removed from the abdominal cavity, cleaned of mesenteric fat and divided into four segments as shown in **Figure 1C**. Each section was opened longitudinally and the gut content collected for analysis of dry matter, crude protein, fatty acid composition, and carbohydrates for calculating nutrient apparent digestibility coefficients (ADCs). For digestibility evaluation, three pooled samples of gut content from 20 randomly selected fish were collected from each of the four tanks. Intestine and liver tissues from six fish per each tank were sampled for RNA extraction (submerged in RNAlater solution, incubated at 4°C for 24 h and stored at −20°C) and histomorphological evaluation (fixed in 4% phosphate-buffered formaldehyde solution for 24 h and transferred to 70% ethanol for storage).

Chemical Analyzes

Concentrations of markers (Experiments 1 and 2), crude protein, fatty acids, and carbohydrate were measured in both feed and gut content samples (Experiment 2). Yttrium (Y) and ytterbium (Yb) were analyzed using ICP-MS with Chem Station software (Agilent 7500, Agilent Technologies Inc.,

Santa Clara, CA, United States). Crude protein levels were analyzed with an Elemental®Vario Macro Cube instrument (Elementar Analysensysteme GmbH, Hanau, Germany) using the standard conversion factor (× 6.25). The compositions of lipid (total fatty acids - TFA), monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), and saturated fatty acids (SFA) were determined using chromatography with the 19:0 methyl ester as an internal standard according to Lie and Lambertsen (1991). The carbohydrate contents were measured *via* glucose produced from hydrolysis of carbohydrate compounds, based on the Trinder reaction described in Hemre et al. (1989).

TABLE 1 | Experimental fish diet formulations, g kg^{-1} dry matter.

Ingredient	Yttrium	Ytterbium
[1]Shrimp meal	285.0	285.0
[1]Cod meal	248.3	248.3
[2]Krill hydrolysate	64.4	64.4
[3]Reference fishmeal stick water	120.0	120.0
[1]Squid meal	110.0	110.0
[4]Fish oil	35.0	35.0
[5]Krill oil	25.0	25.0
[6]Wheat	70.0	70.0
[7]Vitamin mix	26.5	26.5
[7]Mineral mix	50.0	50.0
[8]Soya lecithin	20.0	20.0
[9]Additives	26.8	26.8
[10]Aquate (prebiotics)	4.0	4.0
[11]Ytterbium		0.5
[11]Yttrium oxide	0.5	
Sum	1000	1000

[1]Provided by Seagarden AS, Norway.
[2]Antarctic krill (Euphausia superba), provided by RIMFROST AS, Norway.
[3]Provided by Pelagia AS, Norway.
[4]Provided by Norsildmel AS, Norway.
[5]Provided by Aker Biomarine Antarctic AS, Norway.
[6]Provided by Norgesmøllene, Norway.
[7]Supplied by Vilomix, Norway.
[8]Provided by Denofa, Norway.
[9]Cholesterol from Grudlita, Lithuania; Choline chloride, Carophyll Pink (10%), Stay-C and Methionine from Vilomix, Norway; Lysine from Agrocorn, Denmark; and Taurine from VWR, Norway.
[10]Provided by Alltech, Norway.
[11] Provided by Metal Rare Earth Limited, China.

TABLE 2 | Proximate composition (%) of nutrients in experimental diets.

	Yttrium		Ytterbium	
	Dry	Moist	Dry	Moist
Protein	61	52	59	50
Lipid	16	14	16	13
Carbohydrate	7	6	7	6
Ash	16	13	18	16
Moisture	0	15	0	15
Sum	100	100	100	100

The yttrium labeled diets contained 0.05% of yttrium oxide and the ytterbium labeled diet contained 0.05% ytterbium.

Plasma

Plasma was analyzed for cholesterol, non-esterified fatty acids (NEFA), total triacylglycerides (TAG), total protein and glucose at the Central Laboratory at the Faculty of Veterinary Medicine, Oslo according to standard, medical procedures.

Histology

Six fish from each tank in Experiment 2 were sampled and analyzed histologically for inflammation. **Table 3** below shows the number of histology sections for each intestinal region per diet group that were suitable for the semi-quantitative histology evaluation.

Histological sections were prepared using paraffin technique and H&E (Haematoxylin and Eosin) staining system according to Baeverfjord and Krogdahl (1996). The histological sections were evaluated for morphological changes associated with inflammatory reaction in the intestinal mucosa. Other morphological features unique to the ballan wrasse were also noted and graded. The degree of change of the various morphological features was graded using a scoring system with a scale of 1–10 where 1 to <3 represented normal; 3 to <5, mild changes; 5 to <7, moderate changes; >7 to <9, marked changes, and 9–10, severe changes.

Evaluation of the liver sections focused on changes to hepatocyte morphology, and presence of specific pathological changes such as degeneration, hemorrhage, or inflammation. The degree of liver changes was graded using a scoring system with a scale of 1–5 where 1 represented normal and 2–5 represented mild, moderate, marked, and severe changes, respectively.

RNA Extraction

Total RNA was extracted and DNA removed from anterior intestinal samples (i.e., Segment 1 in **Figure 1C**) using a BioRobot®EZ1 and RNA Tissue Mini Kit (Qiagen, Hilden, Germany). RNA quantity and purity were assessed using a NanoDrop ND-1000 UV–vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE, United States). All samples had 260/230 and 260/280 ratios above 2.0 and 2.2, respectively, indicating high RNA purity. RNA integrity was determined using the Agilent 2100 Bioanalyzer and RNA 6000 Nano LabChip kit (Agilent Technologies, Palo Alto, CA, United States). The average RNA integrity number (RIN) of all samples was 7.8 ± 0.5.

TABLE 3 | Overview of the number of intestine and liver tissue sections for each diet group that were of suitable quality for semi-quantitative histomorphological evaluation.

Tissue	Dry	Moist
Intestinal segment 1	15	12
Intestinal segment 2	15	12
Intestinal segment 3	15	12
Intestinal segment 4	15	9
Liver	15	12

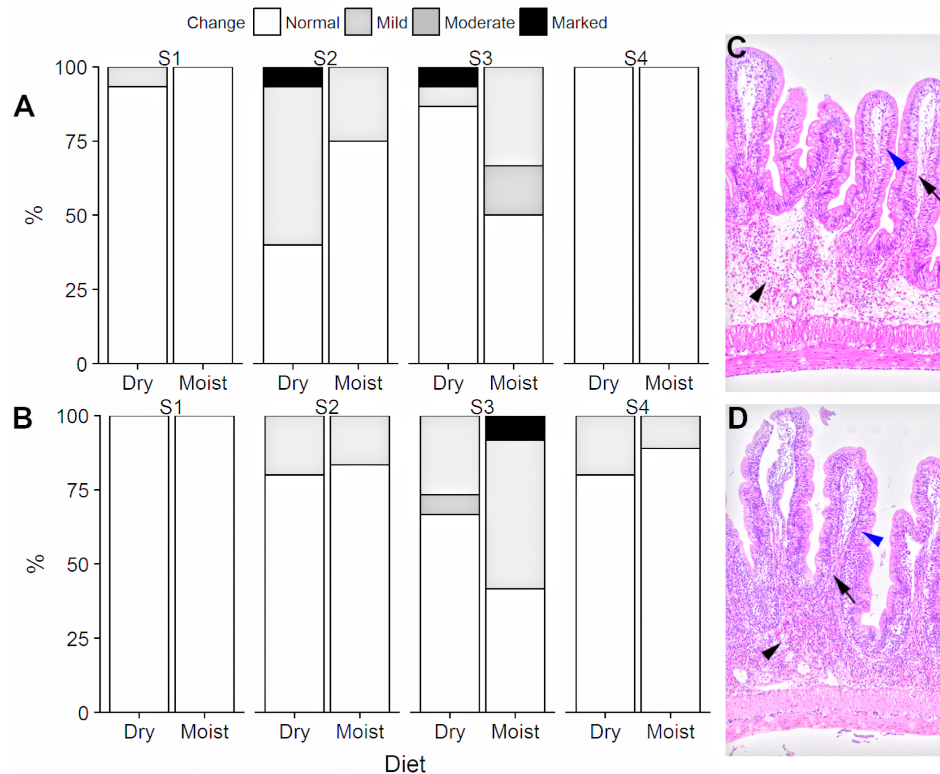

FIGURE 2 | Histological evaluation of intestinal segments in ballan wrasse fed either dry or moist diets. The percentage of each intestinal segment that had normal, mild, moderate, or marked infiltration of mixed populations of inflammatory cells in the intestinal lamina propria **(A)** and intraepithelial lymphocyte infiltration **(B)**. S1, S2, S3, S4 stand for Segments 1, 2, 3, 4. Data were obtained from 15 fish each for all segments for the dry diet, 12 each (S1–S3) and 9 each (S4) fish for the moist diet. **(C)** Normal. **(D)** Moderate submucosa (black arrowhead) and lamina propria (black arrow) increase in cellularity due to infiltration by a mixed population of inflammatory cells and infiltration of the intraepithelial space (blue arrowheads). There was no statistical difference between moist and dry diets (Fisher's Exact test, $p > 0.05$).

Transcriptome Sequencing

Sequencing was performed by the Norwegian Sequencing Centre[1]. DNA sequencing libraries were prepared with an automated NeoPrep platform (Illumina) using 90 ng total RNA input to the TruSeq Stranded mRNA Library Prep Kit (Illumina) and standard Illumina adaptors included in the kit. The libraries were sequenced using HiSeq4000 according to manufacturer instructions, generating pair end libraries with an average library size of 14.2 ± 1.4 million reads.

Calculations

Estimates of Feed Intake

The amount of feed ingested for each treatment was calculated from the sum of markers (Yb and Y_2O_3) in gut content from 4 intestinal segments as such: Feed intake (mg g^{-1}) = [(Yb + Y_2O_3) in 4 segments (mg)]*100*[0.05*BW (g)]$^{-1}$, with 0.05 equalling the % of marker in feed (see **Table 1**).

Gut Passage Rate

Fecal concentrations for each marker were first normalized by dividing them with the dietary marker concentration. The passage rate in each segment was calculated as the percentage between Yb and the total amount of two markers in the gut content, as such: Yb (%) = 100*Yb*(Y_2O_3 + Yb)$^{-1}$.

Moisture of Intestinal Content

The water content (%) of gut content was obtained as the ratio between water weight in each wet sample of gut content collected in digestibility experiment and its wet weight: water content (%) = 100 * (wet weight of gut content − dry weight of gut content) * (wet weight of gut content)$^{-1}$.

Apparent Digestibility Coefficient of Macronutrients

The ADC of macronutrients was calculated as following: ADC (%) = [1 − (nutrient content in gut content * feed marker content) * (marker content in gut content * feed nutrient content)$^{-1}$] * 100.

Data Analysis

The Y_2O_3:Yb ratio within intestinal segments were used to calculate the intestinal evacuation rate between intestinal segments at given time points. Yb % data, as the response variable, were treated as continuous proportions ranging from 0 to 1 as quasibinomial distribution and compared with regards to both

[1] www.sequencing.uio.no

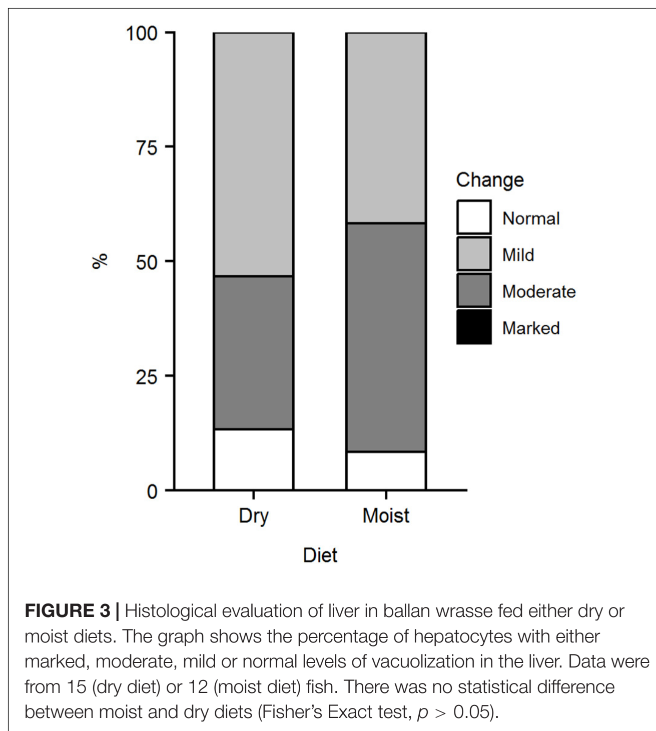

FIGURE 3 | Histological evaluation of liver in ballan wrasse fed either dry or moist diets. The graph shows the percentage of hepatocytes with either marked, moderate, mild or normal levels of vacuolization in the liver. Data were from 15 (dry diet) or 12 (moist diet) fish. There was no statistical difference between moist and dry diets (Fisher's Exact test, $p > 0.05$).

diet and sampling time as predictor variables for each intestinal segment using a generalized linear mixed models (GLMM) (R, version 3.4.2 released 2017-09-28, within RStudio interface (version 1.1.383) for Windows. At some time-points there were large variation differences in the Yb data between the two groups (dry and moist), so the Wilcoxon Rank Sum and Signed Rank Tests (wilcox.test) was applied to test the difference.

Apparent digestibility coefficients of the nutrients as the response variable were also treated as continuous proportions ranging from 0 to 1. The nutrient ADCs for each of the four intestinal segments were clustered in sub-groups of fish from each diet group. The generalized linear mixed models (glmmPQL) test was used to analyze the correlations between diet and intestinal segment as the fixed effect factors, with individual fish as the random effect factor, and the ADC as a response variable with a quasi-binominal distribution. Changing the contrast where each gut segment (S1–S4) became the intercept of the model was applied to determine the variation in ADC between the two diets in each segment. If the full model of fixed effect factors (diet and gut section) showed the diet had no effect on the ADC, the diet factor was then removed from the model. This model was used to compare the ADC among the 4 intestinal segments by changing the contrast. The Wilcoxon Rank Sum and Signed Rank Tests (wilcox.test, paired = TRUE) was applied to test the difference in the digestibility of three lipid classes. The glmmPQL was also applied to the data of water content in gut content with the ratio of water as a response variable and the diet and intestinal section as the predictors. Data of plasma components were analyzed using one-way ANOVA. Differences were considered significant at $p < 0.05$ for all tests in this study. All data are presented as mean ± SEM.

Sequence Analysis

Remaining adaptors were removed using Cutadapt (Martin, 2011) and default parameters. Reads were further trimmed and filtered using Sickle[2] with a 40 bp minimum remaining sequence length, Sanger quality of 20, and no 5′ end trimming. Library quality was investigated using fastQC version 0.9.2 (The FastQC project[3]). Each intestinal RNAseq library was mapped individually to the labrus genome assembly (European Nucleotide Archive accession number: PRJEB13687[4]) using the Tophat2 short read aligner (Langmead et al., 2009). Transcript abundances were estimated using the FeatureCounts software of the Subread package[5]. The Bioconductor - DESeq2 analysis package (Love et al., 2014) was used for differential expression analysis. Genes of which read counts < 10 in all samples were excluded from further analysis prior to normalization and differential expression analysis. An adjusted p-value (p-adjust) of <0.1 was applied for further downstream analysis using the DAVID Bioinformatics Resources 6.8 (Beta) with default settings (GO and KEGG pathway analysis).

Histological Data

Differences in histological scores for the various evaluated morphological characteristics of the gut and liver tissue were analyzed for statistical significance using the Fisher's Exact test. *Post hoc* analysis for significant Fisher's Exact test results was conducted using the chi-squared *post hoc* test. Both statistical tests were run in the R statistical package (version 3.2.4; 2016) within the RStudio interface (version 0.99.892; 2016). Differences were considered significant at $p < 0.05$.

RESULTS

Histology

Fish from Experiment 2 were evaluated for morphological changes associated with inflammatory reactions in the intestinal mucosa. The results of the histological evaluation of the four intestinal segments and the liver are shown in **Figures 2**, **3**. A number of fish displayed mild to moderate changes in lamina propria cellularity and intraepithelial lymphocyte infiltration, predominantly in intestinal segments S2 and S3. None of the chosen indicators of gut health status were significantly different between fish fed dry or moist diets (Fisher's Exact test, $p > 0.05$).

Evaluation of the liver sections focusing on the degree of vacuolization of the hepatocytes showed that most fish had mild to moderate vacuolization of the hepatocytes (**Figure 3**). There was no significant difference in hepatocyte vacuolization between the two diet groups (Fisher's Exact test, $p > 0.05$).

Feed Intake and Gut Evacuation Rate

The fish weighed 100.0 ± 1.8 g at the time of sampling in Experiment 1. The estimated feed intake (feed dry weight/body

[2]https://github.com/najoshi/sickle

[3]http://www.bioinformatics.babraham.ac.uk/projects/fastqc/

[4]http://www.ebi.ac.uk/ena/data/view/PRJEB13687

[5]http://subread.sourceforge.net/

FIGURE 4 | Passage rate (% evacuation) of dry and moist diets through ballan wrasse intestine *in vivo*. The figures show the ratio (mean ± SEM) between Yb marker and the sum of markers [Yb × (Y_2O_3 + Yb)$^{-1}$ × 100] represented in the gut content collected from the 4 intestinal segments (*n* = 12 for each segment) at 4, 6, 8, 10, 12, and 14 h after fish fed Y-labeled feed were switched to those containing Yb. Some error bars are not visible as they are shorter than symbol heights. For each intestinal segment, no significant difference in the percentages of Yb between moist and dry diet treatments (GLMM, $p > 0.05$). The curves were predicted by general linear mixed models in R.

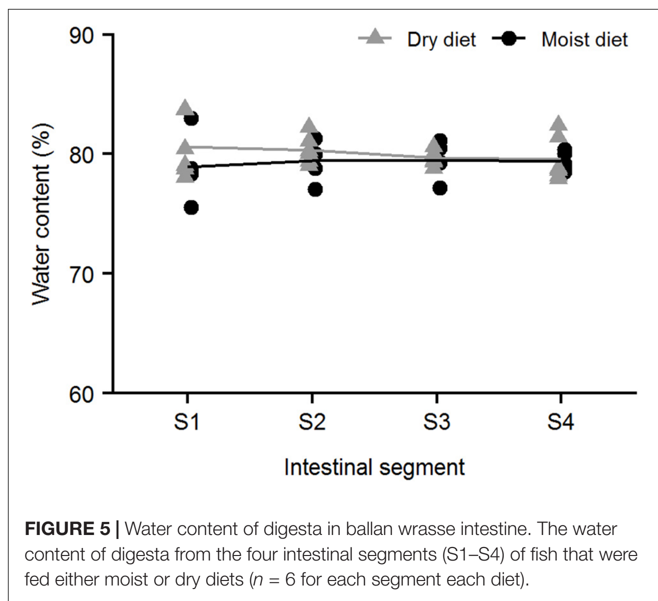

FIGURE 5 | Water content of digesta in ballan wrasse intestine. The water content of digesta from the four intestinal segments (S1–S4) of fish that were fed either moist or dry diets (*n* = 6 for each segment each diet).

wet weight) based on the yttrium marker was 1.3 ± 0.1 mg/g in fish fed the dry diet and 1.1 ± 0.1 mg/g in fish fed the moist diet. There was no significant difference in estimated feed intake between the two groups. Since the fish were fed continuously and feed could be found in most segments in most of the fish analyzed, no evidence for meal-based feeding behavior could be identified.

Segment 1

Four hours post dietary label change (hpc), 90.0 ± 3.0% (*n* = 4) and 97.9 ± 0.3% (*n* = 3) of the initial Y-labeled feed had been replaced by the Yb-labeled feed in the fish fed dry diet or moist diet, respectively. The difference between the two treatments was not significant (GLMM, $p > 0.05$). The amount of Yb in Segment 1 increased to the maximum at 8 hpc and it then plateaued within six following hours for both diet treatments (from 97.0 ± 0.6 to 98.5 ± 0.1% for dry, from 96.2 ± 1.4 to 98.3 ± 0.1% for moist) (Segment 1, **Figure 4**).

Segment 2

Four hpc 86.2 ± 4.9% for dry diet and 86.7 ± 4.1% for moist diet of the Y-labeled diet had been replaced by the Yb-labeled diet. Ten hpc 97.9 ± 0.5% of Y-dry diet and 98.1 ± 0.2% of Y-moist diet were evacuated out of the Segment 2. In both treatment groups, a tiny portion of Y marker still remained (~2% of the total sum of the markers) at 14 hpc (Segment 2, **Figure 4**). The diet type had no effect on the evacuation rate in the Segment 2.

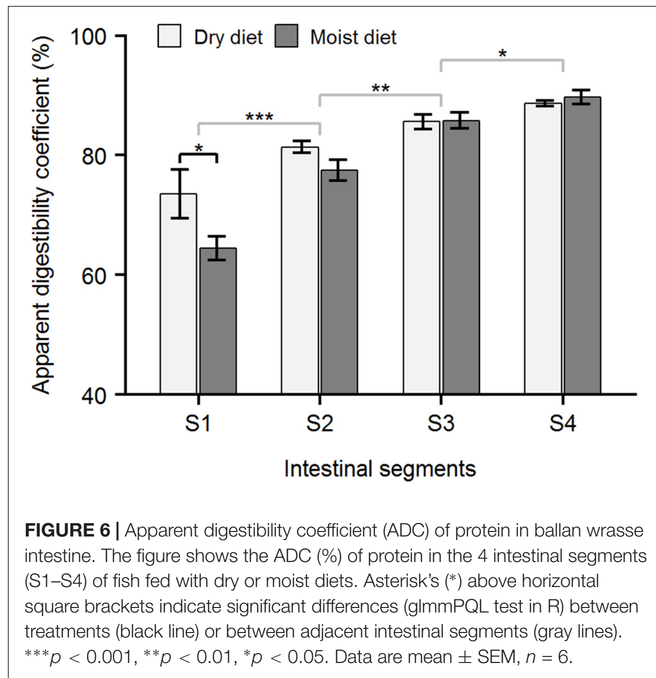

FIGURE 6 | Apparent digestibility coefficient (ADC) of protein in ballan wrasse intestine. The figure shows the ADC (%) of protein in the 4 intestinal segments (S1–S4) of fish fed with dry or moist diets. Asterisk's (*) above horizontal square brackets indicate significant differences (glmmPQL test in R) between treatments (black line) or between adjacent intestinal segments (gray lines). ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$. Data are mean ± SEM, $n = 6$.

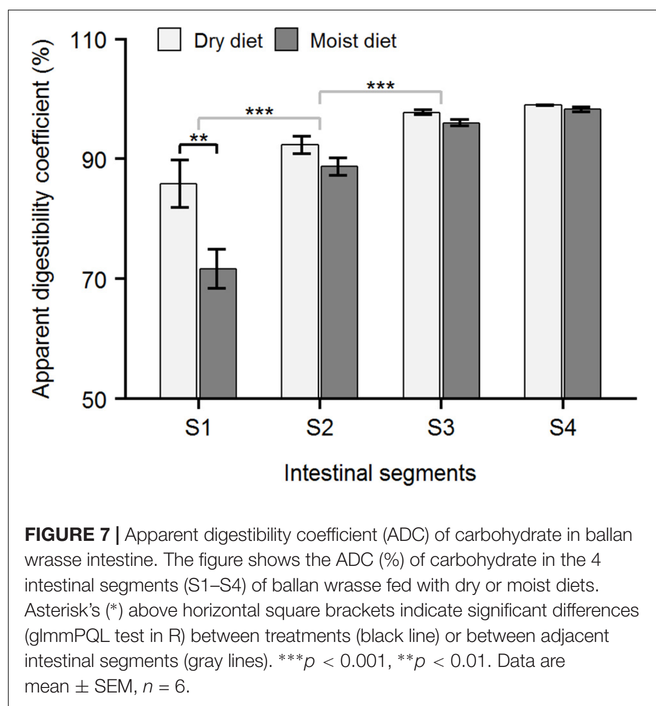

FIGURE 7 | Apparent digestibility coefficient (ADC) of carbohydrate in ballan wrasse intestine. The figure shows the ADC (%) of carbohydrate in the 4 intestinal segments (S1–S4) of ballan wrasse fed with dry or moist diets. Asterisk's (*) above horizontal square brackets indicate significant differences (glmmPQL test in R) between treatments (black line) or between adjacent intestinal segments (gray lines). ***$p < 0.001$, **$p < 0.01$. Data are mean ± SEM, $n = 6$.

Segment 3

In Segment 3, 70.4 ± 10.3% for dry diet and 44.6 ± 22.4% for moist diet of Yb-labeled diet had been taken over the Y-labeled diet at 4 hpc. The proportion of Yb marker then rose significantly and reached to the peak at 98.3 ± 0.1% (in dry group) and 98.1 ± 0.2% (in moist group) at 14 hpc (Segment 3, **Figure 4**). No effect of the moist levels of feed on the passage rate was found in the Segment 3.

Segment 4

14.8 ± 7.8% Y marker from dry diet and 7.5 ± 4.5% Y marker from moist diet were replaced by Yb marker in the last segment at 4 hpc. Over the following 10 h, the amount of Y marker replaced by Yb marker increase to a maximum of 98.3 ± 0.0% for the dry diet group and 98.3 ± 0.1% for the moist diet group at 14 hpc. Only tiny amounts of Y marker still remained in the fourth segment at 14 hpc (Segment 4, **Figure 4**). The proportions of Yb marker were not statistically different between the dry and moist group.

Overall, the feed resided 4–8 h in the foregut and the total passage rate was ∼12–14 h. GLMM for fixed effects (diet and sampling time point) showed that the proportion of Yb in the digesta in each segment increased with time, but diet type had no effect (**Figure 4**).

Moisture of Gut Content

The water content of the digesta was ∼80% by weight and was not statistically different between the four intestinal segments or between fish fed either dry or moist diets (**Figure 5**) (glmmPQL, $p > 0.05$).

Macronutrient Digestibility

Apparent digestibility coefficients for protein and carbohydrates in the foregut (Segment 1, **Figures 6**, **7**) were higher ($p < 0.05$, glmPQL) in the fish fed the dry compared to moist diets. However, in the midgut (Segments 2 and 3) and hindgut (Segment 4), the ADC for proteins and carbohydrates were not significantly different between fish fed dry or moist diets. The digestibility of lipid was not affected by dietary moisture level (glmPQL, $p > 0.05$, **Figure 8**). However, the ADC for fatty acids were different between three fatty acid classes. Polyunsaturated fatty acids (PUFA) had higher ADCs in all the four segments than monounsaturated fatty acids (MUFA) and saturated fatty acids (SFA) (wilcox.test, $p < 0.01$). The ADC for SFA was lower than that for MUFA in Segments 1 and 3 (wilcox.test, $p < 0.05$); but they were similar in Segments 2 and 4. ADCs showed that digestion and absorption of protein, carbohydrate and lipid had similar patterns in the intestine of ballan wrasse. Up to 85.87, 73.57, and 48.97% of carbohydrates, proteins, and lipid, respectively, were digested and absorbed in Segment 1. In addition, the ADC of all macronutrients increased significantly along the intestine, with the ADCs increasing to a maximum for carbohydrate and lipid in Segment 3, and for protein in Segment 4 (glmPQL, $p < 0.05$, **Figures 6–8**).

Nutrient Markers in Plasma

No statistically significant differences were observed for plasma concentrations of cholesterol, free fatty acid, glucose, triacylglycerides or protein between fish fed dry or moist diets (**Figure 9**).

Transcriptomic Effects of Dry and Moist Diets

The transcriptomic expressions of Segments 1 and 4 were analyzed in the two feeding groups. According to the DESeq2

FIGURE 8 | Apparent digestibility coefficient (ADC) of lipid in ballan wrasse intestine. The figures show ADC (%) of lipid (total fatty acids), TFA; monounsaturated fatty acids, MUFA; polyunsaturated fatty acids, PUFA; and saturated fatty acids, SFA in the 4 intestinal segments (S1–S4) of ballan wrasse fed dry or moist diets. Asterisk's (*) above gray horizontal square bracket indicate significant differences between adjacent segments (glmmPQL test in R). ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$. No significant differences occurred in fatty acid ADCs within intestinal segments between fish fed dry and moist diets. Data are mean ± SEM, $n = 6$.

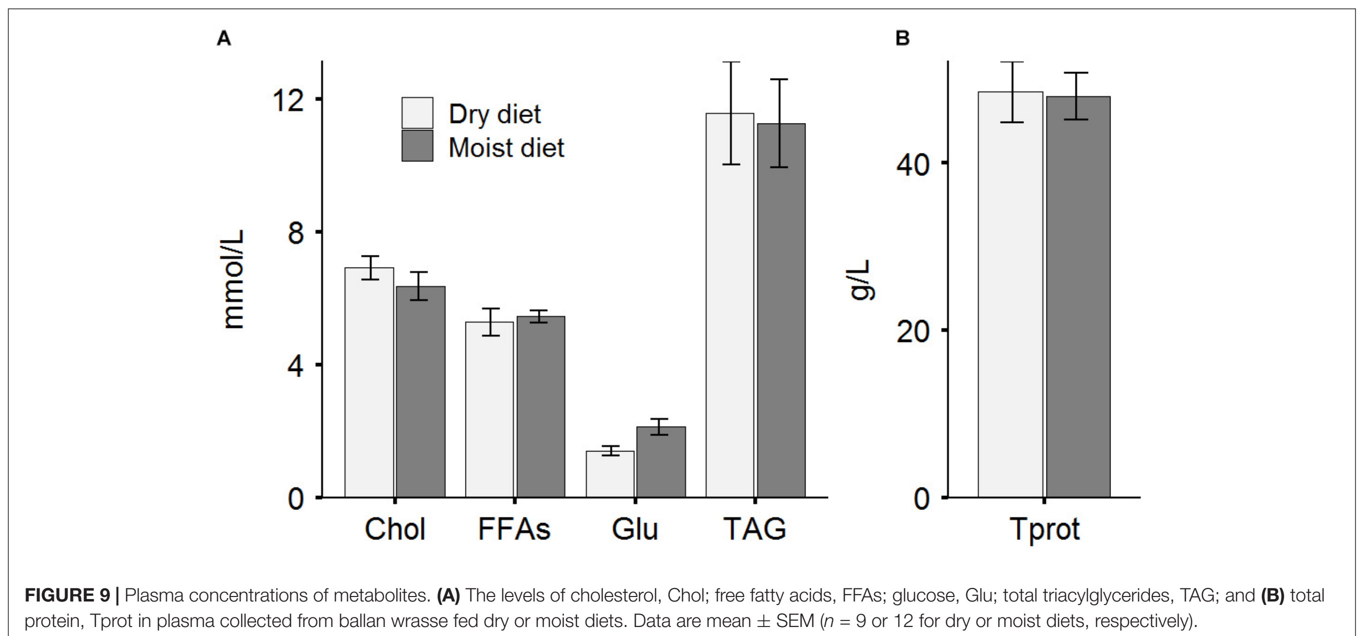

FIGURE 9 | Plasma concentrations of metabolites. **(A)** The levels of cholesterol, Chol; free fatty acids, FFAs; glucose, Glu; total triacylglycerides, TAG; and **(B)** total protein, Tprot in plasma collected from ballan wrasse fed dry or moist diets. Data are mean ± SEM ($n = 9$ or 12 for dry or moist diets, respectively).

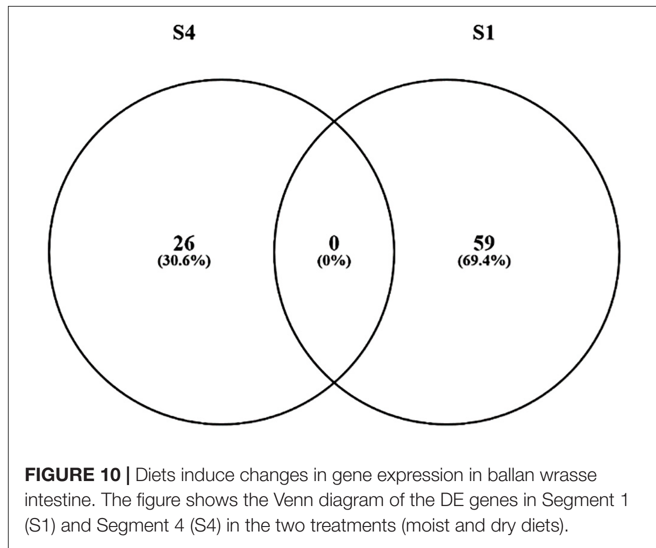

FIGURE 10 | Diets induce changes in gene expression in ballan wrasse intestine. The figure shows the Venn diagram of the DE genes in Segment 1 (S1) and Segment 4 (S4) in the two treatments (moist and dry diets).

analysis, 59 genes were differentially expressed (DE) ($q < 0.1$) between the two treatments in Segment 1 (**Supplementary Table S1**) 26 genes in Segment 4 (**Supplementary Table S2**). However, none of the DE genes in Segment 1 were overlapping with the DE genes of Segment 4 (**Figure 10**).

Of DE genes in Segment 1, 24 were increased and 25 were reduced in fish in the moist diet treatment compared to fish in the dry diet treatment (**Supplementary Table S1**). Downstream analysis of Segment 1 DE genes revealed several enriched pathways of which genes related to cholesterol biosynthetic process were enriched by the moist diet (**Figure 11**). No enriched pathways (FDR > 10%) were observed following pathway analysis using the DAVID Functional Annotation Tool (version 6.8).

Nine aquaporin genes (Aqp1, 3, 4, 7, 8, 9, 10, 11, and 12) were identified in intestinal tissue of ballan wrasse. Aqp1, 8, and 10b had an opposite expression pattern, with relatively low expression in Segment 1 followed by higher levels of expression in more distally located segment/s (**Figure 12**). Aqp11 and Aqp10a were highly expressed in Segment 1 with declining expression levels toward the distal part of the intestine. The expression of the remaining Aqp genes did not differ throughout the intestine. Sodium–dependent glucose cotransporter 1 (Sglt1) gene was highly expressed in three proximal segments with lower expression in the last segment (**Figure 12**). The expression of nine Aqp genes and Sglt1 had no difference between moist and dry diet treatments.

DISCUSSION

Intestinal Health

Wild fish have evolved consuming prey with high moisture contents, whereas cultured fish are generally fed on commercial pellets with low water contents (Buddington et al., 1997). Thus, it is possible that dry hard pellets may physically interact with and damage the epithelial layers of the gut wall (Hedrera et al., 2013; Couto et al., 2015). Earlier reports on gut health in ballan wrasse described severe inflammations in the epithelial tissues [see Krogdahl et al. (2014) in Leppeprod report]. It was hypothesized that the dry and hard feed pellets used in intensive farming

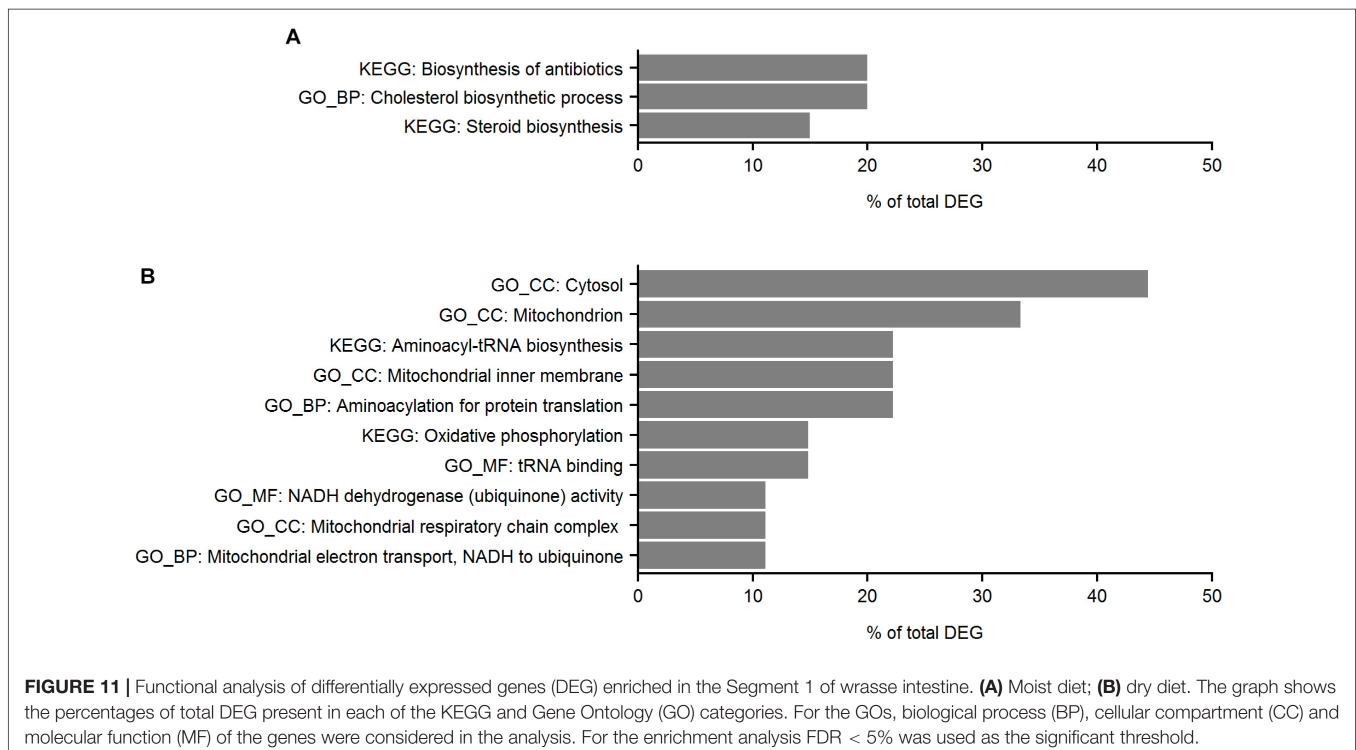

FIGURE 11 | Functional analysis of differentially expressed genes (DEG) enriched in the Segment 1 of wrasse intestine. **(A)** Moist diet; **(B)** dry diet. The graph shows the percentages of total DEG present in each of the KEGG and Gene Ontology (GO) categories. For the GOs, biological process (BP), cellular compartment (CC) and molecular function (MF) of the genes were considered in the analysis. For the enrichment analysis FDR < 5% was used as the significant threshold.

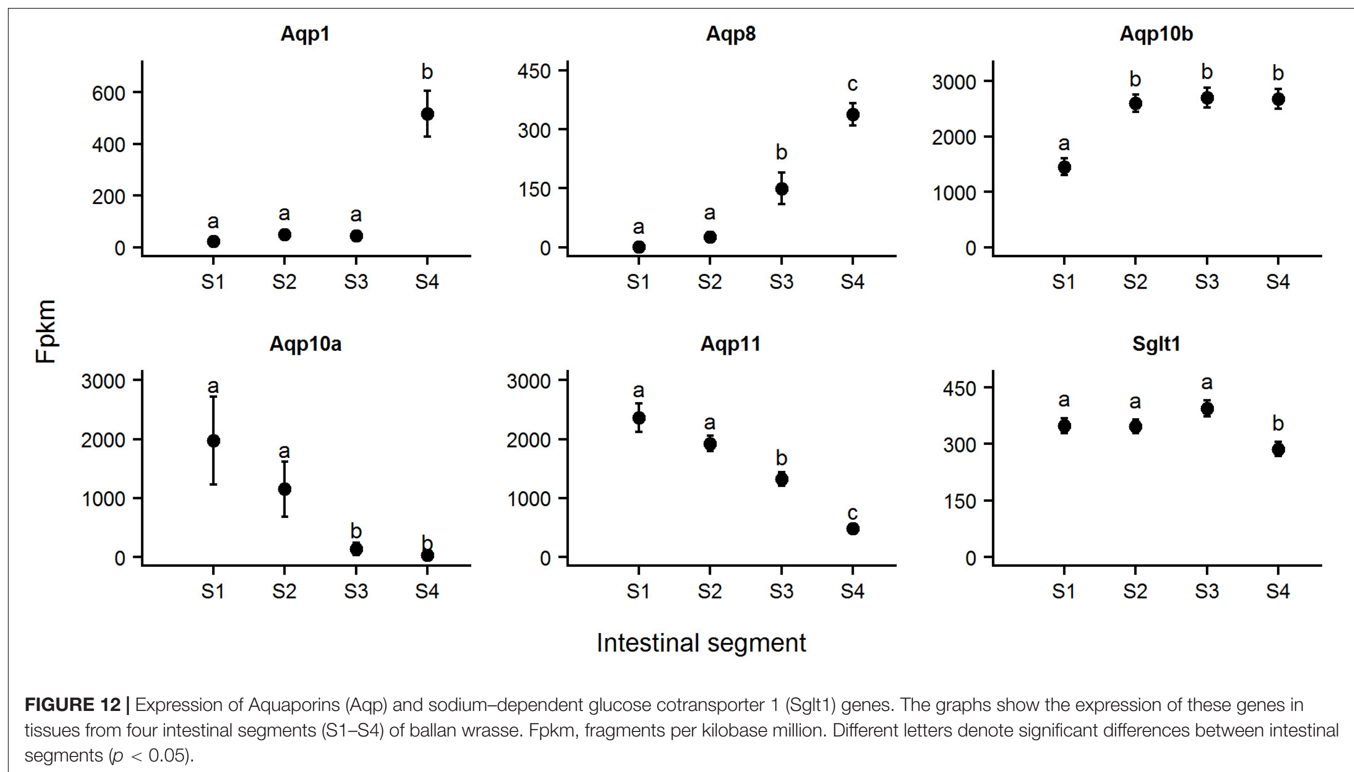

FIGURE 12 | Expression of Aquaporins (Aqp) and sodium–dependent glucose cotransporter 1 (Sglt1) genes. The graphs show the expression of these genes in tissues from four intestinal segments (S1–S4) of ballan wrasse. Fpkm, fragments per kilobase million. Different letters denote significant differences between intestinal segments ($p < 0.05$).

triggered irritations and inflammations. However, in the current study severe intestinal inflammation was not found, nor did diet moisture level has an effect on overall intestinal inflammatory status. Most fish ate the pellets immediately after they were introduced into the tank, but a "docile" species like the wrasse will also feed from the bottom of the tank. Pellets on the tank bottom will be soaked like the moist diet, eliminating the difference in dietary treatment. However, these feeding conditions were the same as for the fish previously observed with intestinal inflammations. It is therefore most likely that previous observed intestinal inflammations were caused by other factors than the hardness of the feed pellets.

Gut Evacuation in the Agastric Fish, Ballan Wrasse

Short feeding intervals and large meals increase the rate of gut evacuation (Grove et al., 1978; Flowerdew and Grove, 1979; Jobling, 1981; Garber, 1983; Lee et al., 2000). The "gastric" evacuation time (time for >90% digesta propelled out of the anterior bulbous – Segment1 to next intestinal sections) in ballan wrasse in the current study was of ~4 h, which was relatively faster than other species, despite relatively low feed intakes [~0.1% body weight in ballan wrasse, compared to ~1% body weight in salmon (Aas et al., 2017)]. For instance, the gastric emptying time (GET) for clownfish (*Amphiprion ocellaris*) fed pellets to satiation is 36 h (Ling and Ghaffar, 2014). Atlantic salmon force fed a single meal of 10 mg/g body weight needed 24 h to evacuate 92–99% of the feed from the stomach to the intestine (Aas et al., 2017). The fast evacuation time in ballan wrasse may be due to the short feeding intervals (10 min) and/or the continuous light regime, which might force wrasse to eat continuously. Increasing feeding frequency can result in faster gut evacuation rates, as a previous meal may physically push the digesta along the gastrointestinal tract. For example, Nile tilapia (*Oreochromis niloticus*) fed at 2–3 h intervals had a faster evacuation rate compared to those fed at 4 h intervals (Riche et al., 2004). A shorter gut transit time due to higher feeding frequencies has also been reported in other gastric fish species, such as; rainbow trout (Grove et al., 1978), Korean rockfish (Lee et al., 2000), Atlantic cod (*Gadus morhua*) (Tyler, 1970), common dab (*Limanda limanda*) (Gwyther and Grove, 1981); and several agastric; shanny (*Lipophrys pholis*) (Grove and Crawford, 1980), rare minnow (*Gobiocypris rarus*) (Wu et al., 2015). To summon up; no effect of dietary moisture level on the passage rate of digesta through different segments, or over the entire length, of the gastrointestinal tract were found the current study. Segment 1 only took 4–8 h to transfer >90% of the digesta (as indicated by the replacement of Y-labeled feed to Yb-labeled) to Segment 2. This indicates that Segment 1, also referred to as the bulbous, plays only a minor, if any, role as a temporary food storage area in ballan wrasse.

We hypothesized, based on pilot studies, that moistening of the pellets prior to feeding would increase digesta moisture levels in the first intestinal segment, and that the dry matter of digesta would decrease by Segment 3 or 4 as seen in European sea bass (Nikolopoulou et al., 2011). However, moisture levels of digesta were not different, nor did water content change in the last intestinal segments, in ballan wrasse fed either dry or moist diet. The missing difference in digesta moisture in the Segment 1 is

probably due to a large variation when the different fish ate the pellets. Only fish that ate relatively soon after the dry pellets were introduced to the tank actually ate dry diet. However, a lot of fish were observed to eat pellets from the bottom of the tanks, pellets that would be moistened by the time they were eaten.

In addition to the functions of digestion and absorption, the intestine is also pivotal in water and electrolyte balance (Buddington et al., 1997). For osmoregulation, marine fish drink seawater, active uptake of NaCl in the esophagus and intestine passively co-transports water, excess NaCl is excreted *via* the gills (Whittamore, 2012). Like this teleost effectively absorbs water in the intestine to compensate for water losses to the hyperosmotic marine environment. In gastric fish, drinking is reduced when the stomach is distended by eating (Takei et al., 1998). However, it is not known how this is regulated in agastric fish. As feed were observed in small quantities in all intestinal segments in most of the fish analyzed in the current work, we hypothesize that this fish eats more or less continuously and have a similar drinking pattern. Thus, a high drinking rate together with frequent eating may play the dominating role for digesta moisture levels diminishing the effects of feed moisture levels. At the cellular level, the stability of digesta water content throughout the wrasse intestine might be regulated by the absorption of water involving aquaporins.

Aquaporins (Aqp), of which 17 isoforms have been identified in fish (Whittamore, 2012; Finn et al., 2014), are cell membrane proteins that function as channels for passive transport of H_2O and/or small organic molecules *via* transmembrane osmotic gradients (Matsuzaki et al., 2004; Finn and Cerdà, 2011; Madsen et al., 2015). The dominant expression of Aqp10a and Aqp11 in Segment 1 may be associated with the major digestion and absorption of macronutrients of this section. In some fish species, including the wrasses, triglycerides (TG) are completely hydrolyzed during digestion to free fatty acids and a free glycerol molecule in the intestinal lumen (Lie et al., 2018; Sæle et al., 2018). We hypothesize that Aqp10a, which is permeable to glycerol (Madsen et al., 2015), may be involved in glycerol absorption to the enterocytes. With the low permeability for water (Yakata et al., 2011), Aqp11 may support the transportation of small organic molecules rather than water uptake in the intestine. Water might be mainly absorbed in the posterior intestinal segments since the increasing expressions of Aqp1 and Aqp10b; those Aqps, in mammals, have been known as "true aquaporins" and have high permeability to water (Madsen et al., 2015). The high expression of Aqp8 in the hindgut suggests this Aqp is involved in the excretion of ammonia in the hindgut, as Aqp8 allows NH_3 to permeate through it channel on the intestinal wall (Saparov et al., 2007). The presence of the "true aquaporins" Apq1, -4 and -10b, and sodium-glucose cotransporters 1 (Sglt1) in the intestinal tracts of wrasse may have contributed to maintaining the stable digesta moisture levels we observed in the wrasse irrespective of whether dry or moist diet was fed. The Sglt1 may also play a role in active water absorption along the teleost gut (Whittamore, 2012) and its gene was constituently expressed along the length of the wrasse intestine. Furthermore, dry diet that is not eaten immediately will moisten in the water, decreasing any difference between moisture levels between dry and pre-soaked feeds prior to ingestion.

Digestion and Absorption of Macronutrients

Apparent digestibility coefficients demonstrated the availability of energy and nutrients in a diet for a given species, and thus facilitate formulation of diets that satisfy the nutritional requirements of a species (Bureau et al., 1999). Increased feed moisture levels can increase (Grove et al., 2001; Baeverfjord et al., 2006), decrease (Adamidou et al., 2009; Venou et al., 2009), or have no affect (Aas et al., 2011; Oehme et al., 2014) on nutrient digestibility. Feed moisture level-induced changes in gastrointestinal transit of digesta can affect the digestibility of proteins and lipids (Adamidou et al., 2009; Venou et al., 2009). In the present study, fish fed moist diet had lower ADCs for protein and carbohydrates than those fed dry diet in Segment 1, however, this difference was evened out in subsequent segments.

Our data demonstrated that ballan wrasse, despite the lack of stomach and pyloric caeca, is able to digest and absorb efficiently protein from high protein diets. In gastric fishes, the ADC of protein in Senegalese sole (*Solea senegalensis*) is ∼94% for ∼55% protein diet (Dias et al., 2010), in Atlantic halibut (*Hippoglossus hippoglossus*) is ∼83% for ∼41% protein diet (Mundheim et al., 2004; Hatlen et al., 2005), in Atlantic cod (*Gadus morhua*) is >92% for ∼44–60% protein diet (Tibbetts et al., 2006), and in haddock (*Melanogrammus aeglefinus* L.) is ∼73–95% for ∼44–51% protein diet (Tibbetts et al., 2004). Compared to gastric fishes, the ADC of protein at 90% for ∼50–61% protein feed in ballan wrasse is quite high. It illustrates that the stomach conditions of pepsin and low pH are not definitive requirement for the efficient digestion of protein.

Our data showed that, lipid digestibility increased with increasing degree of desaturation; higher unsaturated the fatty acids, more the ADC. This is in line with previous findings for differences in intestinal absorption of saturated and unsaturated fatty acids (Ockner et al., 1972; British Nutrition Foundation, 1992; Ramírez et al., 2001). However, the ADC of SFA and MUFA were similar in intestinal Segments 2 and 4, possibly due to the SFA being more efficiently absorbed in Segments 2 and 4 than Segments 1 and 3. Thus, the lower ADC of SFA than MUFA in Segments 1 and 3 was compensated with higher amount of SFA than MUFA that were absorbed in Segments 2 and 4.

Dietary lipid and amino acids, but not carbohydrate, are the principle source of energy for most fish, especially in carnivorous and omnivorous species (Dabrowski and Guderley, 2003). Modeling of data from a comprehensive feeding trial determined that maximum lengthwise growth of ballan wrasse would occur with diets containing, in the dry matter, 65% protein, 12% lipid, and 16% carbohydrate (Hamre et al., 2013). In the present study, the fish were fed diets with 6% carbohydrate which was lower than the optimal level found by Hamre et al. (2013). Carbohydrate ADCs in carnivorous fish tends to be high (>90%) at low dietary carbohydrate (<10% dry matter) inclusions (Krogdahl et al., 2005). Thus, the low dietary carbohydrate level in the current study may explain why the ADCs for carbohydrate

(∼98% in Segment 4) were higher than for protein (∼89% in Segment 4) or lipid (∼76% in Segment 4) in ballan wrasse.

Effect of Dry and Moist Diets on the Transcriptome

Very few (59) differentially expressed genes were observed in the intestine of moist versus dry diet fed wrasse. It has been known that expression of cholesterol biosynthetic genes is affected by dietary feed composition [e.g., replacement of fish meal or fish oil by plant-based ingredients resulted in the increase in expression of genes involved in cholesterol synthesis (Leaver et al., 2008; Kortner et al., 2013, 2014)]. In this study, we found that the moisture levels of feed also had an effect on genes related to cholesterol biosynthetic processes which were enriched in fish fed moist diet compared to those fed dry diet. However, no significant differences were observed in blood cholesterol values between the two groups. This suggests that despite small differences in expression of genes related to cholesterol biosynthesis, these diets composed of the same ingredients had no further effects on cholesterol homeostasis. Dry diet resulted in increased expression of genes related to mitochondrial activity and protein translation, but these changes cannot easily be linked to other measurements taken in the study. As discussed, digesta moisture contents were similar between treatments, so it is not surprising that few differences in gene expression were observed. In accordance with the histological evaluation, we observed no apparent diet effect on immune- and inflammatory related gene expression.

CONCLUSION

The anterior segment of the ballan wrasse intestine does not appear to function as a food storage region, but it does play a key role in macronutrient digestion. Even though the experimental design in the present study did allow for some moist pellet ingestion in the dry pellet group, moisture levels did affect the speed of protein and carbohydrate digestion and absorption (ADC) in the first intestinal segment where nutrient absorption were highest. However, the higher digestibility of carbohydrate and protein in the first intestinal segment was later compensated for by the following intestinal segments. Lipid digestibility was not affected by feed moisture levels. The macronutrients were digested and absorbed along the whole intestinal tract but mainly in the first segment. The dry diet did not damage the gut tissue or negatively affect fish health.

DATA AVAILABILITY

The dataset for this study can be found in European Nucleotide Archive and the accession number is PRJEB13687.

AUTHOR CONTRIBUTIONS

ØS, ÅK, TK, IL, and KK: planning and preparing experiments. IL, KK, and ØS: participated in carrying out experiments. HL, XS, ÅK, TK, KL, and ØS: analysis. HL, XS, ÅK, TK, IL, KK, KL, and ØS: writing and editing.

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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.2019.00140/full#supplementary-material

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Regulation of Dietary Lipid Sources on Tissue Lipid Classes and Mitochondrial Energy Metabolism of Juvenile Swimming Crab, *Portunus trituberculatus*

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An 8-weeks feeding trial with swimming crab, *Portunus trituberculatus*, was conducted to investigate the effects of different dietary lipid sources on the lipid classes, lipid metabolism, and mitochondrial energy metabolism relevant genes expression. Six isonitrogenous and isolipidic experimental diets were formulated to contain fish oil (FO), krill oil (KO), palm oil (PO), rapeseed oil (RO), soybean oil (SO), and linseed oil (LO), respectively. A total of 270 swimming crab juveniles (initial weight 5.43 ± 0.03 g) were randomly divided into six diets with three replications, each consisted of 45 juvenile crabs. The results revealed that crabs fed KO had highest lipid content in hepatopancreas and free fatty acids in serum among all diets. The anabolic pathway relevant genes: *fas* and *acc* were up-regulated in KO diet. The catabolic pathway relevant genes, *hsl*, was up-regulated in LO diet, while *cpt1* was up-regulated in KO diet. Whereas, the genes involved in the transport and uptake of fatty acids such as *fabp1* and *fatp4* were down-regulated in crab fed PO and RO diets. Furthermore, the gene expression levels of transcription factors: *srebp-1* and *hnf4*α in KO and SO diets were the highest among all diets. FO and KO diets had significantly higher unsaturation index of mitochondrial membrane than others. The genes related to mitochondrial energy metabolism, such as *Atpase6*, *sirt1*, and *sirt3* were significantly up-regulated in KO and SO diets. In summary, dietary KO and SO supplementation could improve the lipid metabolism, promote energy production for juvenile swimming crab and improve physiological process and function including molting. These findings could contribute to deepen the understanding of the physiological metabolism of dietary fatty acids for swimming crab.

Keywords: ***Portunus trituberculatus***, **krill oil, fatty acid, anabolism, β-oxidation, mitochondria, sirtuin, electron transport chain**

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INTRODUCTION

Hepatopancreas, a key organ for absorption and storage of nutrients in various crustaceans, plays an important role in lipid metabolism, nutritional status and energy storage (Wang et al., 2008, 2014). The hepatopancreas stores a large amount of energy-supplying substances, especially lipids, to prepare for energy production and expenditure during molting, starvation, reproduction,

limb regeneration, and other life activities (Huang et al., 2015). Therefore, the hepatopancreas is an ideal organ to study lipid metabolism and energy metabolism during the growth stage in crustaceans.

Lipid plays two major roles, as an important energy supply and as a source of essential fatty acids (EFA) for membrane integrity, which is the main organic reserve regulating the physiological metabolism of many crustacean species, especially the synthetic process of crustacean molting hormones (O'Connor and Gilbert, 1968; Harrison, 1990).

Over the last few decades, due to the effective supply of energy and sufficient levels of EFA, especially adequate omega-3 polyunsaturated fatty acids (n-3 PUFA), fish oil (FO) has become the foremost lipid source used in the feed for most species in aquaculture industry including crustaceans traditionally (Tocher, 2015). However, the global fish oil production may be insufficient to meet future demand in aquaculture owing to sustained reliance on marine fish oil and its own finitude (Betancor et al., 2016). Therefore, the limited supply and the rising demand bringing about rising feed prices in aquaculture industry, environmental pressures to use more sustainable lipid resources, and restrictions on contaminants in feeds demand that, for aquaculture sustainable development, alternative lipid sources to fish oil are required urgently (Tocher, 2009). The proposed lipid sources includes vegetable oil sources such as palm oil (PO), rapeseed oil (RO), soybean oil (SO), and linseed oil (LO). Also, marine oil especially krill oil (KO), which extracted from Antarctic krill, containing a high proportion of EPA, DHA and astaxanthin, have the potential to be an effective lipid source in aquaculture feed (Ulven and Holven, 2015). However, these lipid sources mentioned above have different fatty acid profiles, thus respond differently to genes involved in lipid metabolism including anabolism and catabolism. The variation in the fatty acid profiles of different lipid sources and diets may result in different reactions through the modulation of gene expression of various lipid metabolic enzymes (Price et al., 2000; Ayisi et al., 2018). Many key metabolic enzymes and transcriptional factors play crucial roles in lipogenesis and lipolysis, such as fatty acid synthase (FAS), lipoprotein lipase (LPL), carnitine palmitoyltransferase I (CPTI), sterol regulatory element-binding protein 1 (SREBP1) and many others (Zheng et al., 2013). In addition, long chain fatty acid transporters including fatty acid binding protein (FABP) and scavenger receptor B2 (SR-B2) also play a key role in transporting fatty acid (Nickerson et al., 2009). Most researches related to the effects of lipid sources on lipid metabolism have focused on fish such as *Acanthopagrus schlegelii* (Jin et al., 2017), *Ctenopharyngodon idellus* (Yu et al., 2018), *Larmichthys crocea* (Qiu et al., 2017), *Oncorhynchus mykiss* (Fickler et al., 2018), and *Scophthalmus maximus* L. (Peng et al., 2014). However, a few researches related to lipid metabolism have concentrated on crustaceans, such as *Eriocheir sinensis* (Wei et al., 2017; Liu et al., 2018), *Litopenaeus vannamei* (Chen et al., 2015b), and *Sagmariasus verreauxi* (Shu-Chien et al., 2017). Up to now, researches on this topic still limited.

Lipids which stored in the hepatopancreas are used to produce energy for molting, starvation, limb regeneration in the growth stage of crustaceans through β-oxidation, a major pathway of fatty acid catabolism takes place in mitochondria and peroxisome in the cells (Tocher, 2003). Mitochondria, double-membrane-bound organelle which known as "the powerhouse of the cell," play the most prominent role in producing the energy currency of the cell, adenosine triphosphate (ATP) (Huang et al., 2010; Parihar et al., 2015). Mitochondrial sirtuins which contain three types (namely Sirt3, Sirt4, and Sirt5) are a highly conserved family of proteins and metabolic sensors of cell's energetic status that regulate cellular physiology and energy demands in response to metabolic inputs, including fatty acid input (Osborne et al., 2014; Parihar et al., 2015). These sirtuins regulate mitochondrial metabolic functions mainly through controlling post-translational modifications of mitochondrial protein. Sirt3, the predominant mitochondrial deacetylase, is in charge of the deacetylation in many proteins involved in the pathway of mitochondrial energy metabolism, especially the protein complexes of electron transport chain (ETC) (Ahn et al., 2008; Huang et al., 2010; Parihar et al., 2015). Furthermore, Sirt3 can also regulated the mitochondrial biogenesis (Parihar et al., 2015). The levels of Sirt3 are highly responsive to the prevailing nutrient availability of the cell (Osborne et al., 2014). To date, perhaps only very limited studies on the relationships between dietary lipid sources and mitochondrial energy metabolism in crustaceans were conducted.

Swimming crab (*Portunus trituberculatus*), one of the most important commercial mariculture crustacean species, is widely distributed in the coastal waters of China, Japan, Korea, and Malaysia (Jin et al., 2016). Over the past decade, researches on the effects of different dietary lipid sources on growth, tissue fatty acid compositions, immunity and antioxidant capacity and lipid metabolism in crustaceans have increased significantly (Ramesh and Balasubramanian, 2005; Zhou et al., 2007; Unnikrishnan et al., 2010; Hu et al., 2011; Li et al., 2011; Chen et al., 2015a; Han et al., 2015; Shu-Chien et al., 2017). However, researches related to the lipid and energy metabolism on crustaceans are still relatively few. Thus, we carried out a feeding trial to explore the impacts of the different dietary lipid sources (FO, KO, PO, RO, SO, and LO) on the tissue lipid classes, the expression of some vital lipid metabolism and mitochondrial energy metabolism relevant genes for juvenile swimming crab. This study will provide a novel insight into the influences of dietary lipid sources on the lipid metabolism and energy metabolism in crustaceans.

MATERIALS AND METHODS

Diet Preparation and Feeding Trial

Six isonitrogenous (45% crude protein) and isolipidic (8% crude lipid) experimental diets were formulated to meet the nutrient requirements of swimming crab juveniles based on NRC recommendations (**Table 1**). The major fatty acid compositions of experimental diets containing different lipid sources (% total fatty acids) are presented in **Figure 1**. FO diet contained 7.94% EPA and 7.42% DHA of TFA (total fatty acids), KO diet contained 8.71% EPA and 7.54% DHA of TFA, PO diet contained 29.42% C16:0 of TFA, RO diet contained 38.45% C18:1n-9 of TFA, SO diet contained 42.93% C18:2n-6 of TFA,

TABLE 1 | Formulation and proximate composition of experimental diets (dry matter).

Ingredient (g/100 g)	Dietary lipid sources					
	FO	KO	PO	RO	SO	LO
Peru fish meal	15.00	15.00	15.00	15.00	15.00	15.00
Soybean protein concentrate	26.00	26.00	26.00	26.00	26.00	26.00
Soybean meal	20.00	20.00	20.00	20.00	20.00	20.00
Krill meal	3.00	3.00	3.00	3.00	3.00	3.00
Wheat flour	23.50	23.50	23.50	23.50	23.50	23.50
Fish oil[a]	2.00					
Krill oil[b]		2.00				
Palm oil[c]			2.00			
Rapeseed oil[d]				2.00		
Soybean oil[e]					2.00	
Linseed oil[f]						2.00
Soybean lecithin[g]	3.00	3.00	3.00	3.00	3.00	3.00
Vitamin premix[h]	1.00	1.00	1.00	1.00	1.00	1.00
Mineral premix[h]	1.50	1.50	1.50	1.50	1.50	1.50
$Ca(H_2PO_4)_2$	1.50	1.50	1.50	1.50	1.50	1.50
Choline chloride	0.30	0.30	0.30	0.30	0.30	0.30
Sodium alginate	3.20	3.20	3.20	3.20	3.20	3.20
Proximate composition						
Prude protein	46.42	46.70	46.61	46.52	46.68	46.70
Crude lipid	7.80	7.81	7.79	7.79	7.84	7.80
Moisture	12.59	12.93	12.61	12.04	12.76	12.90
Ash	9.54	9.66	9.54	9.60	9.59	9.54

[a]Fish oil was purchased from Ningbo Tech-Bank Feed Co., Ltd., Ningbo, China. [b]Krill oil was purchased from Kangjing Marine Biotechnology Co., Ltd., Qingdao, China. [c]Palm oil was purchased from Longwei grain oil industry Co., Ltd., Tianjin, China. [d]Rapeseed oil was purchased from Yihai Kerry Co., Ltd., Shanghai, China. [e]Soybean oil was purchased from Yihai Kerry Co., Ltd., Shanghai, China. [f]Linseed oil was purchased from Longshan farm agricultural development Co., Ltd., Gansu, China. [g]Soybean lecithin was purchased from Ningbo Tech-Bank Feed Co., Ltd., Ningbo, China. [h]Vitamin premix and mineral premix were based on Jin (Jin et al., 2016).

and LO diet contained 27.73% C18:3n-3 of TFA, respectively. All experimental diets were sealed in vacuum-packed bags and stored at −20°C until used to maintain good quality for the feeding trail. Disease-free and similar sized swimming crab juveniles were obtained from Xiangshan crab field (Ningbo, China). The feeding trial was conducted in Ningbo Marine and Fishery Science and Technology Innovation Base (Ningbo, China) located at (121.7784°E, 29.6481°N). Prior to the start of the feeding trial, swimming crab juveniles which caught from the pond were acclimated in the cement pool for 1 week and fed with a commercial diet (45% crude protein, 80% crude lipid, Ningbo Tech-Bank Feed Co., Ltd., Ningbo, China). A total of 270 swimming crab juveniles with an initial weight of 5.43 ± 0.03 g were randomly divided into six diets. Each diet had three replicates, each consisted of 15 crabs. Each crab juvenile was assigned to an individual rectangle plastic basket (35 cm × 30 cm × 35 cm) in the cement pool (6.8 m × 3.8 m × 1.7 m, length × width × depth). The plastic basket had two compartments, one section filled with sand (diameter, <0.5 mm; thickness, 5.0–7.0 cm) to mimic the habitat of swimming crab and the other section as the feeding area. Fifteen plastic baskets were placed in a line next to each other in the cement pool provided with continuous aeration through an air stone to maintain dissolved oxygen levels near saturation levels. All of the crabs were fed experimental diets once daily (daily ration was about 6–8% of wet weight) at 17:00 h. Crab in each plastic basket was weighted once every 2 weeks and the feed's daily ration was adjusted accordingly. Feces and uneaten feed were removed and about 60% of seawater in the cement pool were daily exchanged to maintain water quality every morning. Seawater temperature in the cement pool was entirely consistent among all individual rectangle plastic basket used for the experiment. Seawater temperature in the cement pool gradually decreases from 32.3 to 26.2°C with the conduct of feeding trial (8 weeks, from July 25th to September 11th). The variation of 6°C referred to the natural temporal variation of temperature over the duration of the experiment. The salinity ranged between 25.5 and 28.6 g/L, pH was 7.3–7.9, ammonia nitrogen was lower than 0.05 mg/L, and dissolved oxygen was not less than 6.0 mg/L during the feeding trail. Salinity, pH, ammonia nitrogen and dissolved oxygen were measured by YSI Proplus (YSI, Yellow Springs, OH, United States). The feeding trial lasted for 8 weeks.

Sample Collection

In the present study, all procedures complied with Chinese law pertaining to experimental animals. The protocol was approved by the Ethic-Scientific Committee for Experiments on Animals of Ningbo University.

At the end of the experiment, crab in each plastic basket of each replicate was counted and weighted to determine survival, percent weight gain (PWG) and molting ratio (MR). The growth performance are shown in **Supplementary Figure S1**. Hemolymph samples were taken from the pericardial cavity of six crabs in each replicate and using 1-ml syringes, and collected into 1.5 ml Eppendorf tubes. The hemolymph samples were centrifuged at 3500 rpm for 10 min at 4°C by centrifuge (Eppendorf centrifuge 5810R, Germany). Then, the supernatant was collected, packaged, and stored at −80°C until analysis of serum lipid classes. A large portion of the hepatopancreas was collected and stored at −20°C for the determination of lipid content and lipid classes analysis. A small portion of the hepatopancreas was collected, immediately placed in liquid N_2, and stored at −80°C for gene expression analysis (eight crabs from each replicate). Another small fresh portion of the hepatopancreas was used to isolate mitochondria and further analysis.

Lipid Content and Lipids Classes

Lipid content of hepatopancreas was determined gravimetrically after extraction in $CHCl_3/CH_3OH$ (2:1) and evaporation to constant weight with a rotating evaporator (IKA RV10, Germany) according to the method described by Folch et al. (1957). Methanol (CH_3OH, ≥99.7%, CAS 67-56-1) and chloroform ($CHCl_3$, ≥99.0%, CAS 67-66-3) were of analytical grade

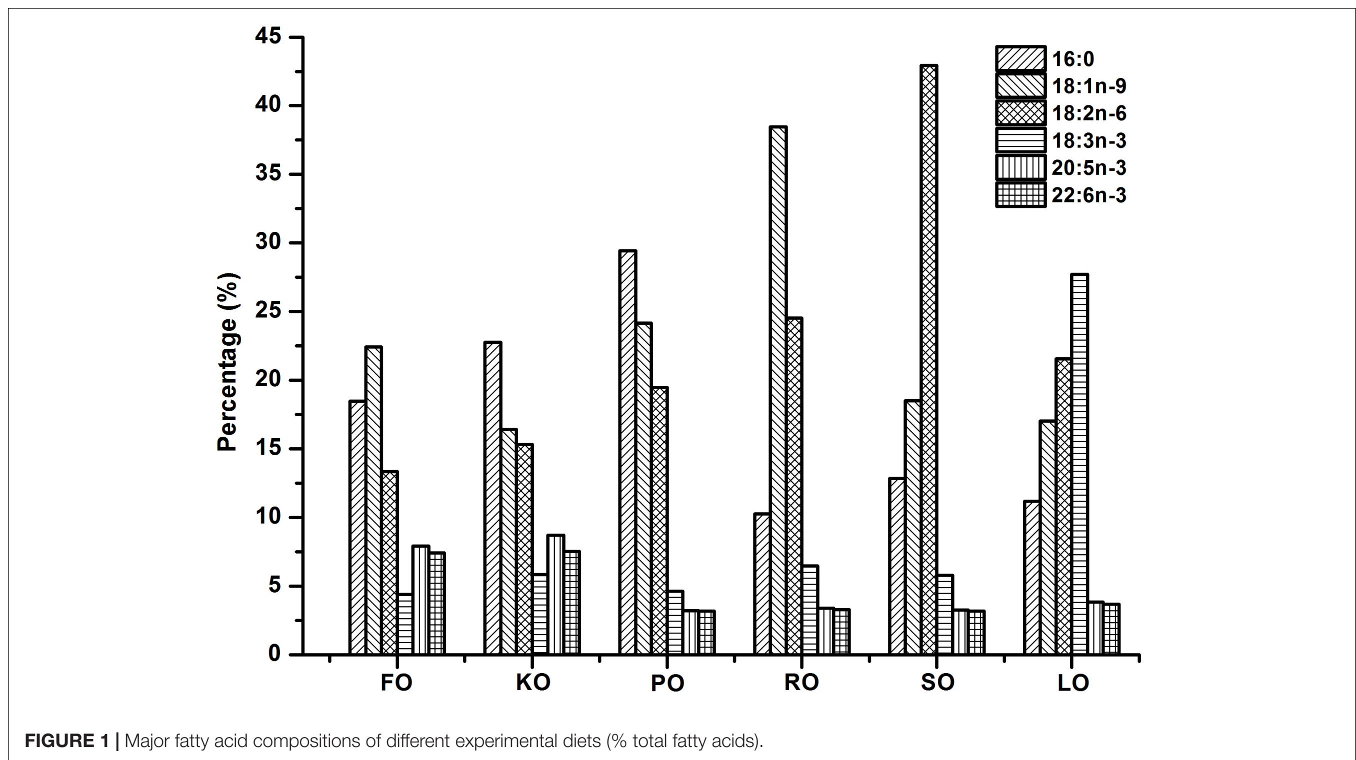

FIGURE 1 | Major fatty acid compositions of different experimental diets (% total fatty acids).

which purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). The triacylglycerol (TG) and total cholesterol (TC) contents in serum were assayed using an automatic biochemistry analyzer (VITALAB SELECTRA Junior Pros, Netherlands). The reagent kits for automatic biochemistry analyzer were purchase from Biosino Bio-technology and Science Inc. (Beijing, China). TG and TC levels in hepatopancreas were determined using Triglyceride GPO-PAP and Cholesterol CHOD-PAP kits (Nanjing Jiancheng Bioengineering Institute, China). High-density lipoprotein cholesterol (HDL-C) and low-density lipoprotein cholesterol (LDL-C) concentrations were determined using a commercial assay kit (Nanjing Jiancheng Bioengineering Institute, China) based on a modification of the cholesterol oxidase method described by Rifai et al. (2000). HDL-C and LDL-C reacted with cholesterol oxidase and cholesterol esterase in the presence of chromogens to produce a colored product (Chen et al., 2007). The concentrations of free fatty acids (FFA) in the serum and hepatopancreas were determined using a commercial assay kit (Nanjing Jiancheng Bioengineering Institute, China) by the method of Duncombe. In brief, FFA reacted with copper reagents to form Cu^{2+} salt, then Cu^{2+} salt reacted with the chromogen diethyldithiocarbamate to give a yellow color (Duncombe, 1963). Each determination was performed in triplicate.

Isolated Mitochondria Preparation

A small fresh portion of the hepatopancreas was used to isolate mitochondria by the method as previously described (Bustamante et al., 1977). Briefly, the tissue fragments were minced by careful shearing, rinsed to remove residual impurities with normal saline, weighed (≈200 mg), put into an ice-cold isolation buffer containing 0.25 M sucrose, 10 mM Tris–HCl, and 0.5 mM EDTA at pH 7.4, and then gently homogenized at 1000 g for 10 min; the supernatant was then centrifuged at 10,000 g for 10 min. The mitochondrial pellets were collected, washed twice and then resuspended in the isolation buffer. The pellets were stored at −80°C. All of the operations were carried out on ice. The mitochondrial protein concentrations were determined using a BCA protein assay kit according to manufacturer's protocol (Beyotime Biotechnology, Shanghai, China).

Mitochondrial Membrane Lipids and Fatty Acid Compositions

Total lipids were extracted from mitochondria (10 mg protein) followed the method of Bligh and Dyer with few modifications (Bligh and Dyer, 1959). The extracts were dried under N_2 flow and resuspended in 1 ml $CHCl_3/CH_3OH/HCOOH$ (1:1:0.1, v/v/v). The mixture was added 0.5 ml 1 M KCl/2 M H_3PO_4 and after shaking for 30 s; the mixture was then centrifuged at 4500 *g* for 5 min. Formic acid (HCOOH, ≥98.0%, CAS 64-18-6) and phosphoric acid (H_3PO_4, ≥85.0%, CAS 7664-38-2) were of analytical grade which purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). The mixture separated into two layers and the subnatant was transfered to a new tube. Added 0.5 ml $CHCl_3/CH_3OH$ (2:1, v/v) to the new tube and the mixture was evaporated to constant weight using a rotating evaporator. The evaporated substance was mitochondrial membrane lipid fatty acid, then collected into the tube, added 200 μl $CHCl_3/CH_3OH$ (2:1, v/v) to be dissolved and stored at −20°C for fatty acid analysis. The

fatty acid compositions were determined using the method described by Zuo et al. (2013) with some modifications. All of the solvents contained 0.005% (w/v) of *tert*-butylhydroxytoluene (BHT) to prevent the oxidation of PUFAs. Diets (approximately 100 mg) and mitochondrial membrane lipid fatty acid solutions (approximately 100 μl) were thawed at 4°C, then added to a 12 ml volumetric glass screwed tube with lid containing a teflon gasket. Followed by adding 3 ml KOH-CH_3OH (1 N) and heated at 75°C in a water bath for 20 min. After cooling, 3 ml HCL–CH_3OH (2 N) was added and the mixture was incubated at 75°C in a water bath for another 20 min. Previous tests were conducted to ensure that all fatty acids can be esterified. Finally, 1 ml hexane was added into the mixture above, shaken vigorously for 1 min, added 1 ml ultra-pure water to promote layering, and then collected supernatant into the ampoule bottle. The solvent contained the FAMEs (fatty acid methyl esters) in the ampoule bottle was reduced to dryness by termovap sample concentrator, and the FAMEs were resuspended in 500 μL of *n*-hexane and stored at −20°C until used for gas chromatography (GC) analysis. HPLC-grade *n*-hexane (≥95.0%, CAS 110-54-3) and the standard mixtures of 37 FAMEs were purchased from Sigma (St. Louis, MO, United States). All FAMEs were separated and analyzed on a gas chromatograph mass spectrometer (GC-MS) (Agilent-GCMS 7890-5975C; Agilent Technologies, Santa Clara, CA, United States). The GC column was a capillary HP-5MS column (Agilent Technologies, Santa Clara, CA, United States). The column length was 30 m with an internal diameter of 0.25 mm. The film thickness was 0.25 μm. Mass spectra were scanned from m/z 50–800. Peaks and fatty acids were identified using retention times from standards by comparing the mass spectra with a commercially available standard library (National Institute of Standards and Technology Mass Spectral Library 2011). Results were presented as relative percentages of each fatty acid (% total fatty acids), calculated using the peak area ratio.

Total RNA Extraction, Reverse Transcription, and Real-Time Quantitative PCR

RNA extraction, cDNA synthesis, and real-time quantitative PCR were performed based on the procedures described by Jin et al. (2017). Briefly, total RNA was extracted from hepatopancreas samples with TRIzol reagent (Takara, Japan) following the manufacturer's protocol and the RNA was treated with RNase-Free DNase (Takara, Japan) to remove DNA contamination. The cDNA was generated from 1000 ng of DNase treated RNA and synthesized by a Prime Script^{TM} RT Reagent Kit with gDNA Eraser (Takara, Japan) using Mastercycler nexus GSX1 PCR (Eppendorf, Germany). Real-time quantitative PCR was conducted by a quantitative thermal cycler (Lightcycler 96, Roche, Switzerland). The complete mitochondrial DNA sequence for swimming crab (*P. trituberculatus*) has been determined as previously described (Yamauchi et al., 2003). All primers were synthesized by BGI (The Beijing Genomics Institute, Shenzhen, China). Specific primers for the candidate genes *fas* (fatty acid synthase), *acc* (acetyl-CoA carboxylase), *g6pd* (glucose 6-phosphate dehydrogenase), *6pgd* (6-phosphogluconate dehydrogenase), *lpl* (lipoprotein lipase), *hsl* (hormone-sensitive lipase), *cpt1* (carnitine palmitoyltransferase 1), *cpt2* (carnitine palmitoyltransferase 2), *fabp1* (fatty acid binding protein 1), *fatp4* (fatty acid transport protein 4), *srb2* (scavenger receptor class 2), *srebp-1*, (sterol regulatory element-binding protein-1), *hnf4*α, (hepatocyte nuclear factor 4-alpha), *nd*, (NADH dehydrogenase), *sdhc*, (succinate dehydrogenase complex, subunit C), *cytb* (cytochrome b), *cox* (cytochrome c oxidase), *sirt* (silent information regulator), *nrf1* (nuclear respiratory factor 1), and β-actin used for qPCR were designed by Primer Premier 5.0 (**Supplementary Table S1**). β-actin was used as a house-keeping gene and the stability of β-actin was confirmed (Pan et al., 2010). The fluorescence data acquired were normalized to β-actin based on the $2^{-\Delta\Delta t}$ method (Livak and Schmittgen, 2001). The relative mRNA expression of target genes in crabs fed FO was selected as the calibrator.

Statistical Analysis

The results are expressed as the means ± SEM ($n = 3$). Data were processed using one-way analysis of variance (ANOVA), followed by Tukey's test. Firstly, all the data were tested for normal distribution and homogeneity of variance. Then, the group means could be further compared using Tukey's multiple range test. All statistical analysis were performed using SPSS 22.0 (SPSS, Chicago, IL, United States). A value of $P < 0.05$ was considered statistically significant. OriginPro 8.5 software (OriginLab, Los Angeles, CA, United States) was used for figure processing.

RESULTS

Growth

Supplementary Figure S1 shows the effects of different lipid sources on growth of juvenile swimming crab. There were no statistical differences in survival of swimming crab juveniles among all diets ($P > 0.05$). It was found that crabs fed KO diet had a significantly higher PWG (percent weight gain) and MR (molting ratio) than those fed other diets ($P < 0.05$), followed by FO diet.

Lipid Content in Hepatopancreas

Figure 2 shows the lipid contents (%, wet matter) in hepatopancreas of swimming crab juveniles fed different lipid sources. Crabs fed KO had a significantly higher lipid content in hepatopancreas than that fed diet supplemented with PO, RO and LO ($P < 0.05$), while no statistic differences were found compared to FO and SO diets.

Lipid Classes in Serum and Hepatopancreas

Table 2 compares the lipid classes levels in serum and hepatopancreas of swimming crab fed with different diet supplements. The levels of TG and TC in serum were not affected by different dietary lipid sources ($P > 0.05$). FO diet

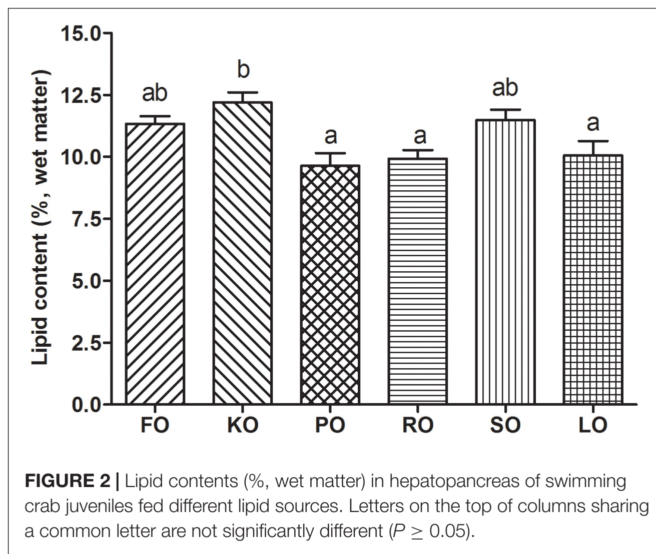

FIGURE 2 | Lipid contents (%, wet matter) in hepatopancreas of swimming crab juveniles fed different lipid sources. Letters on the top of columns sharing a common letter are not significantly different ($P \geq 0.05$).

had a significantly higher HDL-C levels in serum than other diets ($P < 0.05$). The highest level of LDL-C in serum was found in RO diet, while PO diet had the lowest one. The FFA concentrations in serum of crabs fed KO was significantly more abundant than that fed other diets ($P < 0.05$). Different dietary lipid sources could also significantly affect the lipid classes concentrations in hepatopancreas ($P < 0.05$). Hepatopancreas TG concentrations in crabs fed diets supplemented with RO and SO were significantly higher than that fed other diets ($P < 0.05$). The content of TC in FO diet was the lowest, while SO diet had the highest content of TC among all diets. Crabs fed diet supplemented with KO had a high level of LDL-C as well as HDL-C. However, it was found that FO diet had a high level of HDL-C, but a low level of LDL-C. The FFA concentrations in hepatopancreas of crabs fed KO and SO diets were significantly higher than that fed FO and PO ($P < 0.05$).

Expression of Lipid Metabolism Genes in Hepatopancreas

The relative gene expression of some lipid metabolism pathways in the hepatopancreas of juvenile swimming crab including anabolism (A), catabolism (B), transport and uptake (C), and transcription factors (D) are shown in **Figure 3**. Among genes related to anabolic pathway (**Figure 3A**), the expression levels of *fas* and *acc* in crabs fed KO were significantly higher than other diets ($P < 0.05$). The transcript levels of *fas*, *acc*, and *6pgd* in crabs fed PO were all the lowest. There were no significant differences in the expression of *g6pd* in all diets ($P > 0.05$). With regards to the relative expression of genes related to lipid catabolism (**Figure 3B**), the present results showed that the expression levels of *lpl*, *hsl*, and *cpt1* were significantly affected by different dietary lipid sources ($P < 0.05$), while there were no significant differences in the gene expression of *cpt2* in all diets ($P > 0.05$). Crabs fed KO and LO had significantly higher expression levels of *lpl* than other diets ($P < 0.05$). The highest expression levels of *hsl* and *cpt1* appeared in LO and KO diets, respectively, which were both significantly higher than other diets ($P < 0.05$). **Figure 3C** shows the relative expression of genes involved in fatty acid transport and uptake. Crabs fed KO had significantly higher gene expression levels of *fabp1* and *fatp4* than that fed other diets ($P < 0.05$). The expression level of *srb2* was up-regulated significantly by dietary FO, KO and SO ($P < 0.05$). Eventually, the expression levels of genes related to transcription factors are presented in **Figure 3D**. The relative expression levels of *srebp-1* in KO and SO diets were significantly higher than other diets ($P < 0.05$). Crabs fed KO had the highest expression levels of *hnf4α*, followed by SO diet.

Fatty Acid Compositions of Mitochondrial Membrane Lipid

The major fatty acid compositions (**Figure 4A**) (% total fatty acids) and unsaturation index (**Figure 4B**) of mitochondrial

TABLE 2 | Effects of different dietary lipid sources on serum and hepatopancreas lipid classes contents of swimming crab[a].

Item	Dietary lipid sources					
	FO	KO	PO	RO	SO	LO
Serum						
TG (mmol/L)[b]	0.07 ± 0.01	0.09 ± 0.01	0.06 ± 0.02	0.07 ± 0.01	0.06 ± 0.01	0.08 ± 0.01
TC (mmol/L)[c]	0.04 ± 0.01	0.06 ± 0.00	0.05 ± 0.02	0.04 ± 0.02	0.04 ± 0.01	0.05 ± 0.01
HDL-C (μmol/L)[d]	79.15 ± 4.60[b]	49.79 ± 9.64[a]	44.68 ± 1.28[a]	43.02 ± 1.51[a]	45.96 ± 7.97[a]	45.19 ± 1.59[a]
LDL-C (μmol/L)[e]	25.83 ± 2.78[ab]	22.23 ± 2.40[ab]	18.21 ± 1.53[a]	32.18 ± 4.04[b]	25.83 ± 4.88[ab]	21.17 ± 3.05[ab]
FFA (μmol/L)[f]	125.47 ± 16.94[a]	478.62 ± 86.25[c]	116.79 ± 17.84[a]	126.04 ± 5.47[a]	241.69 ± 41.43[ab]	275.02 ± 22.90[b]
Hepatopancreas						
TG (mmol/gprot)	0.10 ± 0.03[a]	0.09 ± 0.02[a]	0.09 ± 0.01[a]	0.16 ± 0.02[b]	0.16 ± 0.02[b]	0.08 ± 0.01[a]
TC (mmol/gprot)	5.64 ± 0.62[a]	20.80 ± 1.56[b]	7.38 ± 1.06[a]	18.82 ± 1.24[b]	31.58 ± 3.68[c]	25.19 ± 1.71[bc]
HDL-C (mmol/gprot)	2.02 ± 0.14[c]	1.20 ± 0.10[abc]	1.12 ± 0.17[ab]	1.41 ± 0.25[abc]	1.68 ± 0.29[bc]	0.57 ± 0.09[a]
LDL-C (mmol/gprot)	0.12 ± 0.01[ab]	0.24 ± 0.02[c]	0.09 ± 0.00[a]	0.15 ± 0.01[b]	0.13 ± 0.01[ab]	0.13 ± 0.01[ab]
FFA (mmol/gprot)	1.49 ± 0.19[a]	2.12 ± 0.22[b]	1.43 ± 0.15[a]	1.81 ± 0.07[ab]	2.08 ± 0.17[b]	1.90 ± 0.23[ab]

[a]Values are means ± SEM of three replicates (n = 3). Values within the same row sharing a common letter in superscripts are not significantly different ($P \geq 0.05$). [b]TG, triglyceride; [c]TC, total cholesterol; [d]HDL-C, high density lipoprotein cholesterol; [e]LDL-C, low density lipoprotein cholesterol; [f]FFA, free fatty acid.

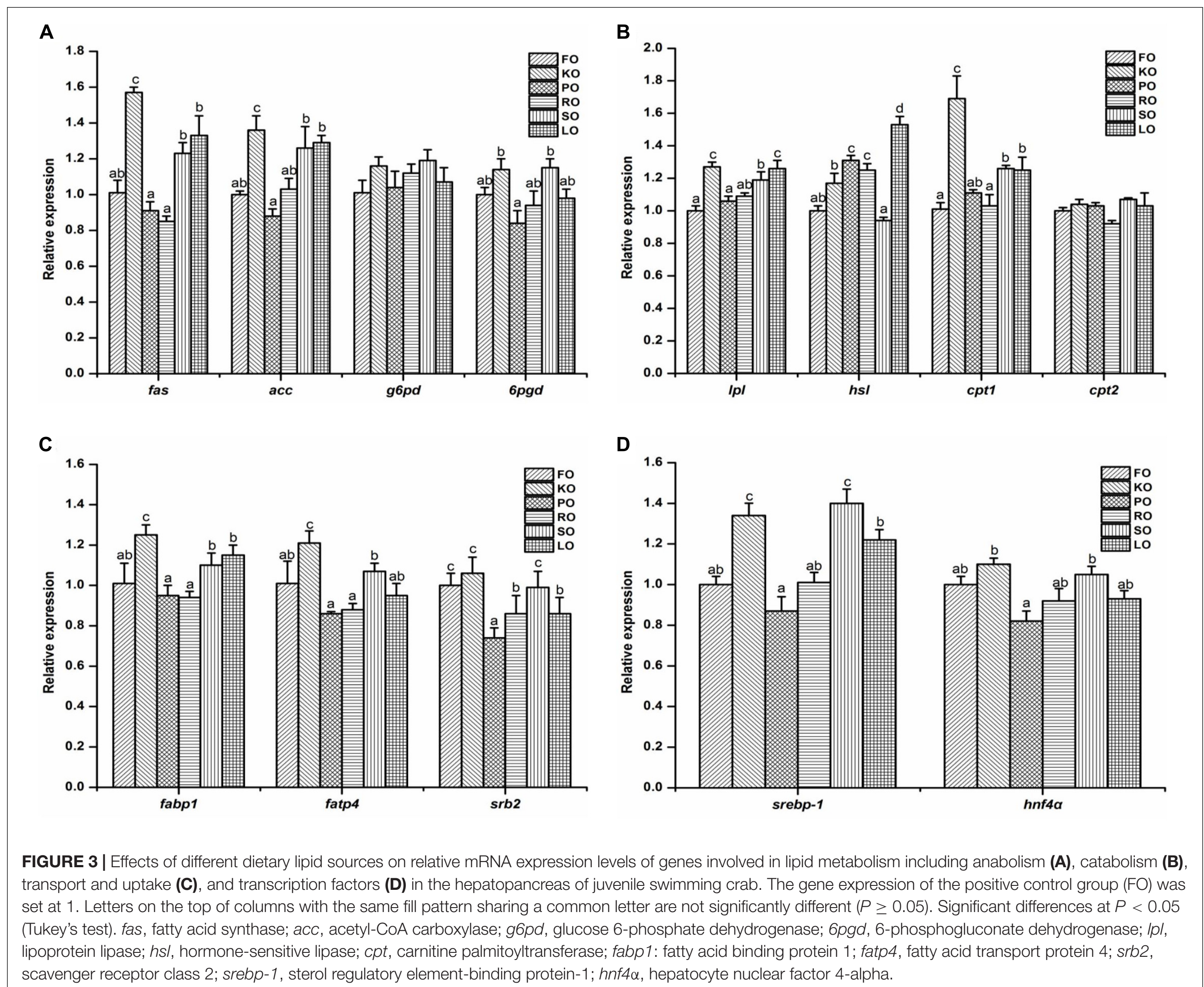

FIGURE 3 | Effects of different dietary lipid sources on relative mRNA expression levels of genes involved in lipid metabolism including anabolism **(A)**, catabolism **(B)**, transport and uptake **(C)**, and transcription factors **(D)** in the hepatopancreas of juvenile swimming crab. The gene expression of the positive control group (FO) was set at 1. Letters on the top of columns with the same fill pattern sharing a common letter are not significantly different ($P \geq 0.05$). Significant differences at $P < 0.05$ (Tukey's test). *fas*, fatty acid synthase; *acc*, acetyl-CoA carboxylase; *g6pd*, glucose 6-phosphate dehydrogenase; *6pgd*, 6-phosphogluconate dehydrogenase; *lpl*, lipoprotein lipase; *hsl*, hormone-sensitive lipase; *cpt*, carnitine palmitoyltransferase; *fabp1*: fatty acid binding protein 1; *fatp4*, fatty acid transport protein 4; *srb2*, scavenger receptor class 2; *srebp-1*, sterol regulatory element-binding protein-1; *hnf4*α, hepatocyte nuclear factor 4-alpha.

membrane lipid in hepatopancreas are presented in **Figure 4**. In general terms, mitochondrial membrane lipid fatty acid profiles reflected the diets, but there were some differences. Each diet had a particularly characteristic fatty acid in mitochondrial membrane. For instance, the percentage of EPA and DHA in crabs fed FO and KO diets were significantly higher than other diets ($P < 0.05$). Moreover, KO diet had a significantly higher content of EPA than FO diet ($P < 0.05$). The maximum value of palmitic acid (PA, 16:0) in mitochondrial membrane was discovered in PO diet. The highest contents of oleic acid (OA, 18:1n-9) and α-linolenic acid (ALA, 18:3n-3) in mitochondrial membrane were found in RO diet and LO diet, respectively. Crabs fed diets containing SO and LO had a significantly higher proportion of linolenic acid (LA, 18:2n-6) than that fed other diets ($P < 0.05$). From **Figure 4B**, it was found that the unsaturation index of mitochondrial membrane lipid in FO and KO diets were significantly higher than other diets ($P < 0.05$).

Electron Transport Chain Complex and Mitochondrial Energy Metabolism

The electron transport chain complex (**Figure 5A**) and mitochondrial energy metabolism (**Figure 5B**) in the hepatopancreas of juvenile swimming crab are shown in **Figure 5**. The gene expression levels of *nd1* and *coI* in KO diet were significantly higher than other diets ($P < 0.05$). The highest expression level of *coII* appeared in SO diet, and it was significantly higher than other diets ($P < 0.05$), followed by FO and KO diets. Besides, the similar trend was found in expression of *coIII* among all diets. Interestingly, the expression trends of genes related to mitochondrial energy metabolism were similar. In brief, crabs fed diet supplemented with KO had significantly higher expression levels of *Atpase6* than that fed other diets ($P < 0.05$), followed by SO diet, then FO diet. The gene expression levels of *sirt1* and *sirt3* were all significantly up-regulated when crabs fed KO and SO diets,

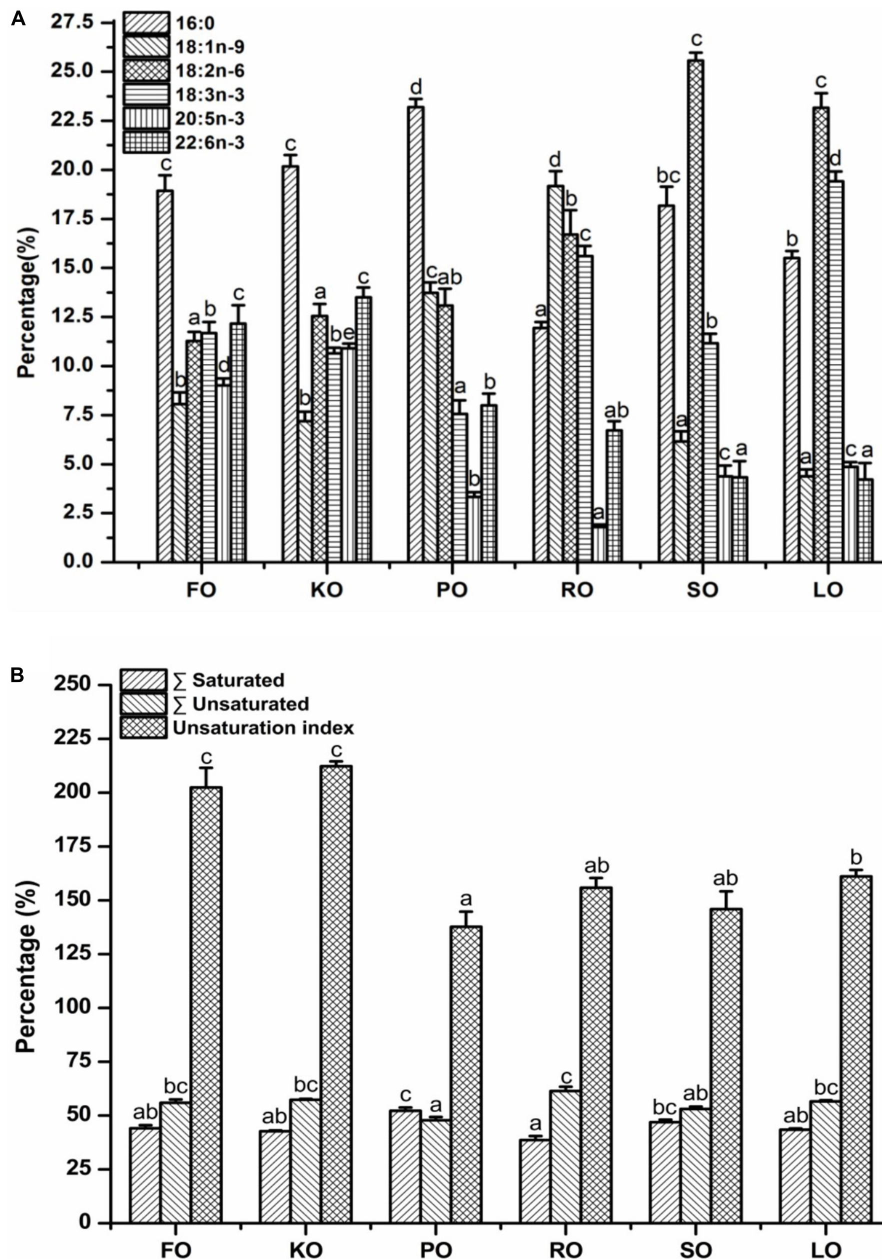

FIGURE 4 | Major fatty acid compositions **(A)** (% total fatty acids) and unsaturation index **(B)** of mitochondrial membrane lipid in hepatopancreas of juvenile swimming crab. Letters on the top of columns with the same fill pattern sharing a common letter are not significantly different ($P \geq 0.05$).

respectively ($P < 0.05$). Crabs fed FO, KO and SO diets had significantly higher expression level of *nrf1* than that fed other diets ($P < 0.05$).

DISCUSSION

It was reported that different dietary lipid sources affected the expression levels of genes involved in various fatty acid metabolic pathways (Minghetti et al., 2011; Martinez-Rubio et al., 2013). In crustaceans, dietary lipids are mainly assimilated in the hepatopancreas, then being distributed to other tissues through the hemolymph (Coutteau et al., 1997). In the present study, significantly higher hepatopancreas lipid deposition in KO group could be partly due to phospholipid (PL) in krill oil. KO contained a high proportion of EPA and DHA, and 30–65% of the fatty acids were esterified in phospholipids (PLs) (mainly phosphatidylcholine) (Ulven and Holven, 2015). However, fatty acids in vegetable oils and fish oil were mostly esterified in triacylglycerol (TAG) (Ulven and Holven, 2015).

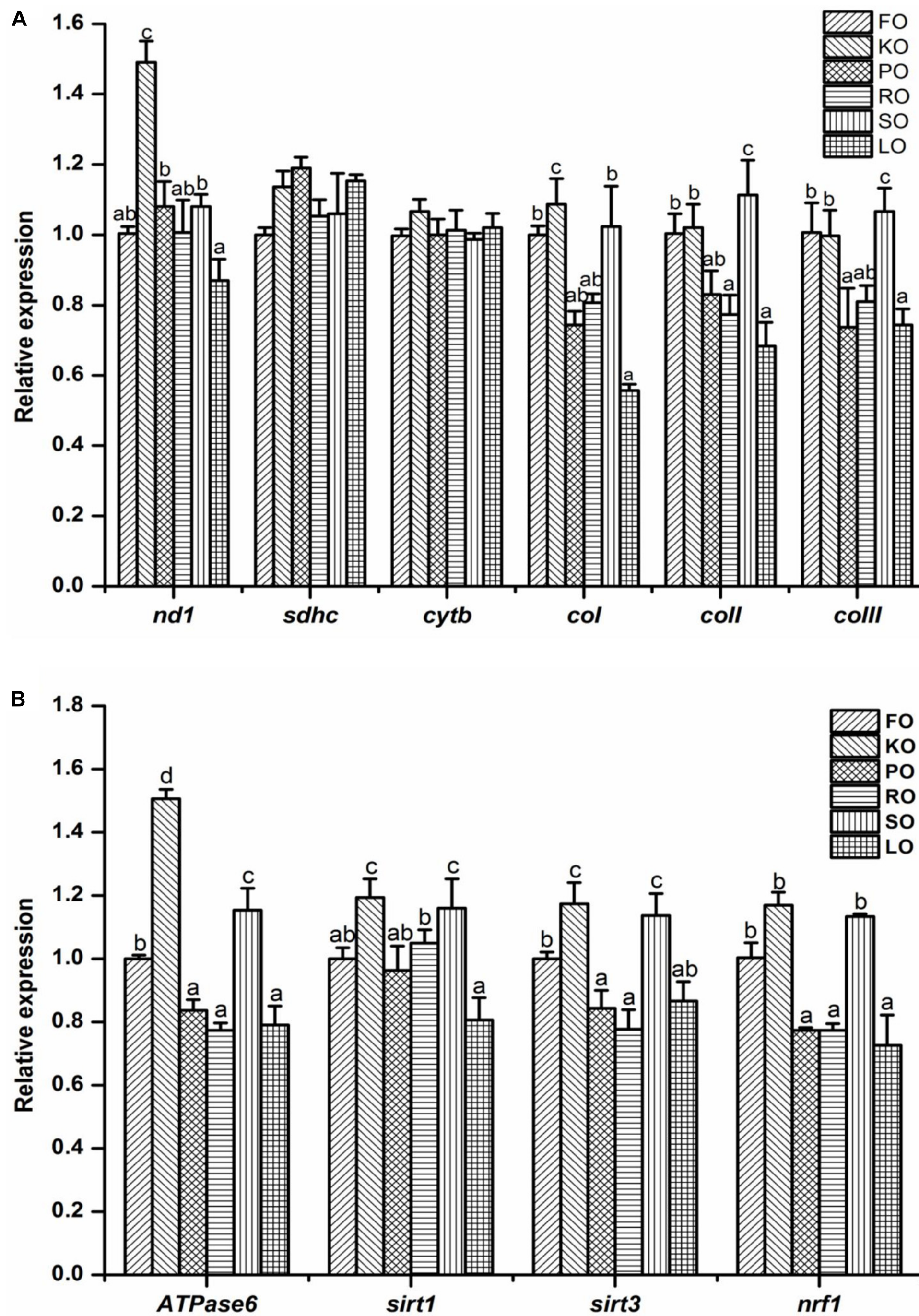

FIGURE 5 | Effects of different dietary lipid sources on relative mRNA expression levels of genes related to electron transport chain complex **(A)** and mitochondrial energy metabolism **(B)** in the hepatopancreas of juvenile swimming crab. The gene expression level of the positive control group (FO) was set at 1. Letters on the top of columns with the same fill pattern sharing a common letter are not significantly different ($P \geq 0.05$). Significant differences at $P < 0.05$ (Tukey's test). *nd*, NADH dehydrogenase; *sdhc*, succinate dehydrogenase complex, subunit C; *cytb*, cytochrome b; *cox*, cytochrome c oxidase; *sirt*, silent information regulator; *nrf1*, nuclear respiratory factor.

Some researches also reported that lipid deposition increased due to PL supplementation in crustaceans, such as *Penaeus japonicus* (Kontara et al., 1997), *Penaeus penicillatus* (Chen and Jenn, 1991), and *P. trituberculatus* (Li et al., 2016). Another reason was that the high expression levels of genes involved in anabolism, such as *fas*, *acc*, *6pgd*, *g6pd*, and *srebp-1* (Ayisi et al., 2018). Some studies demonstrated that FAS is the main lipogenic enzyme which catalyzes successive condensation reactions to form a fatty

acid, playing a key role in energy homeostasis (Chen et al., 2015). ACC is a biotin-dependent enzyme that catalyzes the irreversible carboxylation of acetyl-CoA to malonyl-CoA, the rate-limiting step in fatty acid biosynthetic pathway (Barber et al., 2005). 6PGD and G6PD are major regulatory enzymes involved in the production NADPH and play a role in the biosynthesis of fatty acid (Chen et al., 2013). SREBP is a main regulator of fatty acid/lipid and cholesterol biosynthesis (Menoyo et al., 2004). The results of anabolic gene expression showed that crabs fed KO diet up-regulated the gene expression of *fas*, *acc*, *6pgd*, and *srebp-1*, which may suggest that KO could promote the anabolism of lipid and increase the lipid deposition in hepatopancreas of crabs. This is probably the major reason that crabs fed KO diet had higher lipid content in hepatopancreas. Indeed, some studies about fish demonstrated that dietary lipid sources could regulate expression levels of genes involved in the anabolic process, such as *g6pd* and *fas* (Menoyo et al., 2004; Panserat et al., 2008; Morais et al., 2011; Peng et al., 2014). In one research about spiny lobster, the researchers found that the gene expression of *fas* was significantly up-regulated by dietary KO and VO compared to dietary FO, which was similar to present study (Shu-Chien et al., 2017). It was noteworthy that the vegetable oil of previous study about spiny lobster came from linseed and palm oils, which was a blend. The fatty acid composition of VO diet in previous study combines the characteristic of SO and LO diets with the highest levels of LA and ALA. Therefore, similarity in fatty acid composition led to a high expression level of *fas* in VO diet (spiny lobster), KO, SO, and LO diets (swimming crab). In addition to anabolism relevant genes, the expression of genes related to the catabolic process could also be affected by dietary lipid sources, such as *lpl* and *cpt1* (Peng et al., 2014). LPL is considered as an important factor in lipolysis because it might determine how dietary lipids are partitioned for storage or utilization, and it's a rate limiting enzyme in the provision of fatty acids (Peng et al., 2014; Qiu et al., 2017). The gene expression of *lpl* is regulated differentially according to the nutritional state, hormonal levels and the needs of the tissues for fatty acids (Fielding and Frayn, 1998). The LO and KO diets had a significantly higher expression level of *lpl* than others, followed by SO diet. While, the KO diet had the highest expression level of *cpt1* and was significantly up-regulated. Moreover, the FFA concentration in serum of crabs fed KO also showed the highest, followed by SO and LO diets, which showed a positive correlation between the FFA concentration and the catabolism.

β-oxidation, a major catabolic process of fatty acids, takes place in the cytosol of prokaryotes and the mitochondria of eukaryotes, thus provides acetyl-coenzyme A (acetyl-CoA) as a substrate for the citric acid cycle as well as NADH and $FADH_2$ as co-enzymes used in the electron transport chain (ETC) (Houten and Wanders, 2010). Owing to the negative charge carried by FFA, it cannot penetrate any biological membrane (Voet et al., 2008). Whereas, fatty acids bound to albumin can be transferred across the plasma membrane by the action of plasma membrane fatty acid binding proteins ($FABP_{pm}$), fatty acid transport proteins (FATP), fatty acid translocase (FAT/CD36, namely SR-B2), and caveolins (Anderson and Stahl, 2013). The gene expression of FABP was regulated by PUFA. What's more, FABP can transport and store FA to the mitochondria (Olivares-Rubio and Vega-Lopez, 2016). FATP, a membrane protein, is expressed in tissue which is active in fatty acid metabolism and can effectively promote the transport of long-chain fatty acids (LCFA) (Jeppesen et al., 2012). SR-B2, located on mitochondrial membranes, was found to be involved in the regulation of the rate of cellular fatty acid uptake (Smith et al., 2011). From the result of present study, it revealed that crabs fed diets supplemented with KO and SO had higher gene expression levels of *fabp1* and *fatp4*. The expression levels of *srb2* in FO, KO and SO diets could be up-regulated. The FFA which transported into the mitochondria were used to regulate the key genes and proteins of the energy production.

Mitochondria can produce adenosine triphosphate (ATP) from products of the fatty acid β-oxidation. At the mitochondrial inner membrane, electrons from NADH and FADH2 are transferred from electron donors to electron acceptors, passing through ETC to oxygen eventually (Jonckheere et al., 2012). The above process can release energy, namely oxidative phosphorylation, which is used to form ATP by a series of protein complexes within the inner membrane of the cell's mitochondria in eukaryotes (Voet et al., 2008). This response was largely driven by the expression of genes encoding subunits of Complex I (NADH dehydrogenase). As we can see from the results, KO and SO diets significantly up-regulated the expression level of Complex I. Some previous studies also provided evidences that krill oil supplementation may stimulate mitochondrial respiratory activity (Mootha et al., 2003; Burri et al., 2011). Previous study had found that a coordinated upregulation of nuclear-encoded genes regulating mitochondrial electron transport, and metabolic rate changes were in associated with the content of PUFA in diet (Hulbert et al., 2005). In addition to an increase of gene expression for subunits of complex I, a change of upregulation in the gene expression of *sirt3* was observed in KO and SO diets in present study. Sirt3 deacetylates and activates various target substrates used in oxidation of fatty acids. Sirt3 is a regulator of mitochondrial energy metabolism, particularly Complex I (Ahn et al., 2008). It is important for maintaining basal ATP levels (Ahn et al., 2008). Aside from Sirt3 modulation and changes in gene expression, mitochondrial membrane fatty acid compositions and membrane fluidity have been proposed to play a role in β-oxidation and energy metabolism (Morash et al., 2008; Longo et al., 2016). The unsaturated degree of membrane lipids affects the fluidity of membrane structure and transmembrane transports (Longo et al., 2016). The result showed that KO diet had the highest unsaturated degree of mitochondrial membrane, which partly explain the higher mRNA expression levels of transmembrane transports proteins. Furthermore, nuclear respiratory factor 1, also known as *nrf1*, encodes a protein that homodimerizes, directly regulates the expression of nuclear genes encoding subunits of the respiratory complexes and indirectly regulating the three mitochondrial-encoded COX subunit genes for respiration, mitochondrial DNA transcription and replication (Gleyzer et al., 2005; Wang et al., 2006). High expression of *nrf1* in KO and SO diets demonstrated

a marked effect of KO and SO on the upregulation of genes and pathways involved in hepatopancreas energy metabolism. What's more, the energy produced by mitochondrial could used to maintain a steady state after molting of crabs, which can partly explain the reason why KO diet has the highest molting ratio among all diets when all of these results were considered together.

CONCLUSION

In conclusion, swimming crab fed diet supplemented with KO improved lipid metabolism, increased mitochondrial respiration and strengthen energy metabolism of hepatopancreas. Surprisingly, some of these effects were not observed in swimming crab receiving a diet supplemented with FO, emphasizing the influence that the structural form of n-3 PUFAs (esterified to either PL or TAG) has on exerting a biological response. More researches will be needed to find the direct relationships between physiological metabolism and characteristic fatty acid in swimming crab, which can strengthen the exploitation of lipid sources in aquatic feed and deepen the understanding of the physiological metabolism for the fatty acids utilization in swimming crab. These findings could contribute to optimize feeds for swimming crab during its different growth and developmental stages.

ETHICS STATEMENT

In the present study, all procedures complied with Chinese law pertaining to experimental animals. The protocol was approved by the Ethic-Scientific Committee for Experiments on Animals of Ningbo University.

AUTHOR CONTRIBUTIONS

QZ and YY conceived and designed the research. YY and XW conducted the research. YY, PS, and MJ performed the statistical analysis. YY wrote the first draft of the manuscript. All authors contributed to manuscript revision and approved the submitted version.

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frontiers in Environmental Science

REVIEW
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Integrated Remediation Processes Toward Heavy Metal Removal/Recovery From Various Environments-A Review

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Addressing heavy metal pollution is one of the hot areas of environmental research. Despite natural existence, various anthropomorphic sources have contributed to an unusually high concentration of heavy metals in the environment. They are characterized by their long persistence in natural environment leading to serious health consequences in humans, animals, and plants even at very low concentrations (1 or 2 μg in some cases). Failure of strict regulations by government authorities is also to be blamed for heavy metal pollution. Several individual treatments, namely, physical, chemical, and biological are being implied to remove heavy metals from the environment. But, they all face challenges in terms of expensiveness and *in-situ* treatment failure. Hence, integrated processes are gaining popularity as it is reported to achieve the goal effectively in various environmental matrices and will overcome a major drawback of large scale implementation. Integrated processes are the combination of two different methods to achieve a synergistic and an effective effort to remove heavy metals. Most of the review articles published so far mainly focus on individual methods on specific heavy metal removal, that too from a particular environmental matrix only. To the best of our knowledge, this is the first review of this kind that summarizes on various integrated processes for heavy metal removal from all environmental matrices. In addition, we too have discussed on the advantages and disadvantages of each integrated process, with a special mention of the few methods that needs more research attention. To conclude, integrated processes are proved as a right remedial option which has been detaily discussed in the present review. However, more research focus on the process is needed to challenge the *in situ* operative conditions. We believe, this review on integrated processes will surely evoke a research thrust that could give rise to novel remediation projects for research community in the future.

Keywords: integrated approaches, heavy metal, environment, toxicity, review, remediation

INTRODUCTION

Environment comprises of complex variables that includes air, water, and land. Their positive correlation forms a basis for the existence of humans along with other living creatures, namely, plants, animals, and microbes (Kalavathy, 2004). But, the science and technological advances in the form of industrial societies has contributed to severe environmental pollution of air, soil, and water, which are considered to be the indispensable part of human life. Increasing population, urbanization and rapid industrialization are recognized as significant challenges to the groundwater resources management in developing countries. Many research reports have confirmed the heavy metals pollution existence in several countries, thus signifying it as a worldwide problem. Significant concentrations of toxic heavy metals (Cd, As, Fe, Cr, Zn, Cu, Mn, Pb, Ni, etc.) in soil, surface, and ground water have been reported in various countries like China, Italy, Germany, Hong Kong, India, Turkey, Bangladesh, Greece, Iran etc. (Wuana and Okieimen, 2011; Kaonga et al., 2017). Above all these, lack of knowledge on the proper effluent disposal and failure to imply strict regulatory standards has added to the cause of environmental deterioration (Khalid et al., 2017). Therefore, these factors have ended up in generation of huge amounts of solid waste in various toxic forms which ultimately pollute the entire ecosystem. The disposed wastewaters will also affect the quality of surface water and soil, which on continuous proceeding without proper care may cross permissible limits prescribed by international regulatory agencies (E.P.A, 1992, 2002).

Heavy metals are regarded as significant environmental pollutants due to high density and high toxicity even at low concentrations (Lenntech Water treatment Air purification, 2004). According to United States Environmental Protection Agency (USEPA) compilation, eight heavy metals, namely, lead (Pb), chromium (Cr), arsenic (As), zinc (Zn), cadmium (Cd), copper (Cu), mercury (Hg), and nickel (Ni) are listed to be the most widespread heavy metals in the environment (Moore and Ramamoorthy, 1984; Wang and Chen, 2006). According to coordination chemistry of heavy metals, the above said heavy metals are also categorized as class B metals that are non-essential (highly toxic) trace elements (Nieboer and Richardson, 1980; Rzymski et al., 2015). Broad classification of heavy metals with examples is tabulated in **Table 1**. Heavy metals constitute an ill-defined group that is most commonly found at contaminated sites. They are characterized by their long persistence in natural environment leading to serious health consequences in humans, animals, and plants even at very low concentrations (1 or 2 μg in some cases) (Atkinson et al., 1998). A wide array of toxic heavy metals like Cr, Cd, Hg, Pb, etc., disposed by industries will remain as non-degradable and contaminate the soil and water to a greater extent (Aksu and Kutsal, 1990). Because of the high propensity nature of the heavy metals, they tend to accumulate in various environmental matrices, resulting of misleadingly higher concentrations than the prescribed average safety levels (Järup, 2003; Rzymski et al., 2014). According to Comprehensive Environmental Response Compensation and Liability Act, USA, the maximum permissible limit of heavy metals in aqueous medium is as follows, Cr-0.01 mg/L, Ar-0.01 mg/L, Cd-0.05 mg/L, Hg-0.002 mg/L, Pb-0.015 mg/L, and Ag-0.05 mg/L, respectively (Jaishankar et al., 2014). If the heavy metal concentration exceeds than those recommended, it can be major sources of many human life-threatening complications such as atherosclerosis, cancer, Alzheimer's disease, and Parkinson's disease, etc. (Muszynska and Hanus-Fajerska, 2015).

This has urged various researchers to develop many technological processes of remediation to bring these contaminant levels within the regulatory limit in the environment (**Table 2**). Most of the industrial scale remediation involving, physical, chemical, and biological methods are employed as single methods remediation strategies. Despite the success of these processes, they do face certain disadvantages like low efficiency, high cost and toxic sludge generation, etc. However, this can be overcome by upgrading them as integrated processes, which has exhibited more efficiency for heavy metal remediation as reported by many researchers in recent years (Huang et al., 2012; Mao et al., 2016; Selvi and Aruliah, 2018).

During recent years, many treatment options like physical, chemical, and biological were implied to remediate heavy metal contaminated soil, water, and sediments. Such methods include thermal treatment, adsorption, chlorination, chemical extraction, ion-exchange, membrane separation, electrokinetics, bioleaching etc. (**Table 3**). As reported, most of the above said processes are implied as single methods of remediation only. Despite the success of these processes, they do face certain disadvantages like efficiency, cost and failure during large scale implementation, etc. (Volesky, 1990; Selvi et al., 2015). However, these can be

TABLE 1 | Classification of heavy metals with examples.

Class of heavy metals	Examples
Macro-nutrient elements	Cobalt, Iron
Micro-nutrient elements	Copper, Nickel, Chromium, Iron, Manganese, Molybdenum
Highly toxic elements	Mercury Cadmium, Lead, Silver, Gold, Palladium, Bismuth, Arsenic, Platinum, Selenium, Tin, Zinc
Precious elements	Platinum, Silver, Gold, Palladium, Ruthenium
Radio nuclides	Uranium, Thorium, Radium, Cerium, Praseodymium

TABLE 2 | Indian and European standards (EU) standards for heavy metals in soil, food and drinking water (Source: Awashthi, 2000).

Heavy metal	Soil (μg/Kg)	Food (mg/Kg)	Water (mg/L)	EU standards soils (μg/g)
Cd	3–6	1.5	0.01	3
Cr	–	20	0.05	150
Cu	135–270	30	0.05	140
Fe	–	–	0.03	–
Ni	75–150	1.5	–	75
Pb	250–500	2.5	0.1	300
Zn	300–600	50.0	5.0	300
As	–	1.1	0.05	–
Mn	–	–	0.1	–

TABLE 3 | Existing methods of heavy metal removal.

Type of remediation	References
Adsorption	Feng et al., 2010; Hu et al., 2011; Gomez-Eyles et al., 2013
Chlorination	Fraissler et al., 2009; Nowaka et al., 2010; Nagai et al., 2012
Ion exchange	Vilensky et al., 2002; Lin and Kiang, 2003; De Villiers et al., 2005
Chemical extraction	Marinos et al., 2007; Sigua et al., 2016
Membrane separation	Qdais and Moussa, 2004; Al-Rashdi et al., 2011
Electrokinetics	Virkutyte et al., 2002; Zhou et al., 2004; Violetta and Sergio, 2009
Bioleaching methods	Pathak et al., 2009; Peng et al., 2011
Phytoremediation	Gómez-Sagasti et al., 2012; Shabani and Sayadi, 2012

overcome by upgrading them as integrated processes, which has various advantages, such as effectiveness, economic feasibility, short duration, versatile, eco-friendliness, on-site adaptability, and large scale treatment options etc. (Huang et al., 2012; Mao et al., 2016). Correlating to these factors, combined or integrated treatment processes were reported to be more effective by many researchers worldwide (Wick, 2009; Kim et al., 2010; Peng et al., 2011). But, integration of two different processes needs careful understanding and the purpose of the processes. Two processes should to be integrated in such a way that, they should be experimentally feasible even under large scale applications, economically viable and relatively efficient than the individual processes. Owing to these outcomes, integrated processes are gaining popularity toward heavy metal removal from various environmental matrices (Huang et al., 2012; Chen et al., 2013). Therefore, we, here in this review have focussed to discuss on various integrated or combined treatment options implied for heavy metal removal in soil, sediment, sludge, and aqueous matrices. To the best of our knowledge, this is the first review that summarizes different integrated remediation options for heavy metal removal.

METALS AS ENVIRONMENTAL POLLUTANTS

Heavy metals are naturally occurring elements that are found throughout the earth's crust. Heavy metal pollution is caused as a result of both natural and anthropomorphic activities like mining, smelting, industrial production, using of metals, and metal containing compounds for domestic and agricultural applications. These sources were reported to contribute to human exposure and environmental contamination by various researchers (Herawati et al., 2000; Goyer, 2001; Zouboulis et al., 2004; He et al., 2005; Rahman and Bastola, 2014). The potential sources of environmental contaminations are shown in **Figure 1**. Toxicological properties of heavy metals are characterized by persistence of metal (long half-life), soil residence time (>1,000 years), chronic, and sub-lethal effects of the metal, bioaccumulation, biomagnification, teratogenic, and carcinogenic properties of the metal (Manzetti et al., 2014).

HEAVY METALS DISTRIBUTION IN ENVIRONMENT

Natural Sources

Heavy Metals in Rocks

Rocks are one of the natural sources for heavy metals in the environment. Rocks are classified into magmatic rocks, sedimentary rocks, and metamorphic rocks. Magma is a molten rock that contains various chemical elements transported to the earth surface by geological process such as volcanism or plate tectonics (Press and Sievers, 1994). Heavy metals are incorporated via isomorphic substitution into the crystal lattice of primary minerals while magma cools down. Variations in natural weather conditions cause physical damage to the rocks and disintegrate into particles as sediment that can hold water, gas, and oil since it is porous in nature. Mineral calcite present in the sediment is precipitated by living organisms or chemical reaction. This isomorphic substitution is decided by ion radius, charge, and electro negativity. The most common heavy metals occur in rock are Ni, Co, Mn, Li, Zn, Cu, Mo, Se, V, Rb, Ba, Pb, Ga, Sr, F, etc. (Mitchell, 1964).

Heavy Metals in Soils

Rocks disintegrate into fine particles or soil by the influence of ice, water, temperature, etc. The soil matrix is a major reservoir or transporting media for heavy metals, because soil and heavy metals associations have rich and diverse binding characteristics. Metals do not biodegrade like organic pollutants, rather they bioaccumulate in the environment. Soil matrix may adsorb, oxidize, exchange, catalyze, reduce, or precipitate the metal ions (Hashim et al., 2011). These processes depend on several factors such as pH, water content, temperature, particle size distribution, nature of metal, and the clay content. This composition will determine the mobility, solubility, and toxicity of heavy metals present in the soil.

Generally, the minerals are dissolved by interacting with carbonic acid and water. The insoluble minerals are dispersed into fine particles. Soils are contaminated by metals and metalloids from metal wastes, gasoline, animal manure, sludge, waste water irrigation, atmospheric deposition, etc. (Khan et al., 2008). Typical sources of ground water contamination are given in **Table 4** (Spiegel and Maystre, 1998). The most common heavy metals found in soils are Pb, Cr, Zn, Cd, and Hg. Due to bioaccumulation and biomagnification, these metals decrease the crop production and affects the food chain. The soil concentration ranges and regulatory guidelines for some heavy metals are given in **Table 5** (Wuana and Okieimen, 2011).

The heavy metals present in the soil become contaminant due to the following reasons, (i) Rapid generation via man made cycle, (ii) Direct exposure of mine samples due to transportation from mines to environmental location, and (iii) High metal dispose, etc. The heavy metal balance in the soil can be expressed in the form of equation shown below,

$$M_{\text{total}} = \left(M_p + M_a + M_f + M_{ag} + M_{ow} + M_{ip}\right) - \left(M_{cr} + M_l\right) \quad (1)$$

where "M" is the heavy metal, "p" is the parent material, "a" is the atmospheric deposition, "f" is the fertilizer sources, "ag" are the

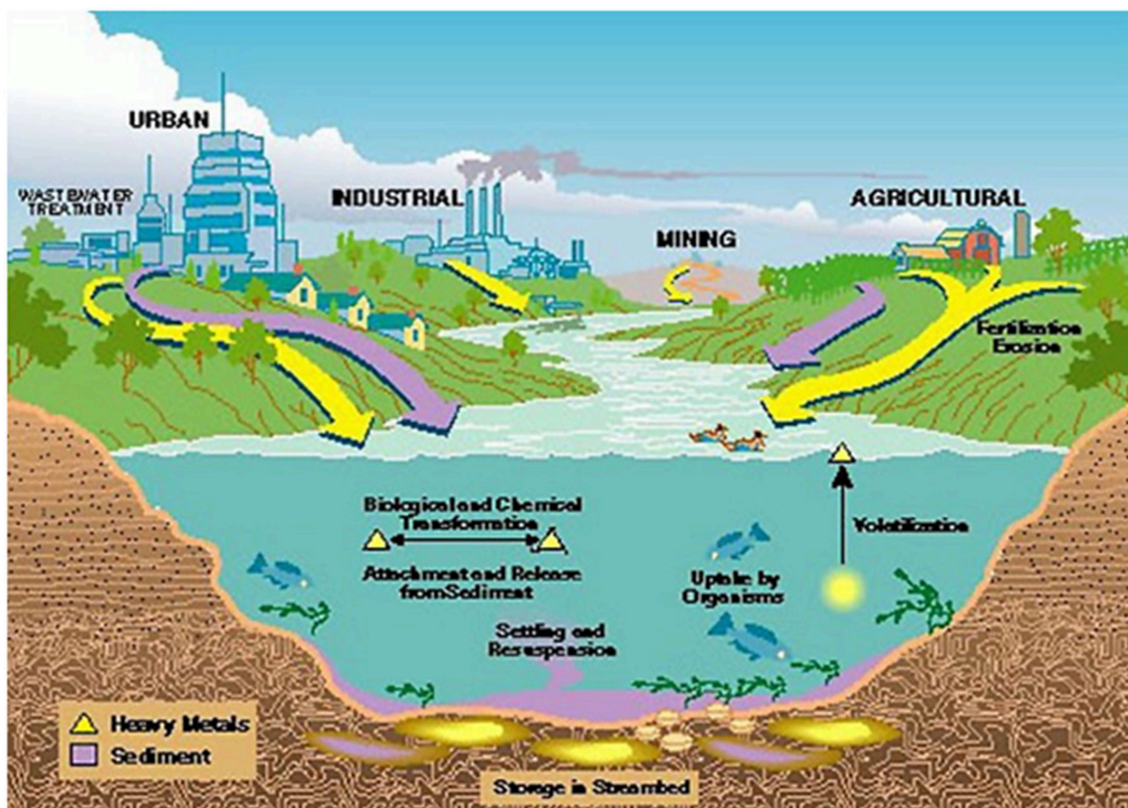

FIGURE 1 | Potential sources of heavy metals in the environment (Source: Garbarino et al., 1995).

TABLE 4 | Typical sources of inorganic substances contributing for ground water contamination (Source: Spiegel and Maystre, 1998).

Source	Inorganic contaminants
Agricultural areas	Heavy metals Salts (Cl^-, NO_3^+, SO_4^{2-})
Urban areas	Heavy metals (Pb, Cd, Zn) Salts
Industrial sites	Heavy metals, metalloids, Salts
Land fills	Salts (Cl^-, NH_4^+) Heavy metals
Mining disposal sites	Heavy metals, Metalloids, Salts
Dredged sediments	Heavy metals, Metalloids
Hazardous waste sites	Heavy metals, Metalloids
Leaking storage tanks	–
Line sources (Motorways, sewerage, railway systems, etc.)	Heavy metals

agrochemical sources, "ow" are the organic waste sources, "ip" are other inorganic pollutants, "cr" is the crop removal, and "*l*" is the losses by leaching, volatilization (Alloway, 1995; D'amore et al., 2005).

Heavy Metals in Water

Metal composition in surface water like rivers, lakes, ponds, etc. is influenced by the type of soil, rock and water flow.

TABLE 5 | Soil concentration ranges and regulatory guidelines of heavy metals (Wuana and Okieimen, 2011).

Metal	Soil concentration range (mg/kg)	Regulatory limits‡ (mg/kg)
Pb	1.00–69,000	600
Cd	0.10–345	100
Cr	0.05–3,950	100
Hg	<0.01–1,800	270
Zn	150–500	1,500

‡*Non-residential direct contact soil clean up criteria.*

Metals present on the surface of soil are carried out from its path, which ends up in sewage and reservoirs (Salem et al., 2000). The rain water gets contaminated while passing through the atmosphere. Water sources get contaminated by the flow of various industrial effluents into it. These industrial effluents were reported to contain many heavy metals as stated in **Table 6**. Ground waters are contaminated from landfill leachates, deep well liquid disposal, industrial wastes, etc. (Oyeku and Eludoyin, 2010). Factors such as temperature, pH, living organism, cation exchange, evaporation, absorption, etc., will also influence the metal composition in the water.

TABLE 6 | Occurrence of metals or their compounds in effluents from various industries (Source: Nagajyoti et al., 2010), Copyright 2018 Springer Nature.

	Al	Ag	As	Au	Ba	Bi	Cd	Co	Cr	Cu	Fe	Hg	Mn	Mo	Pb	Ni	Sn	Zn
Mining	x		x				x					X	x	x	x			
Metallurgy		x	x			x	x		x	x		X			x	x		x
Dyes and Pigments	x		x				x			x	x				x			
Alloys																		
Leather	x		x		x				x	x	x	X						x
Textiles	x		x		x		x			x	x	X				x		
Petroleum	x		x				x		x		x	X			x	x		x
Fertilizer	x		x				x		x	x	x	X	x			x		x

Heavy Metals in Atmosphere

Heavy metals are released into the atmosphere as gases and particulates by surface erosion and colloid loss. Sources of heavy metals in the atmosphere include, mineral dusts, sea salt particles, volcanic eruption, forest fires (Colbeck, 1995). Other than these natural sources, heavy metal air pollution can also originate from various industrial processes that involve the formation of dust particles, e.g., metal smelters and cement factories. Volatile metals such as Se, Hg, As, and Sb are transmitted in gaseous and particulate form in the atmosphere. Metals such as Cu, Pb, and Zn are transported as particulate form. The presence of heavy metal depends upon number of site-specific factors such as (1) the quantity and characteristics of the industrial pollutants, (2) environmental sensitivity, (3) potential for environmental release, (4) proximity of these heavy metals in humans and its effect on their health (Hassanien, 2011).

Anthropogenic Sources of Heavy Metals

Heavy metals are released into environment by various anthropogenic activities. The introduction of heavy metals due to continuous input of pesticides and fertilizer for food production is transported to surface water by infiltration (Darby et al., 1986). Zn and Cd are commonly present in phosphate fertilizers and the input of these fertilizers is directly proportional to the concentration of heavy metals. In addition to Zn and Cd, pesticides used in agriculture have elements such as Hg, As and Pb too. Thought he metal based pesticides are no longer in use, the earlier unregulated pesticide application has led to increased accumulation of heavy metals in various environmental matrices. Added to these, various industrial activities such as mining, coal combustion, effluent streams, and waste disposal has increased the heavy metal contamination in the environment (Herawati et al., 2000; Goyer, 2001; He et al., 2005). The most common anthropogenic sources contributing to heavy metals into the environment are listed in **Figure 2**.

Need for Remediation of Metals in the Environment

The presence of heavy metals released from various sources is either directly or indirectly released into the environment that affects humans, animals, and plants. The main pathways of exposure are through inhalation, ingestion, and dermal contact. Due to increased risk of human exposure to heavy metals, it leads to serious health implications and environmental deterioration (Rzymski et al., 2015). Hence, these metals are categorized as systemic toxicants that can induce adverse health effects in humans that include cardiovascular diseases, developmental abnormalities, neurologic and neurobehavioral disorders, diabetes, hearing loss, hematologic and immunologic disorders, and various types of cancer (IARC, 1993; Mandel et al., 1995; Hotz et al., 1999; Steenland and Boffetta, 2000; WHO, 2001; Järup, 2003). Human health implications of heavy metal are shown in **Figure 3**. The severity of adverse health effects differs with the type of heavy metal, the chemical form, time of exposure, and the dosage. These heavy metal contaminants in soil were also reported to affect the ecosystem by disturbing the food chain, reducing the food quality due to phytotoxicity, and loss of soil fertility etc. (McLaughlin et al., 2000a,b).

In India, the heavy metal concentration in industrial areas is much higher than the permissible level as reported by World Health Organization (WHO), thus exposing humans to occupational hazards (Manivasagam, 1987). This scenario of the serious health hazards due to heavy metal pollution can be contributed to negligence of the industries in the form of direct discharge of untreated effluent into environment, failure to imply strict regulations by government environmental protection agencies in developing countries, and the non-reliability of the present individual remediation methods toward *in situ* and large scale applications.

TYPES OF INTEGRATED PROCESSES

Chemical-Biological Remediation Approach

This process of chemical–biological integrated treatment is considered to be a highly economical and eco-friendly alternative to treat heavy metals containing wastewater. Implementation of this integrated treatment than the individual chemical or biological treatment has been reported to be advantageous and has shown significant results of heavy metal removal by many researchers worldwide (Rahman and Murthy, 2005; Abdulla et al., 2010; Rahman and Bastola, 2014; Greenwell et al., 2016; Mao et al., 2016; Pradhan et al., 2017). When implied alone, both the treatments face, their merits, and demerits. In case of chemical method of remediation, its simple operation and quick results have made this method as one of the most widely used remediation worldwide. However, the production of insoluble metal precipitates and toxic by-products has greatly

FIGURE 2 | Anthropogenic sources of heavy metals. (Source: https://www.slideshare.net/tutan2009/heavy-metal-pollution-in-soil-and-its-mitigation-aspect-by-dr-tarik-mitran).

limited this method (Fu and Wang, 2011). On the other hand, biological treatment is considered advantageous due to its environmental friendliness and economic feasibility. But their limitations include, long acclimatization time, changes in the biodegradable efficiency of the isolate and generation of sludge (Lohner and Tiehm, 2009). However, these limitations can be ruled out by integrating both the methods with a proper understanding of individual method's mechanism. Generally, this type of integrated system involve biological treatment followed by chemical treatment and vice-versa, that acts as a polishing step due to its effectiveness and economic feasibility as reported by few researchers (Ayres et al., 1994; Goswami and Mazumder, 2014). In one of the study by Ahmed et al. (2016), a combined approach of chemical precipitation and biological treatment toward Cr(VI) removal from tannery effluent was reported with a successful recovery of 99.3 and 98.4% of total Cr and Cr(VI), respectively. It was also shown to reduce 77% of chemical oxygen demand (COD) and 81% of turbidity. A similar study of combined process of chemical precipitation and biological system using *Fusarium chlamydosporium* was reported to reduce 64.69% of turbidity, 71.80% of COD, and 62.33% of total chromium (Sharma and Malaviya, 2014). Though this method has gained popularity among researchers, a responsible and an eco-friendly choice of non-toxic chemicals will surely aid in the success of this method.

Electro-Kinetic Microbial Remediation Approach

In this kind of remediation process, the organic matter is electrochemically converted to generate useful by-products, produce bioelectricity, and fuel by the action of microbial metabolic processes (Logan and Rabaey, 2012). As soil contains the majority of heavy metal in insoluble form, their removal rate was minimum, so the solubility can be achieved by coupling electrokinetic with other techniques. On the other hand, if the metal ion was in "soluble" form in the soil, then the remediation rate will be maximized. Based on these implications, electro-kinetic (EK) technique was introduced around 1980s and was widely employed to manage heavy metal contaminated fine-grain soils of low hydraulic conductivity (Maini et al., 2000).

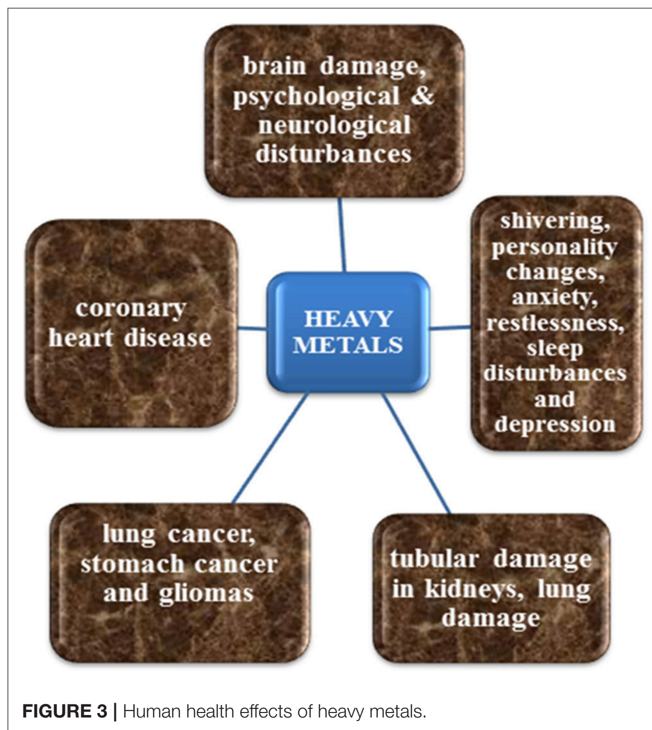

FIGURE 3 | Human health effects of heavy metals.

Here a direct electric current was used to remove fine and low permeability heavy metal particles from the soil with minimum disturbance to the surface. As voltage was applied between two sides of the electrolytic tank containing contaminated soil, an electric field gradient was created. This low-level electric current aid as a cleaning agent by stimulating the pollutants to transport toward recovery wells involving mechanisms such as electro-osmotic flow, electromigration, and electrophoresis thereby inducing electrochemical reactions (Acar and Alshawabkeh, 1993). The main advantage of this method is its simple operation, cost-effective, and no subsequent pollution (Zhou et al., 2004; Deng et al., 2009; Violetta and Sergio, 2009; Ma et al., 2010). But, EK method has also certain restrictions like, bioavailability of the heavy metal and mass transfer between the electrode and pollutants (Simoni et al., 2001; Lohner and Tiehm, 2009).

In order to increase its overcome these restrictions and to achieve high efficiency, an interesting idea of integrating EK remediation with biological method was used and got succeeded by many researchers. This integration was reported to promote increased bioavailability of the pollutants, enhancement in biodegradation efficiency by generating oxidization and reduction zones, releasing of soil/sediment bound pollutant, improved nutrient transport, improved performance, and availability of terminal electron acceptors (Maini et al., 2000; Luo et al., 2005; Wick, 2009; Kim et al., 2010; Peng et al., 2011; Selvi and Aruliah, 2018). As a biological counterpart, both acidophilic and alkalophilic microbes were employed. If the acidic bacterium is involved, it will favor EK, whereas the alkalophilic will aid in metal precipitation. In a few instances, few microbes may require additional nutrients as an energy source (glucose, starch etc.) to survive in the EK cell (Choi et al., 2013). Some of the interesting works on Bio-EK integrated system, implied by the scientific community were discussed in detail here. One such study by Rosestolato et al. (2015) on bio-electrokinetic method was reported in which, 400 kg of mercury contaminated soil was successfully remediated by with a maximum removal of 60%. In a study of EK assisted bioremediation carried out by Azhar et al. (2016a) removal of mercury from the contaminated soil was reported. Electrokinetic study was conducted using electric current of voltage 50V for a period of 7 days, which was followed by microbial remediation using *Lysinibacillus fusiformis* bacteria. The result concluded that higher removal rate of mercury to 78% was achieved within a shorter period of 7 days. In another study of zinc removal, EK assisted bioremediation using *Pseudomonas putida* showed 89% removal in 5 days (Azhar et al., 2016b).

With a future perspective of symbiotic combination strategies using electrochemically active bacterial cells and electrified interfaces, Varia et al. (2013) reported on bioelectrochemical remediation of gold, cobalt, and iron metal ions using gamma Proteobacteria, *Shewanella putrefaciens* CN32. Their demonstration concluded on microbial influenced electronation thermodynamics of the metal ion, with an outcome of prospective energy savings. A similar study by Kim et al. (2012) demonstrated removal of heavy metals such as arsenic, copper, and leads using an integrated system of bioelectrokinetics (bioleaching-electrokinetic). They have employed *Acidithiobacillus ferrooxidans* species to carry out bioleaching process as it was capable to oxidize the reduced sulfur and ferrous ions. This creates an acidic environment in the soil, which was reported to as a suitable condition for removal of heavy metals (Nareshkumar et al., 2008). Peng et al. (2011) too reported on significant reduction of 296.4 to 63.4 mg/Kg of Cu and 3,756 to 33.3 mg/kg of Zn in sewage sludge, within 10 days using an indigenous iron-oxidizing bacteria and EK remediation.

In this Bio-EK integrated remediation, bioleaching process was carried out initially to convert the metal to soluble form which favors a faster and higher rate of remediation in electrokinetic method a follow up process in bioelectrokinetics. From the obtained results they have concluded that the maximum removal of heavy metal was achieved with minimal power consumption, than used for conducting individual EK remediation. A similar bioelectrokinetics remediation work was reported by Huang et al. (2012) to remove copper, zinc, chromium and lead from the polluted soils. In this experiment, soil samples were collected and oxidized using iron containing bacterial species and the soil was further treated by electrokinetic method, by which, the metals will start to eliminate with change in the pH of soil. The corresponding elimination of metal ions of copper, zinc, chromium, and lead was monitored and reported with maximum removal rate.

Dong et al. (2013) used electrokinetic coupled biostimulation method to remove lead from Pb-oil co-contaminated soil. A pilot study was conducted for a period of 30 days in which surfactant (Tween 80) and chelating agents (EDTA) was added to enhance EK operating conditions. The addition of EDTA was found to play a role in eliminating the heavy metal toxicity in soil and this coupled technique reported 81.7% removal of lead from the soil.

Electrokinetic-Phytoremediation Approach

This is an emerging method of remediation that has proved to be more effective in terms of metal recovery and being more economical than the other integrated approaches discussed previously. This combination was initiated with the outstanding results of EK remediation and its compatible operation with phytoremediation (**Figure 4**). When phytoremediation is employed as an individual process, they may offer an economical solution, but, its *in situ* application is limited by climatic conditions, metal bioavailability, and shallow depths (Barber, 1995). The recovery yield and process rate also require a significant improvement. However, this can be enhanced by combining phytoremediation with different strategies like transgenic technology, bioaugmentation, remediation with electrokinetics, permeable reactive barrier (Cameselle et al., 2013). Laboratory studies on EK and phytoremediation approach has exhibited a respectable vision in heavy metal remediation of Zn, Pb, Cu, Cd, and As. Electrokinetics was also found to play an important role in phytoremediation. A direct current passed between electrodes which placed vertically in soil separates organic and inorganic molecules (Cao et al., 2003; Santos et al., 2008). Depending on the plant's uptake mechanisms, different strategies like, phytoextraction, phytoevaporation, phytostabilization, rhizodegradation, and rhizofiltration were employed for phytoremediation (Halim et al., 2003; Cui et al., 2007; Kotrba et al., 2009; Ghosh, 2010; Lotfy and Mostafa, 2014; Mao et al., 2016).

Bhargavi and Sudha (2015) used an electrokinetic assisted phytoremediation process to reduce the levels of chromium and cadmium. In their study, the samples were taken from Bharathi Nagar and Tandalam village of the Ranipet Industrial area. The collected samples were first remediated using EK method, followed by phytoremediation by extruding the remediated soil samples from the electrokinetic cell. The EK remediated soil was potted to grow the plant *Brassica Juncea*. For electrokinetic treatment, 50 V of electric current was applied and the removal rate was monitored at a regular interval of time from 5 to 25 days. They reported on 67.43 and 59.78% removal efficiency of cadmium and chromium after 25 days of treatment. This EK remediated soil, employed for phytoremediation showed a promising accumulation of cadmium and chromium in single harvest, which was further increased in subsequent harvests. A similar study was conducted by Lim et al. (2004) to remove lead from polluted soil using mustard plant. Compared to controls, the electric field assisted phytoremediation showed 2–4 times effective removal of lead in the soil. Cang et al. (2012) have remediated cadmium, copper, lead and zinc from the soil by using integrated methods of electrokinetic assisted phytoremediation. They concluded that the property of the soil was directly influenced by the voltage applied and the growth of plant increased the enzymatic activity of soil to achieve a maximum heavy metal remediation.

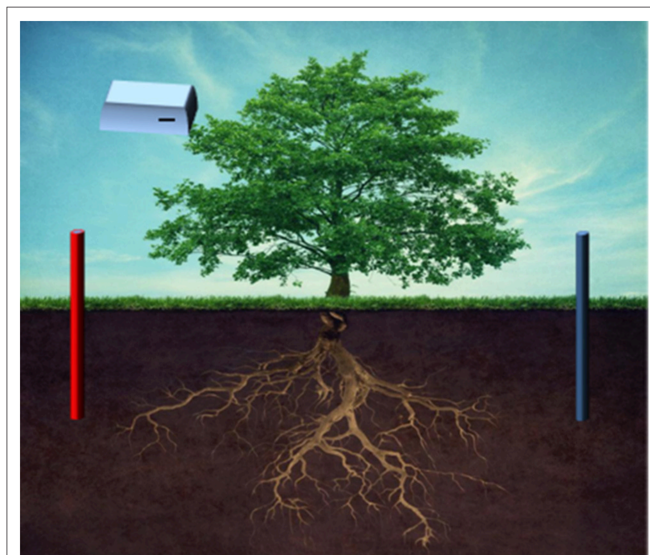

FIGURE 4 | Schematic diagram of electro-kinetic coupled/enhanced phytoremediation (Source: Mao et al., 2016) Copyright 2018, Elsevier.

Electrokinetic coupled phytoremediation using species *Lemna minor* was tested by Kubiak et al. (2012) to remediate toxic arsenic in water. For this test, artificial arsenic water was prepared using sodium arsenate at a concentration of 150 $\mu g\ L^{-1}$. Their preliminary results showed a higher removal rate of 90% at the end of the experiment. In an another study of lead removal from soil was reported by Hodko et al. (2000), in which the EK remediation was carried out by applying several electrode configurations to enhance phytoremediation by increasing the depth of soil to prevent the leaching of mobile metals on the ground surface.

Phyto-electrokinetic remediation under laboratory scale was studied by O'Connor et al. (2003) in which the soil samples were contaminated artificially with metal ions followed by the measuring of removal rate. One of the soil samples was contaminated by copper and the other by cadmium with arsenic. The test soils were filled in the reactor in two separate chambers. An electric current of 30 V was applied simultaneously by seeding with rye grass. A significant removal rate was reported over a period of 98 and 80 days for Cu and Cd-As soil, respectively. EK enhanced phytoextraction demonstrated by Mao et al. (2016) removed lead, arsenic and caesium from soil by lowering the pH of soil to 1.5, which resulted in dissolution of heavy metals to a larger extent with an increased solubility and bioavailability of heavy metals. It was then followed by phytoextraction using plants that enhanced the effectiveness of metal removal from the soil.

Phytobial Remediation Approach

Phytobial remediation is an efficient and eco-friendly solution to remove heavy metals from soil and water. Phytobial remediation utilizes plants as well as microbes to remove heavy metals from soil and water. As mentioned in literature, phytobial based remediation utilizes the plants to uptake the heavy metals and the microbes will help in degradation of those metallic substances (Lynch and Moffat, 2005). **Figure 5** portrays different mechanism viz., (i) Bioprecipitation of metals, (ii) Bioaccumulation of metal by metal binding proteins, (iii) Binding of metals on the cell surface, (iv) Biotransformation of metals,

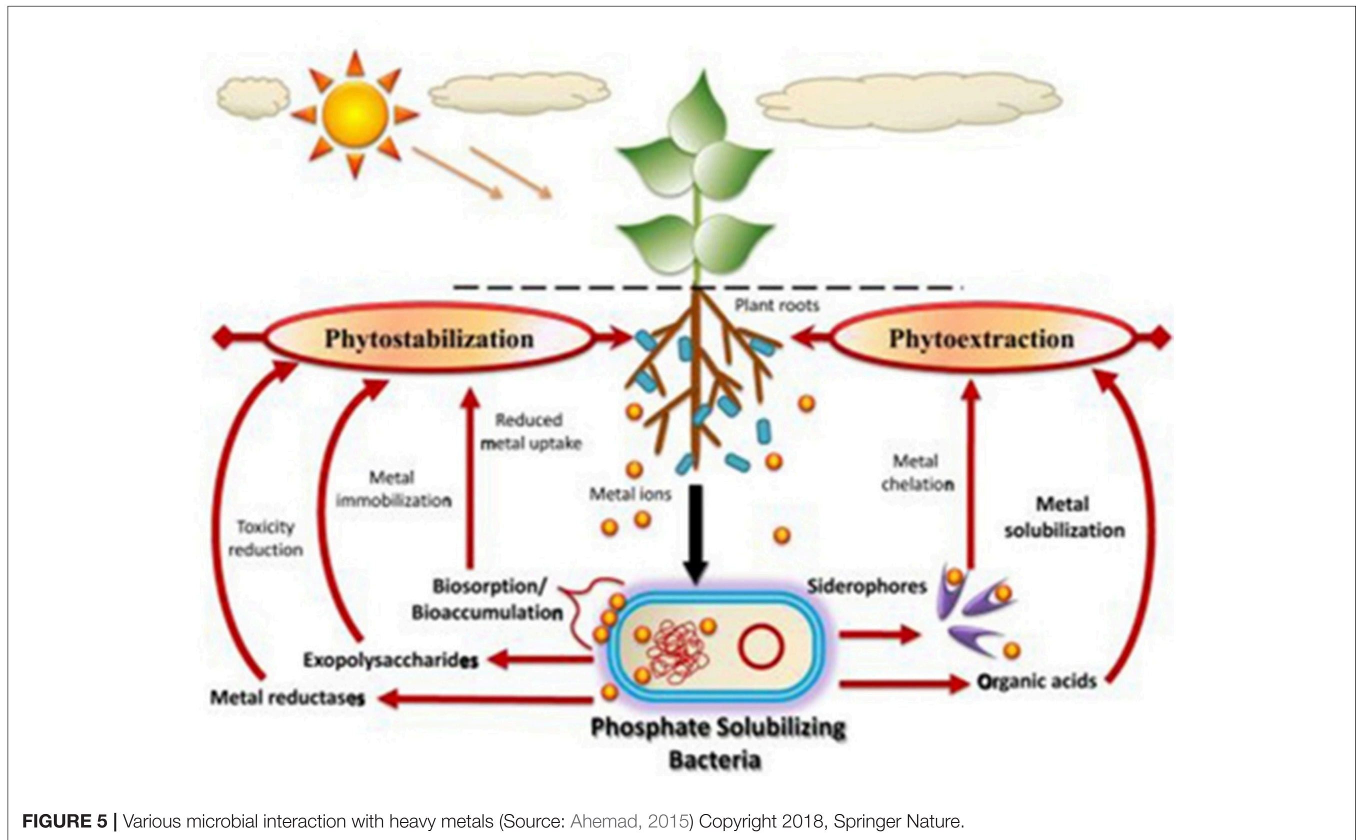

FIGURE 5 | Various microbial interaction with heavy metals (Source: Ahemad, 2015) Copyright 2018, Springer Nature.

(v) Methylation of metals, (vi) Solubilisation of metals, (vii) Biosorption of metals, (ix) Metal reduction, (x) Siderophores secretion, (xi) DNA-mediated interaction toward heavy metal removal (Ahemad, 2015). These mechanisms can be enhanced by integrating a suitable bacterium that can secrete multiple plant growth promoting substances (PGPS) (Martin and Ruby, 2004). These substances include organic acids, ACC deaminase, siderophores, and biosurfactants that will transform the metals into a bioavailable form (Roy et al., 2015). **Table 7** summarizes the PGPS secreted by various phosphate solubilizing bacteria (PSB).

Phytobial remediation is recognized as cleanest and cheapest approach unlike other invasive technologies. It also has an advantage of being applied to vast areas of contaminated groundwater, soil and sediment. In addition, its *in situ* application option was found to decrease the heavy metal distribution in the soil and aids in preserving the top soil. Despite these advantages, this method is restricted to shallow aquifer and soil due to plant root length restriction, potential fear of transfer of heavy metals to the food chain, long duration (may require several seasons), regular monitoring (due to litter fall), lack of safe proper disposal method, tough metal recovery procedures, and high recycle economy. Roy et al. (2015) has offered few solutions to overcome these issues by using deep rooted plants, designing of transgenic plants that distracts herbivores, development of suitable evaluation methods, to integrate with other methods like bioremediation, EK, and bioaugmentation, etc. Different types of microbes involved in phytobial remediation are discussed here in detail.

Phytobial Remediation Using Free Living Organism

Free living microbes assist phytoremediation by mobilization, immobilization, and volatilization. Mobilization of metals occurs by different reactions such as volatilization, redox transformation, leaching, and chelation. The microbes like *Sulfurospirillum barnesii*, *Geobacter,* and *Bacillus selenatarsenatis* are used for arsenic removal. Lee et al. (2009) developed a hybrid method by using anaerobic bioleaching and electrokinetics. The plant used for phytoremediation accumulates heavy metals as harvested tissue, which can be disposed off. Introducing mobilizing microbes into contaminated water speed up the process of heavy metal accumulation (Wang et al., 2005). During the immobilization process, the mobility of the contaminant is prohibited by altering the physical and chemical properties (Leist et al., 2000). The oxidase enzymes present in the microbes oxidize the metals and make them immobilize and less toxic. The microbes such as *Sporosarcina ginsengisoli, Candida glabrata, Bacillus cereus,* and *Aspergillus niger* were used in immobilization technique to remove heavy metals (Littera et al., 2011; Giri et al., 2012). In a biotransformation process, a large number of bacteria, fungi, and algae were employed in heavy metal removal using biomethylation process (Frankenberger and Arshad, 2002).

Endophyte Remediation

Certain bacteria and fungi that live within the plants are called endophytes. They live within the plant for at least a part of their life cycle without damaging the host. They are ubiquitously

TABLE 7 | Plant growth promoting substances realeased by phosphate solubilizing bacteria (Source: Ahemad, 2015), Copyright 2018 Springer Nature.

PGPR	Plant growth promoting traits
Pseudomonas aeroginosa strain OSG41	IAA, siderophores
Pseudomonas sp.	IAA, HCN
Acenetobacter haemolyticus RP19	IAA
Pseudomonas putida	IAA, Siderophore, HCN, ammonia
Pseudomonas fluorescens strain Psd	IAA, Siderophore, HCN, antibiotics, biocontrol activity
Bacillus thuringiensis	IAA
Pseudomonas aeroginosa	IAA, Siderophore, HCN, ammonia
Pseudomonas sp. TLC 6-6.5-4	IAA, Siderophore
Bacillus sp.	IAA, HCN
Klebsiella sp.	IAA, Siderophore, HCN, ammonia
Enterobacter asbariae	IAA, Siderophore, HCN, ammonia
Bacillus species PSB10	IAA, Siderophore, HCN, ammonia
Arthrobacter sp. MT16, *Microbacterium* sp. JYC17, *Pseudomonas chloraphis* SZY6, *Azotobacter vinelandii* GZC24, *Microbacterium lactium* YJ7	ACC deaminase, IAA, Siderophore
Pseudomonas sp.	IAA, Siderophore, HCN, biocontrol potentials
Enterobacter aerogenes NBR1 24, *Ravanella aquatilis* NBRI K3	ACC deaminase, IAA, Siderophore
Enterobacter sp.	ACC deaminase, IAA, Siderophore
Burkolderia	ACC deaminase, IAA, Siderophore
Pseudomonas aeroginosa	ACC deaminase, IAA, Siderophore

ACC 1- aminocyclopropane 1-carboxylate, HCN-hydrogen cyanate, IAA-Indole-3-acetic acid.

associated with most of the plant, of which some can promote plant growth (Ryan et al., 2008). Few fungal endophytes will produce secondary metabolites too. Methylobacterium strains from *Pteris vittata* herb was reported to exhibit heavy metal tolerance (Dourado et al., 2012). However, this endophyte remediation needs more research to explore the potential of unstudied endophytobiome.

Rhizomicrobe Remediation

Rhizosphere refers to the root region of the plant. Certain microbes present in this region forms a symbiotic association with the plant by secreting exudates, secretions, mucilages, mucigel, and lysates that help in plant growth (Kirk et al., 1999). For example, siderophores secreted by microbes will help in chelation and solubilisation of metals. Based on these secretions, rhizo-remediation can induce plant growth, immobilize heavy metals, and accumulation of metals. Siderophores having different ligand binding groups can bind to different metals. Siderophores produced by *Pseudomonas azotoformans* reported to mobilize and remove arsenic (Díaz de Villegas et al., 2002). Since the root microbes are aerobic in nature, the increased pH at the rhizosphere zone favors the mobilization and uptake of heavy metals. The increased pH is due to the cation and the anion uptake ratio in rhizospheric region (Nair et al., 2007). Yang et al. (2012) reported that the plant-microbial consortium secretes biosurfactants that helps in immobilizing metals by increasing the pH of the rhizosphere.

Fungal Phytoremediation

Many plants have an association with mycorrhizal fungi which increase the surface area of plant roots and help them to get more water and nutrients (Sylvia et al., 2005). Recent studies demonstrated that the mycorrhizal fungi can enhance the accumulation and uptake of heavy metals by plants. *Glomus mosseae, Glomus geosporum,* and *Glomus etunicatum* are mycorrhizal fungi present in *Plantago lanceolata* L, that were reported to enhance arsenic (As) accumulation by few researchers (Wu et al., 2009; Orłowska et al., 2012).

Algal Phytoremediation

Algae are regarded as an important component of aquatic system that plays a significant role in bio-geochemical cycle. It has received immense attention of researchers worldwide due to their exceptional absorption and sequestration capability. It also possesses high tolerance to heavy metals, selective removal, ability to grow both autotrophically and heterotrophically, synthesis of metallothioneins and phytochelatins, and can serve as potential agents for genetic alterations (Hua et al., 1995). Algal species such as microalgae (e.g., *Dunaliella salina*), macroalgae (*Ulva* sp., *Enteromorpha* sp., *Cladophora* sp., and *Chaetomorpha* sp), green algae (*Enteromorpha, Cladophora*), and brown algae (*Fucus serratus*) were extensively reported to accumulate appreciable quantities various heavy metals (Rainbow, 1995; Gosavi et al., 2004; Al-Homaidan et al., 2011). Aquatic plants such as *Eichhornia crassipes, Pistia stratiotes, Colocasia esculenta, Spirodela polyrhiza, and Lemna minor* have also been widely studied toward heavy metal remediation.

Enhanced Phytoremediation Approaches

It is quite obvious that an extensive technology is needed to remove heavy metals from the environment to bring them down to the permissible levels. Though it can be achieved by various integrated processes as discussed above, recombinant genetic engineering of bacteria and plants has also proved to be worthy in terms of heavy metal removal applications. Microbes have tremendous remediation potential when they are subjected to genetic modification, by which they can perform better than the wild type. Similarly, phytoremediation can also be triggered by genetic engineering to enhance the accumulation and uptake of heavy metals. The "ars" operon in "arsR" gene code for a regulatory protein which aid in sensing arsenic contamination. Kostal et al. (2004) prepared a recombinant *E. coli* with "ars," gene which accumulated 60-fold higher level of arsenic than the control organism. "Ars" operon incorporated recombinant strain is best suited for *in situ* remediation option to perform bioremediation under real conditions (Ryan et al., 2007). Transgenic canola plants incorporated with *Enterobacter cloacae* CAL2 has accumulated four times more heavy metals than the control cells. Introduction of transgenic plant was reported to enhance the capacity of the plant toward heavy metal removal from soil (Eapen et al., 2003).

Other Integrated Approaches

With the successful remediation of the integrated processes discussed above, there are few other novel research attempts on integrated processes. Jones (1996) was the first person to conduct an electrokinetic-geosynthetic approach to remove metals from the contaminated soil. Geosynthetic material increases the mobility of pollutants and so the remediation rate using electric current will also be increased. This method was also proven to be successful for heavy metal removal from the soil. An integrated approach of using permeable reactive barrier along with microbes is a technique where the dissolved contaminants filter out as it flows. The removal occurs when the contaminated water flows through the permeable reactive barrier treated area in its flow path (Köber et al., 2005). This material is incorporated with microbes and/or plants which have the capability to absorb heavy metal present in ground water. Peng et al. (2015) have conducted integrated electrokinetic remediation coupled with membrane filtration to reduce the level of iron, zinc and calcium. They have made a comparative study and reported on the nanofiber assisted removal, which showed a maximum efficiency of metal ion removal than the individual electrokinetic method. An electric voltage of 25 V and 50 V were applied to carry out the electrokinetic study followed by filtration using polyacrylonitrile nanofiber (PANN) membrane. The removal rates of Zn^{2+}, Fe^{3+} and Ca^{2+} were about 99.15, 98.03, and 99.73%, respectively. Vocciante et al. (2016) have conducted electrokinetic coupled soil washing to remediate heavy metal as it will convert insoluble metal ions in the soil to mobile forms and thereby facilitating the rate of metal removal to a greater extent. Using this coupled technique heavy metals such as antimony, arsenic, cadmium, chromium and mercury was removed effectively. The process occurs based on *in situ* soil washing. However, this technique needs to be validated in large scale (Aboughalma et al., 2008).

Future Projections

Implementation of biotechnological approaches is gaining increasing prominence in the field of remediation, as they are often considered as a promising strategy for the eventual treatment of contaminated sediments. As far as heavy metal removal is concerned, a detailed understanding of metal-induced mechanisms are imperative to devise an effective remediation option, because the heavy metals are known to cause serious health implications such as fertility impairment, genetic, epigenetic, and biochemical alterations as discussed in above sections of this review (Rzymski et al., 2015). This is due to the complexity and uniqueness of the contaminated sites caused by heavy metals.

Remediation methods in general use include physical separation, isolation, immobilization, toxicity reduction, and extraction. But, implementation of two or more techniques in a synergistic mode had resulted in better results, which were quite evident with the results discussed in the present review. Based on the wide literary review, any integrated processes involving EK processes had shown promising results. However, the more research focus is needed on the right remedial option that can challenge *in situ* operative conditions such as site characteristics (geographical location, pH levels, particle size, clay, soil type, depth, water content, climate, types of co-contaminants, etc.). Hence, remediation projects of the future should be capable of assessing the ecological impact, an important environmental criterion. And research innovations in terms of more integrated processes are in great demand. Owing to their wide application, effectiveness, and economic feasibility, few processes viz., phytobial remediation, chelate extraction, and chemical soil washings processes needs more research evaluations. Therefore, more attention should be paid to the evaluation methods for assessing the remediation effectiveness while developing new remediation technologies in future research. Above all, a strict implementation of standard regulations by government agencies and stern action against industries that are responsible for toxic environmental discharges will certainly make a noticeable change in levels of heavy metals in the environment.

CONCLUSION

This review discusses on different sources, need for removal, and related health hazards due to heavy metal in the environment. From the study, it is quite obvious that the anthropogenic activities have been significantly contributing to high concentrations of heavy metal discharge into the environment. Therefore, a serious and strict monitoring of these activities is suggested as an effective solution to address heavy metal pollution. However, a complete background knowledge on the sources of heavy metal, their chemistry, and potential risks posed to environment and humans are needed to select an appropriate remedial option. In this regard, many research investigations of various integrated options that are available for heavy metal removal/recovery from the contaminated environment are systematically summarized in this review. Based on our reviewed literature, processes with an integrated approaches were found to a serve as an effective alternative for removal of toxic heavy metals and recovery of valuable metals from highly contaminated industrial sites. Therefore, we conclude that the integrated processes involving EK processes and phyto-remediation had shown astonishing results of considerable reduction in the level and toxicity of heavy metals, with minimal disturbance to the natural environment. We also believe that these integrated technologies can be highly applicable for *in situ* operations in both developed and developing countries where, urbanization, agriculture, and industrialization are leaving an inheritance of environmental degradation.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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frontiers in Marine Science

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Offshore Crude Oil Disrupts Retinoid Signaling and Eye Development in Larval Atlantic Haddock

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The aim of this study was to examine the impact of dispersed crude oil on retinoid metabolism and signaling in developing haddock larvae (*Melanogrammus aeglefinus*). Retinoids and its active metabolites are involved in the regulation of several developmental processes as cell differentiation, growth and limb patterning. Thus, disruption of retinoid signaling, especially during early developmental stages, may have detrimental effects on the organism. In the present study, crude oil exposure during embryonic development and early larvae development disrupted vitamin A signaling by affecting retinoid levels and genes involved in retinoid metabolism. The disruption of retinoid signaling was also evident when looking at the gene expression pattern at several time-points during and after exposure, demonstrating the complexity and temporal factor of retinoid signaling disruption. Exposure during the embryonic period (pre-hatch) resulted in reduced eye size and increased incidence of abnormal eye morphology. The observed changes in expression of retinoid metabolic genes related to the visual cycle indicates that there is a link between the modulated retinoid levels (all-*trans* retinoic acid and retinol) and increased incidence of abnormal eye development as a result of crude oil exposure. This is the first study to demonstrate disrupted retinoid signaling in fish following crude oil exposure at environmental relevant levels, giving novel insight in to the mechanism of toxicity.

Keywords: retinoid, contaminant, vitamin A, teleost, development, retinoic acid, crude oil, eye development

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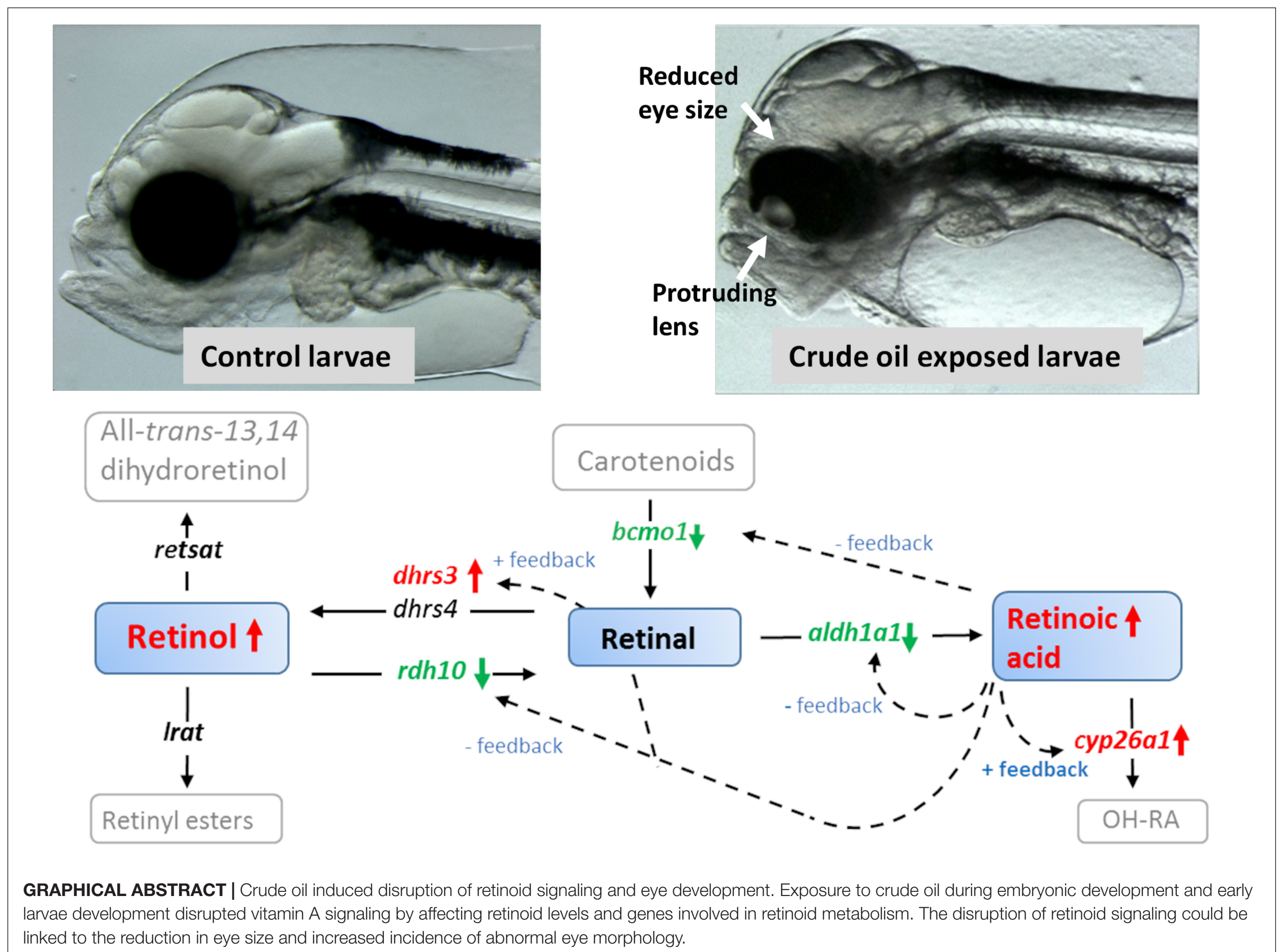
INTRODUCTION

Oil spills, as a consequence of offshore oil production, constitute a threat to the marine environment. The toxicity of crude oil to teleosts and its effects on natural stocks have been thoroughly documented through field and laboratory studies (Hodson, 2017). The recent Deepwater Horizon accident in the Gulf of Mexico in spring 2010 was a strong reminder of the ecological and economical implications of uncontrolled oil discharges on natural fisheries and stocks.

The studies in the aftermath of the Exxon Valdez and the Deepwater Horizon oil spills demonstrated the teratogenic and ultimately lethal effects oil compounds can have on fish (Norcross et al., 1996; Dubansky et al., 2013; Incardona et al., 2013, 2014) The Exxon Valdez oil



GRAPHICAL ABSTRACT | Crude oil induced disruption of retinoid signaling and eye development. Exposure to crude oil during embryonic development and early larvae development disrupted vitamin A signaling by affecting retinoid levels and genes involved in retinoid metabolism. The disruption of retinoid signaling could be linked to the reduction in eye size and increased incidence of abnormal eye morphology.

spill in Alaska overlapped with the spawning seasons of pink salmon (*Oncorhynchus gorbuscha*) and Pacific herring (*Clupea pallasii*). Field and laboratory studies in the aftermath of this disaster disclosed high occurrence of severe developmental abnormalities such as craniofacial deformities, spinal curvature deformities, yolk sack edema, pericardial edema and increased mortality following exposure to crude oil (Carls and Rice, 1990; Norcross et al., 1996; Carls et al., 1999; Incardona et al., 2004, 2014; Carls and Thedinga, 2010; Hicken et al., 2011). The detrimental effects of oil exposure on cardiac function and craniofacial development was recently also demonstrated in Atlantic haddock (*Melanogrammus aeglefinus*) exposed to crude oil (Sorhus et al., 2016b, 2017). It was proposed that crude oil influence transcription related to calcium signaling, resulting in abnormal development (Sorhus et al., 2017). Single PAH components and crude oil from various sources have been shown to interrupt ion signaling, which is linked to effects on cardiac development (Incardona et al., 2004, 2009; Brette et al., 2014, 2017; Sorhus et al., 2016b).

In one of our previous studies we observed that exposure to crude oil modulated the expression of several genes related to vitamin A metabolism, such as cytochrome P450, family 26, subfamily A (*cyp26a*), retinol dehydrogenases and retinoid binding proteins in Atlantic cod (*Gadus morhua*) larvae (Olsvik et al., 2012). Disturbance of vitamin A homeostasis has been observed in both fish and mammals following exposure to persistent organic pollutants (POPs) (Novák et al., 2007, 2008) and heavy metals (Alsop et al., 2007; Defo et al., 2012) and contaminated sediments. Vitamin A levels have thus been used as markers of contaminant exposure in environmental monitoring. However, the mechanisms behind these disturbances seem to be heterogeneous and remain to be fully elucidated. Many of the observed deformities following crude oil exposure, such as heart, limb and craniofacial deformities, coincide with malformations that may be caused by disruption of vitamin A signaling (Cahu et al., 2003; Haga et al., 2003; Laue et al., 2008; Spoorendonk et al., 2008; D'Aniello and Waxman, 2015). Thus, knowledge on how retinoid signaling is affected by crude oil might give novel insight in to mechanisms of toxicity.

Retinoids is a generic term used for a class of compounds chemically related to vitamin A. The name also reflects its importance for retina function and eye health. The retinoids comprise the three mother compounds, retinol,

retinal and retinoic acid, and all their metabolic products and isomers. However, retinol (often referred to as vitamin A) is physiologically inert and must be converted to other biological active metabolites of which all-*trans* retinoic acid (atRA) is regarded as the most potent. During development, retinoids are controlling patterning and limb development [reviewed by Ross et al. (2000)]. In adult organisms, retinoids and their active metabolites are essential for normal cell homeostasis, growth, remodeling, reproduction and vision. Several studies have demonstrated that both excessive and deficient intake of vitamin A can affect development, causing severe morphological abnormalities (Azaïs-Braesco and Pascal, 2000) including heart defects (Colbert, 2002; Pan and Baker, 2007) and bone development in fish (Takeuchi et al., 1998; Mazurais et al., 2008, 2009).

Our knowledge on the effect of environmental contaminants on retinoid signaling and the underlying mechanisms of toxicity are mainly derived from studies using organochloride compounds such as dioxins and polychlorinated biphenyls (PCBs). However, the mechanistic effects might be compound-specific (Widerak et al., 2006; Benisek et al., 2008; Novák et al., 2008; Benisek et al., 2011). Aryl hydrocarbon receptor (AhR) activation followed by cytochrome P450, family 1, subfamily A (CYP1A) induction is the mode of action for several known toxic compounds such as the organochlorides and polyaromatic hydrocarbons (PAHs). This is also believed to be one of the most important factors disrupting retinoid signaling (Murphy et al., 2007; Berntssen et al., 2015). AhR agonists have been shown to affect retinoid signaling on multiple levels in the retinol metabolic pathway. It is postulated that the activation of CYP1A, which is capable of retinoic acid hydroxylation, increases the metabolization and excretion rate of atRA. This will in turn increase the demand for atRA precursors such as retinol and retinal, and result in depletion of retinoid stores as demonstrated in adult zebrafish (*Danio rerio*) (Alsop et al., 2007) and Atlantic salmon (*Salmo salar*) (Berntssen et al., 2016) following benzo(a)pyrene (BaP) exposure. In salmon, this retinoid disruption was accompanied by reduced growth. Other routes of retinoid signaling disruption have also been suggested (Widerak et al., 2006; Benisek et al., 2008, 2011).

The aim of this study was to examine the disruptive impact of dispersed crude oil on vitamin A metabolism and signaling in developing Atlantic haddock larvae. Atlantic haddock were exposed to crude oil during two important periods of organogenesis and growth. The first group (embryo group) was exposed during the embryonic period from 2 to 10 days post-fertilization (dpf). The second group (larvae group) was exposed to crude oil from 0 to 18 days post-hatch (dph). Retinoid levels, gene expression, eye size and eye deformities were analyzed in the developing haddock larvae.

MATERIALS AND METHODS

Fish Husbandry and Exposure

For detailed procedures on fish collection and breeding, and larval maintenance and exposure, see Sorhus et al. (2016b). In brief, a wild broodstock population spawning voluntarily in captivity provided the eggs. Eggs were collected from the broodstock tanks and incubated in egg incubators until transfer to exposure tanks. Two days post-fertilization, eggs were transferred to 50 L circular exposure tanks. At 4 dph, the larvae were fed with natural zooplankton (van der Meeren et al., 2014; Karlsen et al., 2015) and the tanks were further supplemented with marine microalgae concentrate (Instant Algae, Nanno 3600, Reed Mariculture Inc., Campbell, CA, United States) until termination of experiments.

The crude oil used in the experiments was an artificially weathered crude oil from the Heidrun oil field in the Norwegian Sea. To generate an oil in water dispersion an oil droplet generation system described elsewhere was used (Nordtug et al., 2011). This system generates an oil dispersion of droplets in the low μm range with a nominal oil load of 26 mg/L that again are diluted in the exposure tanks. The experimental exposure system consisted of four replicates of three treatments and control: 60 μg oil/L (Low dose), 600 μg oil/L (High dose), 600 μg oil/L for 2.4 h in a 24 h period (Pulse dose), and no oil (Control). Water Chemistry and embryo and larvae body burden of PAHs are shown in Sorhus et al. (2016b). The oil doses correspond to water doses of PAHs from 0.7 to 7 μg/L ΣPAH.

A schematic view of the exposure periods and sampling time points is presented (**Figure 1**). In the embryo experiment, the exposure was terminated at 10 dpf (8 days of exposure), and all surviving embryos were transferred to new tanks with seawater without crude oil. At 13 dpf, 50% hatching was observed and set to 0 dph. In the Larvae experiment, the exposure started at 0 dph, and ended at 18 dph (18 days of exposure). Haddock larvae used for retinoids were deprived of food prior to sampling. Sampling time points were chosen from different stages in development in relation to Sorhus et al. (2016a) and in relation to time of exposure and hatching. Since retinoid depletion has previously been shown to be the outcome of PAH exposure in adult fish, we collected samples for retinoid just after hatching (before start feeding) for the embryo exposure study and ca 12 h post-exposure for the larvae exposure study.

All animal experiments within the study were approved by NARA, the governmental Norwegian Animal Research Authority (http://www.fdu.no/fdu/, reference number 2012/275334-2). All methods were performed in accordance with approved guidelines.

Quantification of Retinoid Metabolites in Fish Larvae Using LC-APCI-MS/MS

Retinoid analysis was performed by VITAS AS (Norway, Oslo). Pools of about 150 Atlantic haddock larvae (about 150 mg tissue in total) were homogenized with 600 μl acetonitrile containing a C_4 labeled internal standard (C_4-atRA) (n = 4 for all groups except Low (n = 3) and Pulse (n = 2) group from the Larvae exposure). After thorough homogenizing (30 s), centrifugation (20 min, 4000 rpm, 9°C), some of the supernatant were transfer to an HPLC vial with insert (500 μl) and centrifuged again (20 min,

4000 rpm, 9°C), 10 μl of the supernatant was then injected into the HPLC system (**Table 1**). HPLC was performed with a HP 1200 Liquid Chromatograph (Agilent Technologies, Palo Alta, CA, United States) with a 6460 Triple Quad LC-MS/MS detector using Multiple Reaction Monitoring (MRM). All-*trans* retinol, all-*trans* retinal, all-*trans* retinoic acid, 9-*cis* retinoic acid and 13-*cis* retinoic acid were separated on a 25 cm × 3 mm, 5 μm reversed phase column (Supelco, Suplex PKB-100). The column temperature was 40°C. Four-point calibration curves were made for the respective retinoids. **Table 1** shows the run parameters for HPLC and MS/MS. Due to the amount of sample material needed for the retinoid analysis we were only able to collect material from one timepoint for each of the groups. Also due to the high mortality in the High crude oil embryo group, we did not get enough material to measure the retinoid levels in this group. The larvae were sampled from replica tanks for each group.

Measurements of Eye Size and Deformities

Digital still micrographs of live larvae were obtained with an Olympus SZX-10 stereo microscope equipped with a 1.2 Mp resolution video camera (Unibrain Fire-I 785c) controlled by BTV Pro 5.4.1 software[1]. Image magnification was calibrated with a stage micrometer. Measurements of eye diameter and body length at 3 dph (embryonic exposure) and 9 dph (larval exposure) were performed using ImageJ (ImageJ 1.48r, National Institutes of Health, Bethesda, MD, United States[2]) with the ObjectJ plugin[3]. To correct the eye diameter measurements to compensate for differences in size, the eye diameter was also expressed as % of standard body length. Developmental abnormalities of the eye were analyzed by observing individual differences in eye shape. Fish were registered and categorized according to four observed phenotypes: (1) no phenotype, normal round shape, (2) bend shape, (3) irregular shape, and (4) protruding lens. The number of analyzed animals

[1] www.bensoftware.com
[2] http://rsb.info.nih.gov/ij
[3] https://sils.fnwi.uva.nl/bcb/objectj/index.html

TABLE 1 | Run parameters for LC-APCI-MS/MS retinoid analysis.

HPLC	
Flow rate	1.2 ml/min
Injection volume	10 μl
MS/MS source parameters	
Polarity	Positive
Gas Temp (°C)	350
APCI Heater	450
Gas flow (l/min)	6
Nebulizer (psi)	60
Capillary (V)	4500
APCINeedlePos	4
Fragmentor	130
CID	15
Dwell	250 (200 for internal standard)
MS/MS multiple reaction monitoring	
Standard	Transition
Retinol	269.3→213.1
Retinal	285.2→161.0
13-*cis* and 9-*cis*	301.4→120.9
All-*trans* retinoic acid	301.4→120.9
Internal standard	305.4→129

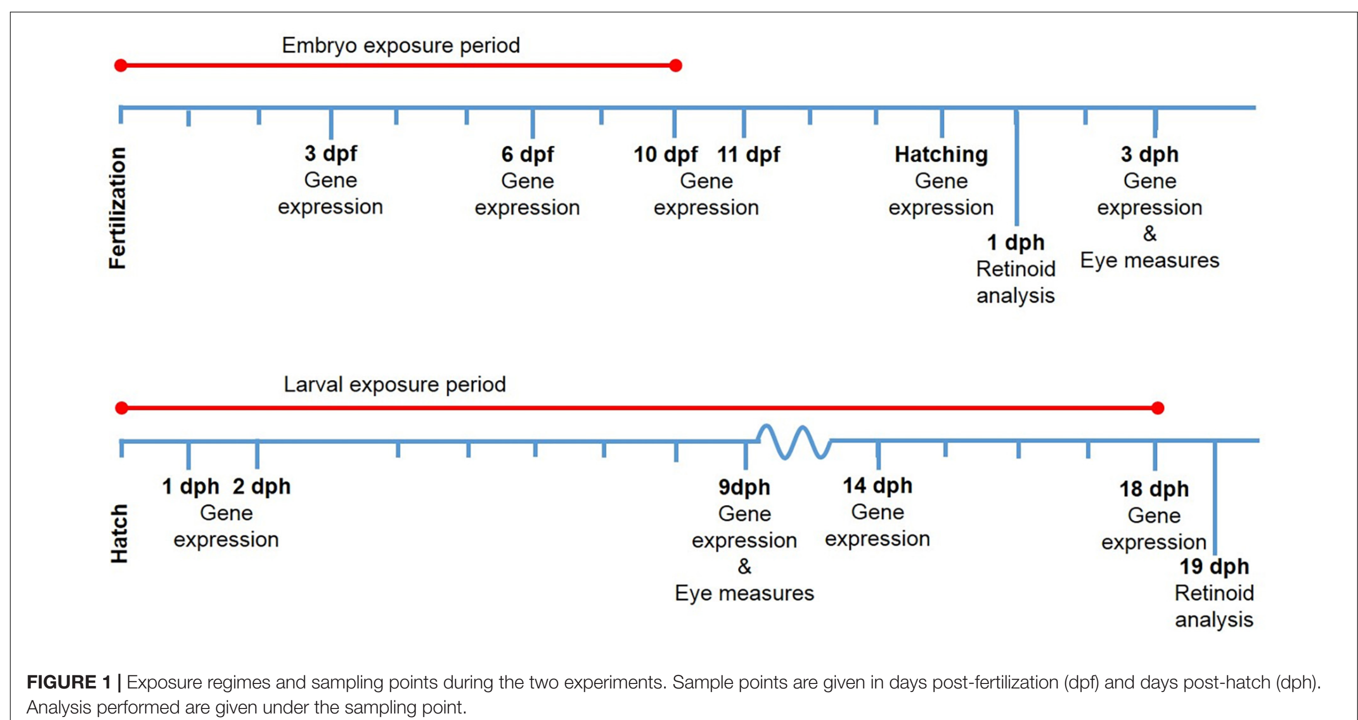


FIGURE 1 | Exposure regimes and sampling points during the two experiments. Sample points are given in days post-fertilization (dpf) and days post-hatch (dph). Analysis performed are given under the sampling point.

per treatment was 80 (Control, High dose) or 60 (Pulse and Low dose) in the embryonic exposure and 48 for all in the larval exposure.

RNA Extraction and RNA-Seq Analysis

For detailed procedure for extraction of mRNA, RNA sequencing (RNA-seq), and bioinformatics, see previous studies (Sorhus et al., 2016a, 2017). In brief, pools of live embryos (15–25) and larvae (10) were inspected and imaged under microscope before snap frozen in LN2. Total RNA was isolated from the pools using Trizol reagent (Invitrogen, Carlsbad, CA, United States), followed by a DNase treatment step using TURBO DNA-free kit (Life Technologies Corporation) according to manufacturer's instructions. cDNA library preparation was performed by the Norwegian Sequencing Centre (NSC, Oslo Norway) using the Illumina TruSeq RNA sample preparation kit. Illumina HiSeq2500 platform was used to sequence the paired end libraries. The raw data are available at the Sequence Read Archive (SRA), NCBI (Accession ID: PRJNA328092). The RNA sequencing data were mapped to the coding sequences of the cod genes (Star et al., 2011) using the Bowtie aligner (Langmead et al., 2009) and annotated as described in Sorhus et al. (2016b). Samtools idxstat (Li et al., 2009) was used to extract the number of mapped reads which were then normalized to the total number of mapped sequences.

Statistical Analysis

The retinoid levels were analyzed using a one-way ANOVA followed by Dunnett's multiple comparison test in GraphPad Prism v 7.02 for Windows (Windows, GraphPad Software, La Jolla, CA, United States). Statistical analysis of RNAseq data was conducted using the Qlucore omics explorer v3.2 software (Qlucore AB, Lund, Sweden). Microsoft® Excel ® 2013 (Microsoft Corporation, Mountain View, CA, United States) was used to construct a heat map based on fold change values of differentially expressed genes ($p < 0.05$). Statistical difference in eye diameter/body length % between groups were tested in R with one-way ANOVA using the Tukey–Kramer multiple comparison test.

RESULTS

Quantification of Retinoids in Pooled Whole Larvae Homogenates

While most of the retinoids were in the form of retinal in the 1 dph larvae and very little as retinol (**Figure 2**), this changed in the 18 dph larvae showing higher levels of retinol. Altered levels of retinoids were observed following continuous and pulse exposure to crude oil during embryogenesis (**Figure 2A**). Both the Pulse exposure and Low exposure caused significant increase ($p < 0.05$) in retinol levels in the larvae 3 days after exposure had ended and the embryos had been transferred to clean water. Due to high mortality in the High exposure group, samples for retinoid analyses were therefore not available. No difference ($p > 0.05$) was observed in retinal levels between the embryo exposed groups at this point. Both retinol and retinal was significantly modulated compared to Control in larvae exposed after hatching (**Figure 2B**). While crude oil exposure caused an increase in the retinol levels, the retinal levels were decreased.

In the embryo experiment, retinoic acid levels were increased in the Low exposure group but not in any of the exposed groups in the larvae experiment (**Figure 3**). Both 13-*cis* retinoic and 9-*cis* retinoic acid were below the detection limits of 10 and 2 ng/g, respectively. The detection limit for 13-*cis* retinoic acid was set at 10 ng/g due to an unidentified interfering component. No differences in total levels of retinoids (retinol + retinal + retinoic acid) were observed between the groups when summing up all analyzed retinoids in the present experiment (**Figure 4**).

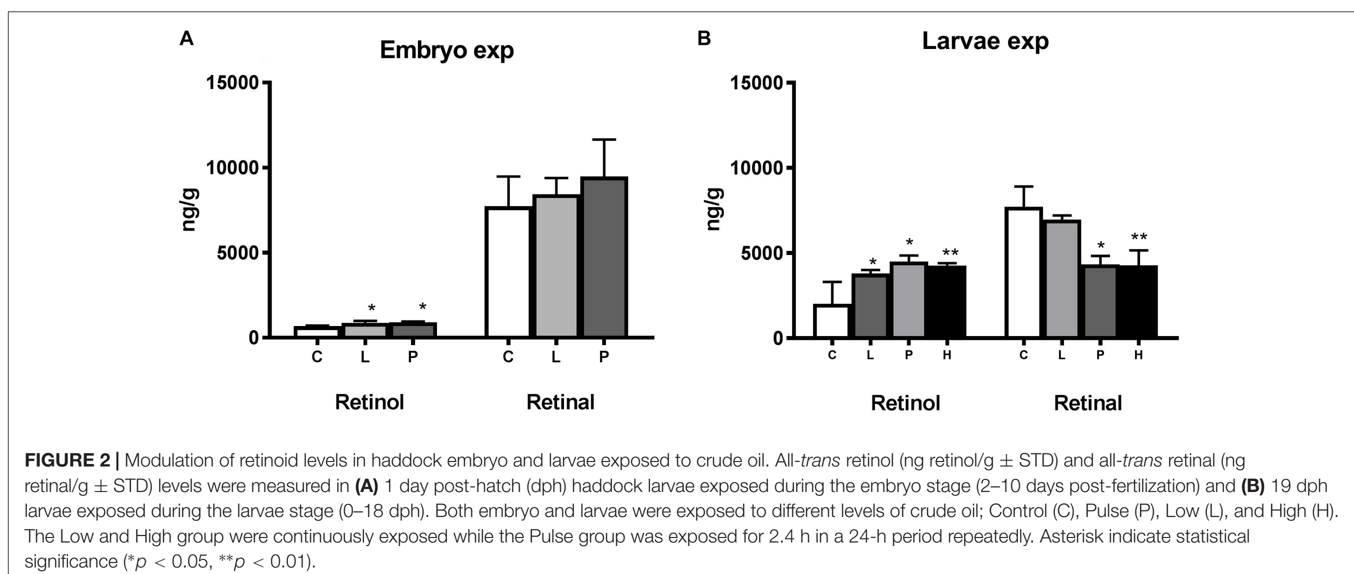


FIGURE 2 | Modulation of retinoid levels in haddock embryo and larvae exposed to crude oil. All-*trans* retinol (ng retinol/g ± STD) and all-*trans* retinal (ng retinal/g ± STD) levels were measured in **(A)** 1 day post-hatch (dph) haddock larvae exposed during the embryo stage (2–10 days post-fertilization) and **(B)** 19 dph larvae exposed during the larvae stage (0–18 dph). Both embryo and larvae were exposed to different levels of crude oil; Control (C), Pulse (P), Low (L), and High (H). The Low and High group were continuously exposed while the Pulse group was exposed for 2.4 h in a 24-h period repeatedly. Asterisk indicate statistical significance (*$p < 0.05$, **$p < 0.01$).

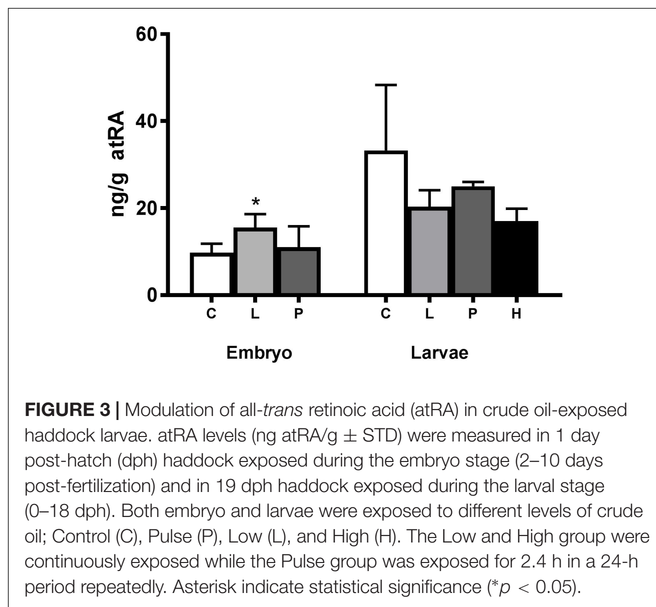


FIGURE 3 | Modulation of all-*trans* retinoic acid (atRA) in crude oil-exposed haddock larvae. atRA levels (ng atRA/g ± STD) were measured in 1 day post-hatch (dph) haddock exposed during the embryo stage (2–10 days post-fertilization) and in 19 dph haddock exposed during the larval stage (0–18 dph). Both embryo and larvae were exposed to different levels of crude oil; Control (C), Pulse (P), Low (L), and High (H). The Low and High group were continuously exposed while the Pulse group was exposed for 2.4 h in a 24-h period repeatedly. Asterisk indicate statistical significance (*$p < 0.05$).

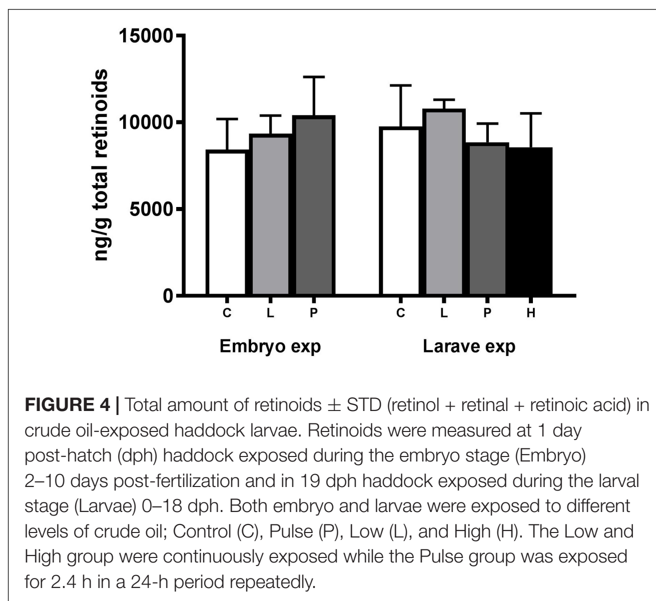


FIGURE 4 | Total amount of retinoids ± STD (retinol + retinal + retinoic acid) in crude oil-exposed haddock larvae. Retinoids were measured at 1 day post-hatch (dph) haddock exposed during the embryo stage (Embryo) 2–10 days post-fertilization and in 19 dph haddock exposed during the larval stage (Larvae) 0–18 dph. Both embryo and larvae were exposed to different levels of crude oil; Control (C), Pulse (P), Low (L), and High (H). The Low and High group were continuously exposed while the Pulse group was exposed for 2.4 h in a 24-h period repeatedly.

Expression of Genes Related to Retinoid Metabolism

Differential expressed genes (DEGs) involved in retinoid metabolism was determined at several time-points during the two experiments. For the embryo experiment, gene expression was measured from 24 h after exposure start (3 dpf) until 3 dph, 6 days after exposure ended. For the larvae exposure, gene expression was measured at 1 dph, 1 day after exposure start, until 18 dph at the end of exposure period. The heat map (**Figure 5**) indicates distinct differences in gene expression profiles between the two exposure regimes (Embryo and Larvae) following 600 μg oil/L (High) crude oil exposure. While 51 out of 68 DEG observations were counted as down-regulated in the Embryo experiment, only 5 out of 41 DEG observations showed down-regulation in the Larvae experiment. Very few of the investigated genes were differentially expressed ($p < 0.05$) in the other exposure groups (**Supplementary Table S1**).

Differential Expression of Genes Related to Retinoid Metabolism Following Embryonic Exposure

For the embryonic exposure group, none of the investigated genes were differentially expressed the first day following exposure start. Number of genes that were differentially expressed increased as the exposure experiment progressed. Interestingly, the differential expression of several of the genes involved in retinol metabolism and signaling was still evident 6 days after the eggs had been transferred to clean water for hatching and further development. Aldehyde dehydrogenase 1 family, member a1 (*aldh1a1*) showed even stronger down-regulation as the experiment progressed, peeking several days after transfer to clean water.

There were also clear differences in regulation of several genes during exposure (2–10 dpf) compared to post-exposure (0–3 dph). Although, the exposure was ended at 10 dpf, there was a continued exposure at 11 dpf due to remaining oil droplets bound to the eggshell. Genes coding for key enzymes which catalyzes the conversion of retinal to retinol such as dehydrogenase/reductase 3 (*dhrs3*) was up-regulated post-exposure, while the gene encoding the protein catalyzing the conversion of retinol to retinal, retinol dehydrogenase 10b (*rdh10b*), was up-regulated in the during exposure and down-regulated post-exposure. Two possible homologous genes encoding *rdh10* have been identified in haddock, of which one *rdh10a* did not show any differential expression between High dose and control. Two homologs were also found for all-*trans*-retinol 13,14-reductase (*retsat*) and lecithin retinol acyltransferase (*lrat*).

In the High group, *cyp26a1* showed down-regulation at 6 and 10 dpf during exposure and up-regulation post-exposure (0 dph (hatch) and 3 dph) in the Low exposure group ($p < 0.05$) (**Figure 6**). *Cyp26a1* tended also to be increased in the High group ($p < 0.07$) post-exposure.

Differential Expression of Genes Related to Retinoid Metabolism Following Larvae Exposure

Similar as during the embryo exposure, genes related to retinol metabolism such as *retsat*, *lrat*, and *rdh10* were up-regulated in larvae (**Figure 5**). Different from during embryo exposure, Beta-Carotene 15,15′-Monooxygenase 1 (*bcmo1*) and *cyp26a1* were both up-regulated and no differential expression was observed for *aldh1a1*.

Effects on Eye Size and Deformities

Eye diameter (**Figures 7A,C**) and eye/length ratio (eye diameter divided by the standard length of the larvae) (**Figures 7B,D**) were measured in 3 dph haddock larvae exposed during the embryonal period (Embryonal exposure) and in 9 dph haddock larvae exposed during the larval stage. Reduced eye diameter was observed for all the exposure groups. However, when the eye diameter was normalized using total length of the larvae, effects of crude oil exposure on eye/length ratio were

	Embryo exposure						Larvae exposure				
	Exp start		Exp end				Exp start				Exp end
Gene name	3 dpf	6 dpf	10 dpf	11 dpf	0 dph	3 dph	1 dph	2 dph	9 dph	15 dph	18 dph
abca4			-1.5	-1.3	-1.9	-1.9	-1.1				
abca4				-1.5	-2.0	-1.4					
aldh1a1		-1.3	-1.6	-2.1	-5.3	-8.1					
aldh1a2			-1.4								
aldh1a3			1.3						1.3		
bcmo1				-4.3						1.5	1.3
bcmo1			-2.0		-2.0	-2.0			2.8		
crabp1				-1.4							
crabp2		1.4									1.1
crabp2										-1.4	
cyp1a1	5.4	134.3	151.2	95.5		1.4	20.9	18.3	39.4	38.4	24.3
cyp26a1		-2.2	-1.9							1.7	
dhrs3					1.6					1.6	
dhrs4											1.5
lrat										2.3	
lrat			1.4	1.3							
rarb							-1.4			1.3	
rarb		-3.8	-2.1								
rbp1											
rbp2			1.6	1.7					1.4		
rbp3					2.7					6.5	
rbp3				-2.2	-1.4					1.5	1.5
rbp4a					1.2	1.8					1.4
rdh10			-3.0	-3.2	-2.9				1.6		1.6
rdh10a							1.3				1.2
rdh10b		9.7			-1.5	-1.7		1.4	1.5	1.5	
rdh11			2.1	2.1							
rdh12				-2.0	-1.5						
rdh12										-1.3	
rdh12				-1.4						2.0	
rdh12				1.4							
rdh7		-1.8		-1.3					1.3		
rdh8		1.9		-1.3							
rdh8			-7.9	-5.2	-5.5	-2.6					1.3
retsat			12.1						2.8	3.3	1.5
retsat		-1.3	-1.1								
rlbp1			-1.3	-1.3	-1.2	-1.3	-1.1			1.5	1.4
rlbp1		-1.6	-2.7	-2.6	-2.0				1.4		1.3
rpe65			-2.9	-3.3					1.2		1.2
rpe65									-1.3		
stra6			-1.6	-2.3							
stra6			-2.1	-2.2	-1.8	-1.6				1.4	1.5

FIGURE 5 | Significantly differentially expressed genes ($p < 0.05$) related to vitamin A homeostasis in haddock larvae and embryo following 600 μg oil/L (High) crude oil exposure. Blue colors suggest down-regulation while red color suggests up-regulation. Fold change is given for each significant reading ($p < 0.05$). Two exposure experiments were conducted of which one group were exposed during the embryonic stage (Embryo exposure) 2–9 days post-fertilization (dpf) and one group during the larval stage (Larvae exposure) 0–18 days post-hatch (dph). 0 dph is defined as just after hatching at 14 dpf.

only observed in the Low and Pulse group compared to Control in haddock exposed during the embryonal period. No differences were observed for the 9 dph haddock exposed during larval period. The variation in ratio was also notably larger in the High embryo exposure group compared to the Low and Pulse group.

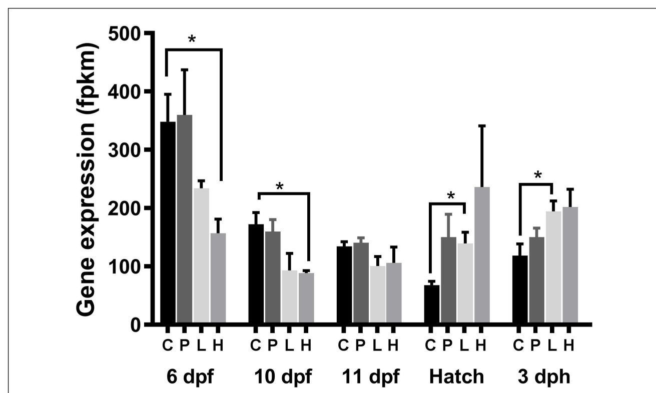


FIGURE 6 | Expression of *cyp26a1* in haddock exposed during the embryonic period. Data was derived from the RNA-seq analysis. Both embryo and larvae were exposed to different levels of crude oil; Control (C), Pulse (P), Low (L), and High (H). The Low and High group were continuously exposed while the Pulse group was exposed for 2.4 h in a 24-h period repeatedly. Asterisk denotes significant differential expression between groups ($p < 0.05$) following Qlucore Omics differential expression analysis.

Presence of eye deformities and irregularities were observed in embryo exposed to crude oil. Three phenotypes were observed in addition to the normal state (no phenotype) (**Figure 8** and **Table 2**). While 95% of the Control group had no abnormal eye morphology, 59% of the High and 58% of the Low group showed abnormal eye morphology. Protruding lens was the most prevalent phenotype in both the High (42%) and Low group (34%). The Pulse group seemed less affected.

DISCUSSION

Disruption of Retinoid Metabolism

Exposure to off-shore crude oil resulted in a marked disruption of retinoid signaling and metabolism following exposure during both the embryonic stages and during early larvae development in haddock. Increased retinol levels were observed in all exposure groups. In addition, decreased retinal levels were observed in the High exposure larvae group. The present findings suggest that crude oil exposure shunts the retinoid metabolism toward retinol following embryonic and larval exposure (**Figures 9A,B**). This is further supported by the gene expression data which shows increased *dhrs3* and decreased *rdh10b* pointing toward increased reduction of retinal (**Figure 9A**). Both enzymes encoded by these genes are key regulators of retinol metabolism acting as important safe guards against excessive levels of retinoic acid.

Exposure During Embryogenesis Caused Post-exposure Effects on Retinoic Acid Signaling

In addition to increased levels of the parent compound, we observed increased levels of the retinoid metabolite atRA in the haddock exposed during embryonal development. Retinoic acid is regarded as a potent nutrient hormone with teratogenic potential [reviewed by Ross et al. (2000)] and the tissue concentration act as a signal to individual cells modulating gene expression, cell function and limb patterning (Ross et al., 2000; Niederreither et al., 2002; Pan and Baker, 2007). Thus, disruption of atRA signaling can have detrimental effects on the developing embryo in both mammals (Ross et al., 2000) and fish (Takeuchi et al., 1998; Haga et al., 2002, 2003). The disruption of retinoid homeostasis and signaling was also evident when looking at the gene expression data. The increased expression of *cyp26a1* at hatch and 3 dph supports the results showing increased atRA levels post-hatch following crude oil exposure during the embryonal period. This key enzyme is directly regulated by atRA through positive feedback mechanism in both fish and mammals (Thatcher and Isoherranen, 2009; Lie and Moren, 2012). The expression patterns of *bcmo1*, *aldh1a1*, *dhrs3*, and *rdh10b* further supports the observed increase in retinoic acid post-exposure (**Figure 9A**). They are all regulated through feedback loops in order to control the levels of the potent nutria-hormone atRA (Elizondo et al., 2000; Strate et al., 2009; Feng et al., 2010; Lobo et al., 2010). Strict local regulation of atRA is crucial for normal development of both heart and craniofacial structures (Ross et al., 2000). The present results indicate that the feedback machinery is trying to counteract the increased retinoic acid levels post-exposure (**Figure 9A**). In contrast to the embryo exposure study, no significant changes in atRA levels were observed following exposure during early larval stages. This probably reflects the difference in sensitivity of the embryonic stage vs. the larval stage. In line with this, a recent parallel study focusing on heart and skeletal defects (Sorhus et al., 2017), showed that haddock was more vulnerable to crude oil exposure during the embryonal period compared to the larval period.

Gene Expression Indicate Decreased Retinoic Acid Signaling in Embryos During Exposure

It was originally hypothesized that an increase in cytochrome P450 enzymes (i.e., CYP1A, CYP1B, and CYP2B) following crude oil exposure, as observed in the present study, could cause increased unspecific atRA hydroxylation with subsequent decrease in atRA levels (Berntssen et al., 2015, 2016). Although we observed an increase in atRA post-exposure rather than a decrease, atRA was not measured during the actual exposure period in the embryo group. However, the down-regulation of *cyp26a1* during exposure, indicates reduced atRA levels during the embryonal exposure at 6–10 dpf. This is also supported by the strong up-regulation of *rdh10b* (**Figure 9A**) and the downregulation of retinoic acid receptor b (*rarb*), both regulated by retinoic acid (Strate et al., 2009; Topletz et al., 2015; Shabtai et al., 2018). Similar to the present study, *cyp26a1* was down-regulated in conjunction with up-regulation of *cyp1a* in Atlantic cod larvae exposed to mechanically dispersed crude oil in a previous study [in **Supplementary Files**, Olsvik et al. (2012)]. Regulation of CYP26 enzymes have been shown to

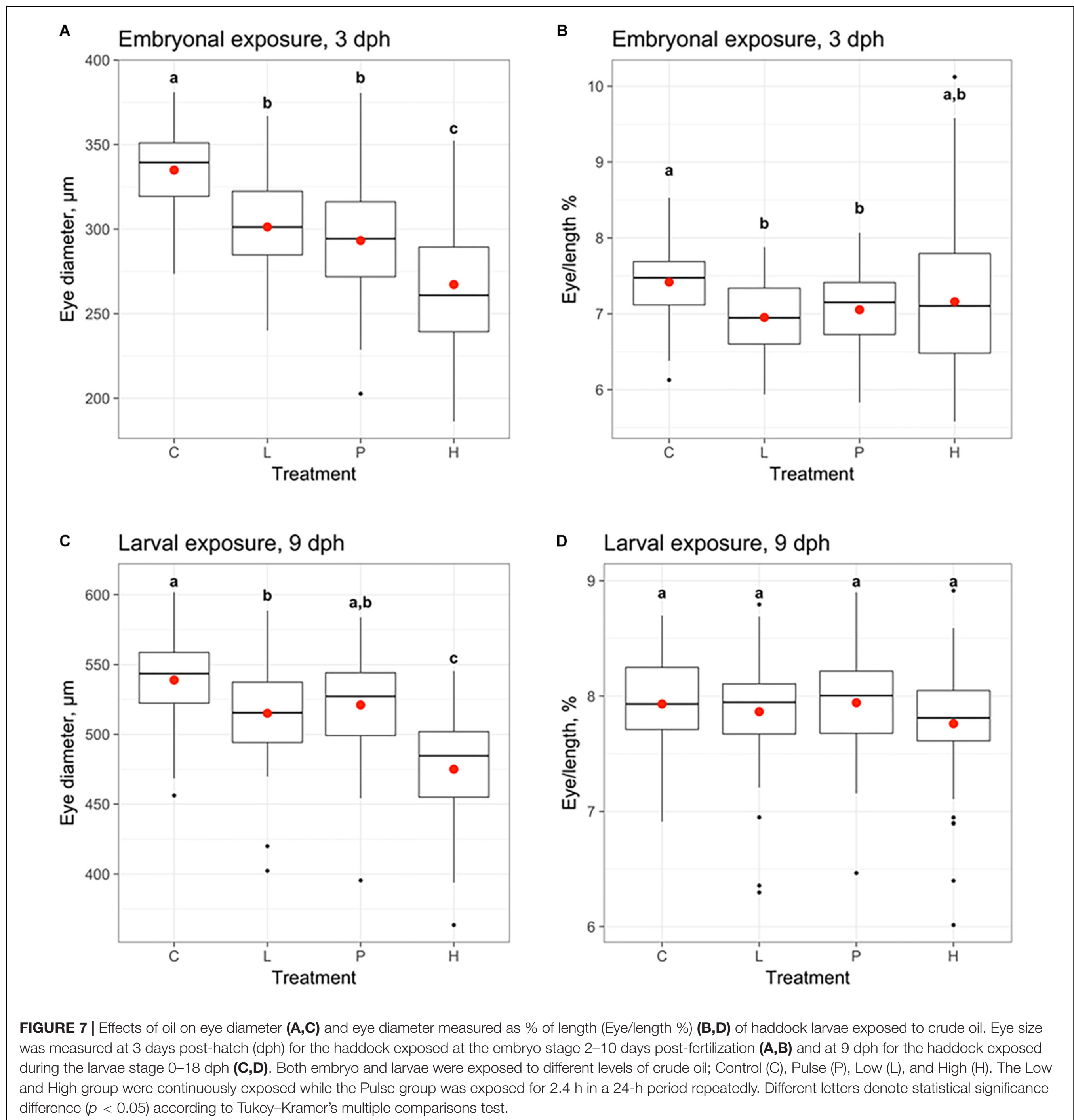


FIGURE 7 | Effects of oil on eye diameter **(A,C)** and eye diameter measured as % of length (Eye/length %) **(B,D)** of haddock larvae exposed to crude oil. Eye size was measured at 3 days post-hatch (dph) for the haddock exposed at the embryo stage 2–10 days post-fertilization **(A,B)** and at 9 dph for the haddock exposed during the larvae stage 0–18 dph **(C,D)**. Both embryo and larvae were exposed to different levels of crude oil; Control (C), Pulse (P), Low (L), and High (H). The Low and High group were continuously exposed while the Pulse group was exposed for 2.4 h in a 24-h period repeatedly. Different letters denote statistical significance difference ($p < 0.05$) according to Tukey–Kramer's multiple comparisons test.

be a key mechanism forming the morphogen atRA gradient necessary for limb patterning during embryonic development (White and Schilling, 2008).

Crude Oil Exposure Affect Eye Development in Embryos but Not Larvae

A reduction in atRA levels with subsequent disruption of retinoid signaling at 6–10 dpf could also be a contributing factor to the observed eye deformities and reduced eye size in the High and Low embryo exposure group of the present study. Retinoids have derived their name from the retina of the eye and plays a key role in both eye development and function (Saari, 2016). The large variation in eye diameter ratio in the High embryo exposure group is most likely related to the gross developmental defects observed in this group (Sorhus et al., 2016b). In the present study, crude oil exposure increased incidence of developmental abnormalities of the eye,

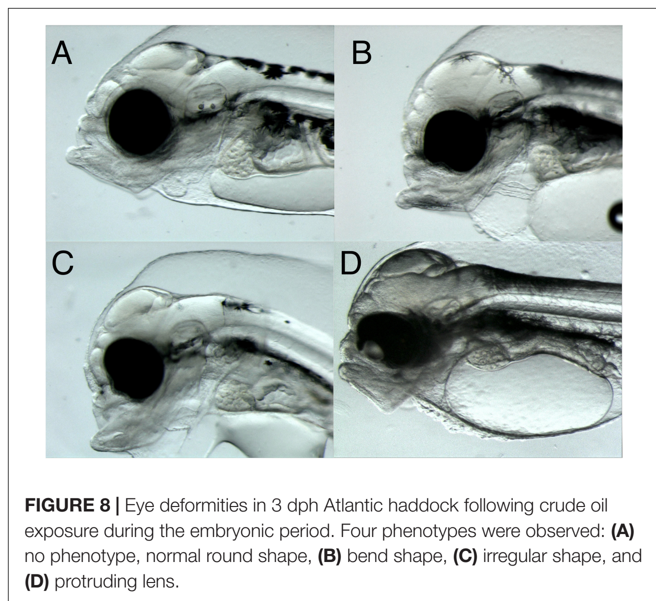


FIGURE 8 | Eye deformities in 3 dph Atlantic haddock following crude oil exposure during the embryonic period. Four phenotypes were observed: **(A)** no phenotype, normal round shape, **(B)** bend shape, **(C)** irregular shape, and **(D)** protruding lens.

TABLE 2 | Eye abnormalities (%) registered in 3 dph haddock following embryonic exposure to crude oil.

Treatment	% No phenotype	% Bend	% Irregular	% Protruding lens
Control	95	5	0	0
Low dose	42	17	7	34
Pulse dose	63	22	7	8
High dose	42	14	3	42

especially related to protruding lens in the High and Low embryonal exposure group. Similar effects on eye development has been demonstrated following disruption of atRA production at critical time points during eye development in zebrafish (Le et al., 2012). Two hour inhibition of atRA production induced microphthalmia (reduced eye ball size), retinal abnormalities, irregular eye shape, reduced pigmentation and severe cardiac edema in zebrafish embryos. Similar phenotypes including cardiac edema and craniofacial abnormalities was also observed in crude oil exposed fish from the present study [cardiac edema and craniofacial abnormalities has been presented previously (Sorhus et al., 2016b, 2017)]. Also excess of RA could have damaging effect on eye and limb development (Alsop et al., 2004; Rydeen and Waxman, 2014).

The effects on eye development was also evident from the gene expression data showing effects on eye-related retinoid signaling especially in the embryo group. Genes such as *rdh8*, *rdh10*, ATP binding cassette subfamily A member 4 (*abca4*), stimulated by retinoic acid (*stra6*) and Retinal pigment epithelium-specific 65 kDa protein (*rpe65*), which are crucial for the visual cycle and eye development, were all affected by crude oil exposure. Although many of the retinol dehydrogenase (RDH) enzymes are present in many tissues and cell types, RDH8 expression is limited to the retina (Kiser et al., 2012) Reduction of atRA with subsequent downregulation of genes related to eye development, could be part of the disruptive effects causing the observed eye abnormalities. Especially the downregulation of *stra6*. STRA6 is a key transporter of which mutation has been previously linked to microphthalmia and heart defects in mammals (Golzio et al., 2007; White et al., 2008). Morpholino knock down of *stra6* in zebrafish embryos led to multiple developmental disorders such as microphthalmia, curved body axis, cardiac edema and craniofacial defects (Isken et al., 2008).

Interestingly, the Pulse group had fewer individuals with protruding lens compared to the Low and High group. This coincides with both atRA not being elevated in Pulse and only in the Low group (High was not measured) and that Pulse did not affect *cyp26a1* expression. This indicates that Pulse exposure affect the retinoid and eye development differently to continuous dosing and it supports the link between changes in atRA and abnormal eye development following crude oil exposure.

A recent study on Atlantic cod showed that starvation had a big impact on the adverse outcome of crude oil exposure (Hansen et al., 2016). Whether this was caused by impaired vision was not investigated. However, observed effects on eyesight from the present study could have severe consequences for the prey capturing success of the larvae, which in turn would affect the chance of survival for the individual. The Pulse and Low group fish were exposed to 0.7 μg/L PAH. This concentration is environmentally relevant in relation to what have previously been measured following major oil spills like the Exxon Valdez spill in Alaska (Boehm et al., 2007) and the Deepwater Horizon spill in the Gulf of Mexico (Diercks et al., 2010).

No Indication of Retinoid Depletion Following Crude Oil Exposure

Despite a clear disruption of retinol homeostasis in both groups exposed to crude oil, there were no apparent differences in total retinoid levels between the High, Low and Control when summing up the measured retinol metabolites. This suggests that the strong *cyp1a* induction did not cause a depletion of total retinoids as we initially hypothesized, at least not following short term exposure. Long term feeding of adult zebrafish to 150 mg BaP per kg feed reduced total retinol levels and retinal levels compared to Control after 100 and 200 days of feeding, respectively (Alsop et al., 2007). A depletion of vitamin A levels was also demonstrated in Atlantic salmon following long term oral BaP exposure (Berntssen et al., 2016). In both studies the authors observed a negative effect on esterification. Alsop et al. (2007) demonstrated a complete depletion of the esterified retinoids after 260 days of BaP feeding, while Berntssen et al. (2016) observed decreased *lrat* expression indicating a reduction in the esterification potential. LRAT together with DGAT convert retinol to retinyl esters. In the present study, *lrat* was only found to be up-regulated at one point during the embryo and larvae exposure, contrasting the findings in salmon but similar to the effects observed following dioxin exposure of male rats (*Rattus norvegicus*) (Hoegberg et al., 2003). It should be noted that the retinyl esters, the most common liver storage form,

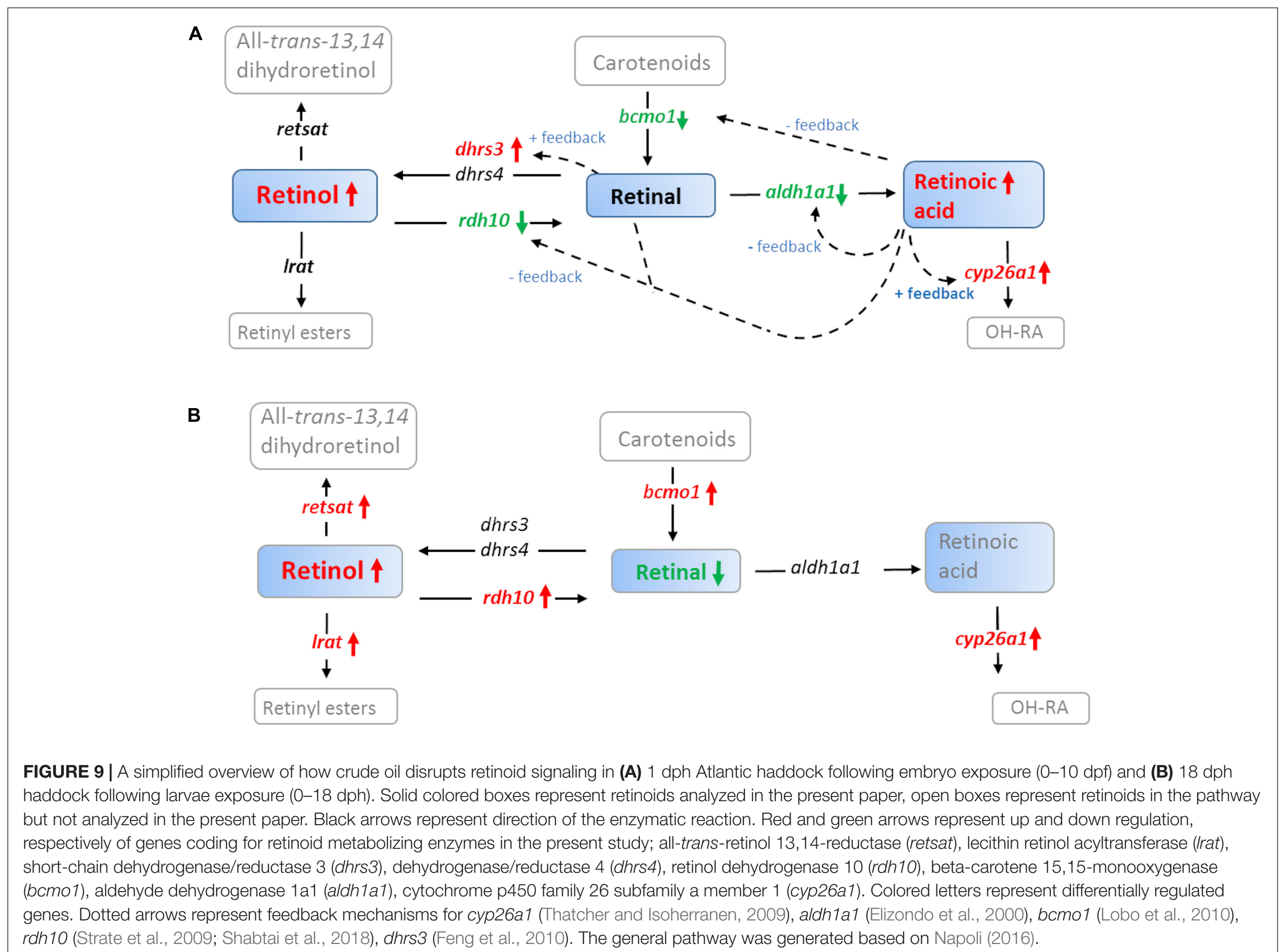


FIGURE 9 | A simplified overview of how crude oil disrupts retinoid signaling in **(A)** 1 dph Atlantic haddock following embryo exposure (0–10 dpf) and **(B)** 18 dph haddock following larvae exposure (0–18 dph). Solid colored boxes represent retinoids analyzed in the present paper, open boxes represent retinoids in the pathway but not analyzed in the present paper. Black arrows represent direction of the enzymatic reaction. Red and green arrows represent up and down regulation, respectively of genes coding for retinoid metabolizing enzymes in the present study; all-*trans*-retinol 13,14-reductase (*retsat*), lecithin retinol acyltransferase (*lrat*), short-chain dehydrogenase/reductase 3 (*dhrs3*), dehydrogenase/reductase 4 (*dhrs4*), retinol dehydrogenase 10 (*rdh10*), beta-carotene 15,15-monooxygenase (*bcmo1*), aldehyde dehydrogenase 1a1 (*aldh1a1*), cytochrome p450 family 26 subfamily a member 1 (*cyp26a1*). Colored letters represent differentially regulated genes. Dotted arrows represent feedback mechanisms for *cyp26a1* (Thatcher and Isoherranen, 2009), *aldh1a1* (Elizondo et al., 2000), *bcmo1* (Lobo et al., 2010), *rdh10* (Strate et al., 2009; Shabtai et al., 2018), *dhrs3* (Feng et al., 2010). The general pathway was generated based on Napoli (2016).

were not measured in the present study. However, previous studies have shown that retinyl esters are not detected in fish eggs (Alsop et al., 2007) and the dominating retinoid is retinal (Costaridis et al., 1996; Lubzens et al., 2003; Alsop et al., 2007), in line with our results seen in newly hatched larvae. While the average retinal levels in the Control group were stable between 3 and 18 dph, retinol was increasing. Thus, the present data suggests that depletion of retinoids following crude oil exposure is not the biggest concern for cod embryo and larvae but rather the disruption of retinoid signaling.

CONCLUSION

This study shows that levels of crude oil equivalent to an oil spill is capable of disrupting the vitamin A signaling, affecting gene expression and eye development in developing haddock embryos. It also shows that the mechanisms do not necessarily involve depletion, but rather a direct effect on the vitamin A homeostasis and the active metabolite atRA. The effects on embryonal retinoid signaling persisted several days after exposure. Despite crude oil exposure during embryonic and larval development display similar effects on retinol levels, there is a striking difference in expression of retinoid related genes between the two exposure regimes. The observed effects on eye development may also be associated with disrupted retinol metabolism in the exposed embryos and possibly linked to developmental abnormalities as observed in other studies. We still need more knowledge on the dynamics affecting the retinoic acid and retinol levels in fish larvae during and after crude oil exposure. In addition, the effect on retinyl esters during early development needs clarification.

DATA AVAILABILITY

Publicly available datasets were analyzed in this study. This data can be found here: https://www.ncbi.nlm.nih.gov/bioproject/?term=PRJNA328092.

ETHICS STATEMENT

All animal experiments within the study were approved by the governmental Norwegian Animal Research Authority (NARA; http://www.fdu.no/fdu/, reference number 2012/275334-2). All methods were performed in accordance with approved guidelines.

AUTHOR CONTRIBUTIONS

KL and PO designed the research questions and wrote the main manuscript. ES and SM contributed to the text. KL, PO, ES, and RE analyzed the data. KL, PO, SM, and ES were actively involved in interpreting the results. KL, PO, SM, ES, and ØK conducted sampling of fish. SM, ES, and ØK ran the main experiments. SM was primus motor for the project, designing the exposure study, and getting funding. All authors contributed with proofreading of the text.

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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.2019.00368/full#supplementary-material

TABLE S1 | Gene expression related to vitamin A homeostasis in haddock larvae and embryo at several time points during development following High, Low and Pulse crude oil exposure. Experiments were conducted of which one group were exposed during the embryonic stage (Embryo exposure) 2–9 days post-fertilization (dpf) and one group during the larval stage (Larvae exposure) 0–18 days post-hatch (dph).

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Effects of Dietary Inclusion of Shrimp Paste on Growth Performance, Digestive Enzymes Activities, Antioxidant and Immunological Status and Intestinal Morphology of Hybrid Snakehead (*Channa maculata* ♀ × *Channa argus* ♂)

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A nutritional feeding experiment was conducted to evaluate the effects of shrimp paste on feeding attractiveness, growth performance, digestive enzyme activities, immune-related genes and intestinal morphology in hybrid snakehead (*Channa maculata* ♀ × *Channa argus* ♂). Two diets were formulated with or without shrimp paste supplementation (D1:0% and D2: 3%) to feed fish for 8 weeks. Results showed that growth performance (FBW, WG and SGR) and feed intake (FI) significantly increased with shrimp paste supplemented ($P < 0.05$), while FCR and SR of hybrid snakehead fed diets supplemented with shrimp paste or not showed no significant difference ($P > 0.05$). Gut lipase and amylase activities were significantly higher in diet supplemented with shrimp paste than that in control one ($P < 0.05$). Hepatic antioxidant statuses of hybrid snakehead fed dietary shrimp paste or not showed no significant differences in total antioxidant capacity, malondialdehyde and superoxide dismutase of fish ($P > 0.05$). Results showed that fish fed diet with shrimp paste supplemented did not show significant difference in expression of GR, IκB, P65 and IL8 than that in control group ($P > 0.05$). There are significantly more goblet cells in shrimp paste supplemented diet than that in control diet ($P < 0.05$). However, villi length and muscle thickness showed no significant difference compared to control diet ($P > 0.05$). The results indicated that dietary 3% shrimp paste supplementation improved the growth performance of hybrid snakehead by enhancing feed intake (FI) while made no difference to antioxidant capacity and immunity.

Keywords: shrimp paste, hybrid snakehead, growth performance, intestinal morphology, feed intake

INTRODUCTION

Carnivorous fish usually require well above 30% protein for optimum growth (Lindner et al., 1995) and fishmeal is the main protein source for carnivorous fish in commercial feed. As a primary high-quality protein source, fishmeal consists of more than 60% crude protein and full of vitamins, minerals and other nutrients (Riche, 2015). One of the reasons that fishmeal become the main source of protein ingredient in aquatic feed is because of its palatability for aquatic animal (Alexis and Nengas, 2001). However, the use of fishmeal is limited in aquafeed production because of the high demand and lack of fish stocks, which constrain the continued development of aquaculture (Tacon et al., 2011). Since it is unlikely to produce fishmeal by a large margin beyond the current need, aquaculture production may depend on inclusion of alternative protein sources, like plant protein sources (El-Haroun et al., 2012). Nevertheless, the use of the plant materials is mainly restricted by the presence of anti-nutritional ingredients and lower protein quality (Zhang et al., 2012). Besides, high content of plant protein sources can result in lower feed intake (FI) caused by low feed palatability (Nunes et al., 2006). To solve these problems, attractants was mainly used to enhance the utilization of feed (Tusche et al., 2011). Currently, there were lots of studies about the feed attractants published for aquatic animals, such as krill meals, fish and krill hydrolysates, squid meal, betaines, amino acids, AMP, or other animal based meals (Coman et al., 1996; Nunes et al., 2006; Derby et al., 2016). However, use of shrimp paste as a feed attractant is rarely reported in aquatic animals. Shrimp paste, as one of feed attractants, might be significant dietary sources of long chain n-3 polyunsaturated fatty acids, rich in free amino acids, nucleotides, amines and nucleosides (Montaño et al., 2001) and has strong shrimp odor (Adnan, 1984).

Snakeheads has been one of the most commercially important fish species for aquaculture in China for the fast growth, delicious taste, tolerance to inferior water quality and resistance to diseases (Hossain et al., 2008). The main farmed snakehead species in China include northern snakehead (Channa argus), blotched snakehead (*Channa maculata*) and hybrid snakehead (*Channa maculates* × *Channa argus*) (Sagada et al., 2017). Northern snakehead (*C. argus*) is native to the Yangtze River (Zhou et al., 2012), while blotched snakeheads (*C. maculata*) is native to Pacific coastal drainages in northern Vietnam and southern China, mainly located in Guangdong Province (Gong et al., 2014). Recently, the hybrid snakehead has gained popularity because of its rapid growth compared to those of *C. argus* and *C. maculate* (Shu-Ren et al., 2013). As a carnivorous fish, hybrid snakehead requires high content of protein in feed, being fishmeal usually considered as the most adequate protein source (Zhang et al., 2017). Currently, researches on better use of protein supplementing with feed attractant on hybrid snakehead are quiet fewer. Therefore, the current study was conducted to evaluate the effect of dietary shrimp paste in practical diets on the feed attractiveness, growth performance and digestive enzyme activities, antioxidant and immunological status and intestinal morphology of hybrid snakehead (*C. maculata* × *C. argus*).

MATERIALS AND METHODS

Diet Preparation and Dietary Treatments

In this study, two isonitrogenous and isoenergetic practical diets were formulated supplementing with or without shrimp paste (D1: 0%; D2: 3%) (**Table 1**). The proximate composition of shrimp paste was shown in **Table 2**. The method of diet preparation was the same as described by Niu et al. (2014). The diets were air dried and stored −20°C until fed.

Animal Rearing and Experimental Procedures

The feeding trial was conducted at an experimental station of Sun Yat-sen University (Guangzhou, Guangdong). Prior to the start of the trial, (*C. maculata* × *C. argus*) were acclimated to a commercial diet for 2 weeks and were fed twice daily to apparent satiation. At the beginning of the feeding trial, the fish were starved for 24 h, weighed after being anesthetized with 10 mg L-1 eugenol (Shanghai Medical Instruments Co., Ltd., Shanghai, China), and then fish with similar size (initial body weight 73.16 ± 0.40 g) were randomly allotted to 6 tanks (170L; three cages per diet treatment); each tank was stocked with 20 fish. Each experimental diet was randomly assigned to three tanks. The feeding frequency was twice daily at 8:00 and 16:00 and lasted for 8 weeks.

Sample Collection

At the end of the feeding trial, fish were starved for 24 h, anesthetized with 10 mg L-1 eugenol (Shanghai Medical Instruments Co., Ltd., Shanghai, China) and then weighed and counted the total number. Eight fish from each tank were randomly collected. Two fish were collected for measuring the whole body composition. Six fish were used to obtain weights of liver, viscera and whole body for the biometric parameters. Livers and foreguts were rapidly removed and frozen in the liquid nitrogen for analysis of enzymes and gene expression. Foreguts were collected in Bouin's solution for paraffin sectioning.

Biochemical Analysis

Feed and whole fish were frozen dried and then grounded. Moisture, crude lipid, crude protein and crude ash of the feed and fish were determined using standard methods (Kavanagh, 2010).

Antioxidant Capacity Analysis and Digestive Enzymes Analysis

Hepatic and intestinal samples were homogenized in ice-cold phosphate buffer (1:10 dilution) (phosphate buffer; 0.064 M, pH 6.4). The homogenate was then centrifuged for 15 min (4°C, 1200 g), and aliquots of the supernatant were used to quantify antioxidant status and digestive enzymes analysis. All indices were measured with commercial assay kits (T-AOC, A015-1; SOD A001-1-2, MDA, A003-1-2, Lipase, A054-2-1; Amylase, C016-2-1) (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) in accordance with the instructions of the manufacturer.

TABLE 1 | Ingredients and proximate composition of the two experimental diets (%).

	D1	D2
Fish meal[1]	25	25
Soy protein concentrate[2]	6	6
Wheat flour[3]	17.46	20.47
Dehulled soybean meal[4]	22	22
Chicken powder[5]	6	6
Brewer yeast[6]	5	5
Peanut bran[7]	6	6
Soya lecithin[8]	1.5	1.5
Soya oil[9]	1	1
Fish oil[10]	2	2
Choline (50Ca(H_2PO_4)$_2$[12]	2	2
Vitamin C[13] 1	0.1	0.1
Vitamin premix[14]	1	1
Mineral premix[15]	1	1
DL-Met[16]	0.05	0.05
Lys-HCL (78%)[17]	0.2	0.2
Threonine[18]	0.19	0.18
Shrimp paste[19]	0	3
Sum	100	100
Nutrient levels		
Moisture	10.28	10.51
Crude protein	40.78	41.13
Crude lipid	6.83	7.95
Ash	11.27	11.73

1: Imported from Australia. Protein: 68.74%; lipid: 7.56%. 2: Guangzhou Chengyi Company Ltd., Guangzhou, China. Protein: 65.3%; lipid: 3.24%. 3: Guangzhou Chengyi Company Ltd., Guangzhou, China. Protein: 12.49%; lipid: 2.8%. 4: Guangzhou Chengyi Company Ltd., Guangzhou, China. Protein: 47.86%; lipid: 1.7%. 5: Guangzhou Chengyi Company Ltd., Guangzhou, China. Protein: 63%; lipid: 10%. 6: Guangzhou Chengyi Company Ltd., Guangzhou, China. Protein: 46%; lipid: 1%. 7: Guangzhou Chengyi Company Ltd., Guangzhou, China. Protein: 50%; lipid: 7%. 8-18: Guangzhou Chengyi Company Ltd., Guangzhou, China. 14: Vitamin premix (mg or g/kg diet) thiamin 25 mg; riboflavin, 45 mg; pyridoxine HCl, 20 mg; vitamin B12, 0.1 mg; vitamin K3, 10 mg; inositol, 800 mg; pantothenic acid, 60mg; niacin acid, 200mg; folic acid, 20 mg; biotin, 1.20 mg; retinol acetate, 32 mg; cholecalciferol, 5mg; alpha-tocopherol, 120 mg; ascorbic acid, 2000mg; choline chloride, 2500 mg; ethoxyquin 150 mg, wheat middling, 18.52 g (Zhang et al., 2017). 15: Mineral premix (mg or g/kg diet): NaF, 2 mg; KI, 0.8 mg; CoCl2 · 6H2O (1%), 50 mg; CuSO4 · 5H2O, 10 mg; FeSO4 · H2O, 80 mg; ZnSO4 · H2O, 50mg; MnSO4 · H2O, 60 mg; MgSO4 · 7H2O, 1200 mg; Ca(H2PO3)2 · H2O, 3000 mg; NaCl, 100 mg; Zoelite, 15.45 g (Zhang et al., 2017). 19. The shrimp paste is offered by Shunde Xinfuda Bioengineering Company Ltd., Shunde, China. The ingredients and proximate analysis are presented on a dry matter basis.

Quantitative Real Time PCR Analysis

Total RNA was extracted from liver using Trizol® reagent (Invitrogen, United States). The cDNA was synthesized using a PrimeScript™ RT reagent kit with gDNA Eraser (Takara, Japan), according to the manufacturer's instructions. Real-time PCR for the target genes were performed using a SYBR® Premix Ex Taq™ II (Takara, Japan) and quantified on the LightCycler 480 (Roche Applied Science, Basel, Switzerland). The primers were showed in **Table 3**.

Intestinal Morphology

Samples fixed in Bouin solution were dehydrated in ethanol, equilibrated in xylene and embedded in paraffin according to the method described by Krogdahl et al. (2003). The paraffin blocks was sectioned (5 μm) in serial sagittal section using a Leica RM 2135 rotary microtome and stained with hematoxylin and eosin (H and E). The sections were examined using a light microscope with villi length and muscle thickness measured. Photographs were taken with an Olympus digital camera attached to the microscope. 10 random villi from each segment were measured.

TABLE 2 | Proximate composition of the shrimp pasted used in the present study.

Composition	Content
Moisture	40%
Crude protein	32–35%
Crude lipid	8%
Ash	6–15%
Animo acid	30%
Cholesterol	56 mg/kg
EAA/TAA	40.5%
EAA/NAA	68.08%
Glu	6.14%
Asp	3.77%
Gly	2.57%

The shrimp paste is offered by Shunde Xinfuda Bioengineering Company Ltd., Shunde, China. Briefely, shrimp shells were removed and the carcases were enzymatic hydrolyzed. The zymolyte were separated, concentrated and sterilized to be the used shrimp paste. EAA: essential amino acid; TAA, total amino acid; NAA, non-essential amino acid; Glu, glutamic acid; Asp, aspartic acid; Gly, glycine.

TABLE 3 | Sequences of primers used in this study.

Primers	forward/reverse (5′to 3′)
GR-F	GGGAAAGACCAGGACTCATA
GR-R	TTCTTGGTTTTCCGTGCTTC
HSP70-F	ATTTTGAATGTGTCTGCGGT
HSP70-R	ACTTGCTGATGATGGGGTTA
IL-8-F	GAGTCTGAGCAGCCTGGGAGT
IL-8-R	CTGTTCGCCGGTTTTCAGTG
NF-κB p65-F	CAGCCAAAACCAAGAGGGAT
NF-κB p65-R	TCGGCTTCGTAGTAGCCATG
IκBα-F	AAAATGTTACCGTGCCAGGAC
IκBα-R	ATGTATCACCGTCGTCAGTC
β-actin-F	CACTGTGCCCATCTACGAG
β-actin-R	CCATCTCCTGCTCGAAGTC

GR, glucocorticoid receptor; HSP70, heat shock protein 70; IL-8; interleukin-8; NF-κB p65, NF-κB p65 subunit; IκBα, inhibitor of NF-κBα.

Calculations and Statistical Analysis

The following variables were calculated:

Weight gain rate (WG, %) = 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;

Feed conversion ratio (FCR) = dry diet fed/wet weight gain;

Survival rate (%) = 100 × (final number of fish)/(initial number of fish);

Viscerosomatic index (VSI,%) = 100 × (viscera weight, g)/(whole bodyweight, g);

Hepatosomatic index (HSI,%) = 100 × (liver weight, g)/(whole body weight, g);

Condition factor (CF, g/cm3) = 100 × (body weight, g)/(body length, cm3);

All data are presented as means ± S.E.M. and subjected to independent-sample *t*-test to test the effects of experimental diets using the software of the SPSS for windows (ver 16.0, U.A.S). Statistical significance was examined at $P < 0.05$ unless otherwise noted.

RESULTS

Growth Performance, Feed Utilization, Survival Rate and Biometric Parameters

Growth performance, feed utilization and biometric parameters of hybrid snakehead fed dietary shrimp paste are shown in **Table 4**. Results showed that growth performance (FBW, WG, and SGR) significantly increased with shrimp paste supplemented ($P < 0.05$). Survival rate showed the same trend as the growth performance but without significant difference ($P > 0.05$). Feed conversion ratio (FCR) of snakehead fed diet supplemented with shrimp paste showed no significant difference with that in control group ($P > 0.05$), while FI of snakehead fed diet supplemented with shrimp paste was significantly higher than that in control group ($P < 0.05$). There was no significant difference among hepatosomatic indices (HSI), visceral somatic indices (VSI) and condition factor (CF) between the two different diet treatments.

Whole Body Composition

Whole body composition of hybrid snakehead fed dietary shrimp paste is shown in **Table 5**. There were no significant difference in whole body composition of fish between the two diet treatments ($P > 0.05$).

TABLE 4 | Growth performance and biometric parameters of hybrid snakehead (*Channa maculata* × *Channa argus)* fed diets with or without supplementation of shrimp paste.

	D1	D2
IBW/g	72.7 ± 0.59	73.6 ± 1.23
FBW/g	98.7 ± 4.98a	122.5 ± 8.68b
SGR/% · d-1	0.54 ± 0.10a	0.91 ± 0.11b
WG/%	35.8 ± 7.96a	66.5 ± 10.9b
SR/%	93.3 ± 5.77	98.3 ± 2.89
FI(g/100gBW/d)	1.01 ± 0.98a	1.38 ± 0.26b
FCR	1.77 ± 0.07	1.56 ± 0.13
VSI/%	4.80 ± 0.74	4.42 ± 1.19
HSI/%	1.45 ± 0.35	1.19 ± 0.43
CF/%	1.34 ± 0.07	1.29 ± 0.11

Values are mean ± SEM of three replicates, and values in the same row with different letters are significantly different (P < 0.05). Initial body weight (IBW,%) = 100 × initial body weight/initial number of fish. Final body weight (FBW,%) = 100 × final body weight/final number of fish. Weight gain rate (WG,%) = 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. Feed conversion ratio (FCR) = dry diet fed/wet weight gain. Survival rate (%) = 100 × (final number of fish)/(initial number of fish). Viscerosomatic index (VSI, %) = 100 × (viscera weight, g)/(whole bodyweight, g). Hepatosomatic index (HSI, %) = 100 × (liver weight, g)/(whole body weight, g). Condition factor (CF, g/cm3) = 100 × (body weight, g)/(body length, cm3).

TABLE 5 | Whole-body compositions (% dry weight) of hybrid snakehead (*Channa maculata* × *Channa argus)* fed diets with or without supplementation of shrimp paste.

	D1	D2
Moisture	73.34 ± 0.45	71.93 ± 1.15
Crude lipid	25.16 ± 1.94	24.53 ± 0.80
Crude protein	61.38 ± 1.16	61.96 ± 0.68
Ash	16.04 ± 0.27	15.62 ± 0.37

Values are mean ± SEM of three replicates, and values in the same row with different letters are significantly different (P < 0.05).

TABLE 6 | Hepatic antioxidant statuses of hybrid snakehead (*Channa maculata* × *Channa argus)* fed diets with or without supplementation of shrimp paste.

	D1	D2
T-AOC (U/mg protein)	0.21 ± 0.03	0.24 ± 0.02
SOD (U/mgprot)	95.83 ± 8.29	91.08 ± 11.24
MDA (nmol/ml)	0.23 ± 0.05	0.28 ± 0.02

Values are mean ± SEM of three replicates, and values in the same row with different letters are significantly different (P < 0.05). T-AOC, total antioxidant capacity; SOD, superoxide dismutase; MDA, malondialdehyde.

TABLE 7 | Intestinal digestive enzyme activity of hybrid snakehead (*Channa maculata* × *Channa argus)* fed diets with or without supplementation of shrimp paste.

	D1	D2
Amylase/U*mgprot-1	0.47 ± 0.07a	0.81 ± 0.05b
Lipase/U*mgprot-1	33.47 ± 8.11a	57.01 ± 5.95b

Values are mean ± SEM of three replicates, and values in the same row with different letters are significantly different (P < 0.05).

Hepatic Antioxidant Status Analysis

Hepatic antioxidant statuses of hybrid snakehead fed dietary shrimp paste or not are shown in **Table 6**. There were no significant difference in total antioxidant capacity (T-AOC), malondialdehyde (MDA) and superoxide dismutase (SOD) of fish between the two diet treatments ($P > 0.05$).

Gut Digestive Enzymes Analysis

Gut digestive enzymes analysis of hybrid snakehead fed dietary shrimp paste or not are shown in **Table 7**. Gut lipase and amylase activity were significantly higher in diet supplemented with shrimp paste than that in control group ($P < 0.05$).

Intestinal Genes Expression Level

The relative genes expression level of hybrid snakehead fed diets with or without supplementation of shrimp paste are showed in **Table 8**. Results showed that there were no significant difference in GR (glucocorticoid receptor), HSP70, IL8, IκB and P65 gene expression level between the two diets ($P > 0.05$).

Intestinal Morphology

Intestinal morphology is presented in **Table 9** and **Figure 1**. Results showed that gut morphology was slightly changed by

TABLE 8 | The relative genes expression level of hybrid snakehead (*Channa maculata* × *Channa argus)* fed diets with or without supplementation of shrimp paste.

	D1	D2
GR	1.05 ± 0.26	0.70 ± 0.04
HSP70	1.00 ± 0.07	0.62 ± 0.20
IL8	1.02 ± 0.25	0.83 ± 0.03
IκB	1.02 ± 0.16	0.79 ± 0.05
P65	1.05 ± 0.14	0.78 ± 0.04

Values are mean ± SEM of three replicates, and values in the same row with different letters are significantly different (P < 0.05). GR, glucocorticoid receptor; HSP70, heat shock protein 70; IL-8, interleukin-8; NF-κB p65, NF-κB p65 subunit; IκBα, inhibitor of NF-κBα.

TABLE 9 | Gut morphology of hybrid snakehead (*Channa maculata* × *Channa argus)* fed diets with or without supplementation of shrimp paste.

	D1	D2
Muscle thickness	198.33 ± 46.39	217.72 ± 39.80
Villi length	405.70 ± 69.56	399.78 ± 56.30
Goblet cells	447.00 ± 9.44a	529.00 ± 22.82b

Values are mean ± SEM of three replicates, and values in the same row with different letters are significantly different (P < 0.05).

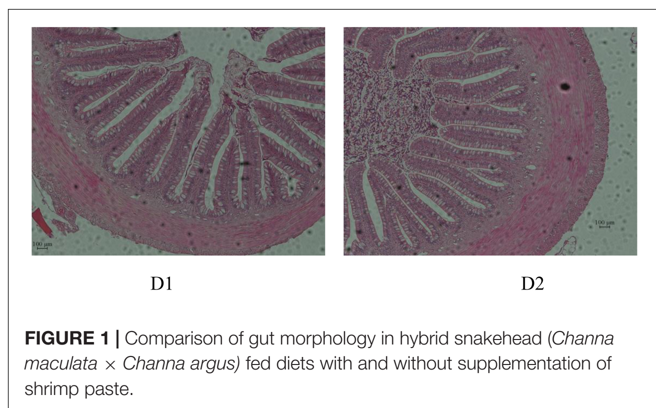

FIGURE 1 | Comparison of gut morphology in hybrid snakehead (*Channa maculata* × *Channa argus)* fed diets with and without supplementation of shrimp paste.

dietary shrimp paste. With shrimp paste supplemented in diet, there were significantly more goblet cells than that in control diet ($P < 0.05$). However, the villi length and muscle thickness showed no significant difference between the two groups ($P > 0.05$).

DISCUSSION

The major components of feed attractants are shown to be water-soluble and relatively small, such as amino acids, mainly alanine, taurine, arginine, glutamic acid, glycine and alanine; small peptides, nucleotides and nucleosides, amines and quaternary ammonium bases, for example betaine (Lee and Meyers, 2010). Research showed that once the ingredients was soluble in water and had a high level of small peptides, especially high proportion of nucleotides and amino acids, the palatability and attractability aspects of the protein source to aquatic animals is better (Suresh et al., 2011). Aquatic animals normally rely on chemosensory systems to identify water-soluble chemicals, locate food and then ingest it (Derby and Sorensen, 2008). The chemical compounds in shrimp paste such as nucleotides and free amino acids are recognizable to the chemosensory systems for fish to locate and ingest food (Suresh et al., 2011). There were previous studies showing that marine animal additives could improve the performance of feed pellets in aquaculture. Williams et al. (2005) showed that krill and shrimp head meal supplemented in a basal diet improved the *Penaeus monodon* growth performance in a dose-dependent manner. Sanchez et al. (2005) showed that krill meal improved attractability of a feed and thus enhance the palatability of the diet for pacific white shrimp. These studies showed that marine animal additives acting as the feed attractants can enhance feed performance via the way of improving the attractability and palatability by stimulating appetitive behavior, for example, arousing, search initiating and locating the food and therefore enhancing feed consumption (Derby et al., 2016). Besides, enterocytes are more likely to digest and absorb the protein hydrolysates which consist of amino acids and low molecular-weight peptides compared to the high-molecular-weight macromolecules (Önal and Langdon, 2009). The low molecular-weight peptides are known to have excellent texture and viscosity and are highly digestible, which facilitate the uptake of nutrients (Bhaskar et al., 2007; Ospina-Salazar et al., 2016). The present results indicated that hybrid snakehead fed the shrimp paste diet had the higher growth performance than the control treatment, mainly by increasing the FI, which can be speculated that shrimp paste acted as the feed attractants. Besides, the better growth performance may also be related to the ability of shrimp paste regulating the production of enzyme activities in fish and thus exerting the effect on the digestion. The ability of aquatic animals to utilize the ingested nutrients mainly relies on the presence of digestive enzymes, which is also considered to be indicators of the fish absorptive and digestive capacity (Deguara and Jauncey, 2010). Lipases are the enzymes which catalyze the hydrolysis of ester bonds in substrates, such as triacylglycerol, thereby releasing free fatty acid and glycerol and providing energy (Wong and Schotz, 2002). In the present study, fish fed diets with shrimp paste supplemented had higher lipase and amylase activities, suggesting that shrimp paste supplemented diets up-regulated the lipid and carbohydrate metabolism in the present study. Increasing activities of digestive enzymes may have effects on the improved growth performance of fish in shrimp paste supplemented treatment.

Except for digestive enzyme activities, the morphology and structure of the intestine are crucial for nutrient absorption and the maintenance of normal intestinal functions (Gao et al., 2013; Vizcaíno et al., 2014). The villi length in a way reflects the function of the intestinal wall (Emami et al., 2012), led to better nutrient absorption and better growth performance (Al-Fataftah and Abdelqader, 2014). Muscle thickness as well plays a role in intestinal digestion and absorption. Increased muscle thickness may enhance intestinal digestion and absorption ability (Chen and Wang, 2013). However, in the present study, fish fed diets with shrimp paste supplemented made no difference to villi length and muscle thickness, indicating that shrimp paste did not improve the morphology and structure of the intestine

and thus enhancing the ability to absorb nutrients. Goblet cells, the major secretory cell in the superficial epithelium, produce and store large amounts of mucus and mucins, which functions for protecting intestine from mechanical damage (Cook et al., 2017). Results in the present study showed that fish fed diets with shrimp paste supplemented had more goblet cells, which suggested shrimp paste may have influence on protective effect of snakehead in intestine.

As for the antioxidant capacity, T-AOC is an overall indicator of the antioxidant status of an individual, on behalf of the level of non-enzyme and enzyme antioxidant in the organism (Xiao et al., 2004). As one of the important antioxidant enzymes, superoxide dismutase (SOD) is an important endogenous antioxidant for protection against oxidative stress and the first enzymes to respond against oxygen radicals (Winston and Di Giulio, 1991). Malondialdehyde (MDA) is a product of lipid peroxidation, through crosslinking with the nucleophilic groups of nucleic acids, amino phospholipids and proteins (Buege and Aust, 1978). The results showed that the MDA between the two diets with or without shrimp paste supplemented were not significantly different when no stress appeared in the present. The present results indicated that snakehead did not suffer from oxidative stress because of the dietary inclusion with shrimp paste.

Reports showed that there are biologically active peptides in protein hydrolysate with immuno-stimulating properties which is produced during the processing procedure (Daoud et al., 2005; Kotzamanis et al., 2007). NF-κB is a pleiotropic transcription factor, which is involved in diverse physiological and pathological processes including infection, inflammation and immunity (Karin and Greten, 2005). NF-κB consists of hetero- and homo-dimeric complexes of members in the Rel family of proteins, composed of p50, p52, p65 (RelA), c-Rel, and RelB (Liu et al., 2008). Activation of NF-κB signal pathway involved in the isolation of the inhibitor of κB (IκB) and led to the NF-κB complex translocating into the nucleus, thus promoted the expression level of NF-κB responsive genes, including IL-6, IL-1β and TNF-α (Moore et al., 1993). As a member of the family of chemokine, IL-8 has the chemotactic properties of leukocyte and lymphocyte and exert an effect on the initiation and amplification of acute inflammatory reaction in the chronic inflammatory process as a pre-inflammatory cytokine (Heinzmann et al., 2004; Hu et al., 2016). As a nuclear hormone receptor, GR is a member of the superfamily of ligand-activated transcription factors (Ning et al., 2019) and can cross talk with NF-κB pathways and thus make difference to the expression levels of inflammatory genes (Helen et al., 2004). The present study showed that fish fed diets with shrimp paste supplemented did not significantly up-regulate expression of GR, IκB, P65 and IL8 snakehead, indicating that shrimp paste exert no effect on immunity.

CONCLUSION

In conclusion, dietary shrimp paste supplementation improved the growth performance of snakehead by enhancing FI, while made no difference to antioxidant capacity and non-specific immunity.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript and/or the supplementary files.

ETHICS STATEMENT

All experimental procedures were conducted in conformity with institutional guidelines for the care and use of laboratory animals in Sun Yat-sen University, Guangzhou, China, and conformed to the National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication No. 85-23, revised 1985).

AUTHOR CONTRIBUTIONS

JN, YL, and LT designed the study. JX, HF, and SL carried out the rearing work. JX and JN analyzed the results. JX wrote the manuscript with contributions from all other authors.

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Diet Supplemented With Synthetic Carotenoids: Effects on Growth Performance and Biochemical and Immunological Parameters of Yellow Perch (*Perca flavescens*)

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The current study assessed the effect of dietary canthaxanthin and lycopene supplementation at different concentrations on growth performance and antioxidant status in yellow perch (*Perca flavescens*). In this regard, fish with initial weight (32 ± 1.0 g) were divided into five groups in triplicate, and fed on carotenoid-free diet (control), canthaxanthin (CTX) (50 and 100 mg/kg diet), and lycopene (200 and 400 mg/kg diet) for 60 days. Growth parameters and antioxidant enzymes were evaluated after 30 and 60 days post feeding. Tissue liver and intestine from six fish per treatment was collected for antioxidant and digestive enzymes analysis. The results revealed a significant increase ($P < 0.05$) of lipid content in the group fed lycopene at a dietary level 400 mg/kg for 60 days, compared to the control. Moreover, dietary carotenoids exhibited no significant effect on growth performance; this was evidenced by no significant up-regulation of growth hormone (*gh*) and insulin-like growth factor 1b (*igf-1b*) genes after 30 and 60 days post feeding. Intestinal lipase and trypsin activities were significantly improved with dietary lycopene especially at a dose of (400 mg/kg diet) for 60 days. Malondialdehyde (MDA) level in liver was also significantly decreased with dietary lycopene (400 mg/kg diet) for 60 days. Hepatic superoxide dismutase (SOD), catalase (CAT), and Glutathione peroxidase (GSH-Px) activities were significantly decreased with dietary CTX, especially at dose (100 mg/kg diet) and lycopene at a concentration of 200 and 400 mg/kg diet after 60 days feeding. Additionally, the immune-related gene interleukin-1 beta (*il-1b*) mRNA expression level revealed up-regulation in groups fed on CTX at different concentrations for 30 days, and fish fed lycopene at a concentration level 400 mg/kg diet for 60 days. The obtained results concluded that dietary supplementation of canthaxanthin and lycopene could enhance immune response and maintain antioxidants defense of fish. Therefore, it considered as a functional aquafeed ingredient for yellow perch.

Keywords: digestive enzymes, antioxidant status, gene expression, carotenoids, yellow perch

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INTRODUCTION

Yellow perch *Perca flavescens*, are considered an important recreational food fish in North America (Brown and Barrows, 2002). Demand for yellow perch is high and varies with its relative availability and regional preference, however declining commercial harvest has created widespread interest in culturing yellow perch in the North Central Region (Brown et al., 1996). Unfortunately, environmental and husbandry stressors such as handling, capture, transport, and environmental quality under intensive aquaculture greatly influence the natural immune system of fish and may result in disease outbreak and, consequently, economic losses (Austin and Austin, 2016). Stress conditions increased the production of reactive oxygen species (ROS) resulting in oxidative stress, which affects fish immune response (Evans and Cooke, 2004). Many previous studies showed that antioxidant molecules such as vitamin C and E and carotenoids are modulators of the stress response in several fish species (Ortuno et al., 2003; Girao et al., 2012; Abd El-Gawad and Abdel Hamid, 2014; Sahin et al., 2014).

Carotenoids, belonging to family of fat-soluble pigments, have various sources including natural source (fungi, yeast and algae), animal source (crustacean), and synthetic source (lycopene, canthaxanthin, zeaxanthin, astaxanthin and β-carotene). They have been ascribed a wide range of biological functions in aquaculture including growth enhancement, improve skin coloration, antioxidant properties, precursors for vitamin A and transcription regulators, immune-system stimulants (Amar et al., 2004; Supamattaya et al., 2005; Zhang et al., 2013a,b, Sallam et al., 2017; Cheng et al., 2018). The physiological mechanism of carotenoids in preventing oxidative stress exerts in different ways as they can act as quenchers of singlet molecular oxygen or convert hydroperoxides into more stable compounds. Additionally, carotenoids can prevent formation of free radicals through the block of free radical oxidation reactions and inhibition of the autoxidation chain reaction (Galasso et al., 2017).

Lycopene is a red colored carotenoid present in tomatoes, watermelon, and carrots. Recently, it has attracted considerable attention as a chemoprotectant agent because of its highly antioxidant scavenging activity (Yonar, 2012; Ural, 2013; Sahin et al., 2014), thus preventing oxidative damage to cells and tissue, toxicity, and disease. Rao and Rao (2007) has been reported that lycopene exert its biological effects via different mechanisms that include gene function regulation, gap junction communication, hormone and immune modulation, carcinogen metabolism, and metabolic pathways involving phase II drug-metabolizing enzymes. Therefore, lycopene is considered as the most effective carotenoids used against biological ROS (Sahin et al., 2014). Canthaxanthin (β, β -carotene-4,4′-dione) is a keto-carotenoid generated naturally in a wide variety of algae (Hertzberg and Liaaen-Jensen, 1966). Canthaxanthin dietary inclusion in fish diet as a feed additive for enhancing growth and skin color of fish has been previously investigated (Storebakken and No, 1992; Kalinowski et al., 2005). As fish cannot synthetize their own carotenoids *de novo* (Gupta et al., 2007), hence, there is a need to incorporate these carotenoids in aquafeed as an essential micronutrient (Baker et al., 2002). To the best of our knowledge, no studies on the effect of these dietary carotenoids on digestive enzymes (lipase, amylase and trypsin enzymes) or immune related gene (*il-1b*) or growth related gene (*gh* and *igf-1b*) expression have been reported in fish. Therefore, the aim of the current study was to evaluate the effect of a diet supplemented with synthetic carotenoids (canthaxanthin and lycopene) on growth performance, immune response, and antioxidant status of yellow perch.

MATERIALS AND METHODS

Experimental Diets

Canthaxanthin powder (Carophyll red 10%)® was purchased from the eBay Company (CA, United States) and lycopene powder was obtained from the Kalyx Company (Camden, NY, United States). A commercial basal diet (AquaMax grower 400)® was crushed and divided into five portions. The first portion (Diet 1, D1) was used as a control without additive. The second (Diet 2, D2) and third (Diet 3, D3) portions were mixed with canthaxanthin (CTX) at a concentration of (50 and 100 mg/kg diet), respectively, while the fourth (Diet 4, D4) and fifth (Diet 5, D5) portions were mixed with lycopene at a concentration of (200 and 400 mg/kg diet), respectively. The diets were reformed into pellets (2 × 3 mm) using an extruder (Brabender Plasti-Corder, Model PL 2000, South Hackensack, NJ, United States). Then pellets were dried in an oven for 24 h at 50°C to maintain the activity of CTX and lycopene in each diet. The dried pellets were kept in dark plastic packs and kept in a freezer at −20°C to avoid degradation of carotenoids and oxidation. The proximate analysis of experimental diets was carried out in Dairyland Laboratories, Inc., Arcadia, WI, United States. The proximate composition of the experimental and control diets are presented in **Table 1**.

Fish and Experimental Design

Adult yellow perch were obtained from, and experiment was conducted in, the Ohio Centers for Aquaculture Research and Development, The Ohio State University, United States. Fish with (mean ± SE) 32 ± 1.0 g and 13.5 ± 0.6 cm were randomly divided into five groups in triplicate in round fiberglass tanks (60 L capacity); each replicate contained 18 fish. All groups were allowed to acclimate to the hatchery conditions for 2 weeks before starting the experiment. During the acclimation period, fish were hand fed commercial pellet (AquaMax 400)® twice per day at 9:00 and 16:00 and tanks were continuously provided with natural

TABLE 1 | Proximate chemical analysis of experimental diets.

Proximate analysis (%)	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5
Dry matter	95.25	95.71	95.28	95.06	94.45
Moisture	4.75	4.29	4.72	4.94	5.55
Crude protein	44.85	45.54	45.62	45.08	44.79
Crude lipid	11.71	11.88	11.73	11.68	11.34
Ash	9.02	9.02	9.12	8.91	8.90

ground water. Temperature was maintained at 15 ± 1.0°C and dissolved oxygen concentration at 6.8 ± 0.5 mg/L.

After acclimation, the first group was maintained as a control and fed with basal diet (D1). The second and third groups received a diet containing 50 (D2) and 100 (D3) mg/kg diet CTX, respectively, while the fourth and fifth groups were fed with lycopene at a concentration of 200 (D4) and 400 (D5) mg/kg diet, respectively. All experimental groups were fed by hand at a rate of 3% of their body weight. Feeding amount was divided into two equal parts twice per day (at 9:00 and 16:00) for 60 days. The rations were kept in small food containers at −20°C to prevent degradation or oxidation of lycopene or CTX. The amount of feed was adjusted every 2 weeks based on weight and the number of fish in each tank. All fiberglass tanks were supplied with throughflow well ground water and the photoperiod during the experimental period was adjusted for 12 h light: 12 h darkness. Fecal matter was siphoned out and 1/3 of the water was exchanged daily to maintain water quality parameters. The water parameters were monitored twice per day throughout the experiment according to the guidelines of Apha. (1998). The temperature ranged from 15.5 to 16.5°C, pH 6.8–7.2, and dissolved oxygen 6.9–8.5 mg/L. This study and all experimental procedures involving the care and use of animals were performed according to the protocol that was approved by The Ohio State University Institutional Animal Care and Use Committee.

Collection of Samples

Fish were sampled at day 0 (initial), 30, and 60. They were firstly anesthetized using MS222 (250 mg/L). The liver samples were quickly removed from six fish per dietary treatment (2 fish/replicate) at each sampling point and preserved in phosphate buffer saline with pH 7.4 at −80°C. Another piece from liver tissue was kept in RNAlater (Ambion, United States) and preserved at −80°C for later gene expression. Also, intestines of treated and control fish were taken, emptied, and washed with an ice-cold phosphate buffer (pH 7.4) two times. After that, a piece from the mid-part of intestine was cut and preserved in phosphate buffer saline at −80°C until assay of the digestive enzymes.

Determination of Growth Performance and Physiological Indices

After 30 and 60 days post feeding, growth performance and physiological indices in experimental groups were assessed according to the following formula: Weight gain rate (WGR%) = [(final body weight- initial body weight)/initial body weight] × 100, specific growth rate (SGR) = [(Ln final body weight − Ln initial body weight)/experimental period (days)] × 100, Feed conversion ratio (FCR) = [amount of feed intake (g)/weight gain]; Condition factor (CF) = [body weight (g)/body length $(cm)^3$ × 100]; Hepatosomatic Index (HSI) = [weight of liver (g)/total body weight (g) × 100].

Analysis of Body Proximate Composition

At the end of the experimental period, three samples of fish per group were randomly collected and kept at −20°C for body proximate composition analysis (moisture, crude protein, crude lipid and ash). Analysis of body proximate was carried out in Dairyland Laboratories, Inc., Arcadia, WI, United States. Moisture content was determined by drying at 105°C and ash content was measured by combustion at 550°C. Crude protein was analyzed according to standard methods (Aoac., 2000). Crude lipid was assessed using a Foss Soxtec 2047 instrument with the use of petroleum ether.

Determination of Lipid Peroxidation and Antioxidant Enzymes

The liver samples were rinsed with phosphate buffer saline to remove any red blood cells, and then homogenized in cooled 50 mM phosphate buffer saline containing 1 μm EDTA (pH 7.4) at a ratio 1:5 (w/v). The procedure was performed on crushed ice. The homogenates were centrifuged at 13,000 × *g* at 4°C for 15 min, and the resultant supernatants were separated and pooled together (*n* = 3) to reduce analytic error and stored at −80°C until analysis. The pooled liver samples were used to estimate lipid peroxidation and antioxidant enzymes.

Malondialdehyde (MDA) as indices of lipid peroxidation in liver tissue was measured using commercial kits (BioVision, United States). The assay depends on reaction between MDA in the sample with thiobarbituric acid (TBA) to generate the MDA-TBA adduct. In this assay, the samples were treated with 600 μl TBA and incubated at 95°C for 60 min then cooled in room temperature for 10 min. The obtained reaction mixture can be easily quantified colorimetrically at OD 532 nm. Antioxidant enzymes activity, superoxide dismutase activity (SOD), Catalase activity (CAT), and Glutathione peroxidase activity (GSH-Px) were assessed spectrophotometrically using (BioTek's Epoch^{TM}, United States) following the procedures of commercial kits purchased from (Cayman Chemical Company, United States).

Superoxide dismutase assay kit utilizes a tetrazolium salt for detection of superoxide radicals generated by xanthine oxidase and hypoxanthine. Standard SOD activity was measured using bovine erythrocyte SOD solution. The total reaction volume of 230 μl was composed of 200 μl tetrazolium salt solution, 10 μl sample, and 20 μl xanthine oxidase, which imitate the reaction. The SOD activity was assessed at 440 nm and expressed as units. One unit of SOD activity is defined as the amount needed to exhibit 50% dismutation of superoxide radical.

The assay of CAT activity is based on the reaction of the CAT enzyme with methanol in the presence of H_2O_2 forming formaldehyde. In this reaction, 20 μl of sample, 100 μl potassium phosphate buffer (pH 7.0), and 30 μl methanol were added to each 96 well plate. The reaction was initiated by adding 20 μl hydrogen peroxide for 20 min, then 30 μl potassium hydroxide was added to terminate the reaction. CAT activity was measured spectrophotometrically at 540 nm and calculated using the following equation [CAT activity = [(μM of sample/20 min × sample dilution)].

The principle of measuring GSH-Px activity depends on reduction of hydroperoxides by GSH-Px enzyme forming oxidized glutathione, which is recycled to its reduced state by glutathione reductase. This assay uses NADPH, glutathione, and glutathione reductase as a co-substrate mixture, and the reaction is started by adding cumene hydroperoxide. The absorbance was read once every minute at 340 nm, and GSH-Px activity was determined using the following formula [GSH-Px activity = [(ΔA340/min/(0.00373 μM^{-1}) × 9.5 ml × sample dilution]. The rate of decrease in the A_{340} is directly proportional to the GSH-Px activity in the sample.

Determination of Digestive Enzymes

Samples taken from intestine were homogenized in cooled 50 mM phosphate buffer saline containing 1 μm EDTA pH 7.4 at a ratio 1:5 (w/v) using a glass homogenizer. The homogenates were centrifuged at 13,000 × *g* at 4°C for 10 min, and the resultant supernatants were separated and pooled together ($n = 3$) then stored at −80°C until analysis.

Lipase activity was assessed using Sigma- Aldrich kit, United States. Briefly, test samples were prepared to a final volume of 50 μl with lipase assay buffer, and then 100 μl reaction mix (93 μl lipase assay buffer, 2 μl peroxidase substrate, 2 μl enzyme mix and 3 μl lipase substrate) was added to each reaction and mixed by pipetting. The plate was incubated at 37°C for 2–3 min and absorbance was measured at 570 nm every 5 min until the value of the most active sample is greater than the value of the highest standard. One unit of Lipase is the amount of enzyme that will generate 1.0 mmole of glycerol from triglycerides per minute at 37°C.

Amylase activity was quantified using a Sigma-Aldrich assay kit, which depends on cleaves of substrate ethylidene-pNP-G7 to *p*-nitrophenol by the amylase enzyme. In this regard, 10 μl of sample was added into a 96 well plate and the final volume was completed to 50 μl with amylase assay buffer. Later on, 100 μl of master reaction mix (50 μl amylase assay buffer and 50 μl amylase substrate) was added and mixed well. Absorbance was measured at 405 nm every 5 min at 25°C. Amylase activity was reported as mU/ml. One unit of amylase is defined as the amount of amylase that cleaves ethylidene-pNP-G7 to generate 1.0 μmole of *p*-nitrophenol per minute at 25°C.

Trypsin activity was determined according to the procedure of commercial kit (BioVision, United States). The samples were prepared at 50 μl with trypsin assay buffer in a 96 well plate, then 1 μl of 50× chymotrypsin inhibitor (TPCK) solution was added and incubated for 10 min at room temperature. After incubation, 50 μl of reaction mix (48 μl assay buffer and 2 μl trypsin Substrate) was added to each well containing samples, and incubated at 25°C while being protected from light. The absorbance was measured initially and after 1–2 h incubation at 405 nm (BioTek's Epoch™, United States). Trypsin activity was expressed as units; one unit is defined as the amount of trypsin that cleaves the substrate, yielding 1.0 μmol of *p*-nitroaniline (*p*-NA) per minute at 25°C.

Gene Expression of Growth and Immune Related Gene

Hepatic gene expression of growth-related genes (growth hormone (*gh*) and insulin-like growth factor (*igf-1b*) mRNAs) and immune-related gene (interleukin 1b (*il-1b* mRNAs) were evaluated at day 0, 30, and 60.

Total RNA was extracted from the liver using RNeasy mini kits (Qiagen, United States). According to the manufacturer's instructions, 20 μg liver tissue was homogenized with 350 μl buffer RLT and 3.5 μl β-mercaptoethanol, then centrifuged for 3 min at high speed. The supernatant was carefully removed and mixed well by pipetting with 350 μl of 70% ethanol. The sample (700 μl) was transferred to a RNeasy mini spin column and centrifuged at 13,000 × *g* for 30 s. Buffer RW1 (700 μl) was added to the spin column and centrifuged. After discarding flow through liquid, 500 μl buffer RPE was added to the samples twice, and then centrifuged for 30 s at 13,000 × *g*. The extracted RNA was re-suspended in RNAs dNase nuclease-free water (Ambion, United States). Total RNAs were quantified at 260/280 nm using a spectrostar nanodrop (BMG LABTECH Inc., Cary, NC, United States) to evaluate their concentration and purity, and also analyzed whether they were degraded on a Gel Doc^{TM} XR (Bio-Rad, United States) using 2% agarose gel electrophoresis. Total RNAs with clear ribosomal band and high RNA ratios ($A_{260}/A_{280} \geq 1.8$) were used for further experiments.

cDNA was synthesized using high capacity cDNA reverse transcription kits (Applied Biosystems, United States). The reaction was prepared by pipetting 10 μl of 2× reverse transcription master mix (RT) and 10 μl of RNA sample into each well of 96-well reaction plate, and mixed well by pipetting up and down twice. The plates were sealed and the reaction conditions were adjusted as follows: 25°C for 10 min, 37°C for 120 min, 85°C for 5 min, and 4°C thereafter. In order to adjust for the variation in quantity of input cDNA, the housekeeping gene β-actin was used as an internal control. Primers for RT-PCR were designed with reference to the known sequences of yellow perch (**Table 2**). Real-time quantitative PCR was performed in total volume 20 μl containing 10 μl SYBR Green Master Mix (Applied Biosystems, Warrington, United Kingdom), 1.5 μl cDNA, 1.5 μl each primer, and 5.5 μl RNAs dNase -free water. RT-PCR was performed as follows: 95°C for 10 min; 40 cycles of denaturizing at 95°C for 15 s, annealing/extension at 60°C for 60 s. We calculated the relative quantification of the target gene *il-1b*, *gh*, and *igf-1b* using the $2^{-\Delta\Delta CT}$ method and the value stood for an n-fold difference according to Zhang et al. (2013b).

Statistical Analysis

The SPSS 16.0 package program was used in analyses. One-way analysis of variance and Duncan test were used for the determination of the significance of differences among the groups at each feeding period. A value of $P < 0.05$ was considered statistically significant. Data was also analyzed by a General Linear Model (Two-way ANOVA) (SPSS 16.0 program) to test the interaction between treated groups and time of feeding on response variables. Statistical significance was considered at $P < 0.05$.

TABLE 2 | Nucleotide sequences of the primers for gene expression in yellow perch.

Target gene	Accession number	Forward (5′ → 3′)	Reverse (5′– 3′)
gh	AY007303	CGGAGGAGCAGCGTCAAC	CCCAGGACTCGACCAAACG
igf-1b	AY332492	CGCAGGGCACAAAGTGGAC	CCCAGTGTTGCCTCGACTTG
il-1b	GO656767.1	ATCTTGAGGTTGTGGAGGCA	GCACATTTCCACTGGCTTGT
B-actin	AY332493.2	GCCTCTCTGTCCACCTTCCA	GGGCCGGACTCATCGTACT

TABLE 3 | Growth performance of yellow perch fed on canthaxanthin and lycopene supplemented diets at different concentrations for 60 days.

Groups*	IBW (g)	FBW (g)	WGR (%)	FCR	SGR (%)	CF	HSI
				For 30 days			
G1	31.51 ± 1.63	49.89 ± 1.87	59.06 ± 9.02	1.61 ± 0.28	0.77 ± 0.1	1.54 ± 0.05	3.03 ± 0.47
G2	32.26 ± 0.94	48.42 ± 1.34	50.20 ± 3.35	1.81 ± 0.13	0.68 ± 0.04	1.52 ± 0.02	2.22 ± 0.49
G3	32.9 ± 0.83	50.41 ± 1.19	53.25 ± 0.92	1.69 ± 0.03	0.71 ± 0.01	1.46 ± 0.04	2.6 ± 0.43
G4	33.68 ± 0.64	52.44 ± 2.04	55.6 ± 3.68	1.63 ± 0.11	0.74 ± 0.04	1.51 ± 0.01	2.49 ± 0.29
G5	32.71 ± 0.25	52.33 ± 1.41	59.94 ± 3.28	1.51 ± 0.08	0.78 ± 0.03	1.46 ± 0.01	3.16 ± 0.23
				For 60 days			
G1	31.51 ± 1.63	73.71 ± 0.99^{ab}	135.0 ± 10.82^{ab}	1.35 ± 0.12^{ab}	1.42 ± 0.08^{ab}	1.38 ± 0.03	1.91 ± 0.68
G2	32.26 ± 0.94	69.6 ± 2.18^{b}	116.0 ± 7.78^{ab}	1.57 ± 0.11^{ab}	1.28 ± 0.06^{ab}	1.3 ± 0.02	1.71 ± 0.55
G3	32.9 ± 0.83	68.92 ± 2.29^{b}	109.46 ± 3.81^{b}	1.65 ± 0.06^{ab}	1.23 ± 0.03^{b}	1.45 ± 0.1	2.66 ± 0.49
G4	33.68 ± 0.64	73.37 ± 1.25^{ab}	118.11 ± 7.7^{ab}	1.54 ± 0.1^{ab}	1.29 ± 0.06^{ab}	1.43 ± 0.07	2.05 ± 0.45
G5	32.71 ± 0.25	78.36 ± 2.22^{a}	139.66 ± 7.86^{a}	1.3 ± 0.07^{b}	1.46 ± 0.06^{a}	1.30 ± 0.03	2.56 ± 0.36
Two-way ANOVA							
Groups		*	*	*ns*	*	*ns*	*ns*
Time		*	*	*	*	*	*ns*
Interaction		*ns*	*ns*	*ns*	*ns*	*ns*	*ns*

*Values are mean (n = 3 replicate) ± SE. Mean values in the same column for each parameter with no letters or same letter are non-significant (P > 0.05). *Group 1, control; Group 2, canthaxanthin (50 mg/kg diet); Group 3, canthaxanthin (100 mg/kg diet); Group 4, lycopene (200 mg/kg diet); Group 5, lycopene (400 mg/kg diet).*

RESULTS

Growth Performance

The results of the effects of CTX and lycopene on yellow perch growth performance are shown in **Table 3**. No mortality occurred throughout the experiment and fish exhibited good health conditions. Growth performance and physiological indices of yellow perch fed CTX or lycopene-supplemented diet recorded no differences ($P > 0.05$) from the control group fed a carotenoid-free diet. Nevertheless, higher growth rates were observed in groups fed a lycopene diet (400 mg/kg diet) for 60 days. In addition, there were no significant interaction ($P > 0.05$) between treated groups and time of feeding.

Body Composition

There was no significant ($P > 0.05$) effect for dietary canthaxanthin or lycopene on body composition of fish compared to the control (**Table 4**). Fish fed lycopene at a dietary level of 400 mg/kg diet exhibited higher lipid content than fish fed the control diet.

Antioxidant Enzymes Activity and MDA Level

After 30 days of feeding, liver MDA level showed no significant difference among treated groups when compared to the control. After 60 days of feeding, MDA level was significantly decreased in yellow perch fed lycopene at a concentration of 400 mg/kg diet compared to other treated and control groups. MDA level was significantly ($P < 0.05$) affected by the interaction between experimentally treated groups and feeding time (**Figure 1A**).

The antioxidant enzymes activity is presented in **Figures 1B–D**. Dietary CTX and lycopene had no significant effect on SOD activity of yellow perch after 30 days of feeding. After 60 days of feeding, groups that received CTX at a concentration 50 mg/kg diet and lycopene at a dose of 200 and 400 mg/diet recorded a significant decrease of SOD activity. Yellow perch fed a diet supplemented with CTX at a concentration of 100 mg/kg revealed

TABLE 4 | Body composition of yellow perch fed on canthaxanthin and lycopene supplemented diets at different concentrations for 60 days.

Groups*	Moisture	Crude protein	Crude lipid	Ash
G1	72.85 ± 0.61	19.88 ± 0.84	4.09 ± 0.17^{bc}	4.38 ± 0.86
G2	71.63 ± 0.93	19.22 ± 0.88	3.86 ± 0.30^{c}	4.68 ± 0.32
G3	70.72 ± 1.23	19.86 ± 0.98	4.52 ± 0.09^{abc}	5.31 ± 0.91
G4	73.07 ± 0.86	19.87 ± 0.72	5.42 ± 0.34^{ab}	3.99 ± 0.21
G5	72.31 ± 1.18	20.02 ± 1.16	5.86 ± 0.83^{a}	4.18 ± 0.76

*Values are mean (n = 3) ± SE. Mean values in the same column for each parameter with no letters or same letter are non-significant (P > 0.05). *G1, control; G2, canthaxanthin (50 mg/kg diet); G3, canthaxanthin (100 mg/kg diet); G4, lycopene (200 mg/kg diet); G5, lycopene (400 mg/kg diet).*

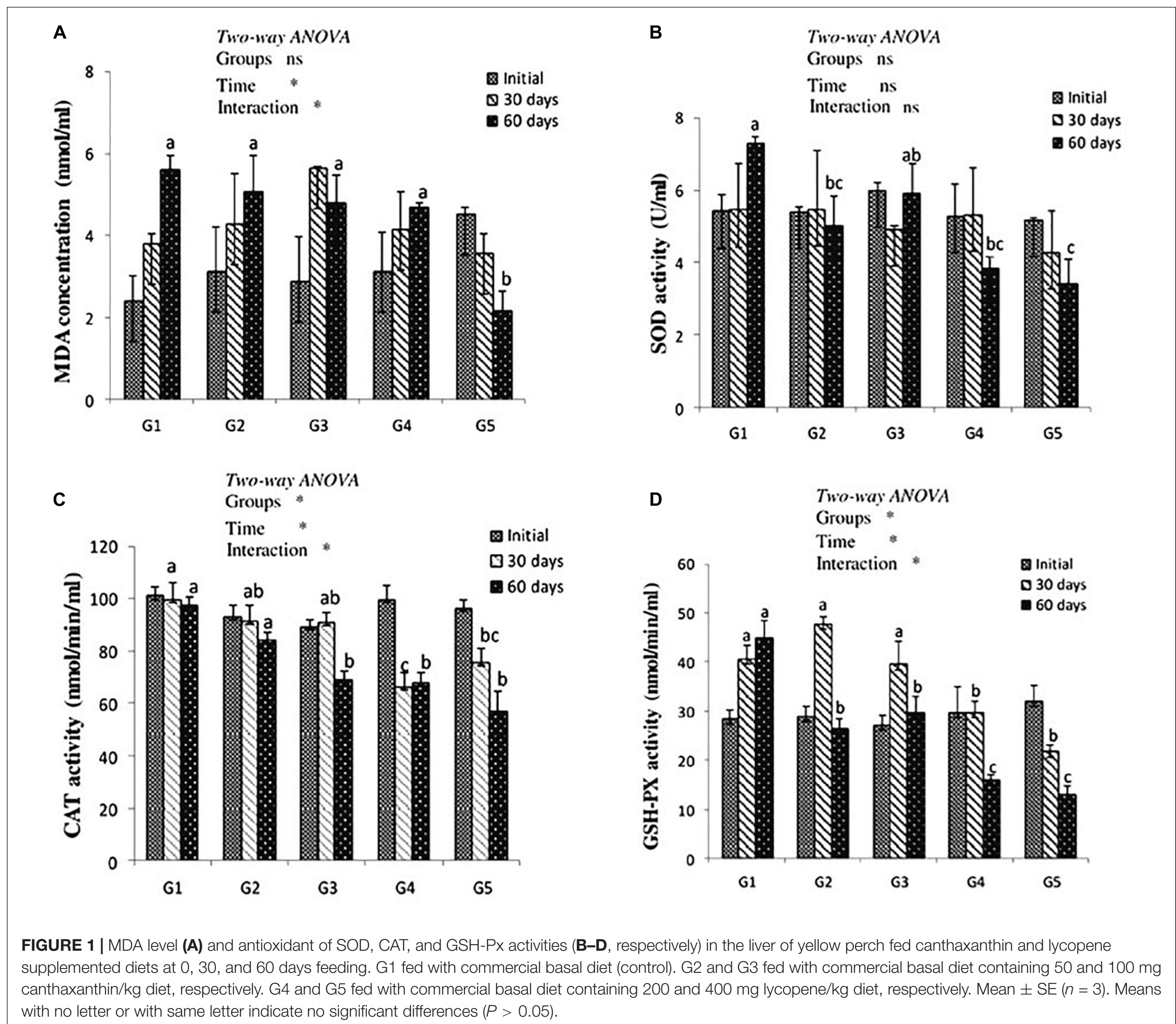

FIGURE 1 | MDA level **(A)** and antioxidant of SOD, CAT, and GSH-Px activities (**B–D**, respectively) in the liver of yellow perch fed canthaxanthin and lycopene supplemented diets at 0, 30, and 60 days feeding. G1 fed with commercial basal diet (control). G2 and G3 fed with commercial basal diet containing 50 and 100 mg canthaxanthin/kg diet, respectively. G4 and G5 fed with commercial basal diet containing 200 and 400 mg lycopene/kg diet, respectively. Mean ± SE (n = 3). Means with no letter or with same letter indicate no significant differences ($P > 0.05$).

a significant decrease of CAT activity after 60 days of feeding. The incorporation of lycopene significantly decreased CAT activity throughout the experimental period. Liver GSH-Px activity of yellow perch showed a significant decrease with dietary lycopene at both concentrations during the experimental period, while fish treated with 50 and 100 mg CTX/kg diet exhibited a significant decrease in GSH-Px activity after 60 days of feeding. The interaction between feeding time and groups was significantly ($P < 0.05$) observed with CAT and GSH-Px activities, while SOD activity showed no significant ($P > 0.05$) effect.

Digestive Enzymes Activity

Results of digestive enzymes activities are shown in **Figure 2**. Amylase activity revealed no significant difference in fish fed CTX or a lycopene-supplemented diet for 30 and 60 days when compared with the control. Lipase enzyme activity recorded significant increases ($P < 0.05$) with dietary lycopene at a concentration of 200 and 400 mg/kg diet after 60 days of feeding. In addition, trypsin activity significantly increased with lycopene dietary supplementation at a concentration of 400 mg/kg diet for 60 days of feeding. Amylase, lipase, and trypsin enzymes activity were not significantly affected by the interaction between groups and feeding time.

Gene Expression

As can be seen from **Figure 3**, there was no significant effect on hepatic *gh and igf-1b* mRNA expression level in yellow perch along the entire period of the experiment (**Figures 3A,B**). In addition, non-significant ($P > 0.05$) interaction between carotenoids treated groups and feeding time was observed. Immune-related gene *il-1b* mRNA expression level revealed up-regulation in groups fed on CTX at different concentrations for

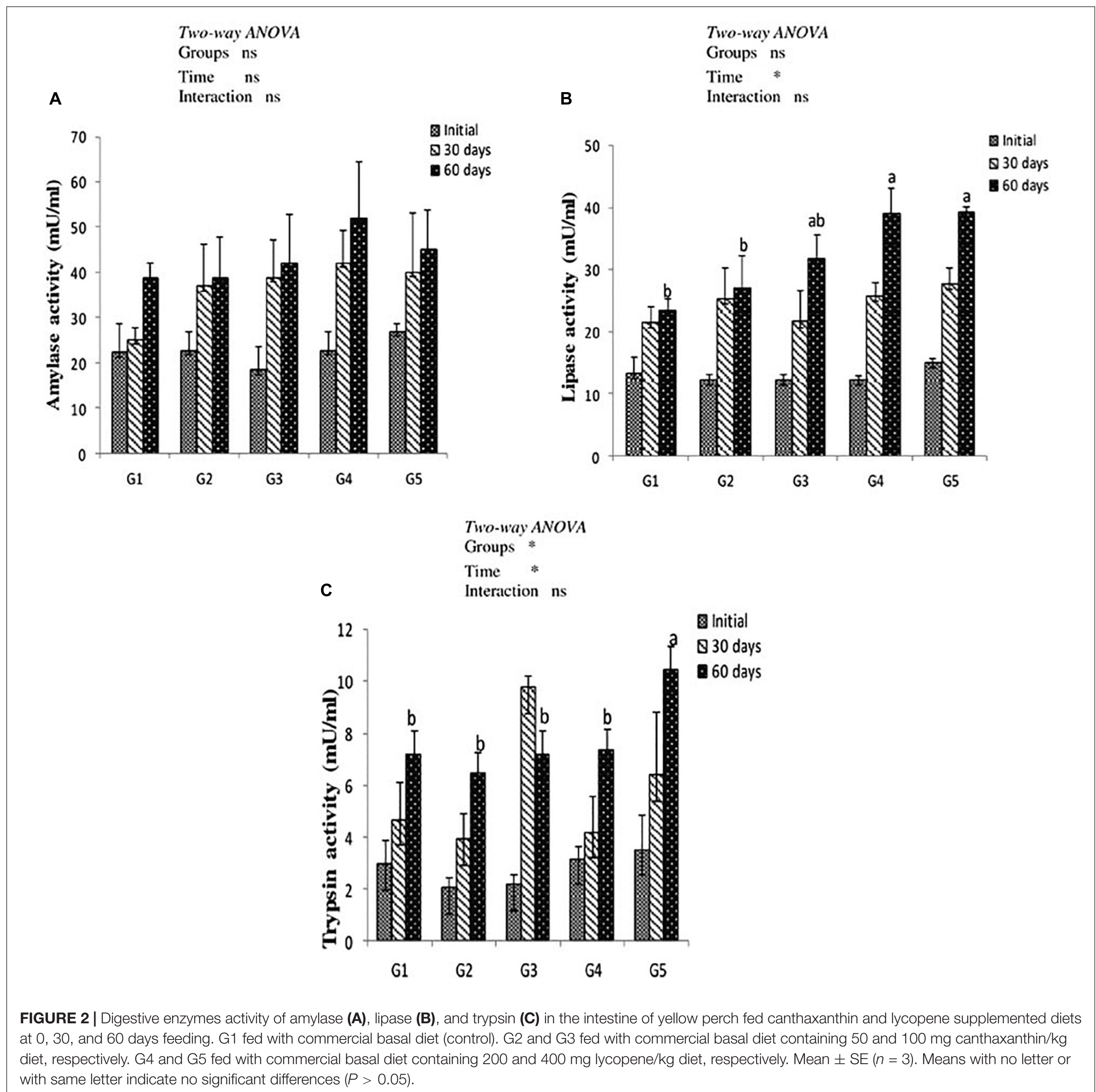

FIGURE 2 | Digestive enzymes activity of amylase **(A)**, lipase **(B)**, and trypsin **(C)** in the intestine of yellow perch fed canthaxanthin and lycopene supplemented diets at 0, 30, and 60 days feeding. G1 fed with commercial basal diet (control). G2 and G3 fed with commercial basal diet containing 50 and 100 mg canthaxanthin/kg diet, respectively. G4 and G5 fed with commercial basal diet containing 200 and 400 mg lycopene/kg diet, respectively. Mean ± SE (n = 3). Means with no letter or with same letter indicate no significant differences ($P > 0.05$).

30 days and fish fed lycopene at a concentration level 400 mg/kg diet for 60 days. Moreover, *il-1b* was significantly affected by the interaction between treated groups and feeding time (**Figure 3C**).

DISCUSSION

In the aquaculture industry, carotenoids are commonly incorporated in farmed fish and shellfish diets as pigmentation source to provide desirable coloration to these cultured organisms (Kalinowski et al., 2005; Kop and Durmaz, 2008; Li et al., 2017). It also has a positive effect on fish reproduction (Vassallo-Agius et al., 2001), antioxidant (Sahin et al., 2014), and immune systems. In addition, the ameliorative effect of synthetic carotenoids against stress such as pesticides exposure (Yonar and Sakin, 2011); antibacterial drug stress (Yonar, 2012); confinement (Girao et al., 2012) and high stocking density (Sahin et al., 2014) have been studied.

In the present study, dietary canthaxanthin and lycopene had no significant effect on the proximate composition of yellow perch compared to the control. This result comes in accordance with rainbow trout, *Oncorhynchus mykiss* that

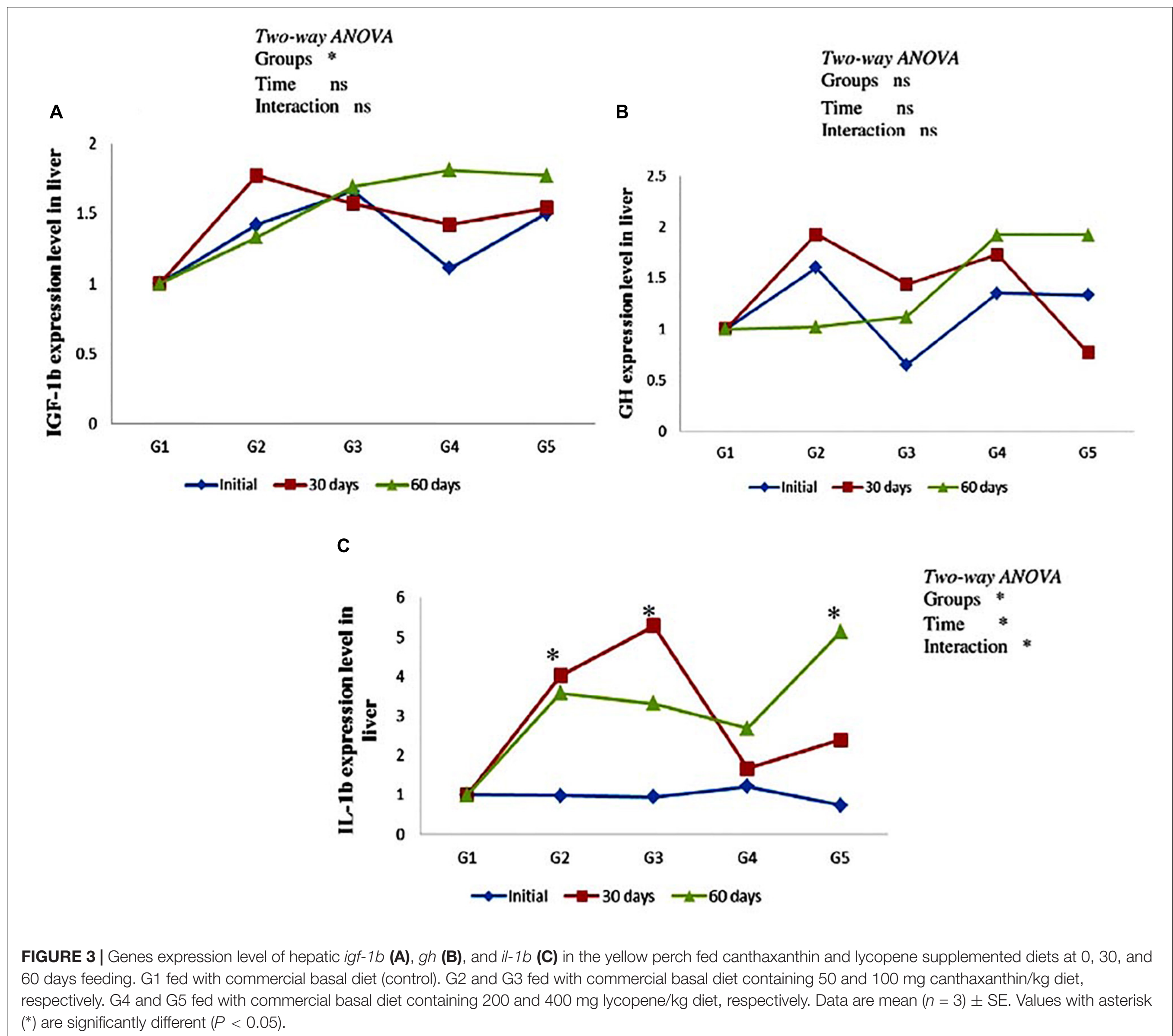

FIGURE 3 | Genes expression level of hepatic *igf-1b* **(A)**, *gh* **(B)**, and *il-1b* **(C)** in the yellow perch fed canthaxanthin and lycopene supplemented diets at 0, 30, and 60 days feeding. G1 fed with commercial basal diet (control). G2 and G3 fed with commercial basal diet containing 50 and 100 mg canthaxanthin/kg diet, respectively. G4 and G5 fed with commercial basal diet containing 200 and 400 mg lycopene/kg diet, respectively. Data are mean (n = 3) ± SE. Values with asterisk (*) are significantly different ($P < 0.05$).

were fed astaxanthin for 60 days (Zhang et al., 2013a) and for 10 weeks (Rahman et al., 2016). Moreover, Zhang et al. (2013b) recorded that Pacific white shrimp, *Litopenaeus vannamei* fed astaxanthin supplemented diet revealed no significant difference in body composition. Carotenoids are lipid-soluble compounds and their absorption into enterocytes occur by passive diffusion or receptor-mediated transport after emulsification into mixed micelles which formed from bile salts, phospholipids, cholesterol, and lipolytic products such as free fatty acids, monoacylglycerols and lysophospholipids (Chimsung et al., 2014). However, micronutrient molecular structure, pH and bile lipid concentration, and the presence of a minimal amount of dietary fat can affect its transfer to mixed micelles (Tyssandier et al., 2001). In this sense, the physiological response of yellow perch growth with dietary lycopene at a level 400 mg/kg diet was better than other treated groups as a result of higher lipid content which might eventually regulate its uptake by intestinal mucosal cells and transportation processes (Castenmiller and West, 1998). In the same manner, Christiansen et al. (1995) recorded high lipid content in Atlantic salmon fed diets containing astaxanthin for 11 weeks. Therefore, the lipid content of a fish indicates the excess energy available for maintenance of growth (Tocher, 2003).

Dietary supplementation of canthaxanthin and lycopene did not affect growth performance, feed utilization, and survival of yellow perch. These findings were further supported by our results of the gene expression in the current study where the growth-related genes *gh* and *igf*-1b recoded no difference among treated and control groups. These results were similar with previous studies that recorded no significant effect for synthetic carotenoids in characins, *Hyphessobrycon callistus* (Wang et al., 2006), juvenile rainbow trout (Zhang et al., 2013a; Rahman et al., 2016), and Atlantic salmon, *Salmo salar*

(Baker et al., 2002). The observation of no effect of dietary canthaxanthin on growth parameters was also recorded in red porgy, *Pagrus pagrus* (Kalinowski et al., 2005) and parrot cichlid (*Amphilophus citrinellus* × *Paraneetroplus synspilus*) (Li et al., 2017). Moreover, Nile tilapia, *Oreochromis niloticus* fed diet supplemented with lycopene 600 mg/kg for 60 days displayed no effect on weight gain, feed conversion ratio, and specific growth rate (Girao et al., 2012). Non-significant effect of carotenoids on yellow perch growth might be due to the lack of its absorption, which differed according to dietary levels and fish species, or might be due to the short period for dietary supplementation. Brown et al. (2009) stated that yellow perch native to North America has a relatively slow growth rate; this might be a possible explanation to our finding. In this study, yellow perch fed CTX-incorporated diets showed lower growth rate than the control group; it could be inferred that fish preferred the non-supplemented diet over those supplemented with carotenoids due to their flavor. Torrissen et al. (1990) recorded that higher inclusion of canthaxanthin with more than 50 mg/kg of feed in rainbow trout must be avoided. However, other studies observed that dietary carotenoids have a positive effect on nutrient utilization, specific growth, and improved weight gain (Amar et al., 2001; Zhang et al., 2013b; Alishahi et al., 2014; Cheng et al., 2018). This difference may be due to a difference of fish species, feeding behavior, as well as type, concentration, and duration of dietary carotenoids supplemented. Kop and Durmaz (2008) reported that the effectiveness of carotenoid is species-specific and that all fish species possess different pathways for the metabolism of carotenoids. Moreover, Kalinowski et al. (2005) suggested feeding fish with carotenoid-supplemented diets for a longer period to observe their significant role on growth.

Physiological indices such as condition factor, hepatosomatic, spleensomatic, and viscerosomatic are considered stress indicators in fish (Barton et al., 2002). All the experimental groups fed either canthaxanthin or lycopene in the current study recorded no significant difference in condition factor or hepatosomatic index, similarly as observed in rainbow trout that were fed astaxanthin (Zhang et al., 2013a). This indicated that dietary carotenoids had no detrimental effects on liver tissue or general fish health conditions.

Lipid peroxidation is considered to be a valuable indicator of cellular membrane damage caused by any stressors (Ferreira et al., 2005). The current study recorded no significant difference in liver MDA level among groups treated with canthaxanthin compared to the control. Meanwhile, the lycopene-treated group, at a dose 400 mg/kg diet, exhibited a significant decrease in MDA level after 60 days. A similar finding was previously reported by Yonar and Sakin (2011), Girao et al. (2012), Ural (2013), and Zhang et al. (2013a). During free radical scavenging, energy is transferred from singlet-oxygen to the lycopene molecule, which converts lycopene to the energy-rich triplet state. This indicates that the inclusion of lycopene decreased the MDA level, due to its free radical scavenging properties that delay lipid peroxidation by inhibiting the initiation or propagation phase of oxidizing chain reactions. Hence, it could protect tissue against the oxidation of lipids, proteins, and DNA (Matos et al., 2000; Wertz et al., 2004; Sahin et al., 2014).

Superoxide dismutase, CAT, and GSH-Px are considered the most important endogenous antioxidant enzymes required to convert superoxide radicals into hydrogen peroxide, and then into water and molecular oxygen. The activities of these enzymes reflect the ability of scavenging oxygen-free radicals, and the changes in redox status of a cell (Zhang et al., 2013a). Our results showed that canthaxanthin and lycopene fed fish had the lowest SOD activity after 60 days of feeding. These findings were in accordance with previous studies of other fish species fed carotenoid treated diets (Wang et al., 2006; Zhang et al., 2013a; Rahman et al., 2016). In addition, Yonar and Sakin (2011) recorded that carp, *Cyprinus carpio* received lycopene for 2 weeks and had no significant effect on hepatic SOD activity compared to control. Lygren et al. (1999) indicated that dietary supplementation of fat soluble antioxidants at high levels reduced the need for the production of endogenous antioxidant enzymes such as SOD and CAT, which are necessary for cell protection against reactive H_2O_2 and O_2^-. It seems that since dietary lycopene and canthaxanthin in yellow perch can already provide protection against ROS and hence there is less stimulation for the production of endogenous antioxidant enzymes.

Superoxide dismutase is part of the critical antioxidant enzyme system, which can convert the intracellular oxygen free radicals (O_2-) into hydrogen peroxide (H_2O_2) and oxygen (O2). In addition, it plays an important role in the enhancement of phagocytic cell activity. The decrease of hepatic SOD activity in yellow perch that were fed carotenoid-supplemented diets in the current study could be attributed to the potential free radical scavenging activity of lycopene and canthaxanthin that effectively eliminate these radicals, or might be due to the inhibition of superoxide radical formation. The antioxidant mechanism of lycopene is a consequence of its chemical structure; it has 11 conjugated double bonds. Hence, due to its polyene structure, it can provide electrons to free radicals or attract unpaired electrons of free radicals, preventing lipid peroxidation and DNA damage. Canthaxanthin antioxidant mechanism occurs through capturing peroxyl-free radicals in its conjugated polyene system (Surai, 2012). Di Mascio et al. (1989) and Palozza et al. (2012) reported that lycopene is one of the most potent antioxidants, with a singlet-oxygen-quenching ability twice as high as that of β-carotene and 10 times higher than that of vitamin E.

Catalase rapidly catalyzes the decomposition of the damaging byproduct H_2O_2 into less reactive gaseous oxygen and water molecules, therefore, its high activity indicates that fish suffered from oxidative stress (Halliwell and Gutteridge, 2007). In the present study, dietary supplementation of lycopene and canthaxanthin significantly decreased hepatic CAT and GSH-Px activities in yellow perch after 60 days of feeding, indicating the strong capability of canthaxanthin and lycopene to quench singlet oxygen and subsequently maintain the redox status of cell. Similarly, significant decrease of CAT activity was also reported in Nile tilapia that were fed lycopene (Girao et al., 2012) and rainbow trout that were fed astaxanthin (Zhang et al., 2013a). Mourão et al. (2009) reported that exogenous antioxidants could decrease CAT activity. In this sense, fish supplemented with natural antioxidants could exhibit a reduction in the activity of CAT enzyme. Moreover, Halliwell and Gutteridge (2007)

indicated that significant decrease of antioxidant activity could be argued to favorable maintenance of the redox state in the cell.

Glutathione peroxidase exists in blood, liver, mitochondria, and cytoplasm and plays a role in the removal reaction of H_2O_2; therefore, it is considered as one of the most important antioxidant defenses against oxygen toxicity inside the cells (Cohen and Doherty, 1987). The detoxifying process of H_2O_2 occurs through catalyzing the reduction in hydroperoxides using glutathione (GSH) producing glutathione disulfide (GSSG), which is reduced to GSH by glutathione reductase. The lower GSH-Px activity in yellow perch indicated that cell protection was activated as a result of dietary canthaxanthin and lycopene at different concentrations, especially for a long period of 60 days. It has been recorded that lycopene can upregulate the antioxidant electrophile/antioxidant response element, thereby stimulating the production of phase II detoxifying antioxidant enzymes that protect cells from reactive oxygen species and other electrophilic molecules (Ben-Dor et al., 2005; Palozza et al., 2012). Antioxidant defenses in fish are dependent on many factors such as age, feeding behavior, and nutritional factors. Radi et al. (1987), in a comparative study between different fish species, reported that carnivorous species had very low GSH-Px activity in the liver compared to other species. In this regard, yellow perch, as carnivore fish, might be exhibit low antioxidant activity. There is positive correlation between dietary supplementation with antioxidants and health status of fish (Küçükbay et al., 2009). Hence, the physiological response of antioxidant enzymes allow fish maintain their homeostasis, which necessary for physiological processes as growth, immunity and activation of stress response in fish (Trenzado et al., 2008).

Digestive enzyme activities among fish species vary by their age and feeding behavior (Péres et al., 1998). The growth of fish relies on nutrient utilization, which is reflected by the development of digestive organs and activities of intestinal enzymes (Yan and Qiu-Zhou, 2006). Therefore, the profile of digestive enzymes indicates the ability of a species to use different nutrients. To the best of our knowledge, there was no study on the effect of synthetic carotenoids on digestive enzymes activity in fish. In the current study, dietary canthaxanthin revealed no significant effect on digestive enzymes activity of yellow perch along the experimental period. This result was similar with findings recorded by Peixoto et al. (2016) in European sea bass (*Dicentrarchus labrax*). As a general assumption, amylase enzyme exhibit lower activity in carnivorous fish fed a diet of low carbohydrate level than omnivorous fish (Harpaz and Uni, 1999). This could be attributed to a non-significant difference of amylase enzyme in yellow perch, as it is a carnivore fish. Intestinal lipase and trypsin activities in the current study recorded a significant increase with dietary lycopene at a high concentration of 400 mg/kg diet for 60 days feeding. This finding was supported by Grosell et al. (2011), who reported that emulsification of fat and fat-soluble compounds into lipid micelles occurs in the small intestine and can increase efficiency of the lipase enzyme. Khani et al. (2017) revealed that natural microalgae *Chlorella vulgaris* supplementation significantly increases lipase enzyme activity in the intestine of koi, *Cyprinus carpio*. This could support our result, in which a significant increase of lipase activity was recorded in yellow perch that were fed diets containing lycopene, compared to canthaxanthin-treated groups. This increase might improve the digestion of fat, which could, in turn, explain the better growth of yellow perch with a lycopene-supplemented diet. The lower digestibility of canthaxanthin, when compared to lycopene, may be due to the presence of keto-hydrocarbon and esterification of the hydroxy-groups, which is the first step in the absorptive process (Young and Fox, 1936).

There was no available data on the dietary effect of synthetic carotenoids on gene expression of growth or immune-related genes in aquaculture. Therefore, this is considered to be the first study in yellow perch fed dietary carotenoids at different levels. Insulin-like growth factor Ib (*igf-1b*) is secreted by the liver and stimulated by *gh*, which in turn enhances growth rate (Suzuki et al., 2004). Growth-related genes [*gh* mRNA and *igf-1b* mRNA] expression level revealed no significant up-regulation in groups fed on canthaxanthin or lycopene for 60 days; this explains, to a certain extent, the non-significant effect on growth performance in these groups compared to the control. This could be attributed to the exposure of fish to acute handling stress, which can down-regulate the expressions of certain genes related to growth (Nakano et al., 2013). Insulin like growth factor-I was down-regulated in the liver of Nile tilapia fingerlings that were fed probiotics and prebiotics (Hassaan et al., 2015). Elabd et al. (2016) reported that incorporating *Astragalus membranaceus* and *Glycyrrhiza glabra* (liquorice) in the diet of yellow perch markedly up-regulated the expression of growth-related genes, insulin-like growth factor-1 (*igf-1*). Cytokines are regulatory proteins secreted by immune cells that initiate and regulate cellular function such as the immune response, inflammation, acute phase response, and tissue repair (Ollier, 2004). Interleukin-1b (*il-1b*) are synthesized by hepatocytes and their production is considered to be indicators of an inflammatory response (Secombes et al., 2001), and they can regulate the production of other cytokines. In our study, the *il-1b* gene revealed up-regulation in groups fed on canthaxanthin at different concentrations for 30 days, and fish that were fed lycopene at a concentration level of 400 mg/kg diet for 60 days, compared to the control. This gene has a crucial role in the host response during microbial invasion and tissue injury due to the enhancement of phagocytic activity, macrophage proliferation, lysozyme synthesis, and leukocyte migration (Magnadóttir et al., 2005). This result coincides with Giri et al. (2015) in *Labeo rohita* that were fed guava leaves and common carp, *Cyprinus carpio* fed *Rehmannia glutinosa* (Wang et al., 2015). Moreover, dietary Spirulina in common carp significantly increases the expression of interleukin-1b (Watanuki et al., 2006). On the other hand, Lee et al. (2003) recorded that astaxanthin inhibits the expression of inflammatory gene of tumor necrosis factor-α (*tnf*-α) and *il-1b* in mice.

It could be concluded that growth and feed utilization are not affected by dietary supplementation of canthaxanthin. However, lycopene-supplemented diets revealed a positive effect on digestive enzymes activity and growth of yellow perch. Both carotenoids have a protective effect by its antioxidant activity, and enhance yellow perch immunity through up-regulation of the immune-related gene (*il-1b*). In this sense,

the inclusion of lycopene or canthaxanthin is recommended as dietary supplements in yellow perch for protection against oxidative stress and disease occurrence.

ETHICS STATEMENT

This study and all experimental procedures involving the care and use of animals were performed according to the protocol that was approved by The Ohio State University Institutional Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

H-PW and EAE-G conceived the experiment. EAE-G conducted the experiment, data analysis, and drafted the manuscript. HY helped and verified the analytical methods. H-PW revised and finalized the manuscript. All authors discussed the results and contributed to the final manuscript.

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Transcriptome Analysis Reveals That Naphthenic Acids Perturb Gene Networks Related to Metabolic Processes, Membrane Integrity, and Gut Function in *Silurana (Xenopus) tropicalis* Embryos

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Naphthenic acids (NAs) are oil-derived mixtures of carboxylic acids and are considered emerging contaminants with the potential to disrupt development of aquatic species. In the Oil Sands Region of Canada, NAs are components of the water released following processing of the bitumen-containing sand. The aim of this research was to identify potential mechanisms of toxicity of NA mixtures. *Silurana* (*Xenopus*) *tropicalis* embryos were raised in water spiked with commercial oil-derived NA extracts (S1 and S2) at a sub-lethal concentration (2 mg/L). The transcriptomic responses of the whole 4-day old embryos following exposure were assessed using a custom oligonucleotide microarray. Both NA mixtures induced embryonic abnormalities that included edema, and cardiac and gut abnormalities. Exposure to NAs also affected morphometric parameters and decreased total length, tail length, and interorbital distance of the embryos. Gene ontology analysis revealed that 18 biological processes, 5 cellular components, and 19 molecular functions were significantly enriched after both S1 and S2 exposures. Sub-network enrichment analysis revealed pathways that were related to phenotypic abnormalities; these included gut function, edema, and cartilage differentiation. Other notable networks affected by NAs included metabolism and cell membrane integrity. In a separate dose-response experiment, the expression of key genes identified by microarray (*cyp4b1*, *abcg2*, *slc26a6*, *eprs*, and *slc5a1*) was determined by Real-Time qPCR in *S. tropicalis* embryos exposed to the commercial NAs and to acid-extractable organics (AEOs) prepared from Oil Sands Process-Affected Water. In general, the RT-qPCR data agreed with the microarray data. In *S. tropicalis* embryos exposed to the AEOs, the mRNA levels of *eprs (bifunctional glutamate/proline-tRNA ligase)* and *slcs5a1 (sodium/glucose cotransporter 1)* were significantly decreased compared to

the controls. Such changes are likely indicative of increased edema and disrupted gut function, respectively. These data suggest that NAs have multiple modes of action to induce developmental toxicity in amphibians. Some modes of action may be shared between commercial NAs and AEOs.

Keywords: acid extractable organics, edema, gut abnormalities, naphthenic acids, oil sands, microarray, toxicity, *Western clawed frog*

INTRODUCTION

Interest in the potential toxicity of oil sands process-affected water (OSPW) emerged in the 1960s after the initiation of oil extraction activities in the oil sands region of Alberta, Canada (Grewer et al., 2010). Crude bitumen from the oil sands contains petroleum mixed with inorganic material (Government of Canada, 2013). Extraction processes require up to 2.5 barrels of water to produce one barrel of crude oil (Natural Resources Canada, 2019). The OSPW is kept in separate tailings ponds to prevent contamination of natural water sources. As of 2015, there was a total surface area of 98 km^2 of tailings pond water (Environment and Parks Alberta, 2015). Frank et al. (2014) compared the chemical profile of the groundwater of the Athabasca River to OSPW and found important similarities, concluding that tailings pond water was leaching out of the ponds and into the surrounding environment.

The toxicity of the OSPW has mainly been attributed to the presence of naphthenic acids (NAs) (MacKinnon and Boerger, 1986). The O_2 family is represented by classical NAs with just one carboxylic acid group or dihydroxy groups, and published data concur that O_2 species are the main toxic and classical NAs in OSPW (Morandi et al., 2015; Hughes et al., 2017; Huang et al., 2018). The term NAs refers to all the carboxylic acids present in crude oil and these are now classified as emerging water contaminants (Richardson and Ternes, 2018). Naphthenic acids have been also detected in sediments after oil spills (Aeppli et al., 2012; Wan et al., 2014), and are also used commercially in a wide range of products such as paint and ink driers, wood and fabric preservatives, fuel additives, emulsifiers and surfactants, and are used in the production of metal salts (Brient et al., 2000). There are only a few published studies that have attempted to address the mechanisms of NA toxicity in aquatic organisms. Frank et al. (2009) proposed narcosis as a mechanism of NA toxicity in *Daphnia magna*. Later, Marentette et al. (2017) suggested oxidative stress as a possible toxic mechanism of naphthenic acid fraction components (NAFCs) of OSPW affecting walleye (*Sander vitreus*) embryos. More detailed studies are needed to assess the effects and potential mechanisms of NA toxicity. Considering that there are more than 100 countries that produce petroleum, the toxicity and biological effects that may be caused by diverse NAs is of international concern.

The interest in frogs as a test organism is due to their importance in the food web, their susceptibility to pollutants and endocrine disruptors in water, and as a model for vertebrate development. Specifically, the African Western clawed frog (*Silurana* (*Xenopus*) *tropicalis*) is a diploid model species commonly used in the field of developmental biology and toxicology (Rosenfeld et al., 2017). A sequenced genome and relatively high number of conserved genes compared to humans, in particular, those controlling development and associated with disease (Hellsten et al., 2010), make *S. tropicalis* an amenable model organism. Moreover, frogs in early developing stages are susceptible to endocrine disruptors, and studies have shown that the exposure of frogs in larval stages to stressors has effects on future morphology, such as body size, locomotor ability, and survivorship (Crespi and Denver, 2005). There have been a few articles published regarding the toxicity of NAs in frogs (Hersikorn and Smits, 2011; Smits et al., 2012; Melvin and Trudeau, 2012a,b; Melvin et al., 2013; Vu et al., 2015). In this study, we report the developmental and transcriptomic responses of *S. tropicalis* embryos to NAs in order to determine potential mechanisms of toxicity.

MATERIALS AND METHODS

Chemicals

Two commonly available commercial NA mixtures were purchased from Sigma-Aldrich (Cat. # 70340; lot numbers BCBC9959V and BCBK0736V), hereafter called Sigma 1 (S1) and Sigma 2 (S2), respectively. The acid extractable organic mixture (AEO) contains organic compounds from the acid extraction of an OSPW sample from Alberta, Canada. Details on the source, handling and preparation of the extract can be found in Gutierrez-Villagomez et al. (2019). The different mixtures were previously described and characterized by electrospray ionization high-resolution mass spectrometry (Gutierrez-Villagomez et al., 2019) and gas chromatography coupled to mass spectrometry (Gutierrez-Villagomez et al., 2017). The S1 and S2 are composed largely of the O_2 and O_4 classes. The AEO extract is composed mainly of O_2, O_3, O_4 (89.3%) and other species in a lower proportion (10.7%). The O_2 species proportion in the AEO extract was estimated as 58.6%. Human chorionic gonadotropin (hCG) was purchased from Millipore. The salts used in the preparation of the FETAX solution (NaCl, $NaHCO_3$, KCl, $CaCl_2$, $CaSO_4$, and $MgSO_4$) were purchased from Fisher Scientific. L-Cysteine was obtained from Sigma-Aldrich.

Animals and Exposure

Adult frogs originated from the University of Ottawa's *S. tropicalis* colony. Adult frogs were fed daily with Nasco pellets and kept under a 12 h light/dark cycle. Embryos were obtained by injection of hCG into the posterior lymph sac of adult *S. tropicalis* as previously described (Langlois et al., 2010). Briefly, the frogs were injected with a priming dose of 12.5 IU of hCG into the posterior

lymph sac and moved into glass tanks with FETAX solution [Standard Guide for Conducting the Frog Embryo Teratogenesis Assay-Xenopus (FETAX)] (ASTM, 2012) with a temperature of 21.4°C and pH of 5.9. After 24 h, the breeding pairs were injected with a boosting dose of 100 IU of hCG and placed in glass tanks with FETAX solution with a temperature of 21.6°C and a pH of 5.8. The exposures were carried out according to the Frog Embryo Teratogenesis Assay Xenopus (FETAX). FETAX is a rapid test for identifying teratogenic compounds or mixtures (FETAX) (ASTM, 2012). After spawning, the embryos were collected and the jelly coat was removed by gently mixing for 3 min in a 2% w/v L-cysteine solution prepared with FETAX solution. Cleaned embryos were then moved to a tank with FETAX solution. According to Nieuwkoop and Faber (NF) staging system (Nieuwkoop and Faber, 1994), embryos at stages 9–10 were individually selected under a light microscope. Four replicates with 10 embryos were used for each treatment and were placed in 10 cm Petri dishes containing 50 mL of the NA solutions. Solutions of NAs for the different treatments were prepared daily from a stock that was kept at 4°C and covered from light. The stock solutions from S1 and S2 were prepared using ethanol as a solvent. The final concentration of ethanol in all of the treatments was 0.0025%. The AEO extract contained 8,050 mg/L NAs in a solution of 0.1 N of NaOH as previously reported (Gutierrez-Villagomez et al., 2019). Glassware preparation also followed (Gutierrez-Villagomez et al., 2019): 15 min soaking with hot water and detergent, rinsed twice with reverse osmosis water, later rinsed in a 10% HCl solution, rinsed twice with reverse osmosis water, rinsed with acetone (Fisher Scientific, Certified ACS grade, 99.8%), rinsed twice with reverse osmosis water and dried in an oven at 120°C for 3 h. The embryos were exposed to S1 and S2 at 2 mg/L as reported (Gutierrez-Villagomez et al., 2019). This concentration was selected as it induces sub-lethal effects in *S. tropicalis* embryos. Control embryos were placed in FETAX solution. Treatment solutions were prepared daily from a respective stock solution that was kept at 4°C and covered from light. Every 24 h the embryos were observed, dead embryos were removed, and the solutions were replaced to ensure concentration and water quality. The exposure was performed in a controlled light/temperature room with a 12:12 light:dark cycle. The water temperature was 25 ± 1°C. After 96 h of exposure, tadpoles were sampled to measure gene expression profiles. The tadpoles were anesthetized with a solution of 0.5 g/L tricaine-S [MS-222, tricaine methanesulfonate (TMS); Western, Chemical, Inc.]. Digital images of the embryos at 96 h were taken under a light stereomicroscope (Nikon SMZ 1500), with a Nikon DS-Fi1 camera and NIS Elements version 3.22.00 software. Images were used for morphometric measurements. The abnormalities were classified according to the FETAX Atlas of Abnormalities (Bantle, 1991). The care and treatment of animals used in this study were in accordance with the guidelines of the Animal Care Committee of the University of Ottawa and the Canadian Council on Animal Care.

Microarray Analysis

Four *S. tropicalis* larval samples for the S1 and S2 treatments were used for microarray analysis. Each sample consisted of a pool of 10 larvae to ensure an adequate quantity of RNA. Total RNA extraction was done using RNeasy Mini Kit (Qiagen) with on-column RNase-free DNase treatment (Qiagen). RNA concentrations were determined using NanoDrop-2000 spectrophotometer (Thermo Scientific). Total RNA integrity was assessed using RNA 6000 Nano Assay Kit with the 2100 Bioanalyzer (Agilent Technologies). The RNA integrity number (RIN) for the *S. tropicalis* samples ranged from 8.0 to 8.8. A custom 4 × 44 K Agilent microarray developed for *S. tropicalis* was used to identify transcripts affected by the exposure to NA mixtures. The microarray platform is deposited in Gene Expression Omnibus (GPL15626) at NCBI and comprised 32,899 unique probes and 1417 Agilent control features (Langlois and Martyniuk, 2013). Microarray hybridization was performed according to the Agilent One-Color Microarray-Based Gene Expression Analysis protocol using Cyanine 3 (Cy3). For the production of complementary DNA (cDNA), 1 μg total RNA per sample was used. The labeling protocol followed that provided by the manufacturer. Microarrays were hybridized for 17 h and washed according to the Agilent protocol. The Agilent G2600D Microarray Scanner was used for microarray scanning at 5 μm. The Agilent Feature Extraction Software (v. 11.0.1.1) was used to extract the signal intensities from the TIFF microarray images. Microarray data have been deposited in the Gene Expression Omnibus (accession # GSE118736) and are MIAME compliant[1]. Intensity data were imported into JMP® Genomics (Version 6.0) and data were normalized using quantile normalization. The microarray limit of detection was 2.5, as determined according to the lowest point in the standard curve and the Agilent negative controls. Thus, probes that had a signal intensity below 2.5 were filtered to a value of 2.5. Following the removal of all the control spots, the differentially expressed genes were identified using a one-way ANOVA test with a false-discovery rate of 5% (FDR = 0.05).

Bioinformatics

Any probe that showed a change with respect to the control ($p < 0.05$) prior to FDR adjustment in one or both treatments of NA mixtures was used to broaden the analysis of the transcriptomic responses. Clustering analysis was performed to determine if there was evidence of separation in transcriptomes among treatments. Two-way hierarchical clustering of differentially expressed probes was performed using the Fast Ward algorithm. Rows were centered to a mean of zero prior to clustering and were also scaled to a variance of one. Gene set enrichment on gene ontology (GO) terms was determined using the parametric analysis of gene set enrichment (PAGE) algorithm, which is a two-sided z-score for GO categories (Kim and Volsky, 2005). Sub-network enrichment analysis (SNEA) was performed using Pathway Studio 9.0 (Ariadne, Rockville, MD, United States) and ResNet 9.0. The SNEA uses known relationships based on expression, binding and common pathways between genes to build networks focused around gene hubs. The GenBank ID was then used for mapping human homologs in Pathway Studio; 4,572 genes were successfully

[1] http://www.ncbi.nlm.nih.gov/geo/info/MIAME.html

mapped. Enrichment *p*-value cut-off was set at $p < 0.05$. The analysis used the function “highest fold change, best *p*-value” for duplicate probes. This bioinformatics method leverages the entire dataset regardless of *p*-value and builds a distribution based on fold change to statistically test for enrichment of processes.

RNA Extraction, cDNA Synthesis, and RT-qPCR for Microarray Validation

The samples obtained in a previous dose-response experiment (Gutierrez-Villagomez et al., 2019) were used for RT-qPCR validation of the microarray. The concentrations used for the S1 treatments for RT-qPCR analysis were 0, 2, 4, 6, and 8 mg/L. For S2, the treatments analyzed were 0, 2, 6, 8, and 12 mg/L. RNA was isolated using the RNeasy mini kit (Qiagen) as described in the manufacturer’s protocol with on-column RNase-free DNase treatment (Qiagen). Before cDNA synthesis, the concentration and quality of all samples were assessed using NanoDrop-2000 spectrophotometer (Thermo Scientific) and on an agarose gel. The quality of the RNA was indicated by the presence of two defined bands. The top band represents 28S ribosomal RNA (rRNA) and the second band represents the 18S rRNA (Aranda et al., 2012). Total cDNA was prepared using Maxima first strand cDNA synthesis kit for RT-qPCR (Thermo Scientific).

Real-time quantitative polymerase chain reaction (RT-qPCR) with SYBR green dye technology was used to validate relative gene expression. Based on the microarray results, six genes were selected for further analysis (**Table 1**); the ribosomal protein L8 *(rpl8)* gene was selected as the reference gene because it did not significantly change with exposure to NAs (**Table 1**) and is a ribosomal gene often used for RT-qPCR normalization (Dhorne-Pollet et al., 2013). Primers were designed using Primer-BLAST[2] and synthesized by Integrated DNA Technologies (**Supplementary Table S1**). To confirm the amplification of the regions of interest, the PCR products were cloned into the pGEM®-T Easy vector (Promega, Madison, WI, United States) and sequenced using 3730 DNA Analyzer (Applied Biosystems). The Rotor-Gene SYBR Green kit (Qiagen) and Rotor-Gene Q (Qiagen) were used to amplify and detect the transcripts of interest. The thermal cycling parameters were as suggested by the manufacturer; an activation step at 95°C for 5 min, followed by 40 cycles of 95°C denaturation step for 5 s and one primer annealing temperature of 60°C for 10 s. After 40 cycles, a melt curve was performed over a range of 60–95°C with increments of 1°C to ensure a single amplified product. The concentration of each primer in the RT-qPCR reactions was 1 μM. The efficiency of all RT-qPCR reactions was 94–107% (100.7 ± 3.4%) and the coefficient of determination (R^2) was ≥ 0.99 (0.995 ± 0.004). Data were analyzed using the Rotor-Gene Q Series software (Qiagen). Analysis of outliers was performed using the ROUT method in GraphPad Prism 6 (Motulsky and Brown, 2006). The relative standard curve method was used to calculate relative mRNA abundance between samples, normalized using NORMA-GENE algorithm (Heckmann et al., 2011) and then presented as fold change of gene expression from replicates (*n* = 5–6; assayed in triplicate) for each group.

[2]https://www.ncbi.nlm.nih.gov/tools/primer-blast/

TABLE 1 | List of differentially expressed genes in *Silurana (Xenopus) tropicalis* exposed to NA S1 and S2 (2 mg/L) from microarray data and selected for RT-qPCR validation.

Gene symbol	Name	Human protein ID	Gene network	Fold change S1	*p*-value S1	FDR adjusted *p*-value S1	Fold change S2	*p*-value S2	FDR adjusted *p*-value S2
cyp4b1	*Cytochrome P450 4B1*	P13584	Metabolism	110.38	2.42E-10	5.0E-06	105.21	2.66E-10	5.0E-06
abcg2	*ATP-binding cassette sub-family G member 2*	Q9UNQ0	Metabolism Gut function	2.83	8.44E-07	5.4E-04	2.62	1.66E-06	8.8E-04
slc26a6	*Solute carrier family 26 member 6*	Q9BXS9	Membrane integrity Gut function	2.27	5.19E-03	9.6E-02	2.23	5.90E-03	1.0E-01
eprs	*Bifunctional glutamate/proline-tRNA ligase*	P07814	Edema	2.36	1.59E-03	5.4E-02	3.54	9.79E-05	1.3E-02
slcs5a1	*Sodium/glucose cotransporter 1*	P13866	Membrane integrity Metabolism Gut function	−2.05	3.02E-04	2.4E-02	−1.78	1.34E-03	4.9E-02
myod1	*Myoblast determination protein 1*	P15172	–	−4.64	4.15E-02	2.6E-01	−5.65	2.50E-02	2.1E-01
rpl8	*Ribosomal protein L8*	P62917	–	−1.01	8.37E-01	9.8E-01	−1.01	9.23E-01	1.0E + 00

Statistical Analysis

Frequencies of abnormalities were analyzed as transformed data using the arcsine square root transformation. To assess data normality and homogeneity of variance, Shapiro–Wilk's test and Levene's test were performed, respectively. Data that failed the normality and/or the equal variance tests were transformed (log10). One-way ANOVA and Tukey's *post hoc* analyses were performed on normally distributed data. Non-parametric Kruskal–Wallis followed by Student–Newman–Keuls (SNK) tests were performed on data that did not pass normality tests. The significance level was set at $\alpha = 0.05$. Statistical analysis was performed using SPSS V21 IBM and Sigma Plot 12.0.

RESULTS AND DISCUSSION

Effects of Exposure to Commercial NAs

The exposures of *S. tropicalis* embryos to NAs at 2 mg/L resulted in a significant reduction in total length (TL), snout-vent length (SVL), tail length (TaL), and interorbital distance (IOD) ($p < 0.05$) (**Figures 1A–C** and **Supplementary Figure S1**). Following exposure to NAs, there was a significant increase of all abnormalities assessed. The most common were cranial abnormalities, edema, gut abnormalities, heart abnormalities, and eye abnormalities (**Figures 1A–C** and **Supplementary Figure S1**). These types of teratogenic effects have been described in detail previously in *S. tropicalis* exposed to commercial extracts and AEOs in a dose-response fashion (Gutierrez-Villagomez et al., 2019). Edema and heart abnormalities have also been described in larval zebrafish (*Danio rerio*) exposed to AEOs extracted from oil sands from the Daqing oil exploring area (Wang et al., 2015) and larval fathead minnows (*Pimephales promelas*) exposed to NA fraction components (NAFCs) of OSPW from Alberta, Canada (Marentette et al., 2015). The 96 h LC_{50} estimates for *S. tropicalis* were 10.4, 11.7, and 52.3 mg/L for S1, S2, and the AEOs, respectively (Gutierrez-Villagomez et al., 2019). The 96 h EC_{50} estimates based on frequencies of developmental abnormalities in *S. tropicalis* were 2.1, 2.6, and 14.2 mg/L for S1, S2, and the AEOs, respectively. In fathead minnow embryos, an EC_{50} for hatching success of 2.5 and 7.5–18.5 mg/L have been reported for a Sigma extract and AEOs, respectively (Marentette et al., 2015). These observations indicate that fish and frogs might have a similar sensitivity with respect to embryotoxicity in response to commercial NAs and AEOs, however, direct comparisons between taxa should be conducted to test this hypothesis.

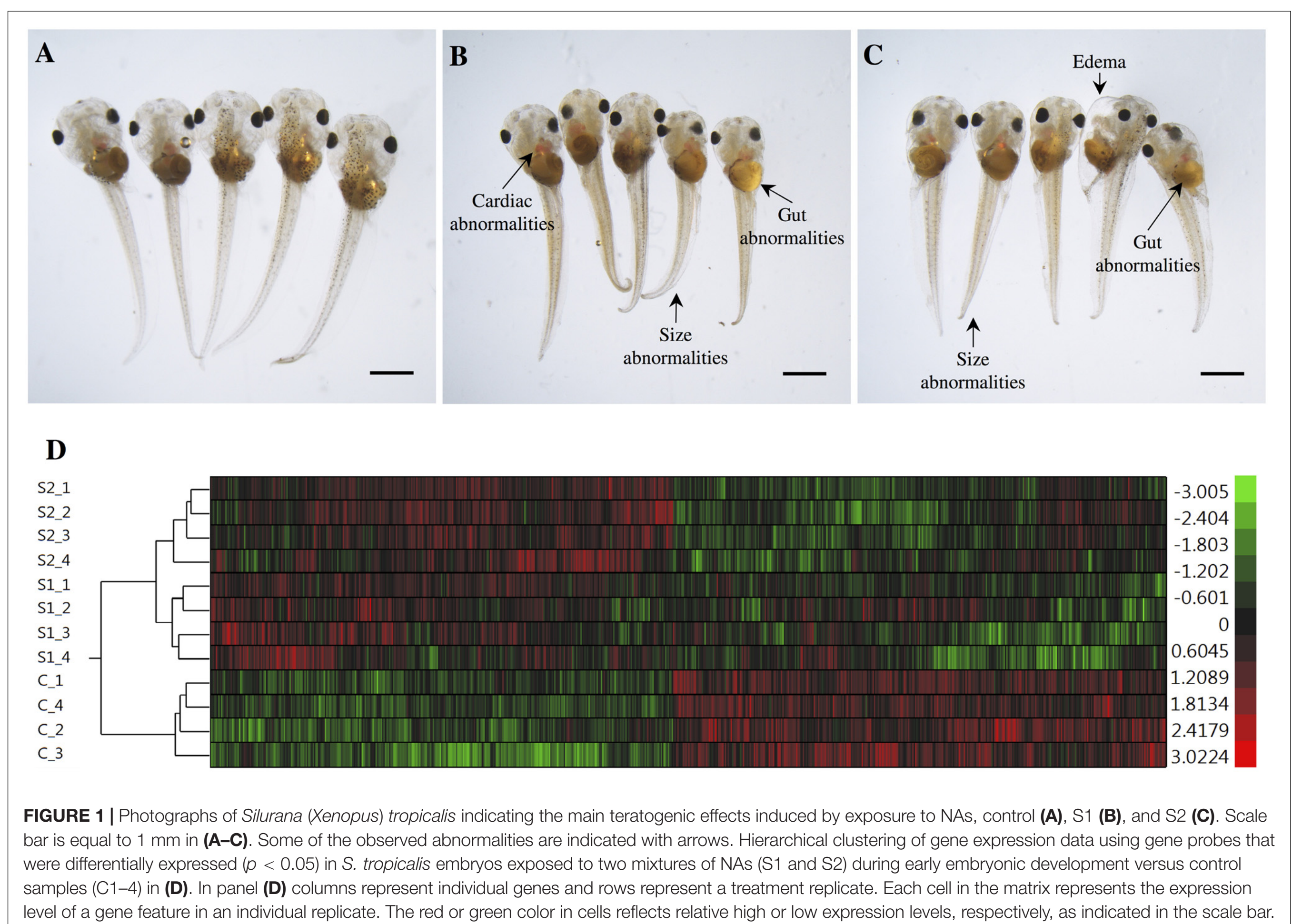


FIGURE 1 | Photographs of *Silurana* (*Xenopus*) *tropicalis* indicating the main teratogenic effects induced by exposure to NAs, control **(A)**, S1 **(B)**, and S2 **(C)**. Scale bar is equal to 1 mm in **(A–C)**. Some of the observed abnormalities are indicated with arrows. Hierarchical clustering of gene expression data using gene probes that were differentially expressed ($p < 0.05$) in *S. tropicalis* embryos exposed to two mixtures of NAs (S1 and S2) during early embryonic development versus control samples (C1–4) in **(D)**. In panel **(D)** columns represent individual genes and rows represent a treatment replicate. Each cell in the matrix represents the expression level of a gene feature in an individual replicate. The red or green color in cells reflects relative high or low expression levels, respectively, as indicated in the scale bar.

Gene Expression Profiling Following NA Exposure

The microarray analysis performed on embryos of *S. tropicalis* exposed to S1 revealed 6,074 differentially expressed genes versus control ($p < 0.05$), of which 2,873 were up-regulated and 3,201 were down-regulated. A total of 894 genes passed the correction for multiple hypothesis testing (FDR < 0.05). This includes 526 up-regulated and 368 down-regulated genes. For S2-exposed animals, the analysis revealed 6,789 differentially expressed genes versus control ($p < 0.05$). Of these, 3,395 were up-regulated and 3,394 were down-regulated by the exposure to S2. A total of 1,177 genes passed the FDR test (FDR < 0.05). Of these, 620 were up-regulated and 557 down-regulated. There were 3,656 common genes affected by S1/S2 ($p < 0.05$). Of these, 564 genes passed the FDR test (FDR < 0.05). The higher number of genes significantly affected ($p < 0.05$) by the exposure to S2 compared to S1, indicates that S2 has a higher impact at the transcriptomic level of *S. tropicalis*. These results highlight that the NA composition has a direct impact on the toxicity of a mixture. The S1 and S2 are composed largely of the O2 and O4 classes. However, the composition and proportion of the components in the two mixtures is different (Gutierrez-Villagomez et al., 2019). We observed that the EC_{50} and EC_{20} for S1 are significantly lower than for S2 (Gutierrez-Villagomez et al., 2019). In this manuscript, we observed that the two mixtures affected the transcriptome of the *S. tropicalis* embryos in a different manner. Thus, it is clear that subtle differences in chemical composition can have different transcriptional effects linked with differing teratogenic outcomes.

Hierarchical Analysis

The hierarchical clustering analysis of the differential expression ($p < 0.05$) data showed two main clades that mark the clear separation of the control samples and the two NA treatments (S1 and S2). The second clade is composed only of expression profiles from embryos exposed to the NA extracts. Treatments S1 and S2 are sister groups, indicating that these two NA mixtures affect the transcriptome of *S. tropicalis* in a similar, but non-identical manner. The different replicates are located in their respective cluster and there are no replicates mixed between groups, indicating a relatively strong transcriptome response to S1 and S2 (**Figure 1D**).

Gene Set Enrichment of Gene Ontology (GO) Terms

In total, 64 biological processes, 25 cellular components, and 52 molecular functions were significantly enriched after S1 exposure ($p < 0.05$) (**Supplementary Table S2**). After S2 exposure, 77 biological processes, 29 cellular components, and 57 molecular functions were significantly enriched ($p < 0.05$) (**Supplementary Table S3**). The number of significantly enriched processes in common between S1 and S2 for each of biological process (p:), cellular component (c:) and molecular function (f:) were 18, 5, and 19, respectively ($p < 0.05$) (**Table 2**). The differences between the number of GO terms significantly enriched with the exposure to S1 and S2 likely reflects the differences in the chemical composition of the two mixtures (Gutierrez-Villagomez et al., 2019). The significant enrichment of GO terms, such as respiratory electron transport chain, respiratory chain, adenylyl-sulfate kinase activity, and pyruvate kinase activity indicates that NAs affect metabolism and that this could be part of the mechanism of toxicity of NAs (**Table 2**). "Protein import into mitochondrial inner membrane" and "mitochondrial intermembrane" GO terms were also significantly enriched ($p < 0.05$). These results support the hypothesis of Frank et al. (2009) who proposed that narcosis as part of the toxic mechanism of NAs. Furthermore, visual perception, structural constituent of eye lens, actin cytoskeleton, and cytoskeleton organization are GO terms significantly enriched ($p < 0.05$) after the exposure to S1 and S2. This may be associated with the multiple abnormalities in *S. tropicalis* induced by S1 and S2, such as eye and morphometric abnormalities (**Figures 1A–C**).

Sub-Network Enrichment Analysis (SNEA)

Using SNEA, a total of 75 cell process and 42 disease pathways were affected by S1 exposure ($p < 0.05$; **Supplementary Table S4**), and 66 cell process pathways and 46 disease pathways were perturbed in the embryos following S2 exposure ($p < 0.05$; **Supplementary Table S5**). There were 38 cell process pathways ($p < 0.05$; **Table 3**) and 14 disease pathways ($p < 0.05$; **Table 4**) in common as perturbed by S1 and S2. Some pathways that were significantly affected by exposure to S1 (**Figure 2A**) and S2 (**Figure 2B**) included networks related to metabolism, such as liver metabolism, cholesterol metabolism, retinoid metabolism, retinoic acid metabolism, steroid metabolism, arachidonic acid metabolism, drug metabolism, and xenobiotic clearance. This corroborates the GO analysis that also indicated that metabolic processes were significantly enriched following exposure. Other pathways detected as significantly enriched were those related to membrane integrity (**Figures 3A,B**), such as membrane depolarization, Cl^- transport, Na^+ influx co-transport, ion transport, anion transport, fluid transport, and HCO_3^- transport. This analysis further supports the hypothesis that toxicity of NAs is at least partially through narcosis, defined as the disruption of cell membrane function (Newman and Clements, 2007; Li et al., 2017). Others refer to this as baseline toxicity and have linked narcosis to changes in membrane potentials (Escher et al., 2002). The morphological analysis showed that NAs significantly induce gut abnormalities (**Supplementary Figure S1J**) in *S. tropicalis* embryos, and the lack of gut coiling was observed in some of the tadpoles (**Figures 1A–C**). Importantly, gene networks related to gut function (**Figure 4**), such as digestion, intestinal absorption, acid secretion, gastrointestinal system digestion, bile acid secretion, and biliary flow were significantly disrupted by exposure to NAs. Supporting this is the identification of disease pathways (**Table 4**) such as constipation and malabsorption syndromes, both related to gut functions. Other pathways related to the abnormalities that were observed in *S. tropicalis* (**Figures 1A–C** and **Supplementary Figure S1**) included cartilage differentiation and edema (**Tables 3**, **4**).

TABLE 2 | List of gene ontology terms (biological process (p:), cellular component (c:) and molecular function (f:) altered after the exposure of *S. tropicalis* to both S1 and S2 using Parametric Analysis of Gene Set Enrichment (PAGE) analysis ($p < 0.05$).

GO term	Frequency	S1			S2		
		z-score	Raw *p*-value	False discovery rate *p*-value	z-score	Raw *p*-value	False discovery rate *p*-value
GO:0050909; p:sensory perception of taste	79	5.07	4.00E-07	2.50E-04	6.21	5.30E-10	3.40E-07
GO:0007601; p:visual perception	76	−3.62	2.90E-04	4.60E-02	−2.90	3.70E-03	1.20E-01
GO:0000103; p:sulfate assimilation	10	−3.55	3.90E-04	4.90E-02	−3.49	4.80E-04	3.50E-02
GO:0048793; p:pronephros development	19	−3.27	1.10E-03	9.90E-02	−2.08	3.80E-02	3.60E-01
GO:0045039; p:protein import into mitochondrial inner membrane	11	−3.13	1.70E-03	1.20E-01	−2.25	2.40E-02	3.10E-01
GO:0042777; p:plasma membrane atp synthesis coupled proton transport	9	−3.05	2.30E-03	1.20E-01	−2.60	9.40E-03	1.80E-01
GO:0022904; p:respiratory electron transport chain	16	2.85	4.40E-03	1.80E-01	3.83	1.30E-04	2.40E-02
GO:0006364; p:rrna processing	45	−2.71	6.70E-03	2.50E-01	−2.93	3.40E-03	1.20E-01
GO:0006744; p:ubiquinone biosynthetic process	11	2.67	7.70E-03	2.70E-01	3.06	2.20E-03	9.40E-02
GO:0006414; p:translational elongation	13	−2.36	1.80E-02	3.70E-01	−2.35	1.90E-02	3.00E-01
GO:0045765; p:regulation of angiogenesis	5	2.31	2.10E-02	3.70E-01	2.37	1.80E-02	3.00E-01
GO:0050852; p: t cell receptor signaling pathway	9	2.27	2.30E-02	3.80E-01	3.82	1.30E-04	2.40E-02
GO:0048260; p:positive regulation of receptor-mediated endocytosis	5	2.24	2.50E-02	3.80E-01	2.13	3.30E-02	3.20E-01
GO:0051225; p:spindle assembly	8	2.16	3.10E-02	4.10E-01	3.21	1.30E-03	8.00E-02
GO:0001522; p:pseudouridine synthesis	12	−2.14	3.20E-02	4.20E-01	−3.12	1.80E-03	8.00E-02
GO:0033504; p:floor plate development	5	2.07	3.80E-02	4.40E-01	2.20	2.80E-02	3.10E-01
GO:0007596; p:blood coagulation	56	2.06	3.90E-02	4.40E-01	2.62	8.90E-03	1.80E-01
GO:0030866; p:cortical actin cytoskeleton organization	5	−1.99	4.70E-02	4.70E-01	−2.00	4.50E-02	3.70E-01
GO:0042719; c:mitochondrial intermembrane space protein transporter	11	−3.12	1.80E-03	1.70E-01	−2.30	2.20E-02	2.60E-01
GO:0070469; c:respiratory chain	13	2.69	7.20E-03	2.20E-01	2.46	1.40E-02	2.20E-01
GO:0000784; c:nuclear chromosome, telomeric region	6	−2.53	1.10E-02	2.20E-01	−2.68	7.50E-03	2.20E-01
GO:0015629; c:actin cytoskeleton	32	−2.53	1.10E-02	2.20E-01	−1.99	4.60E-02	3.60E-01
GO:0032133; c:chromosome passenger complex	6	2.07	3.90E-02	4.30E-01	2.63	8.50E-03	2.20E-01
GO:0004692; f:cgmp-dependent protein kinase activity	9	−3.64	2.70E-04	4.90E-02	−3.13	1.70E-03	7.70E-02
GO:0004020; f:adenylyl-lsulfate kinase activity	10	−3.51	4.40E-04	4.90E-02	−3.47	5.30E-04	4.10E-02
GO:0004781; f:sulfate adenylyl-ltransferase (atp) activity	10	−3.51	4.40E-04	4.90E-02	−3.47	5.30E-04	4.10E-02
GO:0042626; f:atpase activity, coupled to transmembrane movement of substances	19	3.27	1.10E-03	7.60E-02	3.22	1.30E-03	6.20E-02
GO:0042605; f:peptide antigen binding	8	3.26	1.10E-03	7.60E-02	2.48	1.30E-02	2.40E-01
GO:0030955; f:potassium ion binding	9	3.21	1.30E-03	7.60E-02	3.46	5.50E-04	4.10E-02
GO:0070330; f:aromatase activity	14	−3.18	1.50E-03	7.60E-02	−3.76	1.70E-04	3.00E-02
GO:0010181; f:fmn binding	12	2.9	3.80E-03	1.40E-01	2.07	3.80E-02	3.70E-01
GO:0004743; f:pyruvate kinase activity	7	2.77	5.70E-03	1.70E-01	2.85	4.30E-03	1.40E-01
GO:0001567; f:cholesterol 25-hydroxylase activity	9	2.54	1.10E-02	2.20E-01	2.05	4.00E-02	3.80E-01
GO:0043425; f:bhlh transcription factor binding	9	−2.51	1.20E-02	2.30E-01	−2.95	3.20E-03	1.30E-01
GO:0003887; f:dna-directed dna polymerase activity	23	2.22	2.70E-02	3.30E-01	2.18	2.90E-02	3.40E-01
GO:0004864; f:protein phosphatase inhibitor activity	13	−2.16	3.10E-02	3.40E-01	−3.29	1.00E-03	5.50E-02
GO:0004012; f:phospholipid-translocating atpase activity	9	2.16	3.10E-02	3.40E-01	2.25	2.40E-02	3.40E-01
GO:0005212; f:structural constituent of eye lens	24	−2.08	3.70E-02	3.80E-01	−2.54	1.10E-02	2.10E-01
GO:0051864; f:histone demethylase activity (h3-k36 specific)	5	2.04	4.10E-02	4.00E-01	2.22	2.60E-02	3.40E-01
GO:0004402; f:histone acetyltransferase activity	9	−2.02	4.40E-02	4.00E-01	−2.17	3.00E-02	3.40E-01
GO:0001948; f:glycoprotein binding	6	2.01	4.50E-02	4.00E-01	2.15	3.20E-02	3.50E-01
GO:0005085; f:guanyl-nucleotide exchange factor activity	38	1.96	5.00E-02	4.20E-01	3.72	2.00E-04	3.00E-02

RT-qPCR Validation of Differentially Expressed Genes

Five genes were selected for microarray validation and for further study based on the following characteristics: (1) both exposure to S1 and S2 significantly altered their expression; (2) the transcripts had to be annotated and have a human homolog identified; and (3) the transcripts had to appear in the pathway analysis (**Table 1**). The cytochrome *P450 4B1b* (*cyp4b1*) gene encodes a

TABLE 3 | Sub-network enrichment analysis (SNEA) for common cellular processes in *S. tropicalis* exposed to S1 and S2 mixtures of NAs.

Gene set seed (cellular process)	Total # of neighbors	# of Measured neighbors	S1		S2	
			Median change	*p*-value	Median change	*p*-value
Xenobiotic clearance	370	80	1.16	2.20E-05	1.16	2.40E-05
Liver uptake	86	15	1.24	4.40E-04	1.16	3.40E-03
Intestinal absorption	135	34	1.07	8.60E-04	1.16	1.90E-06
Drug metabolism	108	22	1.17	9.40E-04	1.19	1.70E-03
Vascular smooth muscle Relaxation	54	9	−1.18	1.60E-03	−1.09	2.60E-03
Hepatobiliary excretion	18	6	1.31	3.10E-03	1.27	2.40E-02
Endothelial cell production	45	6	1.11	3.80E-03	1.28	1.60E-02
Retinoid metabolism	33	10	1.14	5.00E-03	1.76	2.40E-04
Ion transport	211	34	−1.11	5.70E-03	−1.09	7.80E-03
Digestion	367	87	−1.04	6.30E-03	−1.04	8.60E-03
Arachidonic acid metabolism	87	9	1.06	7.10E-03	1.16	1.50E-02
Gallstone formation	41	9	1.19	7.80E-03	1.23	2.30E-03
Vaso-constriction	320	42	−1.02	8.50E-03	−1.02	3.40E-02
Cl^- transport	228	36	−1.06	8.70E-03	1.05	2.10E-02
Anion transport	38	7	1.24	1.10E-02	1.2	7.00E-03
Retinoic acid metabolism	24	5	1.13	1.20E-02	1.21	5.50E-03
Liver metabolism	116	31	1.08	1.40E-02	1.14	1.70E-03
Membrane depolarization	654	108	−1	1.50E-02	−1.02	3.90E-02
Na^+ influx co-transport	44	8	−1.11	1.50E-02	1.15	8.90E-03
Lipid transport	368	64	1.03	1.60E-02	1.05	5.10E-03
Blood vessel contraction	111	11	−1.08	1.90E-02	−1.13	3.40E-03
Ethanol metabolism	27	8	1.31	2.40E-02	1.19	1.20E-02
Fluid transport	60	17	−1.11	2.40E-02	−1.01	4.80E-03
Bile acid secretion	74	14	1.11	2.80E-02	1.16	1.50E-02
Acid secretion	156	29	−1.02	3.00E-02	−1.1	2.30E-02
Hemato-encephalic barrier	326	52	−1.01	3.00E-02	−1.04	4.70E-02
HCO_3^- transport	116	21	−1.11	3.10E-02	−1.09	1.90E-02
Protein-protein cross-linking via L-cystine	109	24	1.11	3.20E-02	1.09	1.30E-02
Gastrointestinal system digestion	26	6	1.07	3.60E-02	−1.17	3.60E-02
Nucleotide biosynthesis	35	5	−1.04	3.90E-02	−1.08	1.60E-02
Histone ubiquitination	22	5	−1.11	4.00E-02	−1.18	4.50E-02
Cholesterol metabolism	185	35	−1.05	4.10E-02	1.02	3.10E-02
Fluid secretion	102	19	1.04	4.10E-02	1.09	6.20E-04
Cell communication	145	24	−1.09	4.50E-02	−1.07	2.90E-03
Cartilage differentiation	48	9	−1.2	4.70E-02	−1.18	4.90E-02
Kidney filtration	80	13	1.11	4.80E-02	1.16	7.80E-03
Steroid metabolism	392	66	1.06	4.90E-02	1.05	9.30E-03
Biliary flow	56	9	1.07	4.90E-02	−1.09	2.30E-02

All gene set seeds that had > 10% overlap with the annotated pathways are listed ($p < 0.05$). The total number of neighbours refers to the total number of known entities in a sub-network.

monooxygenase enzyme that catalyzes reactions involved in the metabolism of cholesterol, steroids, and xenobiotics (Baer and Rettie, 2006). This gene forms part of a metabolism network that was significantly disrupted by NAs, specifically arachidonic acid metabolism (**Figures 2A,B**). Arachidonic acid is a fatty acid involved in eye and brain development (Uauy et al., 2001). The *solute carrier family 26 member 6* (*slc26a6*) gene encodes an anion transporter protein and regulates acid-base homeostasis (Chernova et al., 2005; Freel et al., 2009). This gene is part of networks that were disturbed by the exposure to NAs, in particular, intestinal absorption and Cl^- transport (**Figures 3A,B**). Furthermore, *Slc26a6*-null mice have defective intestinal oxalate secretion and a higher incidence of formation of stony concretions in the urinary tract (Jiang et al., 2006). The *ATP-binding cassette sub-family G member 2* (*abcg2*) gene encodes a membrane transporter (Allikmets et al., 1998)

TABLE 4 | Sub-network enrichment analysis (SNEA) for diseases in *S. tropicalis* exposed to S1 and S2 mixtures of NAs.

Gene set seed (disease)	Total # of neighbors	# of Measured neighbors	S1 Median change	S1 *p*-value	S2 Median change	S2 *p*-value
Neutropenia	90	9	1.48	6.50E-04	1.35	1.60E-02
Hepatitis	207	27	−1.14	1.40E-03	−1.07	3.40E-03
liver toxicity	627	110	1.06	5.70E-03	1.09	2.40E-03
Constipation	36	6	−1.36	6.20E-03	−1.19	1.00E-02
Cystic fibrosis	125	27	−1.06	7.20E-03	−1.09	4.00E-03
Meningitis, bacterial	33	5	−1.1	8.80E-03	−1.07	3.10E-02
Edema	429	67	−1.02	1.30E-02	1.05	5.00E-02
Mal-absorption syndromes	41	11	−1.13	2.00E-02	−1.19	6.00E-05
Tachy-phylaxis	44	7	1.11	2.70E-02	1.13	1.40E-02
Emphysema	110	24	1.05	4.40E-02	1.05	1.30E-02
Seizures, febrile	34	5	−1.1	4.50E-02	−1.26	2.20E-02
Hyperhomo-cysteinemia	50	5	1.19	4.50E-02	1.1	3.20E-02
Cold ischemia	34	5	−1.11	4.50E-02	−1.16	1.40E-02
Bronchitis, chronic	28	6	−1.04	4.80E-02	−1.09	2.00E-02

All gene set seeds that had >10% overlap with the annotated pathways are listed ($p < 0.05$). The total number of neighbors refers to the total number of known entities in a sub-network.

involved in the gene network of gut function, specifically in the pathway of intestinal absorption (**Figures 4A,B**). The *bifunctional glutamate/proline-tRNA ligase* (*eprs*) gene encodes for the enzyme that charge tRNA with glutamate and proline (Young et al., 2016). It was selected because the SNEA showed that this gene is related to edema in *S. tropicalis* embryos (see **Table 1**). The *sodium/glucose cotransporter 1* (*slc5a1*) gene encodes a sodium-dependent glucose transporter (Wright et al., 1994) and it is involved in several networks affected by NAs, such as liver metabolism, membrane depolarization, Na^+ influx co-transport, and intestinal absorption (**Figures 2–4**). Lee et al. (1994) reported that *slc5a1* is highly expressed in the intestine and in lower levels in liver in rat, where its function is the uptake of glucose (Turk et al., 1991). Also, the mutation of the *slc5a1* gene can induce severe diarrhea and dehydration in humans (Lam et al., 1999). These five genes appear to be associated with the significant increase ($p < 0.05$) of phenotypic abnormalities in *S. tropicalis* embryos, such as gut abnormalities, eye abnormalities, and edema (**Figures 1A–C** and **Supplementary Figure S1**). The *myod1* gene was selected because it changed significantly with both S1 and S2 treatments and is linked to myogenesis and muscle differentiation (Weintraub et al., 1991). Therefore, it may be associated with the significant decrease of TL, SVL, TaL, and IOD of *S. tropicalis* exposed to NAs (**Figures 1A–C** and **Supplementary Figure S1**).

In general, the expression obtained by microarray analysis and RT-qPCR were similar in direction of change (**Figures 5**, **6** and **Table 1**). The genes *cyp4b1* (**Figures 5A,B**), *abcg2* (**Figures 5E,F**), *slc26a6* (**Figures 5G,H**), *eprs* (**Figures 5J,K**), and *slc5a1* (**Figures 6A,B**) were affected in a concentration-response manner and the fold changes were comparable between the S1 and S2 treatment groups. However, some discrepancies were noted for *myod1*. The microarray analysis showed a significant down-regulation of *myod1* by exposure to S1 and S2 (**Table 1**), while the RT-qPCR indicated an upregulation (**Figures 6G,H**).

There are clear differences in the chemical composition of the commercial NA extracts versus the AEOs from OSPW (Gutierrez-Villagomez et al., 2019). However, we had hypothesized that the toxic mechanism would be similar because all extracts contain high levels of O_2 species of NAs, albeit, less in the AEOs. As well, exposures produce the same types of abnormalities. To test this hypothesis, samples of the *S. tropicalis* exposed to AEO concentrations of 0, 2, 12, 24, and 48 mg/L were analyzed by RT-qPCR for the same genes described for microarray validation. The expression of *cyp4b1* (**Figure 5C**), *abcg2* (**Figure 5F**), and *slc26a6* (**Figure 5I**) was not affected in a concentration-response manner in AEO-treated animals. After extraction, the AEOs were dissolved in a solution of 0.1N NaOH. The solvent control did not have a significant effect on morphometric measurements and frequency of abnormalities (Gutierrez-Villagomez et al., 2019). Likewise, Marentette et al. (2015) reported no effect on the frequency of abnormalities up to concentrations of 50 mM NaOH in fathead minnow embryos. However, the RT-qPCR results showed that NaOH at 0.001 N reduced the expression of many of the genes analyzed by PCR (**Figures 5**, **6**, AEO column). It is nevertheless important to use and report the effects of NaOH with the toxicity assessment of AEOs. The expression level of *eprs* (**Figure 5L**) and *slc5a1* (**Figure 6C**). significantly decreased at 48 mg/L AEOs compared to control and NaOH control ($p < 0.05$). As *eprs* and *slc5a1* are part of networks related to edema, membrane integrity, and metabolism and gut function (**Table 1**) and frequency of edema and gut abnormalities was significantly higher in samples

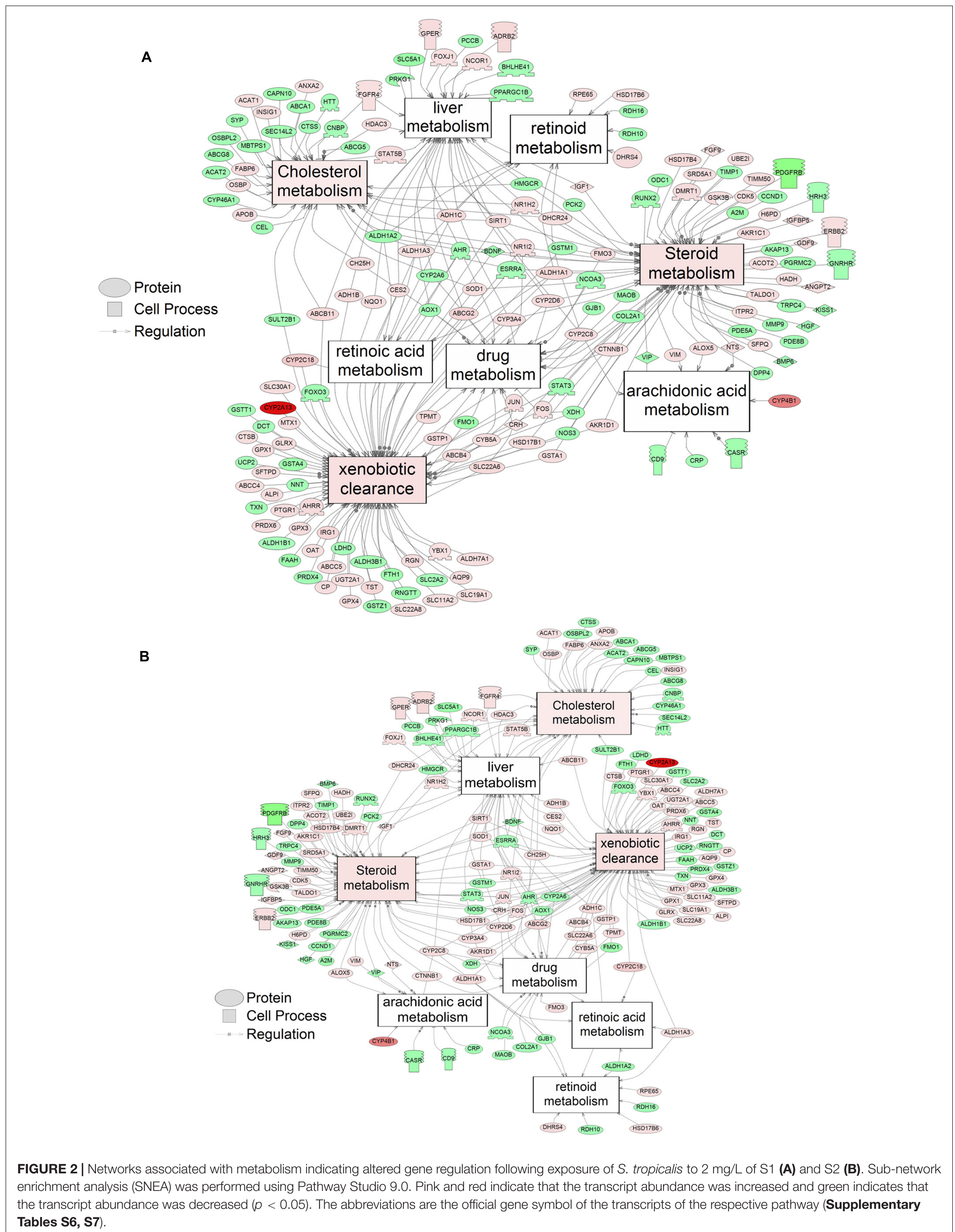


FIGURE 2 | Networks associated with metabolism indicating altered gene regulation following exposure of *S. tropicalis* to 2 mg/L of S1 **(A)** and S2 **(B)**. Sub-network enrichment analysis (SNEA) was performed using Pathway Studio 9.0. Pink and red indicate that the transcript abundance was increased and green indicates that the transcript abundance was decreased ($p < 0.05$). The abbreviations are the official gene symbol of the transcripts of the respective pathway (**Supplementary Tables S6, S7**).

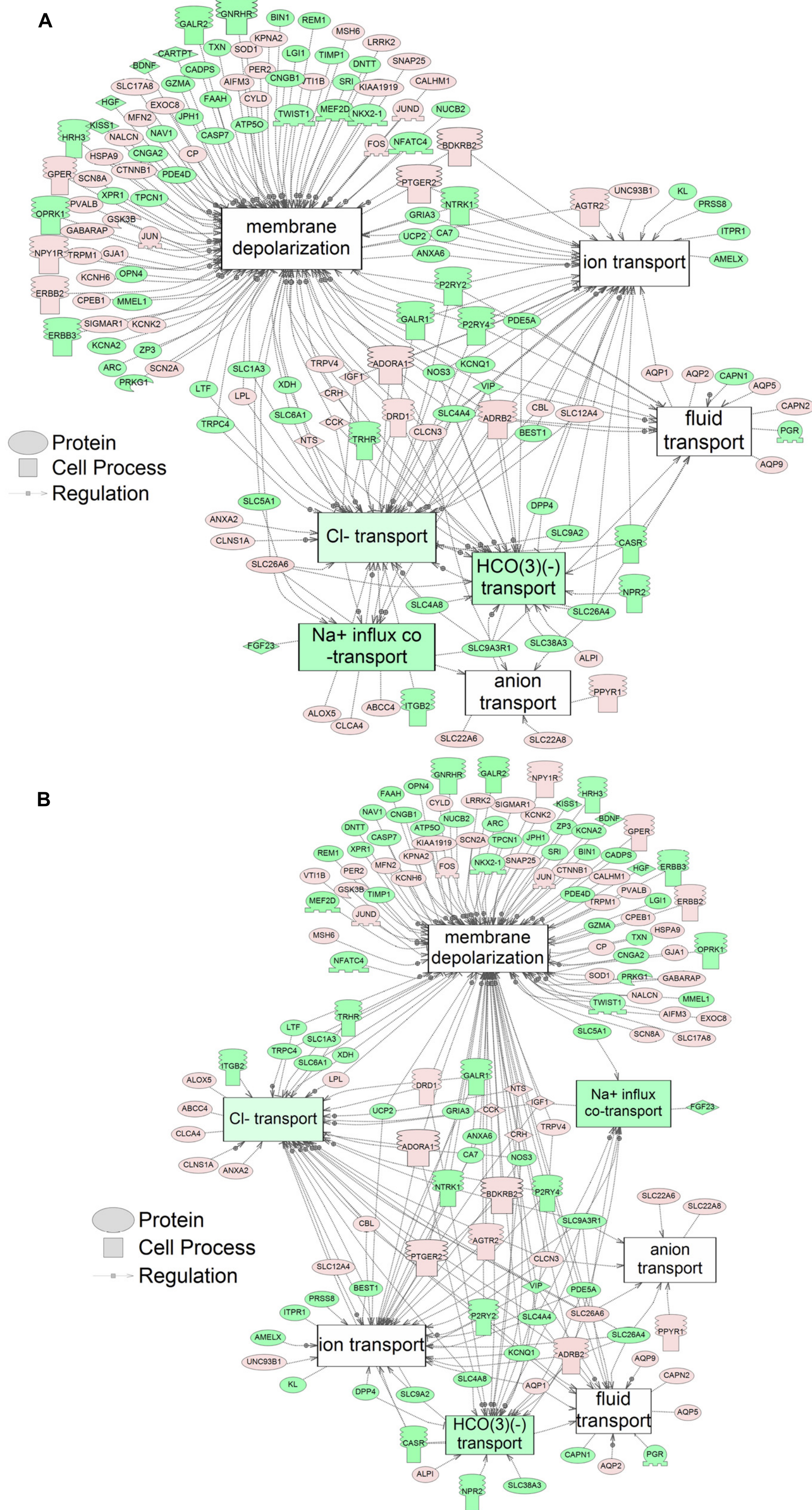
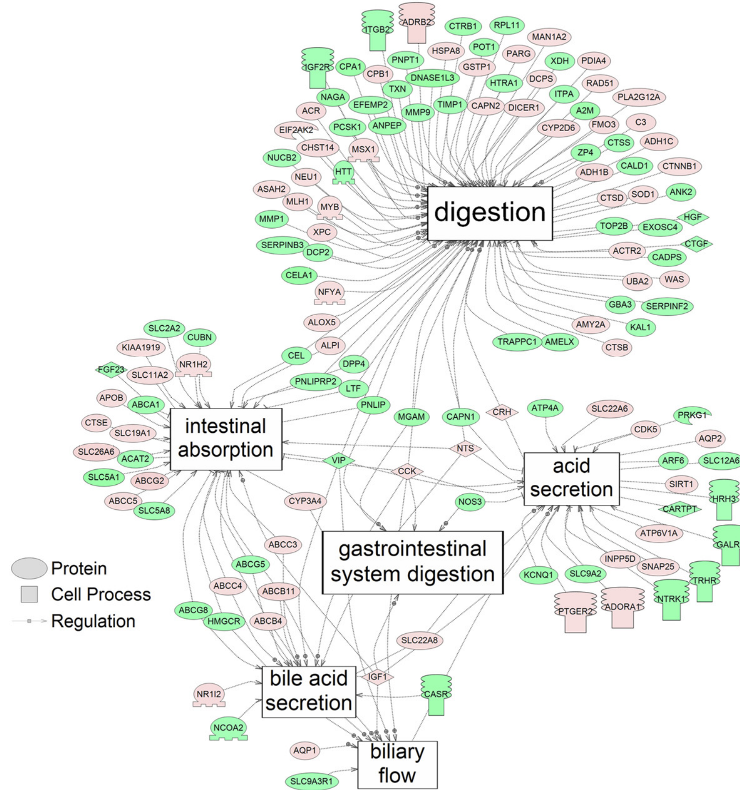


FIGURE 3 | Networks associated with membrane integrity indicating altered gene regulation following exposure of *S. tropicalis* to 2 mg/L of S1 **(A)** and S2 **(B)**. Sub-network enrichment analysis (SNEA) was performed using Pathway Studio 9.0. Pink and red indicate that the transcript abundance was increased and green indicates that the transcript abundance was decreased ($p < 0.05$). The abbreviations are the official gene symbol of the transcripts of the respective pathway (**Supplementary Tables S8, S9**).



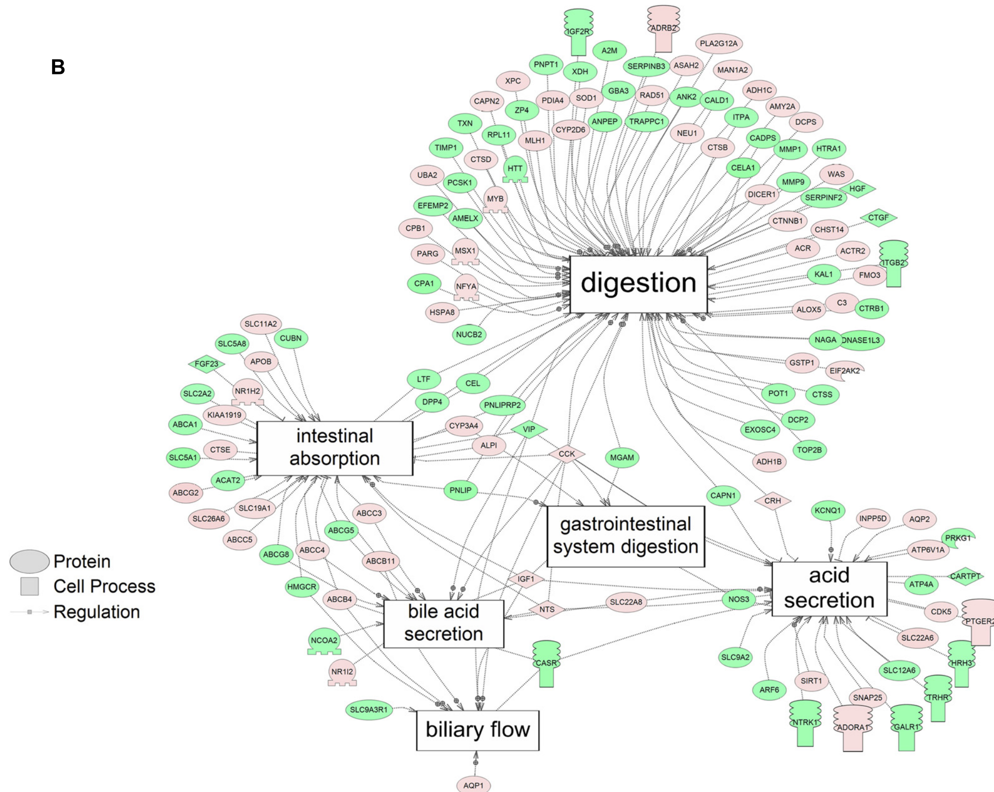


FIGURE 4 | Networks associated with gut function indicating altered gene regulation following exposure of *S. tropicalis* to 2 mg/L of S1 **(A)** and S2 **(B)**. Sub-network enrichment analysis (SNEA) was performed using Pathway Studio 9.0. Pink and red indicate that the transcript abundance was increased and green indicates that the transcript abundance was decreased ($p < 0.05$). The abbreviations are the official gene symbol of the transcripts of the respective pathway (**Supplementary Tables S10, S11**).

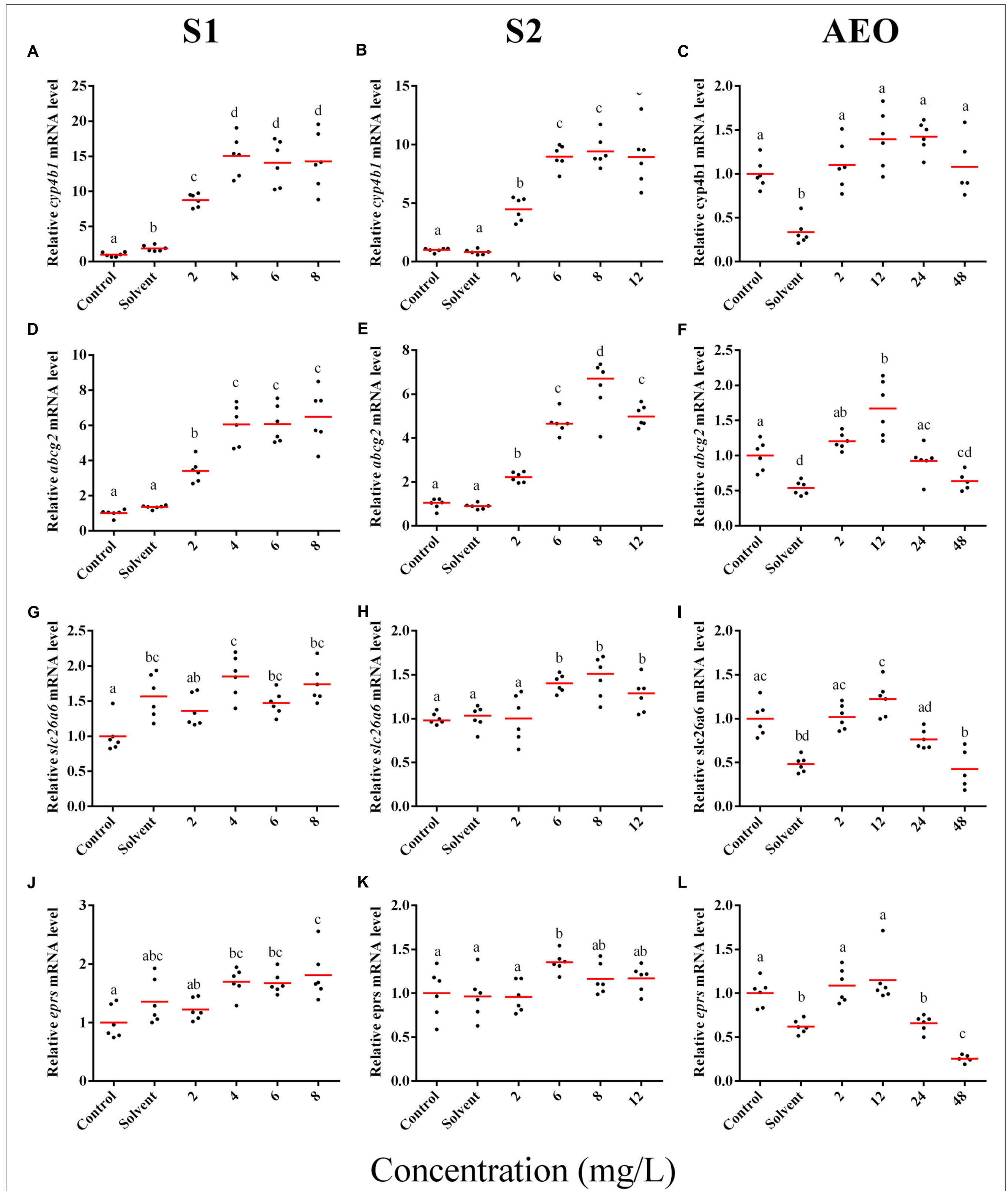


FIGURE 5 | RT-qPCR analysis for *cyp4b1* **(A–C)**, *abcg2* **(D–F)**, *slc26a6* **(G–I)**, and *eprs* **(J–L)**, mRNA in *S. tropicalis* exposed to different concentrations of S1 (first column), S2 (second column), and AEOs (third column). Data were normalized and defined as fold change relative to control. Different letters indicate groups that are significantly different ($p < 0.05$).

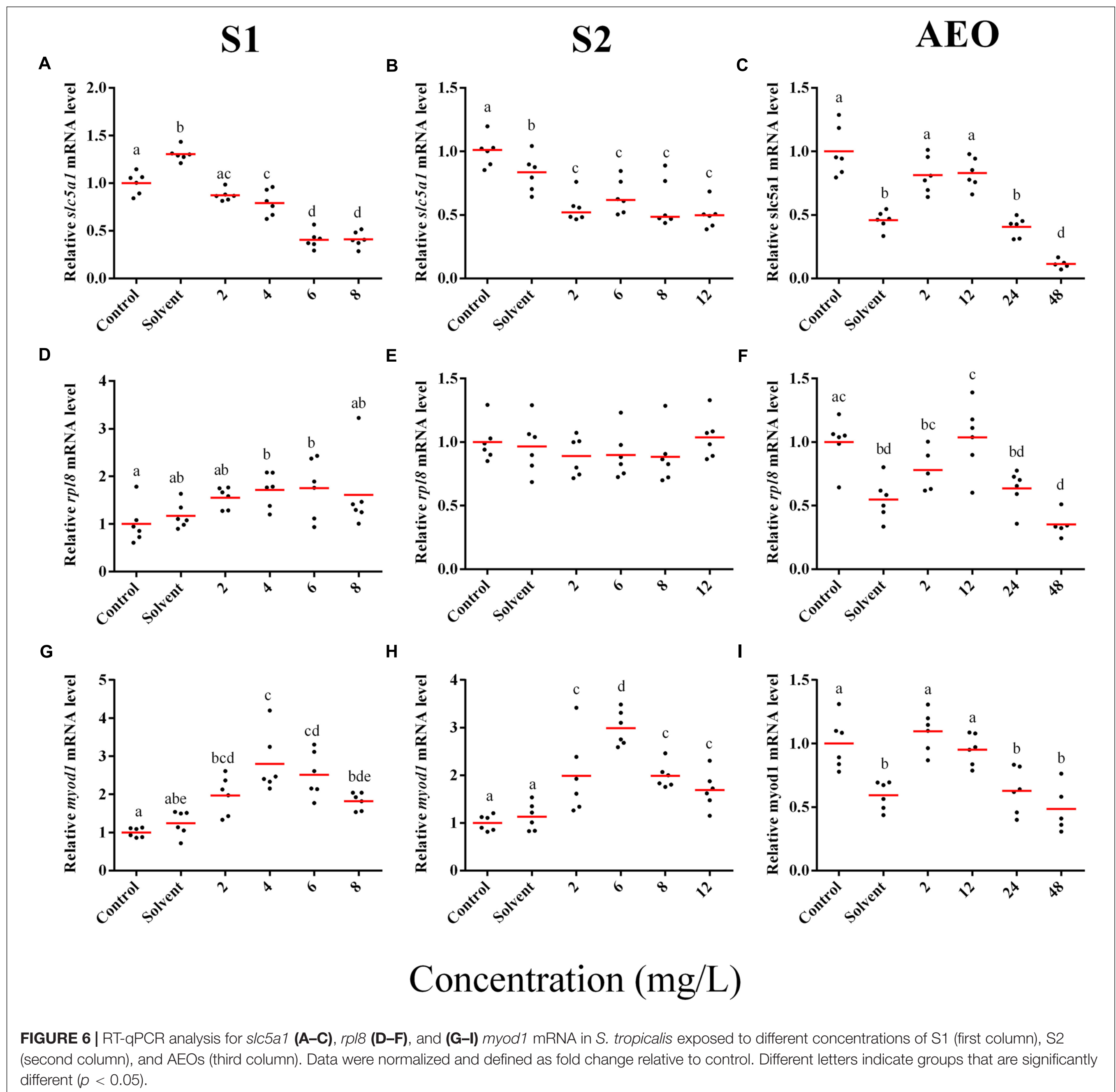


FIGURE 6 | RT-qPCR analysis for *slc5a1* **(A–C)**, *rpl8* **(D–F)**, and **(G–I)** *myod1* mRNA in *S. tropicalis* exposed to different concentrations of S1 (first column), S2 (second column), and AEOs (third column). Data were normalized and defined as fold change relative to control. Different letters indicate groups that are significantly different ($p < 0.05$).

exposed to AEOs (Gutierrez-Villagomez et al., 2019), there appears to be an important relationship between the phenotypic abnormalities in *S. tropicalis* embryos and altered *eprs* and *slc5a1* levels.

CONCLUSION

We report the first comprehensive transcriptomic profiling of the effects of NAs in early *S. tropicalis* embryos. A diverse number of GO terms and pathways were significantly enriched by exposures to commercial extracts S1 and S2, suggesting that there are several mechanisms involved. The results obtained in the microarray analysis and comparison to morphometric abnormalities in *S. tropicalis* exposed to S1 and S2 were highly similar. Metabolism and membrane integrity GO terms and pathways were significantly enriched, indicating that exposure to NAs disrupts metabolism and the functionality of cell membranes in *S. tropicalis*. These data support the hypothesis that narcosis is at least one of the likely mechanisms of toxicity of NAs. Other GO terms and pathways that were also significantly enriched were related to abnormalities observed in the *S. tropicalis* larvae after the early exposure to NAs. These included gut function, cartilage differentiation, actin cytoskeleton, cytoskeleton organization, and

edema. While AEOs contain less of the highly toxic NAs in the O2 class, they still cause similar abnormalities and cause changes in the expression of specific genes. The rank-order potency was S1 > S2 > AEOs (Gutierrez-Villagomez et al., 2019) based on LC50 (classical mortality measurements) and EC50 (incidence of total abnormalities in *S. tropicalis* embryos). Here, we show that both commercial NA mixtures and AEOs extracted from OSPW at concentrations found in the environment affect some of the same genes. What is new in our study is that we were able to screen the activities of the commercial NAs, then test whether the OSPW extract has the same effects on the consistently regulated genes- stepping toward the very first potential biomarkers for NAs. This is especially the case for *eprs* and *slc5a1*, which may be respectively linked to induction of edema and gut abnormalities in exposed embryos. Future research should focus on specific tissues, considering that gut abnormalities were one of the most relevant and unique phenotypic changes observed, and that was strongly supported by the transcriptomic profiling data. It will also be important to determine the relative contributions of AEOs and polyaromatic compounds on the effects of whole OSPW and other effluents associated with petroleum production.

DATA AVAILABILITY

The datasets generated for this study can be found in GEO, GSE118736.

ETHICS STATEMENT

This study was carried out in accordance with the Canadian Council on Animal Care and approved by the University of Ottawa Animal Care and Veterinary Services.

AUTHOR CONTRIBUTIONS

JG-V and VT designed the research project. JG-V conducted the research and wrote the manuscript. CM helped with bioinformatics. LX helped with RT-qPCR analysis. VL and CM designed the microarray platform. BP provided the financial support. All authors read, edited, and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

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frontiers in Marine Science

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Gonadal Histopathological Disorders in *Mytilus galloprovincialis* Male Exposed to Tars Used in Mussel Farms

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Polycyclic aromatic hydrocarbons (PAHs) and trace metals are pollutants widely distributed in the marine environment with toxic effects on live organisms, but few laboratory studies have proved their causal relationship with histopathologic alterations from invertebrates. We analyse the histopathologic effects of a tar mixture used in mussel farms on the gonad of male mussels. Mussels were exposed to water-accommodated fractions from different sub-lethal concentrations of that tar mixture during different times. The accumulation of PAHs and metals in mussels was quantified in each one tar exposure condition, and a comparative histological and morphometric analysis of the male gonad was performed, as well as of the gametogenic disorders and the cellular pathologies induced. Spermatogenesis disruption and alterations of somatic and germinal cells were observed as a direct effect of treatment. The pathologies observed followed an increasing malignity sequence depending on dose and exposure time: spawning induction; arrest of spermatogenesis and spermiogenesis; suppression of immune response; dysplasia of Sertoli and connective cells and finally, severe disorders of germinal cells. We demonstrate for the first time that tar used in waterproofing of floating rafts from the mussel farms causes severe effects on male mussel gonad, constituting a direct risk to the health of these organisms, affecting gametogenesis, quality of gametes and, ultimately, the sustainability of this resource and their quality for human consumption. Moreover, we propose the pathologies described in mussel as biomarkers of marine pollution and the mussel gonad as study model of testicular toxicity mechanisms in male reproduction.

Keywords: ***Mytilus galloprovincialis*, histopathology, tar, mussel farms, gonad**

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INTRODUCTION

Galician Rías (NW Spain) are important aquaculture areas, being mussel farming the main activity (Caballero Miguez et al., 2009). However, the pollution level in these areas rises proportionally to the industrial, tourism and port development. Moreover, the same aquaculture activities interact with the environment and can constitute a source of pollution around farms. Mussel culture is carried out in wood floating rafts, whose cleaning, and waterproofing are usually performed *in situ* with a mixture of petroleum tars and exhausted oil from the diesel engine. This mixture contains

high concentrations of polycyclic aromatic hydrocarbons (PAHs) and transition metals (Ruiz et al., 2014), and filtering organisms, such as mussels, can accumulate them to levels well-above those in the surrounding water (Farrington et al., 2016). So, the spillage of these mixtures around rafts entails a direct pollution risk on the immediate environment of farms, which can affect not only the sustainability of the mussel production but also its quality for human consumption.

Polycyclic aromatic hydrocarbons are considered among the most ubiquitous marine pollutants. Hydrocarbons and their biotransformation metabolites have harmful effects on live organisms, mainly the species with high molecular weight and benzene rings number, which have been identified as highly mutagenic, carcinogenic, and teratogenic (Arcos and Argus, 1975; Lehr and Jerina, 1977; White, 1986; IARC, 1989; Nylund et al., 1992; Boström et al., 2002; Baird et al., 2005; Haines and Hendrickson, 2009; Abbas et al., 2013; Poirier, 2016). Heavy and trace metals are also an extensively studied pollutant group in coastal environments because of their wide distribution, persistence, and toxicity. Some of them, such as Mn, Zn, and Cu, have physiological functions in living organisms but at high concentrations are also toxic (Goyer and Clarkson, 2001). Exposition of live organisms to heavy and trace metals has been related to cellular damages, carcinogenesis, and apoptosis (Gerber et al., 1980; Thompson et al., 1989; Chang et al., 1996; Wang and Shi, 2001; Beyersmann and Hartwig, 2008; Obiakor et al., 2010; Tchounwou et al., 2012; Chandel and Jain, 2014; Fatima et al., 2014). These metals are included in diverse industrial compounds, such as fuels and tars, lubricants, catalysts, paint drying agents, pigments, or pesticides (Laws, 2000; Clark, 2001), so pollution is usually occurring by combination of diverse pollutants whose toxic effects may be additive, suppressive or synergistic (Vakharia et al., 2001; Shen et al., 2006; Wang et al., 2015).

The toxic effects of PAHs and heavy metals and their action mechanisms have been widely studied in vertebrates. Among the main pathological effects usually associated with PAHs and heavy metals, are included toxicity reproductive and metabolic alterations and neoplastic diseases. These pathologies result from direct binding of these chemicals and their metabolites to macromolecules and, secondarily, from toxicity of the reactive oxygen species generated in their biotransformation (Sokol, 1987; Mortelmans and Zeiger, 2000; Adhikari et al., 2001; Ercal et al., 2001; Wang and Shi, 2001; Boström et al., 2002; Pufulete et al., 2004; Flora et al., 2008; Ono et al., 2008; Veeramachaneni, 2008; Sanders et al., 2009; Swedenborg et al., 2009; Balabanič et al., 2011; Tchounwou et al., 2012). Also in marine invertebrates, mainly molluscs, there are studies showing reproductive and neoplastic disorders related to pollution (Viarengo et al., 1990; Bolognesi et al., 1999; Matozzo et al., 2001; Creasy and Foster, 2002; Au, 2004; Pichaud et al., 2008; Jing-Jing et al., 2009; Ji et al., 2011; Ruiz et al., 2011). However, in these, much remains unknown about their biotransformation and toxic mechanisms, and few studies have proved their causal relationship under laboratory conditions (Lavado et al., 2006; Ortiz-Zarragoitia and Cajaraville, 2006; Jing-Jing et al., 2009; Schäfer and Köhler, 2009).

In previous papers, our team has documented in *Mytilus galloprovincialis* the accumulation of PAHs, trace metals and PCBs, relating it with the mutagenicity of seawater, and gonadal neoplasias development (Ruiz et al., 2011, 2013). Likewise, we have previously evaluated the mutagenic capacity of different chemicals used in mussel farms, including the used in the present work (Ruiz et al., 2014). This is a short-term toxicological study in laboratory in order to observe the histopathological effects caused by the more usually tar mixture used in mussel farms on the male gonad of mussel; prove their causal relationship in order to confirm the observed in natural environment; and propose the histological pathologies induced as possible generalist biomarkers of the aquaculture areas quality.

MATERIALS AND METHODS

Mussels were exposed to a mixture of petroleum tars and exhausted oil the from diesel engine, generally used in cleaning and maintenance of the mussel rafts, which was kindly provided by mussel farmers from the Ría of Vigo. Our team previously analysed the trace metals and hydrocarbons content of this tar, showing a significantly high concentration of Mn, Pb, and Zn (in a proportion 1:2:7), as well as a 81.5% of PAHs species with 3 and 4 benzene rings and a 18.5% of species with 5 and 6 benzene rings (Ruiz et al., 2014).

Mussels, Laboratory Conditions, and Acclimation

Mussels (*M. galloprovincialis*), 7–8.5 cm long, were collected from floating rafts in the Ría of Vigo in April, before starting the cleaning and waterproofing tasks of rafts in the culture area. Spring is the reproductive season of mussels in this area (Suárez et al., 2005), but also when we observed a higher PAHs accumulation and prevalence of gonadal histological disorders in these organisms in the wild (up to 739 $\mu g\ kg^{-1}$ dry weight and 62.5%, respectively) (Ruiz et al., 2011, 2013). The reproductive stage seems, therefore, constitute the most sensitive life-stage of mussels and the most suitable to test the toxic effects of pollutants on gametogenesis (Diepens et al., 2017). In the laboratory (Station of Marine Science of Toralla, University of Vigo), mussels were acclimated during 15 days in two polythene tanks of 150 L with 20 μm filtered seawater, in a semi-open system of 2 mL min^{-1} flow and the same out-flow, under natural photoperiod, and constant aeration, temperature and salinity. Animals were fed every 2 days with a microalgal diet (0.0224 g per mussel) composed by *Isochrysis galbana*, *Tetraselmis suecica*, and *Chaetoceros gracilis* in a proportion of 50:30:20.

Preparation of Water-Accommodated Fractions (WAF)

During waterproofing of rafts, a lot of tar falls to water and farmers pour the tar leftovers in the same environment, so high tar concentrations can be achieved around farms. Nevertheless, the dilutive effect of tides and currents makes their lasting become not too prolonged in time. Only the soluble fraction of these compounds, which varies depending on

their concentration and composition, is bioavailable and may exert actual toxicity on aquatic organisms (Singer et al., 2001). The WAF from different concentrations of tar were prepared in 20 µm filtered seawater, following the recommendations by CROSERF (Chemical Response to Oil Spills: Ecological Research Forum) (Singer et al., 2000, 2001; Aurand and Coelho, 2005). Briefly: in a similar way as carried out by the mussel farmers, the tar was heated to make it more fluid. Then, each tar concentration to be tested was prepared in glass Mariotte bottles with 5 L of seawater and maintained at room temperature for 24 h under magnetic stirrer. At the beginning of the experiments, the WAFs from the different tar concentrations were similarly prepared in polythene tanks of 30 L.

Determination of the Median Lethal Concentration (LC_{50})

Based on preliminary experiments, the WAF from 6 concentrations of tar between 60 and 160 mg L^{-1} were used in order to determinate its LC_{50} in an acute toxicity test for 96 h. For mortality test, acclimated mussels were randomly distributed in polythene tanks of 30 L (40 individuals/tank), containing the WAF from 60, 80, 100, 120, 140, and 160 mg L^{-1} tar prepared as above described. Five litres WAF from each tar concentration was prepared daily and supplied to the corresponding tank, at a 2 mL min^{-1} flow, replacing the seawater entry. The assay was performed in duplicate (6×2 tanks), and the mussels from other two tanks were maintained in seawater and taken as reference. Conditions of water flow, nutrition, temperature, aeration, and photoperiod were the same as during acclimation. Water quality in each tank was daily checked by measuring pH, dissolved oxygen (DO), salinity and ammonium concentration, using portable pH meter, oximeter and salinometer, and ammonium kit LCK 304, respectively.

Mortality data were analysed by the PROBIT regression test with IBM SPSS Statistics 23.0 package (IBM Corporation, Armonk, NY) which allowed calculating the LC_{50} of the tar mixture for the mussels, and their associated 95% confidence limits. The adequacy of the probit model to the observed data was evaluated by Chi-square test.

Toxicological Assay

Three sub-lethal WAF from tar concentrations were selected below the LC_5 value (see result section about LC_{50} and probit test) to conduct this study: 10, 40, and 60 mg L^{-1}. The WAF of these tar concentrations, the tanks and the mussels were prepared and disposed of as described in the above sections. The toxicological assay was also performed in duplicate. Two tanks with mussels maintained in seawater without tar were taken as reference. The conditions of the water and the mussels, as well as quality system control, were the above described. The toxic treatment lasted 17 days and samples were taken at 0, 10 and 17 days. After this time, mussels exposed to the WAF from 60 mg L^{-1} tar were transplanted to the other two tanks under non-toxic conditions during 2 weeks before sampling (depuration time). The analysis power of the sample size (320 individuals/4 groups of study) was validated with free software G*power (http://www.psychologie.hhu.de/arbeitsgruppen/allgemeine-psychologie-und-arbeitspsychologie.html), giving a value of 0.96. In each sampling, 20 individuals from each treatment (10 individuals per tank) were collected and, by histological analysis, males were separated for this study.

Biometric Analysis

In each sampling, the size, total weight (Wt), shell weight (Ws), and mantle weight (Wm) of individuals were measured. From these data, the gonadal condition index (GCI) was calculated. This index is an approximation to gametogenic development of an organism. In species of the genus *Mytilus*, the genital papilla proximity to the mantle tissue is used for the expansion of gonad, and the gametogenic development takes place in this tissue. So, in these species, the GCI is determined as the mantle condition index, which can be calculated as the ratio between the mantle weight and the weight of all soft tissues (the difference between total weight and shell weight) (Suárez et al., 2005).

Histological and Morphometric Analysis

A cross-section (1 cm wide) of the central part of one hemimantle of each mussel was fixed in 10% neutral buffered formalin, dehydrated and embedded in paraffin. Sections 5 µm thick were cut, mounted on microscope slides, and stained with haematoxylin and eosin. Additional slides were stained with modified Gomori methenamine-silver nitrate, aniline blue and periodic acid-Schiff (PAS) in order to detect basement membranes and reticulin fibres, basophilic components, and glycogen, respectively.

The slides were examined under a light microscope Nikon 90i Eclipse. Representative photomicrographs were captured by a high-resolution camera digital (Nikon DS-Fi1c) coupled to a microscope and using the image analysis system Nis-Elements BR 4.0 software (Nikon Instruments Inc., Melville, NY). Morphometric analyses were performed using the same image analysis system above: the number of gonadal follicles, vesicular cells (VC), adipogranular cells (ADG), and granulocytes, as well as the area of gonadal follicles and VCs, were quantified in 20 sections 100 μm^2 from each slide at 100 or 400x magnification (for gonadal follicles and cells, respectively). Composition of figures was performed using CorelDraw® Graphics Suite X5 (Corel Corporation, Canada).

PAHs and Metals Extraction and Quantification

After dissection for histological analysis, soft tissues of the individuals sampled from each treatment was pooled, quickly frozen, and pulverised in liquid nitrogen using a laboratory ball mill (Fritsch GmbH, Germany). The analyse of the PAHs and trace metals levels were contracted to the Centre for Scientific and Technological Support to Research (CACTI) of the University of Vigo. PAHs were extracted with methanol in ultrasonication bath for 30 min and separated and quantified by high-performance liquid chromatography (HPLC) within tandem detectors of photodiode array and scanning fluorescence. The 16 most abundant species of PAHs in the tar composition were measured: naphthalene (Nap), acenaphthylene (Acy), acenaphthene (Ace), fluorene (Flu), phenanthrene (Phe),

anthracene (An), fluoranthene (Flt), pyrene (Py), benzo(a) anthracene (BaA), chrysene (Chry), benzo(b)fluoranthene (BbF), benzo(k)fluoranthene (BkF), benzo(a)pyrene (BaP), dibenzo(a,h) anthracene (DBA), benzo(g,h,i)perylene (Bper), and indeno—(1, 2, 3,-c,d) pyrene (IP). The most abundant trace metals in tar (Zn, Pb, and Mn) were determined by optical emission spectroscopy with inductively coupled plasma as the excitation source (ICP-OES) after a hydrogen peroxide:nitric acid (2:5) digestion. All analyses were performed in triplicate and PAHs and metals concentrations were expressed as $\mu g\ kg^{-1}$ fresh weight (fw) and $mg\ kg^{-1}$ fw, respectively.

Statistical Analysis

Statistical analyses were performed with IBM SPSS Statistics 23.0. All histological preparations were comparatively examined in relation to the reference organisms. The distribution and homoscedasticity of biometric and morphometric data were analysed by Kolmogorov-Smirnov and Levene tests, respectively. Data were assessed to one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test or Student's *t*-test to determine the statistical significance between groups. $p < 0.05$ was considered statistically significant.

Multivariate analysis was carried out using the software packages PRIMER6 & permanova + (Anderson et al., 2008). The distribution of the samples based on quantitative parameters variation as the effect of tar exposure was examined by Hierarchical Cluster analysis using Bray Curtis similarity. Previously, the collinearity between variables was analysed by Pearson correlation matrix, eliminating the variables with $r^2 > 0.95$. The main variables explaining this distribution were identified by parsimonious models (DistLM), and represented by distance-based redundancy analysis (dbRDA) (Anderson et al., 2008).

RESULTS

Water Quality and LC_{50} Determination

The water quality parameters remained within a narrow range in all tanks throughout the mortality test and the toxicological experiment: 18 ± 1°C; 7.8 ± 0.4 pH; 34.5 ± 0.5‰ salinity; 87.5 ± 5% dissolved oxygen, and 0.03 ± 0.01 mg L^{-1} ammonium. So, the differences we described in mussels intoxicated relative to reference mussels could be considered as a consequence of tar exposition.

Survival of the reference mussels and those exposed to the WAF from 60 mg L^{-1} tar was 100%. However, mortality of the mussels exposed to WAF from tar concentrations higher than 80 mg L^{-1} increased as tar concentration increased. The log-probit regression analyse of the data allowed estimate the LC_{50} of the tar in 139.84 mg L^{-1} for *M. galloprovincialis* (95% confidence interval: 132.57–149.08 mg L^{-1}; regression equation: $Y = 0.028X-3.922$, Chi-square = 0.182, $P = 0.67$). In the same analyse, the LC_5 (tar concentration whose WAF cause a 5% mortality of mussels) yields a value of 81.20 mg L^{-1} (95% confidence interval: 64.02 – 92.23 mg L^{-1}). The toxicological assay was then performed with the WAF from tar concentrations below this value.

PAHs and Metals Accumulation

Pollutants accumulation in the mussels exposed to tar increased significantly ($p < 0.05$) in relation to reference mussels within the following ranges: 6–106 $\mu g\ kg^{-1}$ fw total PAHs ($\sum 16$ parent PAHs); 0.08–0.71 mg kg^{-1} Mn; 0–1.4 mg kg^{-1} Pb; and 12–75 mg kg^{-1} Zn (**Table 1**). The total PAHs accumulation increased proportionally to tar concentration and exposure time in the mussels subjected to 10 and 40 mg L^{-1} of tar. However, in mussels exposed to 60 mg L^{-1} tar, the accumulation falls concerning that of the exposed to 40 mg L^{-1} tar in both two exposure times. Moreover, contrary to expected based on tar composition, the hydrocarbons with 5 and 6 benzene rings were accumulated in greater proportion (up to 30.8%) than species of 3 and 4 benzene rings. The mussels that were transplanted to non-toxic conditions for 2 weeks, after exposure to the WAF from 60 mg L^{-1} tar during 17 days, showed a small, but significant decrease ($p < 0.05$) in the accumulation of PAHs of 5 and 6 benzene rings and metals, but not of hydrocarbons of 2, 3, and 4 benzene rings.

The heavy metals followed a similar pattern of accumulation, increasing proportionally with the tar concentrations supplied and exposure times ($p < 0.05$). Only the mussels exposed to the highest tar concentration during longer time showed a decrease in metals accumulation. Unlike the proportion of the metals in the tar composition, the ratio Mn:Pb:Zn in the mussels exposed to tar was around 1:2:>100. However, the Zn and Pb proportion decreased significantly ($p < 0.05$) both, in mussels exposed for 17 days to 40–60 mg L^{-1} and 60 mg L^{-1} tar, respectively, as in the mussels transplanted to depuration conditions for 15 days.

Biometric Analysis and Gonadal Condition Index

Biometric and histological analysis was performed individually in each one of 80 mussels collected in each sampling. The histological analysis allowed separating the males for this study. The ratio between sexes was almost 1.2 in all sampling, similar to that found for *Mytilus edulis* and *M. galloprovincialis* by others authors in the wild (Lubet, 1959; Villalba, 1995; Suárez et al., 2005). All male mussels showed similar size (7.05 ± 0.45 cm) and shell weight (8.45 ± 0.8 g) along the experiment. However, the total weight of soft tissues ranged between 15.98 and 5 g, and the mantle weight between 5.68 and 0.18 g, decreasing significantly as tar concentration and exposure time increased ($p < 0.01$; **Figures 1A,B**). The mantle tissue of *Mytilus* has two interrelated physiologic functions: storage of reserve substances in specialised cells and gametogenesis at the expense of these reserves (see **Supplementary Materials**, "Mantle tissue of *Mytilus galloprovincialis*: histological structure"). So, *a priori*, the significant fall in mantle tissue weight could suggest an alteration of these two functions.

The GCI from the reference individuals was similar throughout the experiment (**Figure 1C**). The mussels exposed to 10 and 40 mg L^{-1} of tar mixture for 10 days showed negligible alterations ($p > 0.05$) of mantle and soft tissues weights, as well as similar GCI values, compared to those of the control

TABLE 1 | Accumulation of hydrocarbons, Mn, Pb, and Zn in *Mytilus galloprovincialis* exposed to different tar concentrations and exposure times.

Exposure time tar concentration (mg L^{-1})	0 days	10 days			17 days			15 days depuration
	0	10	40	60	10	40	60	
PAHs (μg kg^{-1})								
Nap	LD	1.0	12.0	2.5	1.6	13.0	1.3	1.5
Acy	LD	5.6	17.2	8.8	16.5	11.2	18.4	16.4
Ace + Flu	1.0	2.5	3.2	2.2	4.3	3.3	1.5	1.6
Phe	0.1	2.2	9.6	2.4	5.0	4.9	1.8	2.0
An	LD	0.4	1.7	0.5	0.9	1.0	0.3	0.9
Flt	0.7	4.7	8.9	7.2	7.5	12.7	5.0	6.9
Py	0.5	6.5	16.8	8.7	9.5	19.3	5.9	7.9
BaA	0.5	1.8	2.9	4.0	1.9	4.5	3.1	2.4
Chry	LD	2.8	5.1	6.7	3.6	8.2	4.5	4.4
BbF	LD	1.2	2.6	3.9	1.5	3.3	2.9	2.3
BkF	LD	0.6	0.8	1.5	0.7	1.8	1.3	0.9
BaP	0.3	1.0	1.3	2.2	1.1	2.8	1.9	1.8
DBA	2.1	9.1	9.1	9.1	10.3	18.1	10.4	7.7
Bper + IP	LD	0.4	0.5	0.9	0.8	1.5	0.8	0.6
ΣPAHs	6	40	92	61	65	106	59	57
% 2 + 3 + 4 rings PAHs		69	84	71	78	74	71	77
% 5 + 6 rings PAHs		31	16	29	22	26	29	23
Metales (mg kg^{-1})								
Mn	0.08	0.37	0.42	0.56	0.40	0.71	0.41	0.35
Pb	LD	0.67	0.75	0.98	0.76	1.4	0.7	0.51
Zn	12	46	63	69	61	75	48	35
Mn:Pb:Zn		1:1.8:124	1:1.8:150	1:1.8:123	1:1.9:152	1:2:106	1:1.7:117	1:1.5:100

Each data is the average of three independent measurements. The values of all groups exposed to tar were significantly higher ($p < 0.01$) than those of the reference individuals (0 mg L^{-1} tar). The values of the mussels transplanted to non-toxic conditions (depuration sample) were significantly lower than those of the exposed to 60 mg L^{-1} for 17 days ($p < 0.05$).

mussels. However, the mussels exposed to 60 mg L^{-1} tar showed a significant decrease in the mantle weight and, consequently, of the GCI ($p < 0.01$; **Figures 1B,C**). After 17 days' tar exposure, the mantle tissue weight and the GCI of the mussels exposed to 10 and 40 mg L^{-1} tar fell ($p < 0.01$) until similar values as those of the mussels exposed to 60 mg L^{-1} tar during 10 days; but the mussels exposed to this last concentration showed similar values to those achieved after 17 days of exposition (**Figures 1B,C**).

Gonadal Stage and Histological Disorders

At the onset of the toxicological assay, the acclimatized mussels were in a pre-spawn stage (see **Figure S1**). This gametogenic stage is characterised by the presence of large gonadal follicles full of ripe gametes occupying the whole mantle tissue and by a scarce storage tissue, mainly formed by vesicular cells (see **Supplementary Materials**, "Reproductive cycle of *Mytilus galloprovincialis*"). Throughout all experiment, the reference individuals showed this same stage, with a slight but significant increase of gonadal follicles number ($p < 0.01$) parallel to decrease of vesicular cells (VC) area and adipogranular cells (ADG) number ($p < 0.0001$; **Figures 2A1,A2**, **3A–E**). In the follicular wall, auxiliary cells and some spermatogonias, spermatocytes and spermatids can be observed (see **Figures S2A,B**). At 17 days, auxiliary cells and eosinophilic granulocytes were observed in the lumen of some follicles from the reference mussels (see **Figures S2C,D**; **Figure 3F**). In the seminiferous tubules, these two cellular types have a phagocytic function of cellular debris after the spawn. In bivalves, the increase in temperature is one of the main natural factors inducing spawning (Suárez et al., 2005), but during our experiment, the temperature was kept constant at 18°C. So, the lack of stimuli for spawn could have caused atresia of the over-riped gametes, inducing the phagocytic function of auxiliary cells and the increase in the number of eosinophilic granulocytes in the follicular lumen (**Figure 3F**) as it is described in the wild under normal conditions (Suárez et al., 2005; Suárez Alonso et al., 2007).

The number and area of gonadal follicles from mussels exposed to 10 and 40 mg L^{-1} tar for 10 days were unchanged in relation to those of the reference individuals (**Figures 3A,B**). However, lumen was partially empty, and spermatozoids were present in gonoducts, evidencing clear images of spawn (**Figures 2B1,C1**). Mussels exposed to 40 mg L^{-1} tar also showed evidence of sloughing of spermatids or emission of immature germinal cells (**Figure 2C1**). After 17 days, spawn had been massive in most individuals, and atrophy of gonadal

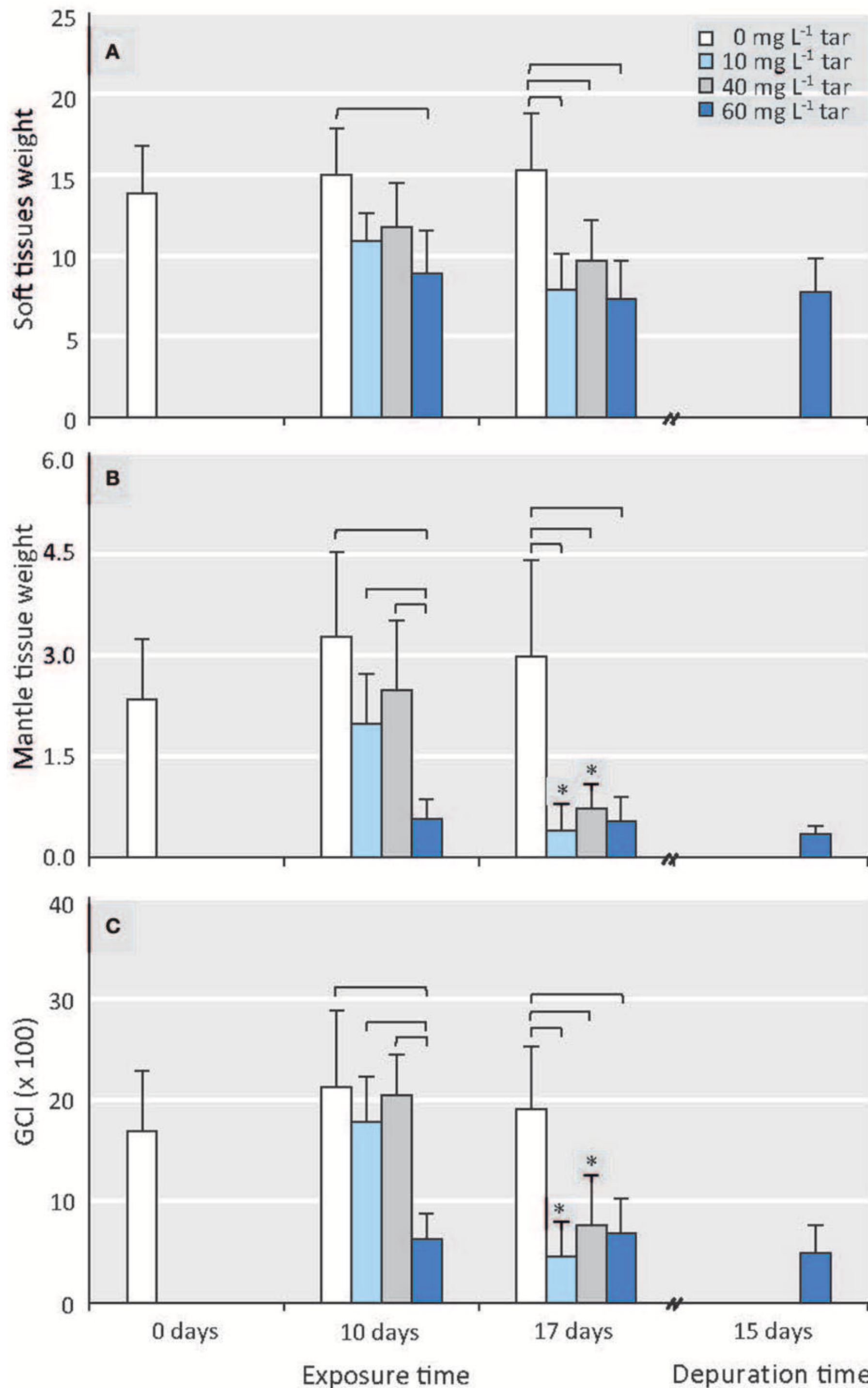

FIGURE 1 | Effect of different tar concentrations and exposure times on biometric parameters. **(A)** Soft tissues weight. **(B)** Mantle tissue (gonad) weight. **(C)** GCI. Data are means ± SD, $n \geq 8$. Reference mussels (0 mg L^{-1} tar). Square brackets represent significance differences between mussels exposed to different tar concentrations during same time ($p < 0.001$). Significance differences between mussels exposed to each of the tar concentrations at different times: *$p < 0.01$.

follicles was observed, decreasing their size and increasing their number per area (**Figures 2B2,C2**, **3A,B**). The basement membrane of follicles appeared thickened acquiring a hyalinised, appearance and the germinal epithelium lost its characteristic radial architecture (**Figures 2B2,C2**). Simultaneously, storage tissue was increased, mainly due to the proliferation and

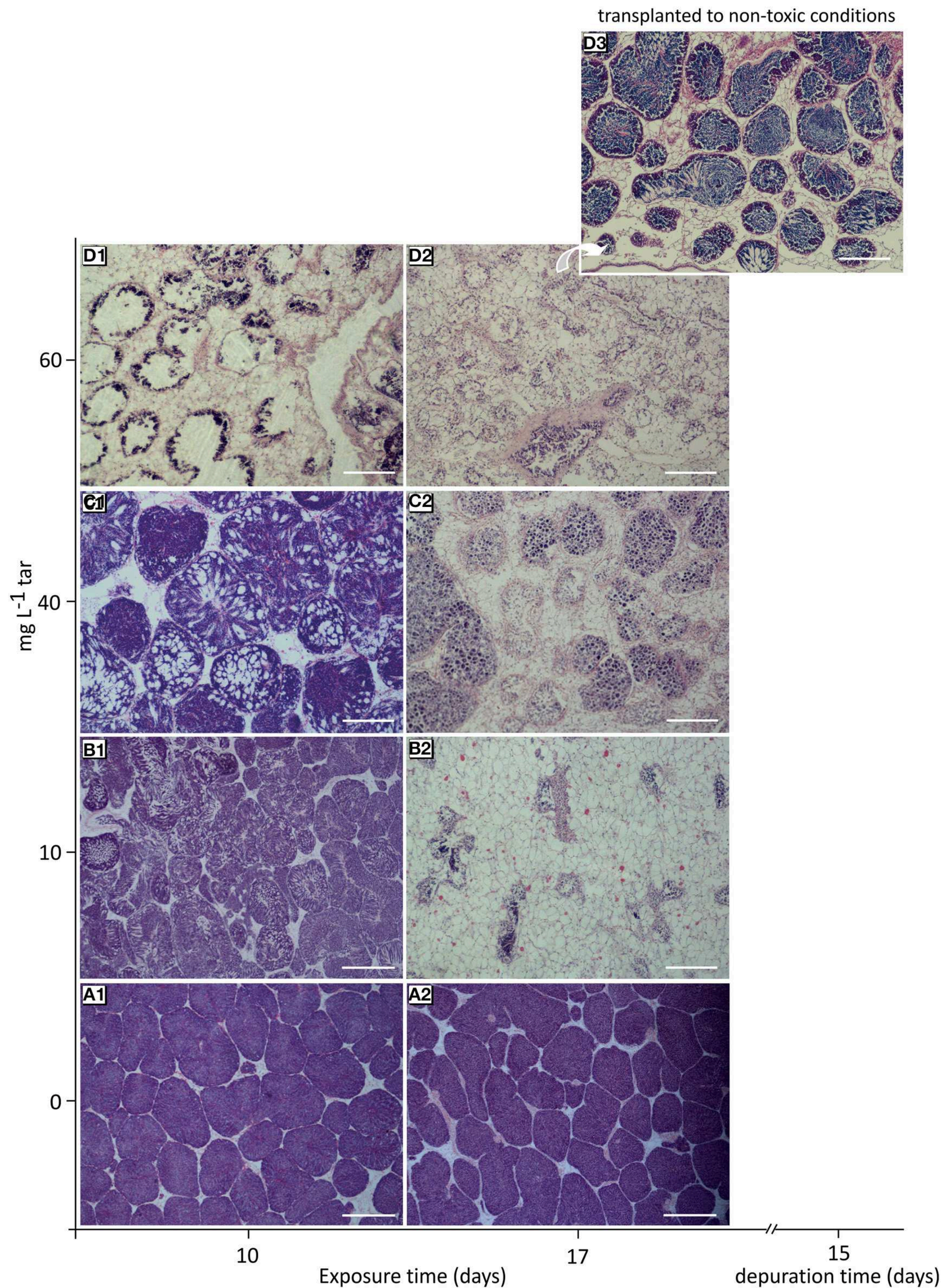

FIGURE 2 | Histological overview of the gonad from mussels exposed to different tar concentrations for 10 and 17 days. **(A1,A2)** Reference mussels (0 mg L^{-1} tar) showing a typical pre-spawn stage. **(B1)** Spawn induction. **(B2)** Atrophy of gonadal follicles in mussels exposed to 10 mg L^{-1} tar. **(C1)** Sloughing of spermatids. **(C2)** Loss of radial architecture of germinal epithelium and hyalinisation of basement membrane of follicles in mussels exposed to 40 mg L^{-1} tar. **(D1)** Disappearance of advanced germ cells. **(D2)** Virtually empty gonadal follicles with hyperchromatic spermatocytes and proliferation of storage tissue cells in mussels exposed to 60 and 80 mg L^{-1} tar. **(D3)** Gametogenesis reactivation in mussels transplanted to non-toxic conditions for 17 days after exposition to 60 mg L^{-1} tar. Haematoxylin and eosin stain. Scale bar: 500 mm **(A1,B1)**, 200 mm **(B2–D3)**.

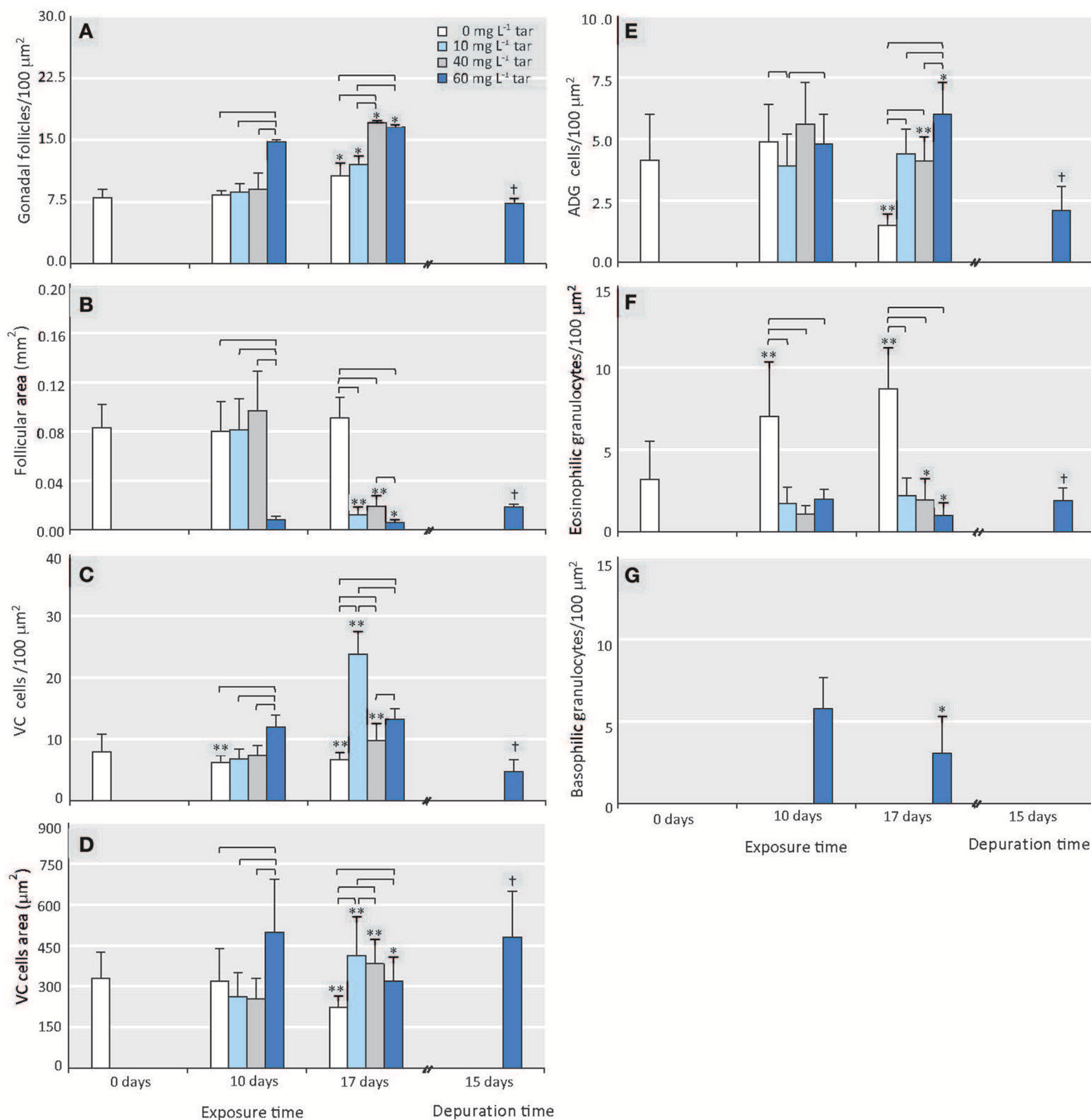

FIGURE 3 | Effect of different tar concentrations and exposure times on histological morphometric parameters. **(A)** Number of gonadal follicles. **(B)** Area of gonadal follicles. **(C)** Number of VC cells. **(D)** Area of VC cells. **(E)** Number of ADG cells. **(F)** Number of eosinophilic granulocytes. **(G)** Number of basophilic granulocytes. Data are represented as means ± SD, $n \geq 8$. Reference mussels (0 mg L^{-1} tar). Square brackets represent significance differences between mussels exposed to different tar concentrations during same time ($p < 0.001$). Significance differences between mussels exposed to each of the tar concentrations at different times: *$p < 0.01$, **$p < 0.0001$. Significance differences between mussels transplanted to non-toxic conditions for 15 days after exposition to 60 mg L^{-1} tar for 17 days: $^{\dagger}p < 0.001$.

hypertrophy of VC cells (**Figures 3C–E**). The mussels exposed to 60 mg L^{-1} tar for 10 or more days showed similar histological morphometric parameters to these described in mussels exposed to 10 and 40 mg L^{-1} tar for 17 days, but in a more obvious way (**Figures 3A–E**). Besides, these individuals showed virtually empty gonadal follicles, devoid of advanced germ cells (spermatids and spermatozoids), with scarce and hyperchromatic spermatocytes, and staining of the storage tissue seemed cloudy and diffuse (**Figures 2D1,D2**). Concerning to immune cells, all concentrations of tar assayed caused a significant decrease of eosinophilic granulocytes ($p < 0.001$; **Figure 3F**), but aggregates of basophilic granulocytes were observed in the mussels exposed to 60 mg L^{-1} tar (**Figure 3G**). More than 80% of the individuals exposed to each one of the tar concentrations assayed showed the histological alterations described.

Cellular Pathological Features

The typical aspect of the different cellular types constituting the mantle tissue of *Mytilus galloprovincialis* is shown in

the **Figure S2** (see **Supplementary Materials**, "Mantle tissue of *Mytilus galloprovincialis*"), which includes representative photomicrographs of the reference individuals from this work. Exposure of mussels to 10 mg L^{-1} tar for 17 days and 40 mg L^{-1} tar during 10 days did not cause observable cellular alterations. However, higher

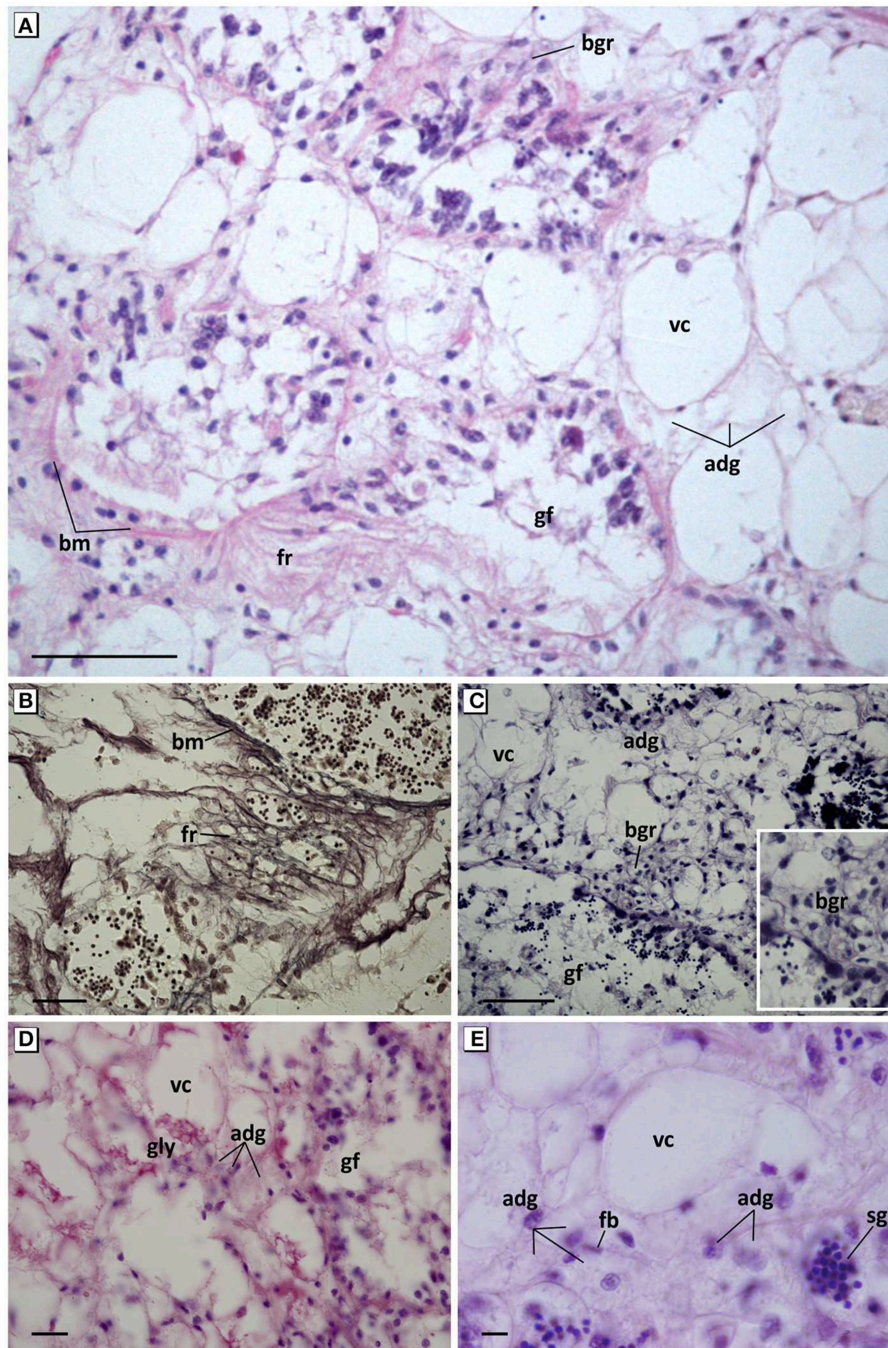

FIGURE 4 | Pathological features of connective tissue cells. **(A)** Overview of hyperplasia, fibrotic reaction of connective tissue, and basophilic granulocytes. Haematoxylin and eosin stain. **(B)** Detail of reticulin fibers and the thickening and break of the basement membrane of the gonadal follicles. Gomori methenamine-silver nitrate stain. **(C)** Hypertrophy and hyperplasia of VC and ADG cells; appearance of cluster of basophilic granulocytes around the gonadal follicles. Aniline blue stain. **(D)** Decrease of accumulated glycogen in VC cells and increase of small granules in ADG cells. PAS stain. **(E)** Foamy aspect with refringent microvesicles and loss of eosinophilia of ADG cells. Haematoxylin and eosin stain. fr, Fibrotic reaction; fb, fibroblast; bm, basement membrane; adg, adipogranular cells; vc, vesicular cells; gly, glycogen; bgr, basophilic granulocytes; sg, spermatic granuloma invading connective tissue; gf, gonadal follicle. Scale bar: 50 µm **(A,C)**, 25 µm **(B)**, 10 µm **(D,E)**.

concentrations and longer exposure times induced cellular disorders of increasing intensity and malignity in 75–82% of individuals.

After treatment with 40 mg L^{-1} of tar for 17 days and with 60 mg L^{-1} during 10 or more days, substantial changes were observed in somatic cells from the storage tissue.

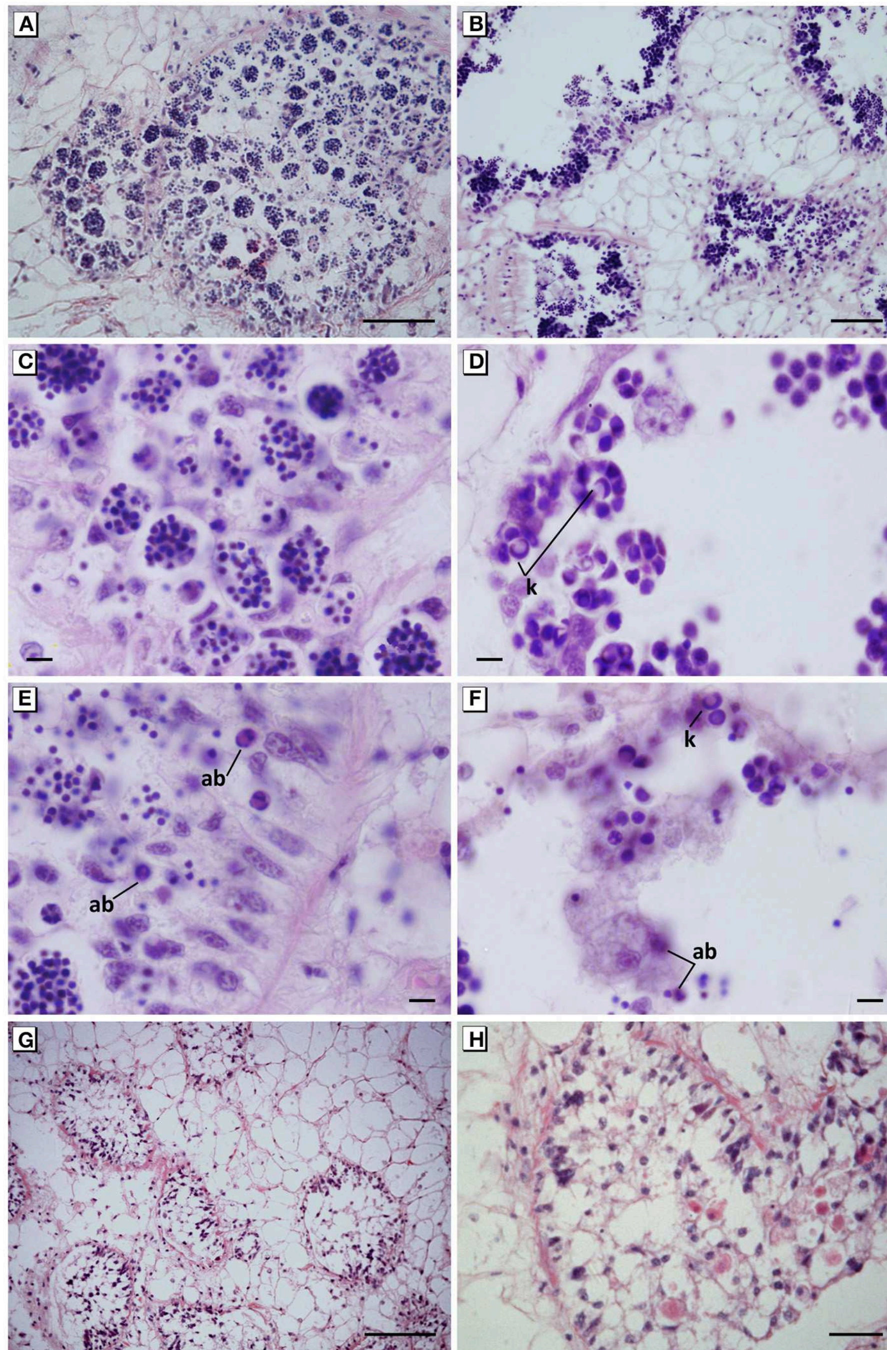

FIGURE 5 | Maturation arrest of the germinal cells and spermatogenesis failure. **(A,C)** Disorganisation of the germinal epithelium and abnormal aggregates of hyperchromatic spermatids in mussels exposed to 40 mg L^{-1} for 17 days. **(B,D)** Disorganisation of the germinal epithelium and abnormal aggregates of apoptotic spermatocytes showing karyorrhexis in mussels exposed to 60 mg L^{-1} for 10 days. **(E)** Hypertrophy and hyperplasia of auxiliary cells (Sertoli-like cells) with an intense phagocytic activity of apoptotic spermatids in mussels exposed to 40 mg L^{-1} tar for 17 days. **(F)** Auxiliary cells in the lumen follicular engulfing apoptotic spermatocytes in mussels exposed to 60 mg L^{-1} for 10 days. **(G,H)** Hyperplasia of pleomorphic and vacuolated auxiliary cells filling the lumen follicular in mussels exposed to 60 mg L^{-1} for 10 days. In all cases, a total absence of spermatozoa in mussels was evident. Haematoxylin and eosin stain. ab, Apoptotic bodies; k, karyorrhexis. Scale bar: 100 µm **(B,G)**, 50 µm **(A)**, 25 µm **(H)**, 10 µm **(C–F)**.

The amorphous extracellular matrix increased and collagen synthesis by fibroblasts and myofibroblasts was stimulated in a possible fibrotic reaction. The fibrosis originated entailed the hyalinisation of tissue, mainly in the basement membrane of the gonadal follicles, which appeared thickened and occasionally broken (**Figures 4A,B**). The VC cells displayed hypertrophy and hyperplasia (**Figures 3C,D**, **4**). Their size and number increased as the accumulated glycogen decreased, and their nucleus acquired a rounded shape and a more central position (**Figure 4**). The ADG cells changed their morphology, their size and number also increased abnormally, and their cytoplasm lost its eosinophilic character, acquiring a foamy aspect with refringent microvesicles, probably lipidic, and small PAS-positive granules (**Figure 4**). As cited above, the number of eosinophilic granulocytes decreased significantly in all mussels exposed to tar. Nevertheless, in individuals exposed to 60 mg L^{-1}, the occurrence of another type of granulocytes with a small and slightly basophilic cytoplasm, clustered around the gonadal follicles was observed (**Figure 4C**).

In gonadal follicles, a disorganisation of the germinal epithelium was observed, with loss of germline and total absence of spermatozoa, manifesting a maturation arrest and spermatogenesis failure (**Figures 5A,B**). In the most of the follicles from mussels exposed to 40 mg L^{-1} tar for 17 days, the only germinal cells present were abnormal aggregates of spermatids showing a large number of hyperchromatic nuclei immersed in one lightly eosinophilic cytoplasm, likely due to an incomplete cytoplasmic division (**Figures 5A,C**). In many cases, these aggregates filled the follicular lumen, and occasionally were seen invading the connective storage tissue (**Figure 4E**), like the spermatic granulomas described in vertebrates (Creasy et al., 2012; Greaves, 2012). Follicles from mussels exposed to 60 mg L^{-1} tar for 10 days only contained spermatocytes, sometimes also forming aggregates, many of them with condensed chromatin in a peripheral position, evidencing fragmentation of the nucleus (karyorrhexis) and apoptotic processes (**Figures 5D,F**). In both cases, apoptotic bodies (fragments intensely stained by haematoxylin within a round mass of intensely eosinophilic cytoplasm) of spermatids or spermatocytes were observed into the cytoplasm of auxiliary cells. These cells (Sertoli-like cells) showed hypertrophy and hyperplasia, and their nucleus changed their original basal position elongating toward the follicular lumen perpendicularly to the follicular membrane. Their cytoplasm appeared swollen and separated from the membrane until remain free in the follicular lumen, showing an intense phagocytic activity of apoptotic germ cells (**Figures 5E,F**). By contrast, the phagocytic response of haemocytes was minimal and very few eosinophilic granulocytes were observed in the follicular lumen. Moreover, Sertoli cells with pleomorphic aspect and swollen and vacuolated cytoplasm filled a large number of gonadal follicles (**Figures 5G,H**). These morphological and positional changes of Sertoli cells hindered their quantification.

The most severe disorders caused by the tar mixture on the germinal cells were observed in mussels exposed to 60 mg L^{-1} for 17 days. In these mussels, auxiliary (Sertoli-like) cells were not observed, or their number decreased drastically. In most individuals, the gonadal follicles were virtually empty, showing large mononucleate and multinucleated germinal cells with prominent and irregular hyperchromatic nuclei (pyknosis), karyorrhexis, and karyolysis (**Figure 6A**). The gonadal follicles of some others individuals (30% of mussels) showed microcytic germ cells with hyperchromatic irregular nuclei, clear cytoplasm and blurred edge, forming encapsulated nodules which also invaded the connective storage tissue. These encapsulated nodules showed fibrosis and deposits PAS-positive, evidencing glycogen accumulation (**Figures 6B–D**).

Depuration Assay

The mussels transplanted to non-toxic conditions for 2 weeks after exposure to WAF from 60 mg L^{-1} tar for 17 days, showed signs of an incipient reversion of the above-described disorders (**Figure 7**). The mantle weight and the GCI were maintained at similarly low values (**Figure 1**). However, a significant increase of follicular area took place, decreasing the number of the gonadal follicles per area (**Figures 2D3**, **3A,B**). Spermatogenesis was reactivated, while the number of vesicular and adipogranular cells decreased significantly ($p < 0.001$; **Figures 2D3**, **3C–E**). Furthermore, aggregates of basophilic granulocytes disappeared at the same time as eosinophilic granulocytes increased significantly ($p < 0.001$; **Figures 4F,G**). The appearance of the storage connective tissue cells was similar to those in the reference individuals, and the ADG cells started again to acquire their characteristic eosinophilia; although the VC cells area remained high and their round nucleus indicated a low glycogen accumulation. However, disorganisation and discontinuity of the germinal epithelium and some disorders of germinal cells, as aggregates of multinucleated and pyknotic spermatocytes were still evident (**Figures 2D3**, **7**).

Multivariate Analysis of the Quantitative Parameters

The Pearson correlation analysis identified a high collinearity ($r^2 > 0.95$) between the PAHs and metals accumulation, and the other hand between the weight of soft tissues and mantle, GCI and follicular area. So, in the Hierarchical Cluster and DistLM analysis, the data of metals accumulation, the weight of soft tissues and mantle and follicular area were not considered.

Cluster analysis showed two significantly different groups. The first group comprised two subgroups: one included reference mussels and the other those exposed to low and medium tar concentrations for 10 days. The second group included the mussels exposed to the highest concentration for 10 and 17 days into a subgroup, and those exposed to low and medium concentrations for 17 days in other. The mussels transplanted to non-toxic conditions for 2 weeks (depuration assay) were included in the second group, although at a considerable distance of mussels exposed to medium and high doses tar. The best variables combination determined by DistLM analyses excluded the GCI, and the first two dbRDA axes explained the 87.7% of the fitted variability (**Figure 8**).

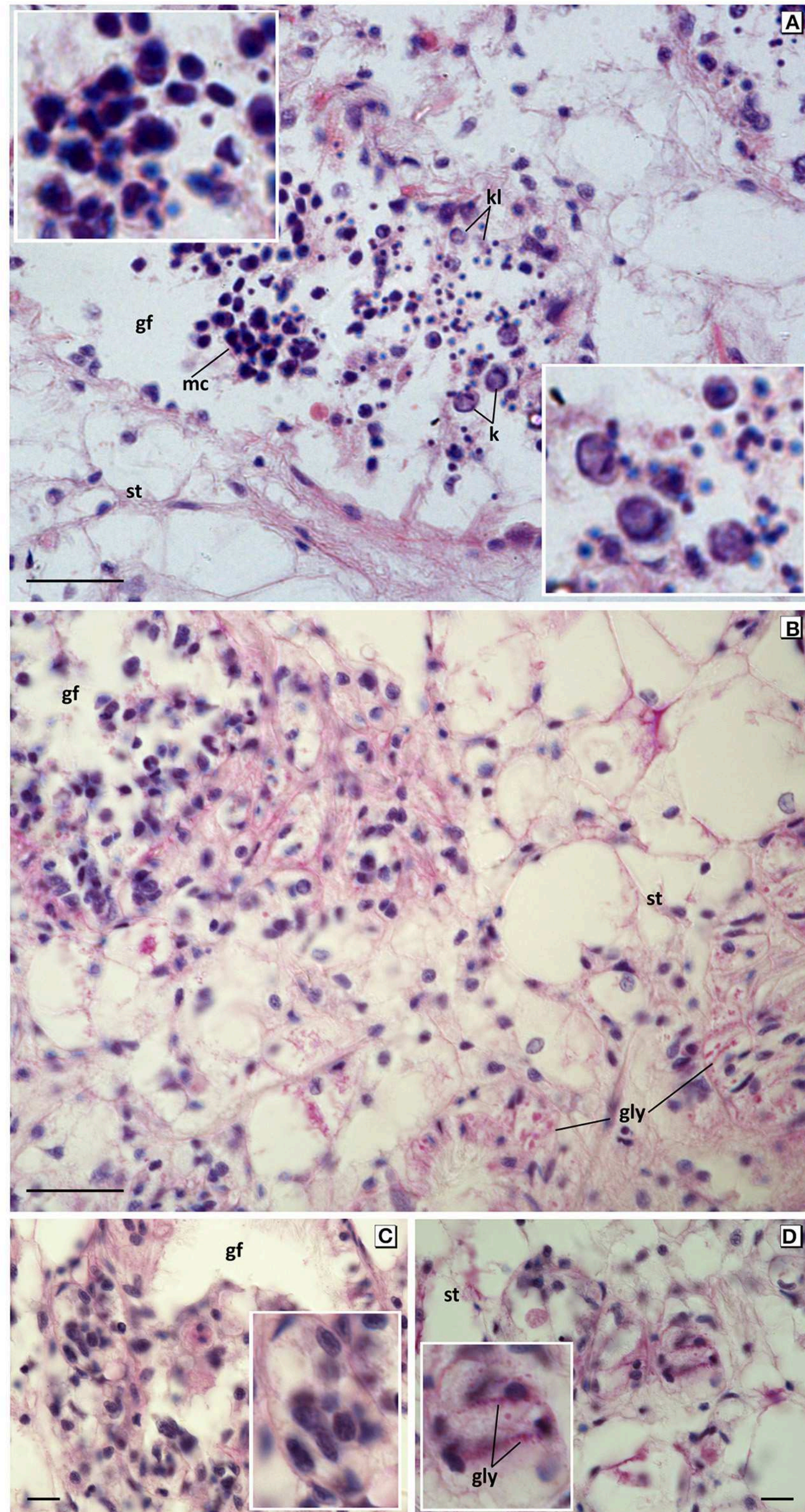

FIGURE 6 | Apoptotic and degenerative disorders in germinal cells from mussels exposed to 60 mg L^{-1} tar for 17 days. **(A)** Large mononucleate and multinucleated germinal cells whit big and irregular pyknotic nuclei (top inset), karyorrhesis and karyolisis (bottom inset). Haematoxylin and eosin stain. **(B–D)** Microcytic germ cells with hyperchromatic irregular nuclei, clear cytoplasm, and blurred edge forming encapsulated nodules, with deposits PAS positive in gonadal follicles and invading the connective tissue. PAS stain. Gf, Gonadal follicle; st, storage tissue; gly, glycogen; k, karyorrhexis; kl, karyolysis. Scale bar: 25 μm **(A,B)**, 10 μm **(C,D)**. Insets: 2x magnification in base image.

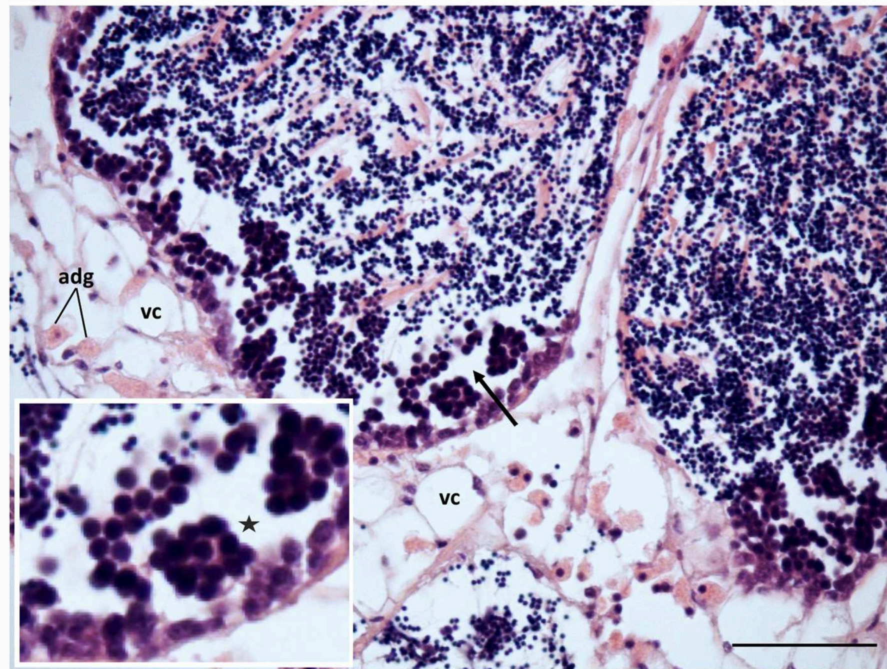

FIGURE 7 | Gametogenesis reactivation and recovery of the connective tissue in mussels transplanted to non-toxic conditions after exposition to 60 mg L^{-1} tar for 17 days. adg, Adipogranular cells; vc, vesicular cells; discontinuity in the germline (*), and aggregates of large and pyknotic spermatocytes (arrow) can still be observed. Scale bar: 50 μm. Inset: 2x magnification in base image.

DISCUSSION

Toxicological studies have demonstrated that hydrocarbons disrupt multiple endocrine pathways affecting hormonal regulation, metabolism and reproduction, and that several congeners have mutagenic effects contributing to carcinogenesis (Creasy and Foster, 2002; DeFur, 2004; Oetken et al., 2004; Pufulete et al., 2004; Dietrich and Krieger, 2009; Sanders et al., 2009; Balabanič et al., 2011; Creasy et al., 2012; De Falco et al., 2015).

Heavy metals have also been related to cellular injury, metabolic disorders, and DNA and proteins damage, that may lead to cell and reproductive cycle disturbance, carcinogenesis, or apoptosis (Gerber et al., 1980; Ronis et al., 1996; Wang and Shi, 2001; Tchounwou et al., 2004, 2012; Beyersmann and Hartwig, 2008; El-Sayed and El-Neweshy, 2010; Obiakor et al., 2010; Ebrahimi and Taherianfard, 2011). Although in invertebrates the number of these studies is lower, and these organisms present different susceptibility to pollutants, several authors have suggested similar effects and mechanisms than that of vertebrates and have propose them as models in endocrine disruption research (Viarengo et al., 1990; Bolognesi et al., 1999; Matozzo et al., 2001; DeFur, 2004; Porte et al., 2006; Zhou et al., 2009; Fatima et al., 2014).

PAHs and Metals Accumulation

Applying, where it was necessary, the conversion suggested by OSPAR (2005) to compare data given in dry or fresh weight, the PAHs, Mn, Pb, and Zn accumulated in mussels in this study are within the range found by other authors in *Mytilus* spp, and are similar to found by our team in mussels from the Ría of Vigo (Broman et al., 1990; Baumard et al., 1998; Piccardo et al., 2001; Besada et al., 2002; O'Connor, 2002; Webster et al., 2006; Monteduro et al., 2007; Bartolomé et al., 2010; Ruiz et al., 2011). Compared to the composition of the tar used, the variation of hydrocarbons and metals in the mussels exposed to WAF of different concentrations of tar seems to indicate that PAHs with a larger number of benzene rings, Zn and Pb are more efficiently accumulated. On the other hand, the decrease of these species of PAHs and metals in the mussels after depuration for 2 weeks suggest their faster elimination, like it is also appointed by other authors (Phillips, 1976; George, 1980; Rainbow, 2002; Glad et al., 2017). These facts seem to reflect the specific metabolism of *Mytilus* and the complex relationship between bioavailability, accumulation, biotransformation, and excretion of these compounds.

Gonadal and Histological Disorders

In the present study, cumulative gonadal disorders were observed in male mussels concerning both tar exposure and accumulation of PAHs and metals. In molluscs, similar disorders have been related to PAHs and metals pollution in wild environments (Pridmore et al., 1990; Viarengo et al., 1990; Bolognesi et al., 1999; Alonso et al., 2001; Tay et al., 2003; Aarab et al., 2004; Smolders et al., 2004; Jing-Jing et al., 2009; Yurchenko and Vaschenko, 2010; Ortiz-Zarragoitia et al., 2011; Ruiz et al.,

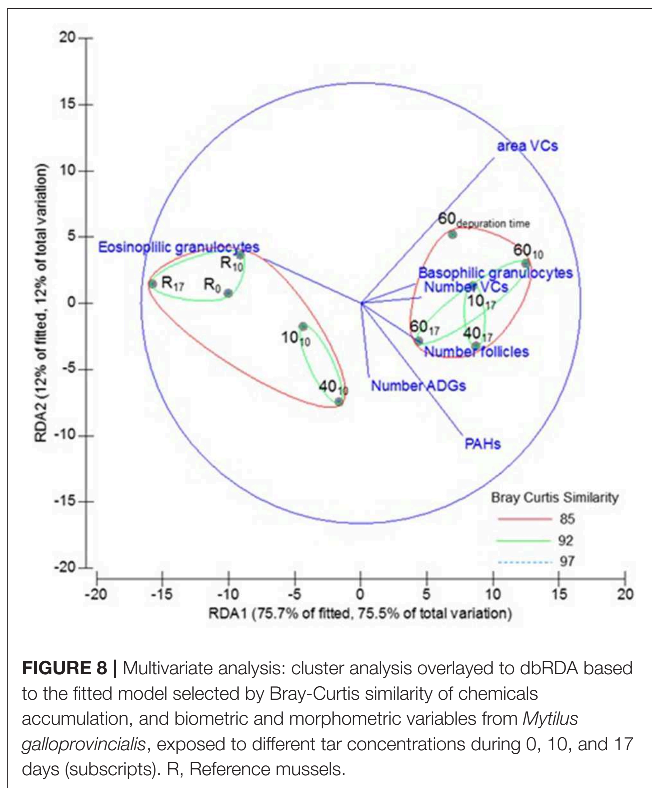

FIGURE 8 | Multivariate analysis: cluster analysis overlayed to dbRDA based to the fitted model selected by Bray-Curtis similarity of chemicals accumulation, and biometric and morphometric variables from *Mytilus galloprovincialis*, exposed to different tar concentrations during 0, 10, and 17 days (subscripts). R, Reference mussels.

2011, 2013; Smolarz et al., 2017). However, this is the first study demonstrating that tar used in the waterproofing of the floating rafts from mussel farms has a direct causal relationship with the histopathological alterations observed in male mussel gonad, which affect the reproductive function, the associate storage tissue metabolism and the immune response.

The germinal epithelium of male gonad and the spermatogenesis process are, in general terms, astonishingly similar in even very different animals, and their control mechanism is also highly conserved (Roosen-Runge, 1977; Working, 1988; Bonilla and Xu, 2008; Cheng and Mruk, 2010; White-Cooper and Bausek, 2010). However, there are scarce studies about cellular disorders and pathologies of this process in invertebrates, contrarily to in vertebrates. In the latter, the testis is a known target of PAHs and metals toxicity, experiencing disruption of spermatogenesis, degeneration, and loss of germ cell, and testicular atrophy (Ronis et al., 1996; Adhikari et al., 2001; Creasy and Foster, 2002; Dietrich and Krieger, 2009; El-Sayed and El-Neweshy, 2010; Mathur et al., 2010; Ebrahimi and Taherianfard, 2011; Creasy et al., 2012; Chandel and Jain, 2014).

In this study, the first histological effect of exposure to tar in mussels was the spawning induction, like described in other invertebrates (Tay et al., 2003; Aarab et al., 2004; Jing-Jing et al., 2009; Ortiz-Zarragoitia et al., 2011). However, as tar concentration and exposure time increases, the effect observed was a gonadal atrophy, manifested as a significant decline of the GCI and a drastic reduction of gonadal follicles size. In vertebrates, diameter reduction of seminiferous tubules is associated with loss of germ cells and constitutes a quantitative index of testicular injury (Creasy and Foster, 2002; Richburg et al., 2002; Dietrich and Krieger, 2009).

Cell Injuries

As dose and exposure time increased, pathological disorders in different cell types were observed. The study of these cellular alterations could subsequently shed light on cellular target of tar in the gonad of molluscs, as well as on the role of these cells in the spermatogenesis regulation of these organisms.

In addition to spawning induction, exposure to 40 mg L^{-1} tar for 10 days provoked a premature release or sloughing of immature spermatids, which resulted in cellular disorganisation of germinal epithelium and loss of its centripetal radial architecture, as has also been described in others molluscs from polluted environments (Yurchenko and Vaschenko, 2010). However, higher concentrations of tar and longer exposure times originated retention of abnormal aggregates of spermatids or spermatocytes with signs of degeneration and apoptosis, which occasionally were seen invading the connective storage tissue, like some spermatic granulomas described in vertebrates (Creasy et al., 2012; Greaves, 2012). These aggregates, have been termed multinucleated giant cells in several organisms where were related with disturbances of Sertoli cells and with the arrest of spermatogenesis and spermiogenesis (Creasy and Foster, 2002; De Falco et al., 2015). Among functions of Sertoli cells are the paracrine signalling, regulation of the material passing to gametes developing, structural support of spermatogenesis and release of the mature spermatids (Creasy and Foster, 2002; França et al., 2015). These functions are accomplished through junctional complexes maintained with the developing germ cells. Several toxicants, including hydrocarbons, disturb these junctions, leading to spermatogenesis failure (Richburg et al., 2002; Salian et al., 2009; Cheng, 2014).

As described above, in mussels exposed to 40 mg L^{-1} tar for 17 days and to 60 mg L^{-1} tar for 10 days, auxiliary or Sertoli-like cells proliferated abnormally, their aspect changed acquiring a swelled and vacuolated cytoplasm, and basal position of their nucleus was modified. Similar injuries have been described in Sertoli cells of vertebrates caused by toxicants and related to rapid and massive degeneration of germ cells, which suggest disruption of their structural and metabolic function (Creasy and Foster, 2002; Dietrich and Krieger, 2009; Cheng, 2014). Another function of Sertoli cells is phagocytosis and removal of residual sperm and degenerating germ cells (Boekelheide et al., 2000; França et al., 2015). This function seemed highly induced in these mussels, in which an inordinate phagocytic activity of apoptotic germ cells was observed, as also has been described in fish (Dietrich and Krieger, 2009). So, the Sertoli cells hyperplasia could be both a response to counteract germinal cells degeneration through their phagocytic capacity, as also the result of a direct endocrine disruptor effect of tar. Moreover, similar as we observed in a large number of follicles from these mussels, seminiferous tubules full of pleomorphic and vacuolated Sertoli cells have been described in neoplastic and degenerative processes of these cells from vertebrates (Henley et al., 2002;

Vegter et al., 2010). According to this latter idea, the absence of Sertoli cells in mussels exposed to 60 mg L^{-1} tar for 17 days seems to support their possible degeneration. The diminution or absence of Sertoli cells cause deregulation of germ cells and their most severe disorders, getting to form encapsulated nodules invading the connective tissue, similar to *in situ* germinal carcinomas described in vertebrates (Guminska et al., 2010).

The storage cells from interstitial connective tissue (VC and ADG cells) of the mussels exposed to 40 mg L^{-1} of tar mixture for 17 days and to 60 mg L^{-1} during 10 or more days showed pathological hyperplasia, hypertrophy and appearance changes compared to those of the reference mussels. These morphologic changes seem to suggest an alteration of their biochemical components and their function. In *Mytilus* spp, the VC cells function is the glycogen storage. These cells contain only a large glycogen vacuole, some mitochondria and a limited metabolic activity. However, in this work, the hyperplasia and hypertrophy of these cells as a consequence of tar exposition was parallel to depletion of glycogen store. Glycogen is the main energetic reserve in adult bivalves, and several authors have also reported its decrease as a result of oil exposure, limiting the energy available for growth and reproduction (Pridmore et al., 1990; Smolders et al., 2004). On the other hand, the ADG cells contain mainly lipid droplets and protein granules and present a high metabolic activity that varies during the gametogenic cycle under hormonal control (Pipe, 1987; Peek et al., 1989). Several authors identify the ADG cells with Leydig cells, but although there are hints on their possible role in steroidogenesis, their function (trophic and/or regulatory) has not yet been fully clarified (Croll and Wang, 2007; Lafont and Mathieu, 2007). In Leydig cells from vertebrates, similar disorders to those we described in the ADG cells from mussels in this work have been identified as pathologic: abnormal enlargement, changes in shape, and internal structure and foamy appearance of cytoplasm due to proliferation of lipid droplets (lipidosis) (Creasy and Foster, 2002; Dietrich and Krieger, 2009; Cheng, 2014). Lipidosis of Leydig cells has been related to their degeneration and with disturbance of their function (Chung et al., 2011; Oh, 2014). The Leydig cells function is the biosynthesis of testosterone, which indirectly regulates spermatogenesis in a complex cascade of chemical interactions with Sertoli and germ cells, resulting in feedback regulation of testosterone synthesis. So, disorders of Sertoli cells, such as those we described above, not only disrupt the interactions with germ cells causing the spermatogenesis failure, but also the interactions with Leydig cells, inhibiting testosterone production, which in turn induces germ cell degeneration (Creasy and Foster, 2002; Boekelheide, 2005). The sequence of disorders that we described in this work seems to point to these complex interrelations. So, the auxiliary cells of mussels, like the Sertoli cells of vertebrate, could be one of the primary targets of PAHs toxicity, whose injuries could induce sloughing of immature germ cells, as well as disturb the possible steroidogenic function of the ADG cells. Moreover, the ADG and germinal cells could also constitute secondary targets of these compounds, inducing the degeneration and the severe injuries we described in germ cells.

Fibrosis is defined as the abnormal proliferation of fibroblast resulting in an increased synthesis and deposition of collagen, which confers a thickened appearance of the connective tissue. This phenomenon is associated to diverse pathologies as degenerative lesions and tumours coursing with inflammatory processes. In the connective tissue lining seminiferous tubules of the vertebrate testis, fibrosis has been equally described associated with injuries and degeneration of germ cells but also following exposure to endocrine disruptors (Creasy and Foster, 2002; Albrecht et al., 2006; Dietrich and Krieger, 2009). The collagen synthesis stimulation in the fibrotic reaction is mediated by the release of fibroblast growth factors from paracrine and/or tumoral cells, whose induction has been related to estrogens and oxidative stress (Walker, 2001; Albrecht et al., 2006; Dietrich and Krieger, 2009; Gómez-Sámano et al., 2017). This process is considered to be a scarring reaction aimed to maintain the structure of the tissue after degenerative injuries (Albrecht et al., 2006; Dietrich and Krieger, 2009). However, the estrogens-induced fibrosis in reproductive tract seems to suggest a different function related to the testicular development and differentiation (Watanabe and Onitake, 1995; Dietrich and Krieger, 2009; Jiang et al., 2013). Also in the gonad of bivalve molluscs, several authors have reported fibrosis associated to gonadal and haemocytic neoplasms, as well as to hydrocarbons and metals pollution and oxidative stress (Yevich and Barszcz, 1976; Peters et al., 1994; Alonso et al., 2001). In our work, the fibrotic reaction observed around gonadal follicles, related to spermatogenesis arrest and the severe disorders that we described, could be caused jointly by possible xenoestrogenic effects of tar and by the oxidative stress and cell damage generated, as described in other organisms.

The immune response is responsible for recognition, oxidation, and phagocytosis of non-self particles. The bivalve immune system is a non-specific defence mechanism known as the innate immune system, which is yet not fully understood. This is a primitive mechanism, evolutionarily conserved, triggered by intracellular molecular signalling cascades (Danilova, 2006; Song et al., 2010). The haemocytes activity from bivalves is not limited to immune response, but they are involved in different physiological processes including tissue repair, shell production, nutrition, and response to environmental and biological stresses (Song et al., 2010). The lineage of bivalve haemocytes is not clear. Several authors have suggested their origin from two different cells, precursors of granulocytes, and hyalinocytes, respectively. However, other authors indicate a single type of precursor cell of hyalinocytes, which would mature to granulocytes (Cheng, 1984; Hine, 1999). Moreover, the different types of granulocytes identified in bivalves (basophils and eosinophils) could be the result of subsequent differentiation and maturation processes (Donaghy et al., 2009).

The bivalve haemocytes seem particularly susceptible to environmental pollution by chemical or biological toxics. However, the bibliographic data are sometimes contradictory because several authors describe an increase of haemocytes number in bivalves exposed to oil spills, whereas others report their decrease (Pichaud et al., 2008; Hannam et al., 2009). Moreover, it has been described that innate immune response in bivalves is accomplished mainly by granulocytes, while hyalinocytes are the main responsible of tissues repair, requiring aggregation at a point of injury (Ruddell, 1971;

Hégaret et al., 2003). According to these assertions and with studies above cited, our results show firstly an evident decrease of the number of eosinophilic granulocytes in mussels undergoing at all tar concentrations and exposition times assayed, evidencing immunosuppression induced by treatment, although no infection was observed. Secondarily, in mussels exposed to the highest tar concentration, which developed the more severe injuries, we observed an increase of basophilic granulocytes forming aggregates around the affected follicles. These data suggest that the immunotoxic effects of PAHs are dependent on concentration and exposure time, resulting in either an increase or decrease of different types of granulocytes, whose specific functions should be addressed in later works. On the other hand, the significant decrease of eosinophilic granulocytes induced by tar intoxication in mussels would explain the absence of these phagocytic granulocytes containing degradation products described by others authors as brown cells (Puy-Azurmendi et al., 2010; Bignell et al., 2011).

Depuration Assay

Several works conclude that hormonal disturbance effects may be reverted as the xenoendocrine disrupters disappear, but it is known that processes of cell death are irreversible (Creasy and Foster, 2002; Creasy et al., 2012). In our study, the mussels transplanted to non-toxic conditions after the toxicity assay showed recovery signs of all cellular injuries.

Hydrocarbons, metals, and other chemicals exert direct effects on the function of Leydig and Sertoli cells in vertebrates, causing, among other, inhibition of testosterone synthesis, cholesterol regulation, lipid homeostasis, and insulin signalling (Jeng, 2014; De Falco et al., 2015). Testosterone inhibition, in turn, induces germ cell degeneration (Creasy and Foster, 2002; Boekelheide, 2005). In our work, the connective tissue cells (VC and ADG cells) from mussels transplanted to depuration conditions, recovered their appearance. So, the alterations described in these cells could be due to a direct xenoestrogenic effect or mediated by endocrine disruption of auxiliary cells. In these mussels, the germinal line was also partially restored, and no signs of apoptosis and degeneration were observed. In vertebrates it has been demonstrated that spermatogonial precursor cells are relatively resistant to injurious stimuli (Creasy and Foster, 2002; Creasy et al., 2012). So, in our study, some spermatogonial stem cells would have remained intact to allow resuming the gametogenesis. Nevertheless, due to the short time of transplant to non-toxic conditions, several disorders, as germinal epithelium discontinuity and aggregations of multinucleated and hyperchromatic spermatocytes were still evident.

As described in other bivalve species (Mayrand et al., 2005), we also observed granulocytes number is recovered, but we have not enough data to know if their phagocytic capacity is recovered too, neither if animals become more sensitive to stress and pathogens. Non-cellular defence responses could also be involved in this granulocytes number recovery as described in some bivalves (Charlet et al., 1996; Roch, 1999; Ortiz-Zarragoitia and Cajaraville, 2006). However, these studies are still scarce to understand the integrated immunomodulation in these organisms.

CONCLUSIONS

The chemicals accumulation, and biometric and morphometric analysis of the mussel male gonad presented in this study indicates quantitative specific patterns of change after exposure to tar as corroborated by multivariate analysis (concentration of PAHs accumulated; number of gonadal follicles, haemocytes, and connective interstitial cells; area of VC cells), which together with the qualitative observations of other histopathological changes (fibrosis, appearance change of the Sertoli, ADG, and germinal cells) could offer a mechanistic understanding of the joint effects of hydrocarbons and metals on reproductive function of invertebrates. This histopathological approach could support further molecular studies in order to clarify the role of the different gonadal cells in the spermatogenesis regulation of invertebrates, and be used in aquatic toxicology.

To our knowledge, this is the first study to demonstrate that tars used in the waterproofing of the floating rafts from mussel farms cause severe effects on male mussel gonad, inducing histopathological alterations, which affect the reproductive function, the associate storage tissue and the immune response. The malignancy of injuries is dependent on the concentration and exposure time and affects to different cell types in a manner comparable to that described in other organisms both vertebrates and invertebrates. So, the spillage of these mixtures around rafts entails a direct pollution risk on the immediate environment of farms, which affect the health of mussels, their reproduction and ultimately, the sustainability of this resource and its quality for human consumption.

Our findings suggest that the histopathological changes produced by the combined effects of heavy metals and PAHs tar that we described could be used as biomarkers in the monitoring of marine pollution and quality of aquaculture areas. Also, the mussel gonad could be considered as an excellent model in studies about the testicular toxicity mechanisms, disruption of spermatogenesis and metabolism in male reproduction.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript/**Supplementary Files**.

AUTHOR CONTRIBUTIONS

AA, YR, and FS designed the study. AA, PS, YR, and VD performed the data collection and wrote the draft of the manuscript. AA performed the histological and microphotographic analysis. PS performed the statistical analysis. AA, PS, YR, VD, and FS contributed to the interpretation of the results. PS and FS contributed to 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.2019.00577/full#supplementary-material

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The Potential Use of Invasive Ascidians for Biomonitoring Heavy Metal Pollution

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Heavy metal (HM) inputs into marine environments and their effect on marine organisms are of major concern. Here, we examined the potential use of two invasive ascidian species, *Phallusia nigra* and *Microcosmus exasperatus,* as bio-indicators of 11 HMs in the Mediterranean and Red Sea coasts of Israel. Individuals were collected on a seasonal basis from three sites over 1 year, and analysis was carried out separately for the tunic and the body. Both species accumulated high levels of HMs, which varied seasonally and spatially. In *M. exasperatus* the majority of HMs were found in the tunic, and in *P. nigra* in the body, suggesting the need to analyze total individuals in future studies. Hepato-Somatic Index values for *M. exasperatus* were significantly lower at the polluted site. Investigation of a popular public beach revealed high levels of certain dissolved HMs in both the water and in the ascidians. The wide geographic distribution and high filtration capacity of invasive ascidians offer great potential for their use in monitoring metal pollution in marine environments.

Keywords: benthic ecology, metal accumulation, biomonitoring Programs, tunicates, Red Sea, Mediterranean Sea

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INTRODUCTION

Biological indicators (bio-indicators) are used to measure and assess the health and quality of the environment, by examining the response of these organisms to exposure to external contaminants and the changes in response to this exposure over time (Holt and Miller, 2010; Chiarelli and Roccheri, 2015). In most cases, the risk to human health constitutes the main motivation for the development and research of bio-indicators to assess environmental health. Biological monitoring of marine pollutants presents an advantage compared to chemical analysis of environmental matrices such as seawater samples, as pollutant concentrations in water are often extremely low and their determination presents significant methodological and analytical challenges (Richir and Gobert, 2014; Bellante et al., 2016). Furthermore, live organisms used as indicators can accumulate different substances over a long period, hence providing a time-integrated evaluation of the past environmental status and its biological effects (Schettino et al., 2012). Consequently, the use of bio-indicators has greatly developed in the last decade as a promising alternative approach for the monitoring of environmental pollution (Holt and Miller, 2010; Abdul Jaffar Ali et al., 2015). For example, in order to monitor the aquatic ecosystems of the Doñana National Park, a UNESCO Biosphere Reserve in Southern Spain, the freshwater crayfish, *Procambarus clarkia*, was used as a bio-indicator

(Vioque-Fernández et al., 2009), and bivalves have been most widely used as bio-indicators of the marine environment (Beliaeff et al., 1997; Rodríguez-Ortega et al., 2003; Hedge et al., 2009).

Anthropogenic activities are responsible for a significant increase above natural background values in metal abundances in the environment. Anthropogenic heavy-metals (HMs), introduced into the oceans mainly by river runoff, atmospheric transport, and groundwater seeps, pose a serious threat to human health, living organisms, and natural habitats. Due to their toxicity, persistence, and bioaccumulation, they lead to a reduction in species diversity and abundance, while also accumulating in living organisms and thus entering the food chain (Abreo et al., 2015).

All aquatic invertebrates accumulate metals in their tissues, whether these metals are essential to the animal's metabolism or not (Rainbow, 2002). Essential trace elements, such as iron (Fe), manganese (Mn), copper (Cu), and zinc (Zn), are vital components of enzymes and play an important role in electron transport reactions. In contrast, aluminum (Al), nickel (Ni), vanadium (V), cadmium (Cd), lead (Pb), and arsenic (As), lack any known biological role in marine invertebrates and exhibit high toxicity if allowed to accumulate in metabolically active sites (Tchounwou et al., 2012; Horwitz et al., 2014). Different invertebrates accumulate trace metals at different concentrations in their tissues, organs, and bodies. Invertebrates dwelling in the same habitat can thus have very different body concentrations of metals, even within closely related taxa (Rainbow, 2002).

There are many factors that influence HMs accumulation in living organisms, with each factor functioning differently, depending on the species and type of metal (Jitar et al., 2014). Aquatic invertebrates take up trace metals either from their surrounding water or from food intake. The bio-concentration of contaminants such as HMs in the bio-indicator marine species can reach up to several orders of magnitude higher than their concentration in the ambient waters (Wang, 2016). The destination of the metal within the organism depends on the physiology of the invertebrate, and whether the metal is used for an essential metabolic purpose. Most metals possess a great affinity to sulfur and nitrogen, which are abundant in every living cell. Due to this affinity, all trace metals are potentially toxic, binding to proteins or other molecules and preventing them from functioning in their intended role (Rainbow, 2002). Studies on the bioaccumulation of HMs in different marine organisms are thus essential in order to expand our knowledge regarding the relative metal bioavailability from different sources (Rainbow, 2002; Aydın-Önen, 2016). Determining the metal concentrations in organisms in the coastal region should constitute an essential part of any assessment and monitoring program of contaminants in the marine ecosystem. Such assessment plays an important contributory role in decision-making regarding an ecosystem's assessment and management (Usero et al., 2005; Jitar et al., 2014). The choice of an appropriate bio-indicator species thus constitutes an integral part of any successful bio-monitoring program (Chiarelli and Roccheri, 2015).

Here, we took advantage of the wide geographic distribution of invasive ascidians (Chordata, Ascidiacea), which are able to thrive in both contaminated and pristine environments (Lambert and Lambert, 1998), as biological indicators of HMs in marine environments. Ascidians are highly efficient filter-feeding organisms, able to accumulate and concentrate harmful toxicants when present even at low concentrations in their surroundings (Jiang et al., 2010; Treberg et al., 2012). Ascidians actively accumulate HMs, including manganese, iron, cadmium, magnesium, molybdenum, niobium, tantalum, chromium, titanium, and vanadium (Odate and Pawlik, 2007; Gallo and Tosti, 2015; Aydın-Önen, 2016). There have been a number of studies in recent years on ascidians as bio-indicators, using different measurements and indices (physiological, molecular, reproductive) the results of which demonstrate the potential of this group to function as a useful bio-indicator of contamination in the marine environment (Bellas et al., 2004; Radhalakshmi et al., 2014; Aydın-Önen, 2016; Bellante et al., 2016).

Here, we report on the first time-series study conducted along both the Red Sea and the Mediterranean coasts of Israel concomitantly to assess the use of the invasive ascidians *Microcosmus exasperatus* Heller, 1878 and *Phallusia nigra* Savigny, 1816 as bio-indicators of HMs. We also sought to determine the distribution of the studied metals in *M. exasperatus* body tissues: tunic, body wall, gonads, and digestive gland, and to examine any potential physiological changes caused by metal pollution, such as the hepato-somatic and gonad indices. We further determined the heavy metal abundances in seawater from each of the study sites in order to quantify their relation to heavy metal content in the corresponding ascidians.

Invasive ascidians such as *M. exasperatus* and *P. nigra* present an ideal combination of both widespread (Shenkar and Loya, 2009) and highly efficient benthic filter-feeders (Jacobi et al., 2018), closely related to vertebrates (Delsuc et al., 2006), thereby enabling us to employ the same organism and methodology on a wide geographic scale, and thus contribute to the development of an ubiquitous method for assessing environmental health.

MATERIALS AND METHODS

Study Design and Sampling Sites

Sampling was performed by snorkeling at three main research sites in the Mediterranean and Red Sea coasts of Israel, located in Akko (32°55′15″N 35°04′22″E, Mediterranean coast), Mikhmoret (32°22′52″N 34°51′41″E, Mediterranean coast), and Eilat (29°32′51″N 34°57′13.47″E, Red Sea coast), between November 2015 and June 2017. Additional sampling was conducted in April 2017 at "Ha'Sela" beach in Bat-Yam (32°01′27″N 34°44′20″E, Mediterranean coast), a popular recreational beach close to several potential anthropogenic contamination sources, and which served as a site for comparison (**Figure 1**). According to Herut et al. (2017), the Akko site is frequently polluted, mainly by industrial discharge outlets, and is in proximity to many anthropogenic contamination sources; the Mikhmoret site is considered less disturbed, and the Red Sea site in Eilat is considered moderately polluted, being located at the North Beach area and highly influenced by

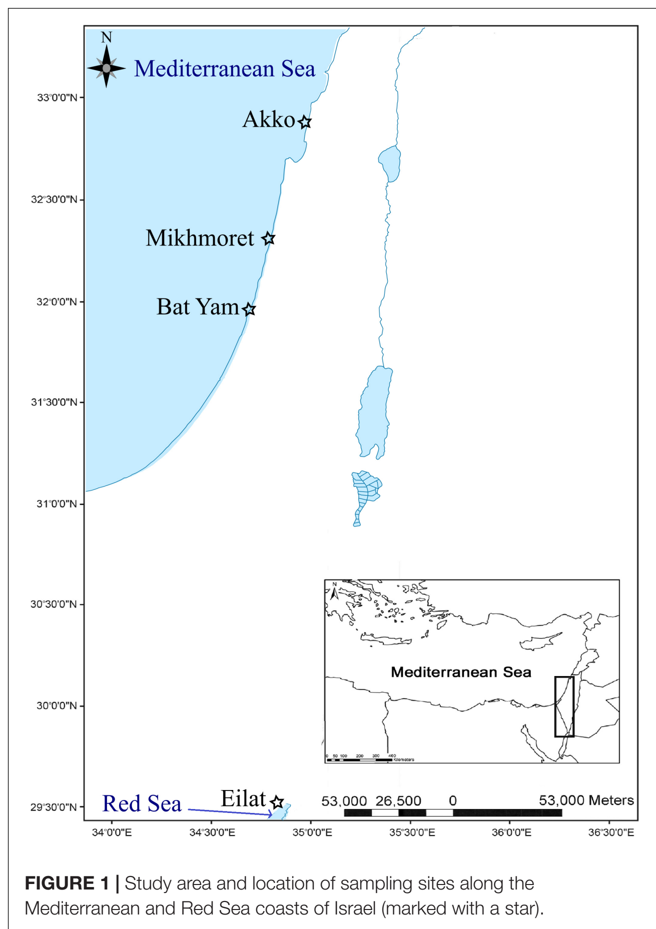

FIGURE 1 | Study area and location of sampling sites along the Mediterranean and Red Sea coasts of Israel (marked with a star).

anthropogenic activity, yet not subjected to industrial discharges as the Akko site (Israel National Monitoring Program at the Gulf of Eilat)[1].

Temperature data for the Akko site was recorded from November 2015 to April 2016, and from June to September 2016 with a data logger device (HOBO pendant® data logger, Onset, MA, United States) attached to a shipwreck in the shallow water area of the beach of "Akko Nautical School." We encountered difficulties in collecting consistent data due to the reoccurring disappearance of our devices, therefore, the rest of the data were obtained from measurements recorded on the "Sea Temperature" website[2]. Temperature data for Mikhmoret were obtained from the National Marine Monitoring Program in the Mediterranean Sea operated by Israel's Ministry of Environmental Protection and by Israel Oceanographic & Limnological Research Ltd. Temperature data for Eilat were obtained from the National Marine Monitoring Program at the Gulf of Eilat operated by Israel's Ministry of Environmental Protection[3] (**Figure 2**). Taxonomic identification of both species was carried out following Shenkar and Loya (2009).

[1]http://www.iui-eilat.ac.il/NMP

[2]https://seatemperature.info

[3]http://www.meteo-tech.co.il/EilatYam_data/ey_data.asp

Heavy Metals in Seawater Sample Collection and Preparation

Seawater samples were collected in acid cleaned bottles (Tarsons, HDPE 250 ml, 584230) by SCUBA or snorkeling from each study site. Sample bottles were pre-cleaned with 10% HNO_3 followed by concentrated double-distilled HNO_3 and a rinse with MQ water (18.2 $M\Omega\cdot cm^{-1}$). After the samples were collected, they were brought to pH < 2 in the laboratory using concentrated (15.5 N) double distilled HNO_3 (J.T.BAKER), and then kept at −20°C until further analysis. For final analysis samples were filtered through a 0.22 μm membrane (Pall AcroPak) and 125 ml of seawater were pre-concentrated using the NOBIAS PA-1 chelate resin (Hitachi High Technologies) in a method modified after Biller and Bruland (2012) and Sohrin et al. (2008). The full pre-concentration procedure is described in **Appendix 1**. Procedural blanks, detection limits and resin recovery are summarized in **Supplementary Table S1**. Procedural blanks comprise 4.2, 10.9, 0.4, 1.9, 1.5, 6.2, 6.4, 5.1, 0.3, and 1.3% of average sample signal for Al, V, Mn, Fe, Co, Ni, Cu, Cd, Ce, and Pb, respectively. Results of metal concentrations in water are reported in μg/g units in order to allow the calculation of the Bio-accumulation factor (BAF). All pre-concentration steps were performed in a trace-metal clean laboratory (class 1000 laboratory with a class 100 workbench), and all acids and reagents used were either purchased ultra-pure solutions or double distilled in the lab.

Heavy Metals in Biota Sample Collection, Preparation, and Analysis

For the seasonal monitoring of heavy metal levels in the tunic and body of the investigated species, specimens were collected by snorkeling every 2–3 months, for a period of 20 months (November 2015–June 2017). In each sampling session at the Mediterranean study sites, five individuals of *P. nigra* and five of *M. exasperatus* were sampled. In Eilat, Red Sea, only five individuals of *P. nigra* were sampled each session, as *M. exasperatus* do not occur at this location. The specimens were placed in Ziploc bags, together with water from their surroundings, and transferred to the lab within 2–3 h of collection. Menthol tablets (Merck, EMPROVE® exp Ph Eur, 105995) were added to relax the animal's muscles prior to dissection. In the laboratory, the specimens were dissected separating the tunic from the body using ceramic scissors and tweezers to avoid heavy metal contamination, preserved in 50 ml Falcon tubes (SARSTEDT, PP, 62.547.004), and kept at −20°C until further analysis.

For the purpose of determining metal accumulation in the different tissues of *M. exasperatus*, an additional 5–9 specimens of *M. exasperatus* (in accordance with their availability in the field on the date of sampling) were collected in September 2016 (Mikhmoret and Akko), February 2017 (Akko), and June 2017 (Mikhmoret and Akko). Upon arrival at the laboratory, samples were dissected and separated into the different tissues: tunic, gonads, liver, and rest of body, and each was put in

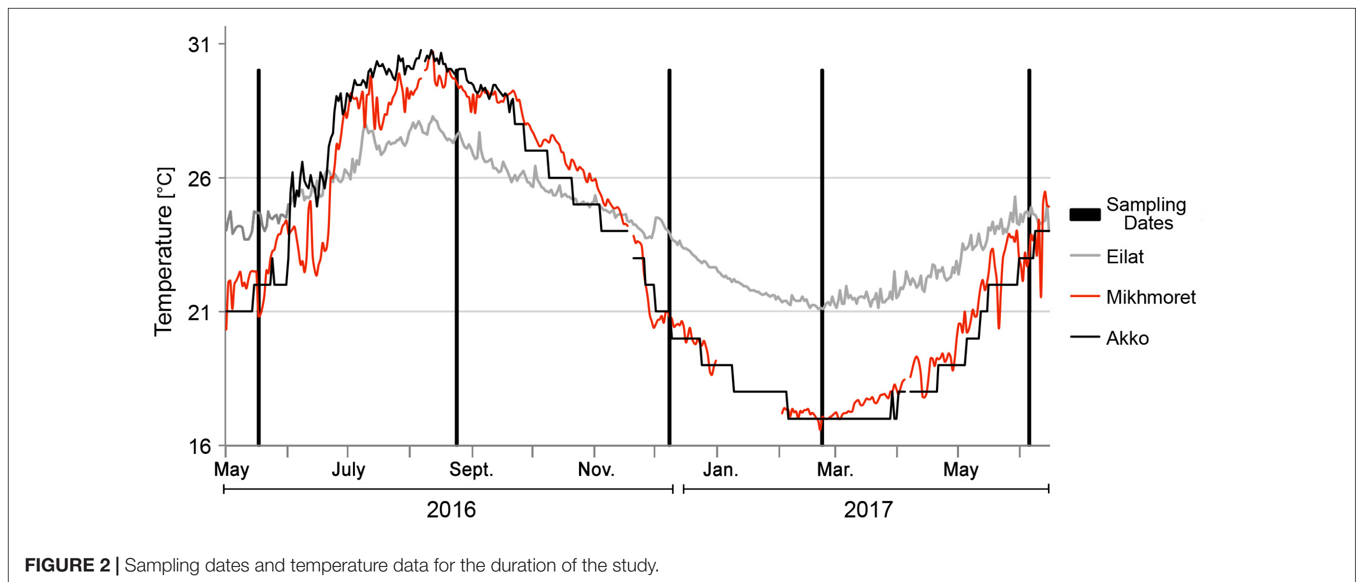

FIGURE 2 | Sampling dates and temperature data for the duration of the study.

a separate Falcon tube and stored at −20°C until further preparation. Epibionts were carefully removed from the tunic before weighing, in order to avoid error. Due to the small size of the dissected organs, two to three organs from different individual samples (but from the same sampling session) were combined into a single sample to obtain sufficient weight and element concentration for later analysis. All samples intended for trace metal analysis (−20°C) were frozen at −80°C for at least 24 h and then freeze-dried for 18–24 h (VirTis benchtop K, Bieleved, Germany) prior to the digestion process.

Samples for heavy metal analysis using ICP-MS were treated as follows: approximately 0.1 g of dry tissue sample was taken from each specimen. When a specimen weighed below 0.1 g, the entire specimen was used for digestion, and the weights were documented for further calibration. The dry-weighed tissue of each sample was added to 2 ml 30% H_2O_2 (J.T.BAKER, ACS, 2186) and 8 ml concentrated HNO_3, as follows: dry tissue sample numbers 1–117 were digested using concentrated 65–69% HNO_3 (J.T.BAKER, CMOS, 9606) double distilled; and dry tissue sample number 117-395 were digested using concentrated 67–70% HNO_3 (Trace Metal Analysis, Fisher Chemical, 7697-37-2). This protocol follows EPA method 3052 (microwave-assisted acid digestion for siliceous and organically based materials). Elemental analysis of seawater and ascidian samples for metals and metalloids was performed by inductively coupled plasma mass spectrometry (ICP-MS, Agilent 7500cx). ICP-MS analyses was conducted at the Freddy & Nadine Herrmann Institute of Earth Science at the Hebrew University, Jerusalem. ICP-MS operating conditions were set at 15, 0.92, and 0.9 L/min for plasma gas flow, carrier gas flow and auxiliary gas flow, respectively.

Bio-Accumulation Factor

The BAF represents an estimate of the potential uptake of the metal from both water and food sources (hence, from the dissolved and particulate phases). The bio-accumulation factor for each metal was calculated based on the metal concentrations determined from the collected samples (μg/g) and then divided by that metal's concentration in the seawater (μg/g) from the same sampling session:

$$\text{BAF} = \frac{\text{concentration in specimen}}{\text{concentration in seawater}}$$

Separate BAFs were calculated for the tunic and for the body of each species.

"Ha'Sela" Beach in Bat-Yam – A Case Study

Following 18 months of study of the seasonal study sites, "Ha'Sela" beach in Bat-Yam, a popular recreational beach in the central region of Israel, was chosen to test the applicability of using *P. nigra* and *M. exasperatus* as bio-indicators of metal contamination in their environment. Five specimens of *M. exasperatus* and five specimens of *P. nigra* were obtained for HMs and trace elements analysis on 18/05/2017 (spring), including a seawater sample (250 ml). Water and specimens were sampled, prepared, and analyzed as described in the previous sections.

Gonadosomatic Index and Hepatosomatic Index Measurements

Individuals of *M. exasperatus* (n = 12–13) were collected by SCUBA or snorkeling, from the Mediterranean study sites during September 2016, February 2017, and April–May 2017. The samples were collected as detailed above, relaxed using menthol tablets, and preserved in 4% formalin solution for several days prior to measurements. For each individual, total wet weight was obtained prior to dissection of the gonads and digestive gland (referred to here as "liver"). The gonadosomatic

Index (GI) and Hepatosomatic Index (HSI) were calculated as follows:

Gonadosomatic Index:

$$GI = \frac{\text{Gonad wet weight (g)}}{\text{Total wet weight (g)}}$$

Hepatosomatic Index:

$$HSI = \frac{\text{Liver wet weight (g)}}{\text{Total wet weight (g)}}$$

Statistical Analysis

Statistical analysis was performed using R software (version 3.4.1, 2017, R Core Team, 2017). For all data, the appropriate test assumptions were determined before selecting the suitable statistical test. When the data of the heavy metal concentration were not normally distributed, log transformation was performed. For the data on metal concentrations in ascidians and for body size, HIS, and GI data, a two-way ANOVA was performed. A t-test was also used to examine differences between the months in the HSI and GI analysis. One-way-ANOVA on transformed data was used to determine differences between the sites (Akko and Mikhmoret) in metal concentrations measured from *M. exasperatus* different tissues (tunic, body, gonads, and liver). One-way ANOVA was also used to determine significant differences among the sites (Bat-Yam, Akko, Mikhmoret, and Eilat) in the analysis of metals in the body and tunic of the research species, and in the analysis of HSI and GI. Data are presented as average and standard deviation unless denoted otherwise.

RESULTS

Concentrations of Heavy Metals and Trace Elements in Seawater

All the studied metals from summer 2016 and winter 2017 showed higher concentrations in Akko (**Figure 3** and **Supplementary Table S2**). In Eilat and Mikhmoret, no steady trend could be observed. In summer 2016, winter 2017, and spring 2017, most of the metals demonstrated lower concentrations in Mikhmoret, except for Mn (in summer 2016 a lower concentration was measured in Eilat); Ni (in summer 2016 the concentration was similar to Eilat and in winter and spring 2017 Ni was lower in Eilat) and Cd (in summer 2016 and winter 2017 Cd was lower in Eilat). In fall 2016, all the metals showed higher concentrations in Akko, except for V (concentrations were almost identical at all the sites), and Cd (higher in Eilat). The lowest concentrations during fall 2016 were in the samples from Eilat, except for V (lower in Akko, but very similar concentrations at all sites); Cd and Pb (lower concentrations in Mikhmoret). In spring 2017, "Ha'Sela" beach in Bat-Yam was also examined for comparison, and revealed higher concentrations of all the studied metals compared to the other sites, except for Cu (higher in Akko), and Pb (higher in Eilat). The lowest metal concentrations in this season (spring 2017) were in Mikhmoret, except for Co, Ni and Cd, which were lower in Eilat (**Figure 3** and **Supplementary Table S2**).

Temporal and Spatial Variations in Heavy Metal and Trace Element Accumulation in the Body and Tunic of the Solitary Ascidians *Phallusia nigra* and *Microcosmus exasperatus*

The concentrations of HMs (Al, V, Mn, Fe, Co, Ni, Cu, Zn, Cd, Ce, and Pb) accumulated in the body and tunic of *M. exasperatus* and *P. nigra*, collected from two study sites in the Mediterranean (Akko and Mikhmoret) and from one study site in the Red Sea (Eilat), between fall 2016 and winter 2017 are presented in **Figures 4**, **5** (full data set is provided in the **Supplementary Excel File**).

The mean metal levels in the body and tunic of *P. nigra* and *M. exasperatus* based on all the measurements conducted throughout the study are presented in **Table 1**, arranged in order of decreasing abundance. For an inclusive assessment of the effects of season and study site on variation in the accumulation of metals in the research species, p-values of a two-way ANOVA test for these variables are summarized in the **Supplementary Table S3**. A significant difference in spatial variation of all the studied metals, apart from Ni, was detected in both the body and tunic of *P. nigra* (two-way ANOVA, $p < 0.05$, **Supplementary Table S3**); while spatial variation in the body and tunic of *M. exasperatus* did not reveal significant differences for all the studied metals, with different trends in the variation being found. The effect of the seasons was significantly more prominent in the bodies of the two species than in the tunics, and was significant for most of the studied metals ($p < 0.05$). The season:site interaction was found to be significant in some of the metals in both species' tunics (Al, Fe, and Ce in *M. exasperatus* and Co, Cu, Zn, and Cd in *P. nigra*).

In many of the metals, variations existed between body and tunic. The concentrations of all the studied metals in the tunic of *M. exasperatus* were higher than those in the body at both research sites, except for Zn, which demonstrated an opposite trend (**Figure 4**).

In *P. nigra*, in contrast, the concentrations of all the metals in the body were higher than in the tunic, with the exception again of Zn, in which concentrations in the tunic were higher. Cadmium results were inconclusive: some of the concentrations were higher in the tunic than in the body but varied inconsistently among sites and seasons (**Figure 5**). Seasonal changes in metal accumulation in the tissues of *M. exasperatus* followed temperature changes in some cases: in the body and tunic of *M. exasperatus* (**Figures 2**, **4**), most of the highest concentrations of metals were recorded in the cold seasons – fall 2016 and winter 2017, and most of the lowest concentrations in spring 2016/2017.

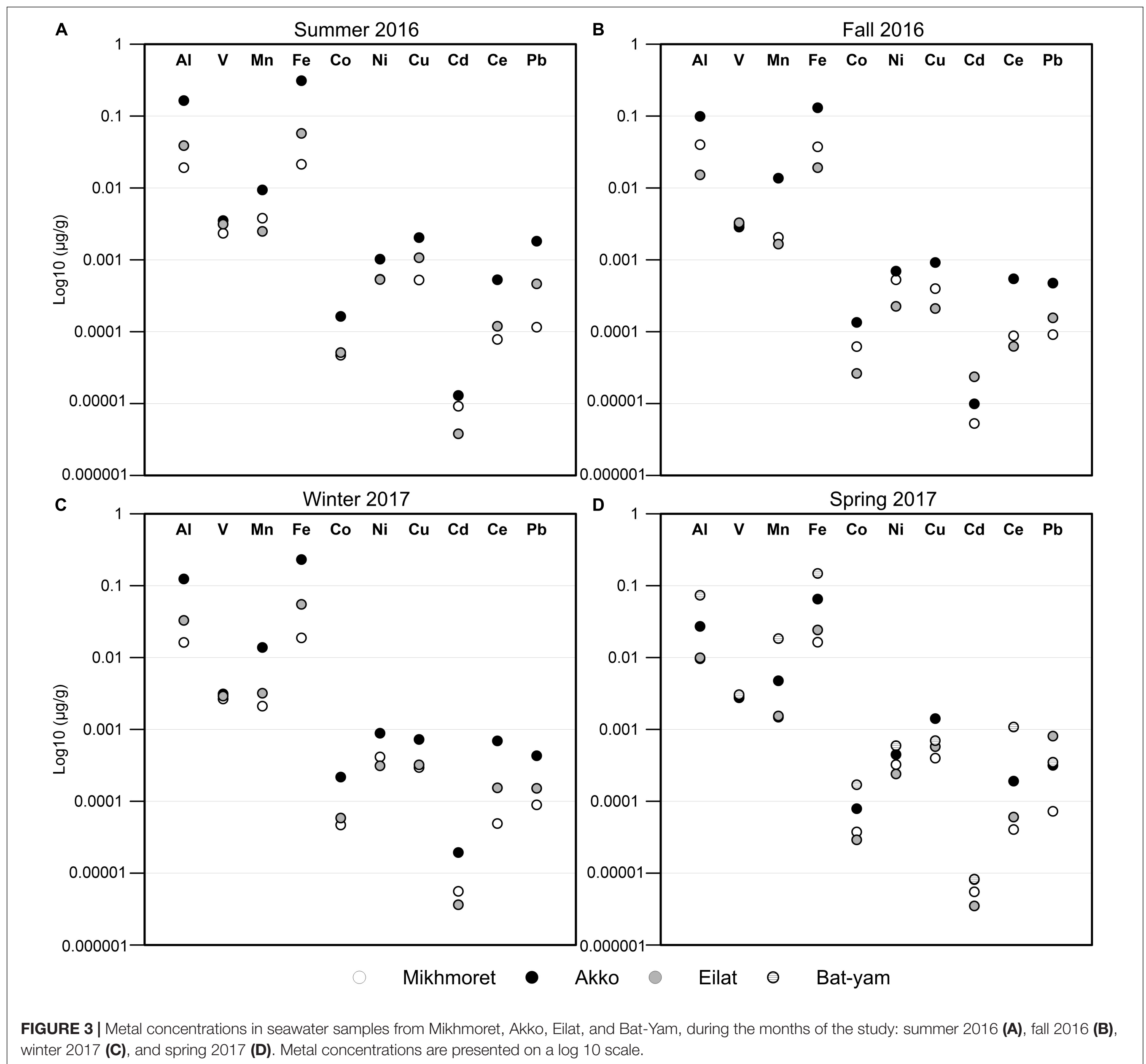

FIGURE 3 | Metal concentrations in seawater samples from Mikhmoret, Akko, Eilat, and Bat-Yam, during the months of the study: summer 2016 **(A)**, fall 2016 **(B)**, winter 2017 **(C)**, and spring 2017 **(D)**. Metal concentrations are presented on a log 10 scale.

Seasonal changes in metal concentrations in the body and tunic of *P. nigra* demonstrated less of a pattern than in *M. exasperatus*. *P. nigra* was sampled from Eilat, Red Sea, in addition to Akko and Mikhmoret. Temperatures in Eilat are characterized by a smaller range of change (21–28°C, **Figure 2**). Some of the metals measured in *P. nigra* tissues did not seem to follow a seasonal trend (V, Cd, and Zn), while others did, albeit demonstrating different patterns of change for body and for tunic. The highest concentrations of metals in the body of *P. nigra* were measured in fall 2016 (similar to *M. exasperatus*), for Al, Mn, Fe, Co, and Ce, for which their lowest concentrations in the tunic were generally in fall and/or winter. For Al, Fe, Co, and Ni, the lowest concentrations were in spring (2016/2017) and summer; and for Al, Mn, Fe, Co, Ni, Cu, Ce, and Pb the highest concentrations were in spring (2016/2017) and summer.

Heavy Metal and Trace Element Accumulation in the Different Tissues of *Microcosmus exasperatus*

The distribution of the metals in the tunic, body, gonads, and liver of *M. exasperatus* presented a steady trend (**Figure 6** and **Supplementary Table S4**). For all of them except Cu, Zn, and Cd, the tunic accumulated higher concentrations than the other tissues (60–78%); for all of them except Zn the gonads accumulated the lowest concentrations (4–14%); the liver accumulated higher concentrations of Cu and Cd compared

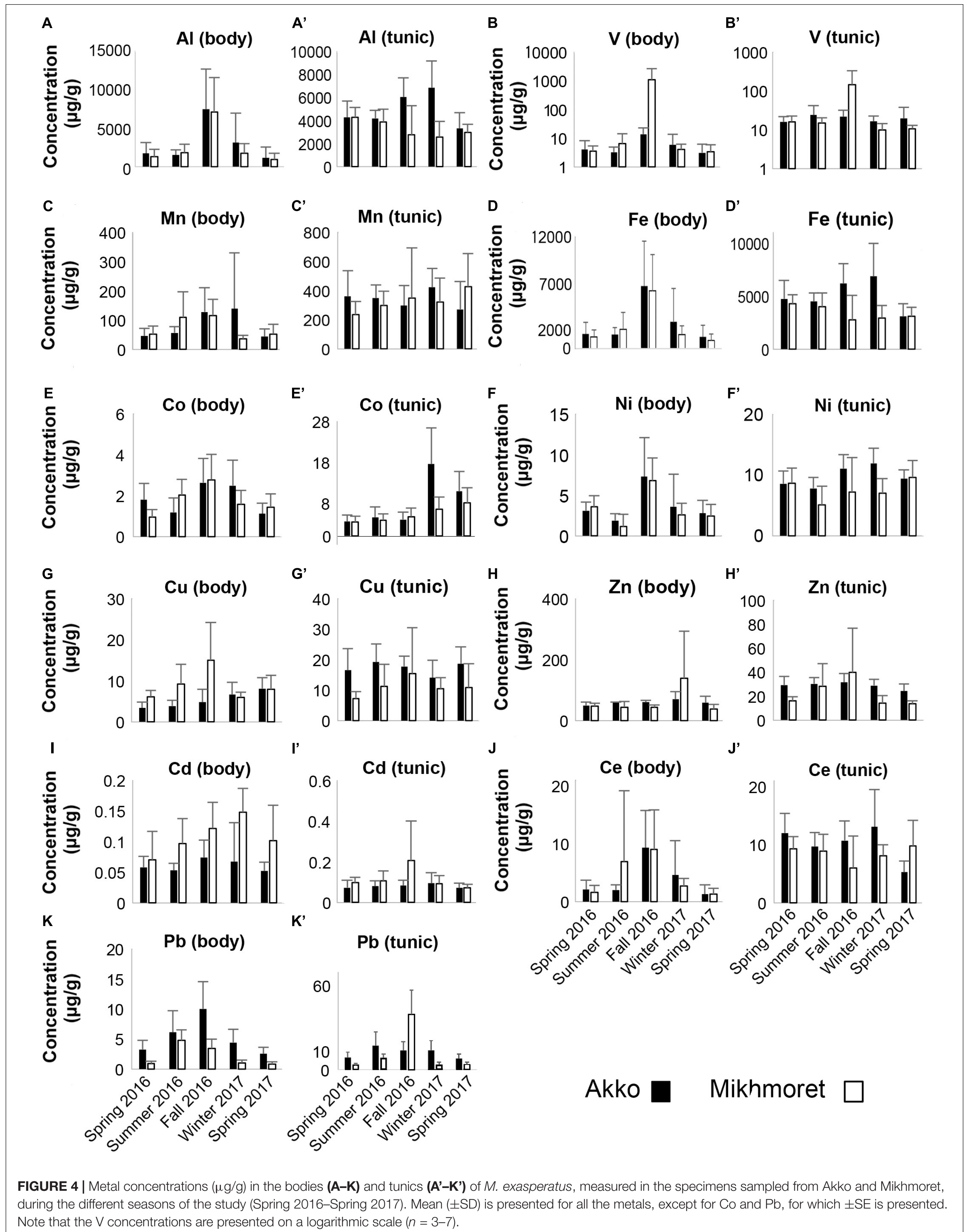

FIGURE 4 | Metal concentrations (µg/g) in the bodies **(A–K)** and tunics **(A'–K')** of *M. exasperatus*, measured in the specimens sampled from Akko and Mikhmoret, during the different seasons of the study (Spring 2016–Spring 2017). Mean (±SD) is presented for all the metals, except for Co and Pb, for which ±SE is presented. Note that the V concentrations are presented on a logarithmic scale (*n* = 3–7).

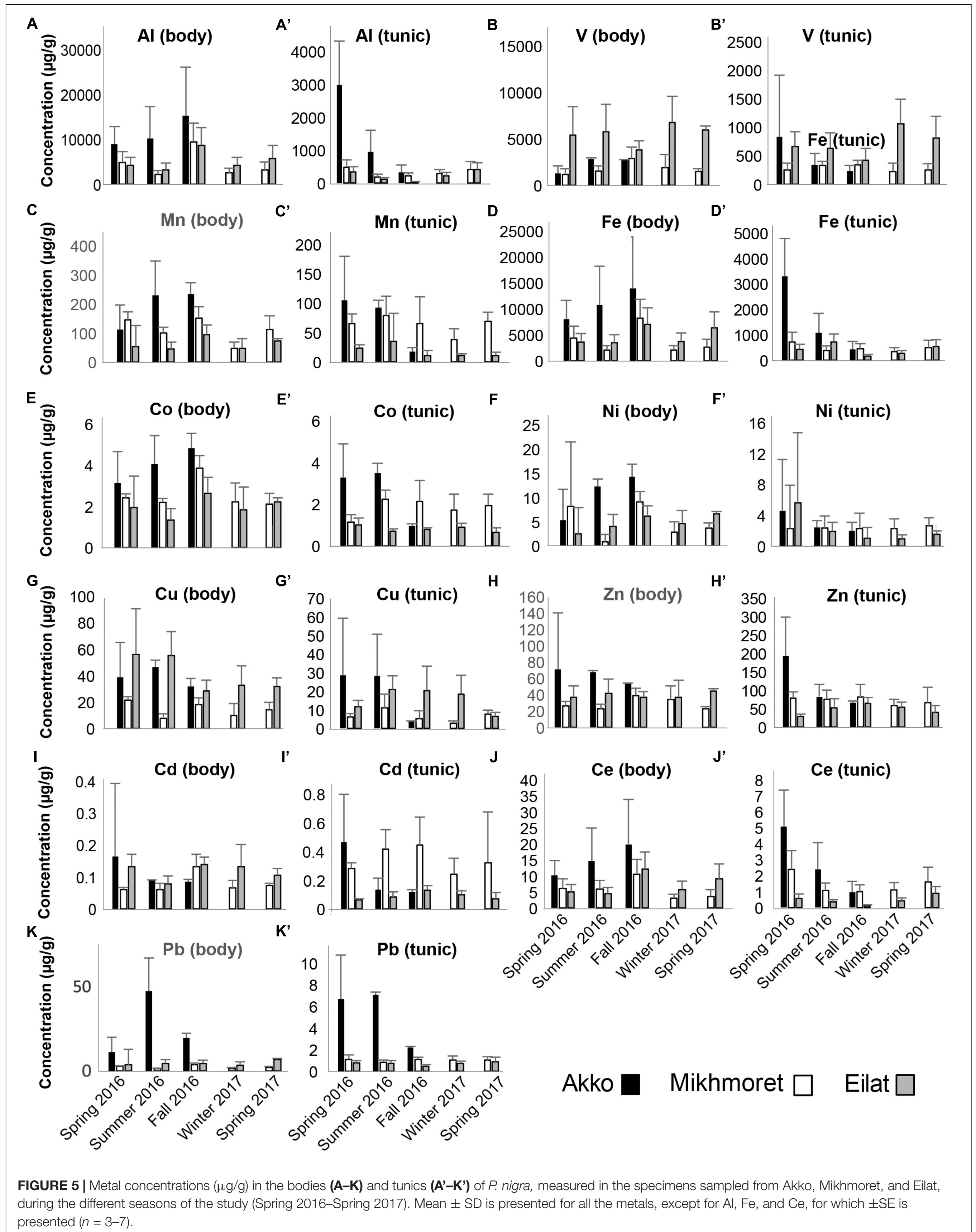

FIGURE 5 | Metal concentrations (µg/g) in the bodies **(A–K)** and tunics **(A'–K')** of *P. nigra,* measured in the specimens sampled from Akko, Mikhmoret, and Eilat, during the different seasons of the study (Spring 2016–Spring 2017). Mean ± SD is presented for all the metals, except for Al, Fe, and Ce, for which ±SE is presented (*n* = 3–7).

TABLE 1 | The mean metal levels (μg/g), and range in the body and tunic of *M. exasperatus* (ME body and ME tunic, *n* = 50) and of *P. nigra* (PN body, PN tunic, *n* = 58) measured throughout the study arranged in order of decreasing abundance (from left to right).

ME body	**Al**	**Fe**	**V**	**Mn**	**Zn**	**Cu**	**Ce**	**Pb**	**Ni**	**Co**	**Cd**
	2763 58–15300	2564 70–13762	112 0–3443	79 15–426	60 16–338	10 2–29	4 0–35	4 0–19	3 0–14	2 0–6	0.08 0–0.2
ME tunic	**Fe**	**Al**	**Mn**	**V**	**Zn**	**Cu**	**Pb**	**Ce**	**Ni**	**Co**	**Cd**
	4235 187–11768	4108 35–10021	330 12–836	29 4–362	25 9–103	14 5–42	11 1–182	9 0–24	9 0–15	7 1–54	0.1 0–0.55
PN body	**Al**	**Fe**	**V**	**Mn**	**Zn**	**Cu**	**Ce**	**Pb**	**Ni**	**Co**	**Cd**
	5697 755–22360	5217 680–19161	3426 352–11150	97 16–313	39 9–190	29 0–100	8 1–24	6 1–61	5 0–28	3 1–6	0.1 0–0.57
PN tunic	**Fe**	**Al**	**V**	**Zn**	**Mn**	**Cu**	**Pb**	**Ni**	**Co**	**Ce**	**Cd**
	716 66–7691	525 0–7651	517 65–2746	72 23–306	47 4–199	13 1–79	5 0–175	2 0–22	2 0–5	1 0–10	0.2 0–0.91

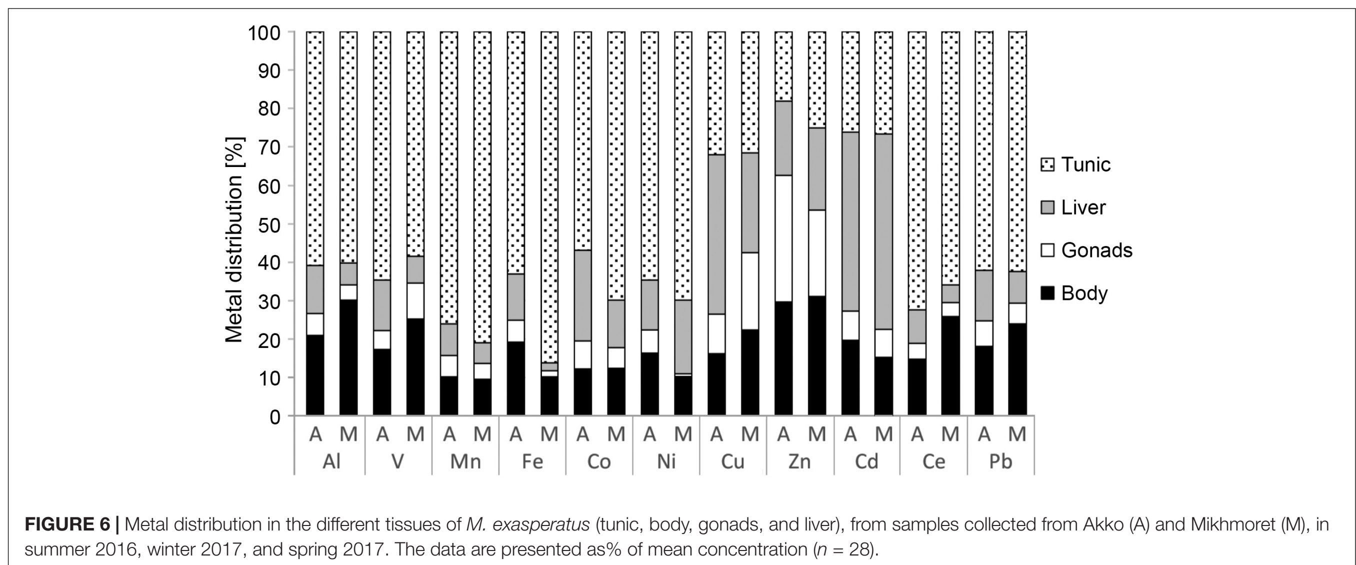

FIGURE 6 | Metal distribution in the different tissues of *M. exasperatus* (tunic, body, gonads, and liver), from samples collected from Akko (A) and Mikhmoret (M), in summer 2016, winter 2017, and spring 2017. The data are presented as% of mean concentration (*n* = 28).

to the other tissues; and the body and gonads accumulated higher concentrations of Zn than the other tissues (30%). There were no significant differences in metal distribution between specimens collected in Akko and in Mikhmoret (one-way ANOVA, $p > 0.05$).

Bio-Accumulation Factor for Heavy Metals in the Tunic and Body of *Phallusia nigra* and *Microcosmus exasperatus*

Accumulation of the studied metals in the tunic of the research species differed among the metals: the BAF of Al, Mn, Fe, Co, Ni, and Ce was significantly higher in *M. exasperatus* tunic than in *P. nigra* tunic (t-test, $p < 0.05$), while the BAF for V, and Cd was significantly higher in the tunic of *P. nigra* than in that of *M. exasperatus*. BAF of the two species' bodies for the studied metals, was significantly higher in *P. nigra* (t-test, $p < 0.05$) except for Cd and Pb in which no significant differences were found.

The BAF values for the tunic and body of both species are presented in **Figures 7A,B** and **Table 2**, arranged in order of decreasing abundance.

Hepato-Somatic Index and Gonadosomatic Index of *Microcosmus exasperatus*

Hepato-Somatic Index (HSI) values revealed a significant difference between Akko and Mikhmoret ($p < 0.05$): mean HSI value in Mikhmoret was significantly higher than in Akko.

A two-way ANOVA analysis also revealed a significant difference in HSI values between average June and September measurements ($p < 0.05$), and a significant site:season interaction ($p < 0.01$). The highest mean HSI measurement was from the June sampling from Mikhmoret (1.94, ± 0.43, $n = 12$, **Figure 8A**), and the September sampling from Akko (1.26, ± 0.43, $n = 12$), although T-test analysis revealed a significant difference

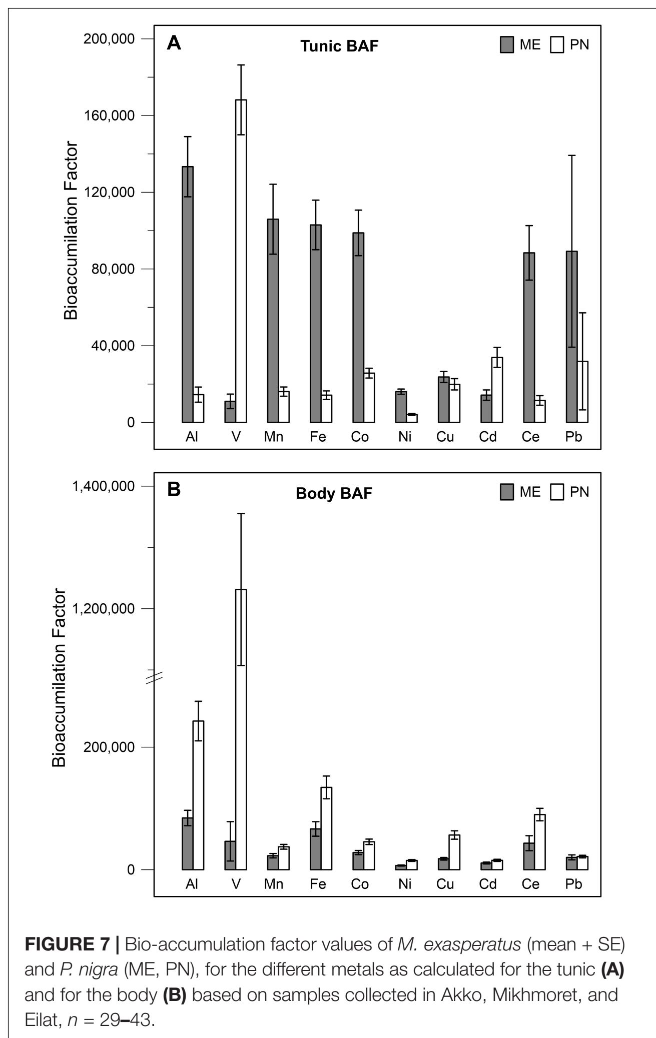

FIGURE 7 | Bio-accumulation factor values of *M. exasperatus* (mean + SE) and *P. nigra* (ME, PN), for the different metals as calculated for the tunic **(A)** and for the body **(B)** based on samples collected in Akko, Mikhmoret, and Eilat, n = 29–43.

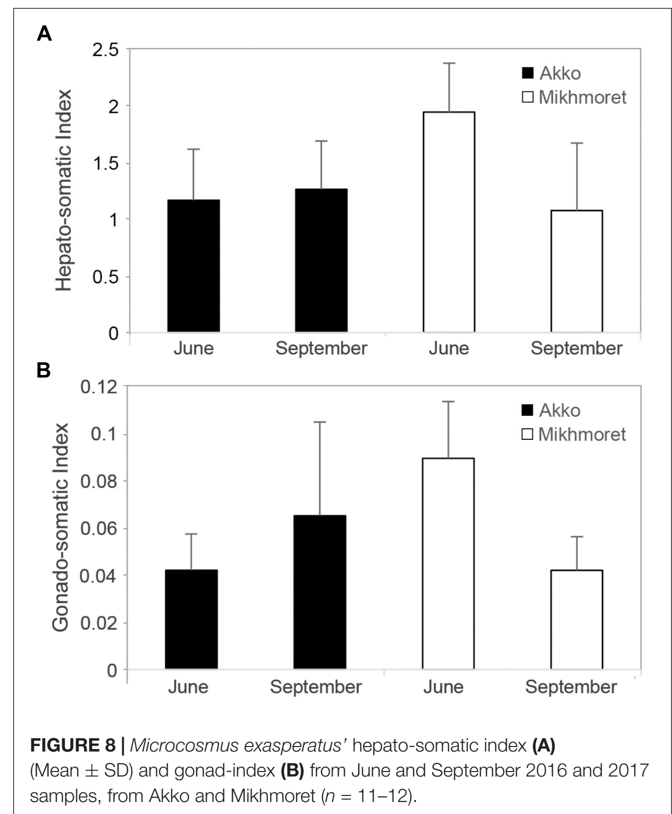

FIGURE 8 | *Microcosmus exasperatus'* hepato-somatic index **(A)** (Mean ± SD) and gonad-index **(B)** from June and September 2016 and 2017 samples, from Akko and Mikhmoret (n = 11–12).

TABLE 2 | The mean BAF of *M. exasperatus* (ME) and *P. nigra* (PN) body and tunic, arranged in decreasing order, based on samples collected in Akko, Mikhmoret, and Eilat.

ME tunic	Al > Mn > Fe > Co > Pb > Ce > Cu > Ni > Cd > V
PN tunic	V > Al > Fe > Ce > Cu > Co > Mn > Pb > Cd > Ni
ME body	Al > Fe > V > Ce > Co > Mn > Pb > Cu > Cd > Ni
PN body	V > Cd > Pb > Co > Cu > Mn > Al > Fe > Ce > Ni

between mean HSI values of June and September only from Mikhmoret ($p < 0.01$).

A two-way ANOVA test for GI revealed similar trends to those found for the HSI analysis (**Figure 8B**). Although there was no significant difference between site and month in GI values, the highest mean GI was measured in the samples collected from Mikhmoret in June (0.09, ± 0.02); and the lowest mean GI in the samples from Akko in June (0.04, ± 0.02). There was a strong and significant site:season interaction (two-way ANOVA, $p < 0.0001$), emphasizing the importance of both geographic and seasonal effect in *M. exasperatus*. For Mikhmoret a t-test analysis revealed a significant difference between Mean GI values in June (n = 12) and September (n = 11, t-test, $p < 0.01$); but no significant difference for the same period in Akko.

"Ha'Sela" Beach in Bat-Yam – A Case Study

Metal concentrations in seawater samples during spring 2017 (**Figure 3**) were higher in Bat-Yam in comparison to the other sites for all metals except Cu, Zn, and Cd. Concentrations of HMs accumulated in *M. exasperatus* and *P. nigra* samples from spring 2017, collected from Bat-Yam and compared to Akko, Mikhmoret, and Eilat, are presented in **Figures 9**, **10**. Similar to the findings presented in the previous section, metal concentrations in the tunic of *M. exasperatus* from Bat-Yam revealed concentrations 1.5–27-fold higher than in the body, for all metals except Cu, which was found in almost equal concentrations in tunic and body; and Zn, which was 4-fold more concentrated in the body than in the tunic of *M. exasperatus*. A similar contrast was found in *P. nigra* samples from spring 2017: metal concentrations were 1.6–18-fold higher in the body than in the tunic, except for Cd and Zn, which were 3.8 and 1.5-fold higher, respectively, in the tunic than in the body.

In *M. exasperatus*, significant differences among the study sites were found in concentrations of V, Co, and Cu in the body and of Co, Cu, Zn, and Ce in the tunic (one-way ANOVA, $p < 0.05$, **Figure 9**). In *P. nigra* significant differences were found in concentrations of V, Cu, and Zn in the body samples, and concentrations of V, Mn, Co, Cd, and Pb in the tunic samples (**Figure 10**, One-Way ANOVA, $p < 0.05$).

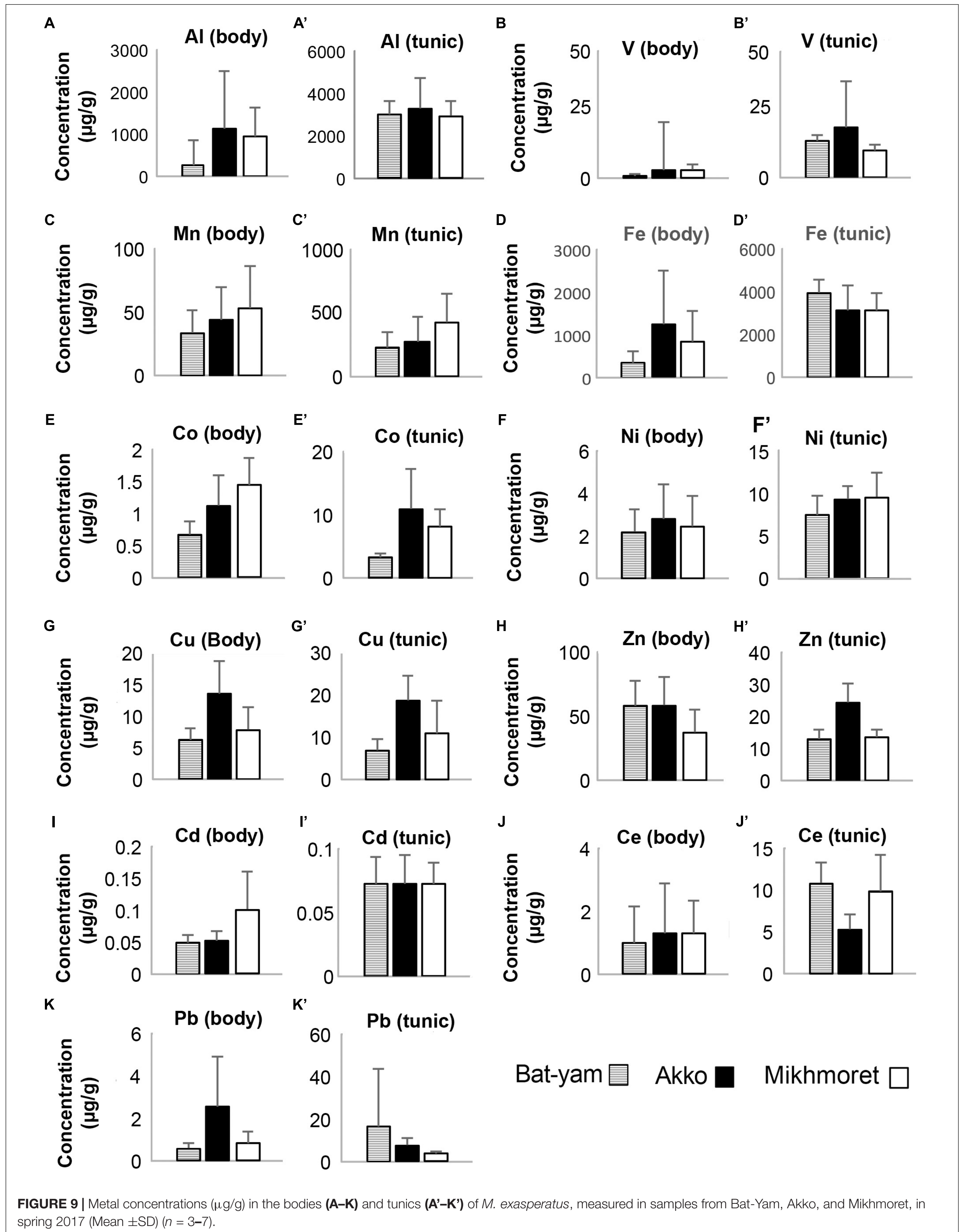

FIGURE 9 | Metal concentrations (μg/g) in the bodies **(A–K)** and tunics **(A'–K')** of *M. exasperatus*, measured in samples from Bat-Yam, Akko, and Mikhmoret, in spring 2017 (Mean ±SD) (*n* = 3–7).

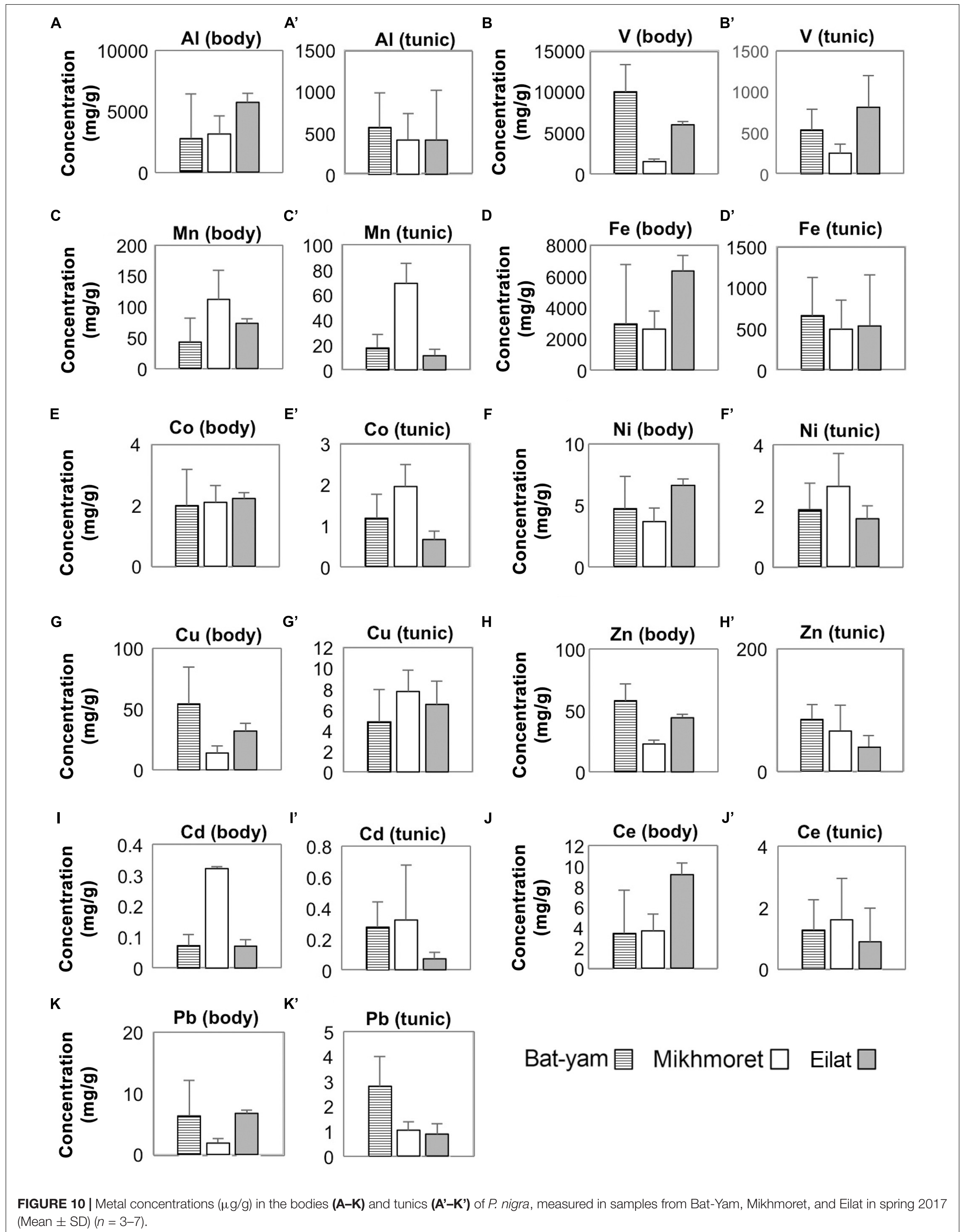

FIGURE 10 | Metal concentrations (μg/g) in the bodies **(A–K)** and tunics **(A'–K')** of *P. nigra*, measured in samples from Bat-Yam, Mikhmoret, and Eilat in spring 2017 (Mean ± SD) (*n* = 3–7).

Mean metal bio-accumulation factors (BAF) of the studied metals, for the samples collected in spring 2017 from Bat-Yam, were found to be lower than the calculated BAF from the other sites, for the majority of metals (**Supplementary Table S5**). GI values in Bat-Yam were significantly lower (0.031 ± 0.009, $n = 12$, one-way ANOVA $p < 0.05$), in comparison to Mikhmoret (0.089 ± 0.023), while HSI values did not significantly differ between Akko (1.17 ± 0.44, $n = 12$) and Bat-Yam (1.22 ± 0.23, $n = 12$, one-way ANOVA $p > 0.05$).

DISCUSSION

Both *M. exasperatus* and *P. nigra* have demonstrated an ability to accumulate large quantities of the studied metals in their tissues (between ~5,000 and 2.5×10^6 times higher compared to ambient seawater concentrations). The concentrations of the metals varied between the research species and among the different tissues examined. The findings from the study at a popular public beach (Ha'Sela Beach, Bat-Yam) further illustrate the feasibility of using *M. exasperatus* and *P. nigra* as biological indicators of metal contamination in a variety of habitats.

Temporal and Spatial Variations in Heavy Metal and Trace Element Accumulation in the Body and Tunic of *Phallusia nigra* and *Microcosmus exasperatus*

By conducting separate analyses for the tunic and the body of our two ascidian study species we were able to detect the differences in the mechanism of metal accumulation between the two. The metal content in the tunic of *M. exasperatus* was higher than in its body, while the opposite was the case for *P. nigra*. A study by Abdul Jaffar Ali et al. (2015) demonstrated a similar trend of accumulation in *P. nigra*. Since all the vital life functions such as absorption, circulation, storage, breeding, etc., take place in the body, the accumulation of metals is expected to be higher in the body than in the tunic (Abdul Jaffar Ali et al., 2015). A similar phenomenon of variation in accumulation pattern in organisms belonging to the same taxon was also documented in the past (Rainbow, 1998). *P. nigra* is characterized by a very smooth tunic, while *M. exasperatus* is densely covered with epibionts (Gewing et al., 2016). Such differences may influence the primary site of metal accumulation and should be taken into consideration according to the particular ascidian tissue used for metal analysis. Radhalakshmi et al. (2014) studied the accumulation of V, Cu, Pb, and Cd in the whole tissue of five species of ascidians, including *M. exasperatus*, and found that Cu accumulated at the highest concentrations in *M. exasperatus* (body and tunic), followed by V, Pb, and Cd in that order, as also reflected in the BAF values. Their results are similar to those of the current research, in which these four metals accumulated in *M. exasperatus* in the following order of concentration V > Cu > Pb > Cd.

In the present study, metal accumulation in the body and tunic of *M. exasperatus* and *P. nigra* was measured at different sites and seasons, revealing significant effects of both parameters. Significant spatial differences in the tunic and body of *P. nigra* were detected for all the studied metals, except for Ni, which showed no seasonal or spatial differences. Chiffoleau et al. (2004) examined the differences in Ni levels over a period of 1 year in mussels (*Mytilus edulis*) and oysters (*Crassostrea gigas*) and did not detect any significant variations in this metal's levels, whereas a significant difference in the levels of V was detected. Ni is a common element and known for its toxicity, persistence, and affinity for bio-accumulation (Eisler, 1998). However, it is considered to be essential for various biological processes, mostly at very low concentrations (Muyssen et al., 2004). It has been shown to accumulate in the tissues of crayfish, primarily in the exoskeleton, which might indicate that this tissue is involved in the excretion of this metal (Kouba et al., 2010). In the current study, Ni was found to preferentially accumulate (64%) in the tunic of *M. exasperatus*, suggesting that the tunic may participate in the excretion of this metal in this species.

Seasonal differences in metal levels were significant only for some of the metals: in the tunic of *P. nigra* – Al, Mn, Fe, Cu, and Ce; and in the body of *P. nigra* – all the metals except Ni, Cu, and Zn. The majority of the metals in the tunic and body of *M. exasperatus* varied significantly in quantity between seasons: in the tunic Al, V, Co, and Zn; in the body? all the metals except Zn and Cd. In a recent study conducted by Aydın-Önen (2016) on heavy metal accumulation by the solitary ascidian *Styela plicata*, only Cu showed seasonal changes. However, in the present study seasonal differences in heavy metal accumulation were evident for many of the metals. In the body and tunic of *M. exasperates* and in the body of *P. nigra*, the highest concentrations of metals were measured in the fall and winter, when water temperatures were relatively low. As established in previous studies, food supply and environmental temperature vary over an annual cycle and, in response to these variations, metal uptake and accumulation by marine organisms is affected (Stacey and Driedzic, 2010; Wang, 2016). Temperature affects the speciation of metals in the water, thus affecting their bio-availability (Tan et al., 2013). Additionally, food availability and temperature influence the organism's water pumping rate and metabolic rate as well as growth rate, all of which affect the metal accumulation process (Stacey and Driedzic, 2010; Wang, 2016).

In the above-noted study by Aydın-Önen (2016), an analysis of Cd, Cu, Pb, V, and Zn was carried out on the tissues of *S. plicata*, an ascidian belonging to the same order as *M. exasperatus* (Stolidobranchia). The accumulated concentrations of these metals were very similar to those of the current study, and of the same order of magnitude.

The bio-accumulation factor (BAF) calculated here should be cautiously interpreted, being based on a single measurement of seawater at each site. It nonetheless provides an estimation of the ability of a particular organism to accumulate metals from its surroundings. BAF values for the body of *P. nigra* were greater than those of *M. exasperatus* for all the metals. The BAF values for the tunics of both species varied among the metals, and were not consistently higher in one species. In general, for the BAF values of the tunic and body together, *P. nigra* showed higher values for most of the metals, revealing its promising potential as a pollutant indicator. However, because *P. nigra* lacks a digestive gland (a major point of interest for the present study), ideally,

a combination of *P. nigra* and *M. exasperatus* would enable a broader assessment of metal contamination in the environment.

Heavy Metal and Trace Element Accumulation in the Different Tissues of *Microcosmus exasperatus*

In the present study accumulation of metals was in the following order: Zn > Cu > V > Pb > Cd, for the liver and the body (which was separated from the gonads and liver and contained the branchial basket). Bellante et al. (2016) examined metal accumulation in the liver and branchial sac of *S. plicata* and found their accumulation to be in the following order: Cu > Zn > V > Pb > Cd for the branchial sac and V > Cu > Pb > Zn > Cd for the liver (only metals relevant for the current study are shown here). Similar findings for body tissue to those provided by Bellante et al. (2016) were also found here. However, the accumulation of metals in the liver differ here: Zn > Cu > V > Pb > Cd. Bellante et al. (2016) also found the accumulation of metals in the digestive gland of the mussel *Mytilus galloprovincialis* to be: Zn > Cu > V > Pb > Cd, similar to that found in the current study for the liver and body of *M. exasperatus*.

There were no significant differences in the distribution of metals in the different tissues between Akko and Mikhmoret. This suggests that the distribution of the studied metals in the tissues of *M. exasperatus* does not depend on the site or the level of contamination at the site, but on the identity of the metal and its regulation and metabolism in the examined tissues in this species.

In the present study, a high accumulation of Cu in the liver of *M. exasperatus* was also apparent. Cu is an essential element for normal growth and metabolism of all organisms, and was found to be a key element in the respiratory protein hemocyanin, present in certain mollusks and arthropods. In crustaceans, for example, relatively high concentrations of Cu have been found in the liver (Rainbow, 2002; Kouba et al., 2010). Cd is considered a non-essential element, and thus has no required minimum concentration in the body of living organisms, and needs to be swiftly detoxified or excreted (Rainbow, 2002). Because the liver has a major role in the detoxification of metals, it is sometimes the major tissue in which non-essential HMs accumulate (Muyssen et al., 2004; Kouba et al., 2010; El-Moselhy et al., 2014). The highest levels of Cd accumulation in the present study were measured in *P. nigra* tunic, suggesting that this tissue also plays a role in the excretion of this metal.

Canli and Atl (2003) examined the distribution of Fe, Cu, Pb, Cd, and Zn in certain tissues of six species of fish. The mean results showed that for most of the species the accumulation in the liver was in the following order: Fe > Zn > Cu > Pb > Cd; and the accumulation in the gills: Fe > Zn > Pb ≈ Cu > Cd. Their results for fish liver are identical to those found in the liver of *M. exasperatus* in the present study. Their order of accumulation in the gills (which, similar to the branchial sac of ascidians, function as the organ of oxygenation and encounter large amounts of seawater), is also very similar to that found here for *M. exasperatus*. The differential distribution of each metal in the different tissues of *M. exasperatus* may be due to the different biological requirements of the tissues and to their possessing different metal-binding proteins. Metal concentrations found in the tunic may also have originated in part from the epifauna on the tunic, despite our attempt to thoroughly remove them. The high metal accumulation capacity of the tunic of *M. exasperatus* should be further examined in order to determine the role of these metals in this tissue, and the reason for their high presence there. It is crucial therefore to consider the differential organ accumulation capabilities when choosing a particular tissue as a biological marker of metal accumulation, or use total individual HMs analysis (body and tunic) as a marker.

"Ha'Sela" Beach in Bat-Yam – A Case Study

"Ha'Sela" beach (Bat-Yam) is a popular recreational beach, situated in the center of Israel. It is highly visited throughout the year, and is subjected to many potential anthropogenic effects. Although the marine environment of Akko is considered as one of the most polluted in the country, the seawater collected at "Ha'Sela" beach revealed the highest concentrations of metals of all four sites sampled in spring. There are sporadic reports of sewage leakage and ground water pollution from this area, which can reach "Ha'Sela" beach with the currents, and demands future monitoring of the high levels reported here. *M. exasperatus* GI values were significantly lower in Bat-Yam compared to the other sites during this period, demonstrating an inverse relationship with the metal concentrations in the water samples. This may indicate seasonal changes in the reproductive cycles of different populations of *M. exasperatus*; or it may be a consequence of the relatively high metal pollution in the area. Studies have shown that some metals can disrupt various enzymatic mechanisms and DNA-related processes (Kouba et al., 2010), influence reproductive processes (Gallo et al., 2011; Gallo and Tosti, 2013), and cause other sub-lethal and lethal effects. For example, cadmium is known to increase atresia (the breakdown of oocytes) in the fish *Oryzias javanicus*, which can accumulate high Cd levels in its liver, resulting in a reduction in GI (Pereira et al., 1993).

Sindhe and Kulkarni (2004) conducted toxicity tests for several metals on *Notopterus notopterus,* and showed that upon exposure to HMs at sub-lethal concentrations, both GI and HSI were reduced. This could be the reason for the low GI and HSI values found for Bat-Yam, compared to the other sites.

Mean bio-accumulation factors (BAF) for the body and tunic of the samples collected for Ha'Sela Beach (Bat-Yam) were lower than the average values for the other study sites for the same season. DeForest et al. (2007) examined the relationship between bio-accumulation factors and exposure concentrations. They observed a statistically significant inverse relationship between these factors in seven out of the eight metals examined (the relevant metals for the current study: Cd, Cu, and Pb). Thus, a relatively high BAF value observed for a metal may also derive from the exposure concentration and from other metal and species-specific factors that influence the bioaccumulation of the metal. This could explain the lower BAF values in Bat-Yam, a site that was shown here to be the most metal-polluted of all four study sites. The Bat-Yam case study demonstrates the potential

of *P. nigra* and *M. exasperatus* to function as bio-indicators on a wide geographic scale.

The present study is the first to examine concomitantly the potential use of two highly common invasive species as bio-indicators in two different bodies of water: the tropical Red Sea and the Mediterranean Sea. The findings have revealed the high potential of both species to serve as bio-indicators of metal accumulation in the marine environment. Both *M. exasperatus* and *P. nigra* accumulated high levels of HMs, up to 2.5×10^6-fold higher than the ambient seawater concentrations. The metal concentrations in the two species varied seasonally and spatially, and their distribution within the tissues of each species also differed. While in *M. exasperatus* the tunic served as the main site of bio-accumulation, in *P. nigra* the majority of metals accumulated in the body. Such physiological differences should be taken into consideration in future studies using ascidians as bio-indicators. A significant association between low HSI and GI indexes and heavy-metal levels was found in *M. exasperatus*, indicating a physiological response. The beach case study chosen for this research further illustrates the feasibility of using *M. exasperatus* and *P. nigra* as biological indicators of metal contamination on a large geographic scale, providing valuable information on the ecosystem's health, and on the presence and bioavailability of pollutants.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the manuscript/**Supplementary Files**.

AUTHOR CONTRIBUTIONS

RT-M, AT, TB, and NS designed the study and analysis and wrote the manuscript. RT-M and NS research team have conducted the field work.

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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.2019.00611/full#supplementary-material

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APPENDIX 1

HMs in Seawater Pre-concentration Procedure

A total of 125 ml of filtered (0.22 μm) seawater were bought up to pH 6.1 ± 0.2 with 3.7M Ammonium Acetate (NH_4Ac), made up from trace metal grade Ammonium Hydroxide and Acetic Acid (Sigma Aldrich). Before loading the sample, resin columns (Biorad) were conditioned with a sequence of 1N HNO_3, MQ water (18.2 $M\Omega \cdot cm^{-1}$) and 0.05M NH_4Ac (pH of 6.1 ± 0.2). After loading the sample, columns were rinsed with 0.05M NH_4Ac to remove major salts and finally eluted with 1N HNO_3. Concentrations were determined by a calibration curve of gravimetric standard addition to trace metal clean seawater (purified three times in NOBIAS PA-1 chelate resin). Concentration factors were calculated gravimetrically by weighing seawater and eluent tubes before and after pre-concentration. To evaluate procedural blanks, concentrated HNO_3 and 3.7M NH_4Ac (same amount as added to the samples) were added to 3 ml of trace metal clean seawater and were pre-concentrated as samples. Resin recovery was assessed by comparing the slopes of the seawater standards (extracted on the resin) to the slopes of standard addition to 1N HNO_3, which represent a 100% recovery.

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Multiple Stressors in the Environment: The Effects of Exposure to an Antidepressant (Venlafaxine) and Increased Temperature on Zebrafish Metabolism

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Aquatic organisms are continuously exposed to multiple environmental stressors working cumulatively to alter ecosystems. Wastewater-dominated environments are often riddled by a myriad of stressors, such as chemical and thermal stressors. The objective of this study was to examine the effects of an environmentally relevant concentration of a commonly prescribed antidepressant, venlafaxine (VFX) [1.0 μg/L], in addition to a 5°C increase in water temperature on zebrafish metabolism. Fish were chronically exposed (21 days) to one of four conditions: (i) 0 μg/L VFX at 27°C; (ii) 1.0 μg/L VFX at 27°C; (iii) 0 μg/L VFX at 32°C; (iv) 1.0 μg/L VFX at 32°C. Following exposure, whole-body metabolism was assessed by routine metabolic rate (RMR) measurements, whereas tissue-specific metabolism was assessed by measuring the activities of major metabolic enzymes in addition to glucose levels in muscle. RMR was significantly higher in the multi-stressed group relative to *Control*. The combination of both stressors resulted in elevated pyruvate kinase activity and glucose levels, while lipid metabolism was depressed, as measured by 3-hydroxyacyl CoA dehydrogenase activity. Citrate synthase activity increased with the onset of temperature, but only in the group treatment without VFX. Catalase activity was also elevated with the onset of the temperature stressor, however, that was not the case for the multi-stressed group, potentially indicating a deleterious effect of VFX on the anti-oxidant defense mechanism. The results of this study highlight the importance of multiple-stressor research, as it able to further bridge the gap between field and laboratory studies, as well as have the potential of yielding surprising results that may have not been predicted using a conventional single-stressor approach.

Keywords: venlafaxine, temperature, metabolism, zebrafish, wastewater, multiple stressors

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INTRODUCTION

Pharmaceuticals and personal care products (PPCPs) are frequently introduced and detected in aquatic environments (Daughton and Ternes, 1999; Metcalfe et al., 2010; Arlos et al., 2014, 2015). PPCPs enter aquatic environments from a variety of sources, such as treated municipal and hospital wastewater, as well as agricultural runoff (Daughton and Ternes, 1999; Bottoni et al., 2010).

The concentrations of most pharmaceuticals in the surface water of aquatic environments are generally in the ng/L to low μg/L range (Fent et al., 2006; Kümmerer, 2010). However, despite the relatively low concentrations of these chemicals, their impact on aquatic organisms can be significant and has become a source of growing concern. This partly stems from the biochemical nature of these chemicals, as they have the ability to alter the physiological and behavioral responses of exposed aquatic organisms due to the highly conserved drug targets and physiological pathways across vertebrates (Arnold et al., 2014; Brown et al., 2014; Margiotta-Casaluci et al., 2014; McCallum et al., 2017; Mehdi et al., 2018; Saaristo et al., 2018). Another concern of these chemicals that is relatively unexplored is their frequent presence with other stressors, especially in environments impacted by wastewater inputs. Wastewater treatment plant (WWTP) effluent-dominated environments typically suffer high nutrient loads, decreased dissolved oxygen levels, increased water temperature, as well the presence of PPCPs and other chemicals of concern (Chambers et al., 1997; Daughton and Ternes, 1999; Environment Canada, 2001; Kinouchi et al., 2007; Odjadjare and Okoh, 2010; McCallum et al., 2019).

Venlafaxine (VFX), a selective serotonin-norepinephrine reuptake inhibitor (SNRI), is a heavily prescribed and readily detectable antidepressant found in many Canadian waterways that receive WWTP effluents (Metcalfe et al., 2010; Arlos et al., 2014). VFX and its active metabolite, O-desmethyl venlafaxine (O-VFX) are mainly introduced into WWTP effluents via human excretion; approximately 5% of the average human daily dose is excreted in urine as the unchanged parent form and 29% in the active metabolite form (Metcalfe et al., 2010). VFX and O-VFX are often detected at higher concentrations than any other antidepressant drug and its active metabolite in WWTP effluents and effluent-receiving environments (Schultz and Furlong, 2008; Metcalfe et al., 2010). In discharged WWTP effluent, VFX and O-VFX have been detected at concentrations ranging from 808 to 2,050 ng/L and 1,637 to 1,927 ng/L, respectively (Metcalfe et al., 2010; Arlos et al., 2015). Whereas in the surface water of effluent-receiving environments in the Grand River watershed (Southern Ontario, Canada), VFX and O-VFX have been detected at concentrations ranging from 61 to 901 ng/L and 167 to 1,472 ng/L, respectively (Metcalfe et al., 2010). Despite these relatively high concentrations, very little is known about the impacts of this drug on the metabolic responses of aquatic organisms, especially in combination with other stressors that may be present in WWTP effluent-dominated environments.

Temperature, known as the "ecological master factor" is one of the most important determinants of life-cycle events in ectotherms, because of its influence on metabolism, energy production and expenditure, development, survival, and growth (Harig and Fausch, 2002; Lee, 2003; Farrell et al., 2008; Schultz and Bertrand, 2011). Various studies in the past have examined the effects of temperature on the metabolic and energetic responses in fish, however, the effects of such an ecologically relevant stressor have frequently been ignored in the field of ecotoxicology, especially in studies examining the effects of PPCPs on aquatic organisms. This is somewhat surprising, especially since the toxicity of various contaminants has been demonstrated to be enhanced by increasing temperatures, as reviewed in Noyes et al. (2009). Studies investigating the interactive effects of temperature and contaminants are critical, especially since many contaminants of emerging concern (CEC) in treated wastewater effluent are often found in the presence of thermally polluted environments produced via WWTPs. Effluent discharged from WWTPs can be a source of thermal pollution in effluent-receiving environments (Environment Canada, 2001), increasing the temperature by as much as 5–9°C (personal observations downstream of Woodward Avenue WWTP, Hamilton, ON, Canada, 2018). Previous studies have demonstrated links between the effects of temperature on the toxicity of chemicals using a variety of fish species and chemicals (Nussey et al., 1996). Zebrafish (***Danio rerio***) exposed to cadmium at temperatures ranging from 12 to 34°C demonstrated increasing cadmium-tissue accumulation and toxicity, measured by mortality, with increasing temperature (Vergauwen et al., 2013). Similar trends have been observed in ***Penaeus semisulcatus*** exposed to ammonia at different temperatures (Kir et al., 2004), as well as ***Prochilodus scrofa*** exposed to copper at different temperatures (Carvalho and Fernandes, 2006). While there are a number of studies examining the effects of temperature on the toxicity of contaminants, to our knowledge, this phenomenon is largely unexplored in studies examining the effects PPCPs on aquatic organisms. This is especially important as temperature may regulate the uptake of PPCPs by aquatic organisms, thereby, increasing their toxicity.

In our study, we were interested in how increased water temperature and VXF exposure interact and alter fish metabolic responses specifically. Metabolic physiology is an important indicator and contributor to fitness as it is linked to various levels of biological organization. Previous studies have demonstrated that exposure to WWTP effluent poses additional metabolic costs demonstrated by elevation in oxygen consumption rates (Du et al., 2018, 2019; Mehdi et al., 2018). VFX, at environmentally relevant concentrations, has also been shown to potentially act as a metabolic disruptor in fish when exposed to secondary stressors (Best et al., 2014). Added metabolic costs associated with contaminant exposure can result in energy-allocation trade-offs, thereby, potentially affecting fundamental basal processes such as growth, reproduction, and behavior (Scott and Sloman, 2004).

This study aimed to investigate the effects of an environmentally relevant concentration of VFX [1.0 μg/L] in combination with a 5°C increase in water temperature on the metabolic responses of zebrafish. We examined the combined and individual effects of the two stressors on whole-body metabolism by measuring routine metabolic rate (RMR). We also assessed tissue metabolic capacity by measuring muscle glucose and activities of enzymes involved in key metabolic pathways including glycolysis [pyruvate kinase (PK) and lactate dehydrogenase (LDH)], β-oxidation of lipids [3-hydroxyacyl CoA dehydrogenase (HOAD)], aerobic capacity [citrate synthase (CS) and cytochrome c oxidase (COX)], and antioxidant defense capacity [catalase (CAT)].

MATERIALS AND METHODS

Adult, mixed sex zebrafish were acquired from a fish wholesale facility (AQUAlity Tropical Fish Wholesale Inc., Mississauga, ON, Canada) and maintained in acrylic tanks (density of <5 fish/L) in a recirculating Habitats®Z-Hab System (Pentair Aquatic Eco-Systems Inc., Apopka, FL, United States). Water supplying the system underwent reverse osmosis, deionization, aeration, biological and chemical filtration, and UV sterilization. Water in the system was maintained at 27°C, pH of 7.5, and conductivity of 670 μS/cm. Fish were kept under a 12h:12h light-dark cycle and fed twice daily. Food consisted of a mixture of ground commercial fish food (TetraMin Tropical Flakes, Blacksburg, VA, United States) and live brine shrimp. This feeding schedule was maintained until start of the exposure experiment. Zebrafish were chosen as an ideal laboratory candidate to assess both the impact of VFX and temperature, with minimal disruption from other stress factors, as they are acclimated to aquarium housing. It should be noted, however, that the responses of lab-reared model organisms may differ from endemic wild organisms. It should also be noted that the responses demonstrated in this study using the tropical/subtropical zebrafish may differ from responses exhibited by temperate species, as fish adapted to different climates may exhibit different responses to temperature. Therefore, proper cross-species comparisons must be considered to solidify our findings. All experimental protocols followed the guidelines of the Canadian Council on Animal Care and were approved by the animal care committee at the University of Waterloo (AUPP #15-03).

Exposure Design

Adult, male zebrafish were exposed to environmentally relevant concentrations of VFX [1.0 μg/L] with or without an additional thermal stress over a period of 21 days. This exposure period was chosen because it has been demonstrated that steady-state is reached within 14–28 days in fish following a temperature change (Sidell et al., 1973; Hazel and Carpenter, 1985). Twenty fish were placed in 12-L aquaria, with three tank replicates per treatment. Each tank was supplied with the same system water that fish had previously been housed in, with sufficient aeration and heating. Fish were exposed to one of four treatments: (i) 0 μg/L VFX at 27°C; (ii) 1.0 μg/L VFX at 27°C; (iii) 0 μg/L VFX at 32°C; (iv) 1.0 μg/L VFX at 32°C; henceforth referred to as *Control*, *VFX*, *Temp*, and *VFX & Temp*, respectively. Fish were slowly acclimated to the temperature conditions over a 1-week period (∼0.7°C increase in temperature per day) prior to the start of the exposure. During the 1-week acclimation period, the tanks were equipped with back-hanging filtration units. The filters were removed when the acclimation period was over and the exposure period had begun. During the exposure period, the respective treatments mentioned above were dosed with 1.0 μg/L VFX (Millipore-Sigma-Aldrich, Oakville, ON, Canada). VFX aliquots dissolved in water were made in advance and stored at −20°C prior to daily dosing. The dosing protocol was adapted from an earlier study where similar static exposures were performed to examine the effects of VFX on neuroendocrine responses to stress in rainbow trout (*Oncorhynchus mykiss*; Melnyk-Lamont et al., 2014). The aforementioned study demonstrated that VFX concentrations are stable and are able to be maintained within the desired ranges throughout the duration of the exposure period.

Fish were fed ground flakes once daily until satiety, and 50% daily water changes were performed 1 h after feeding to remove waste and buildup of nitrogenous products. VFX daily dosing was performed at the same time water changes took place. Once a week, 100 mL water samples were collected from each tank at least an hour after the daily VFX dosing, and frozen at −20°C for later extraction and analysis of VFX concentrations using mass spectrometry. Fish mortality and tank temperature were monitored daily in addition to weekly water quality parameter-testing of ammonia, nitrate, nitrite, and pH. Throughout the duration of the experiment, there were no significant differences in mortality rates nor water quality parameters between treatments (**Supplementary Tables 1**, **2**). Following the 21-day exposure period, feeding was ceased for 24 h and RMR was measured in a subset of fish. Another subset of fish was euthanized with an overdose of MS-222 (Millipore-Sigma-Aldrich, Oakville, ON, Canada; 0.5 g/L). Following euthanasia, fish lengths and weights were recorded and epaxial muscle was removed and snap frozen in liquid nitrogen and stored at −80°C for later analysis. The remainder of fish were used in concurrently occurring studies. Although fish were always acclimated for 1 week and exposed for 21 days, tanks had randomly staggered exposure start dates to facilitate the processing of physiological assays after the end of the exposure period.

Routine Metabolic Rate

Routine metabolic rate was measured using a 170-mL glass swim tunnel respirometer equipped with a polymer optical fiber oxygen dipping probe, DAQ oxygen data acquisition system, Witrox oxygen reader, and AutoResp respirometry software (Loligo System, Tjele, Denmark). A water bath circulator equipped with a submersible water heater and a return pump controlled the temperature in the swim tunnel which was maintained at 27 or 32°C to match the exposure conditions. Throughout the experiment, the swim tunnel was programed to automatically cycle through three phases over 5 min: 60 s of flushing, 20 s of waiting, and 220 s of measuring oxygen concentration. Individual zebrafish were introduced into the swim tunnel and allowed to acclimate for up to an hour at a slow velocity of 5 cm/s to mimic normal activity in the exposure tanks and reduce spontaneous activity that would otherwise be observed in a static water chamber. A one-hour acclimation period was used to limit the depuration of VFX. Further, fish activity and oxygen consumption were continuously monitored during the acclimation and measurement periods, no significant changes in oxygen consumption nor fish activity were seen after the acclimation period. Following the acclimation period, up to six RMR measurements were taken over a period of 30 min. The mean of the three lowest metabolic rate measurements was used to calculate the metabolic rate for each individual. The average mass and total length of fish in each experimental group were as follows: *Control* (0.36 ± 0.02 g; 3.83 ± 0.04 cm);

VFX (0.34 ± 0.03 g; 3.84 ± 0.09 cm); *Temp* (0.34 ± 0.02 cm; 3.78 ± 0.07 cm); *VFX & Temp* (0.32 ± 0.02 g; 3.70 ± 0.04 cm).

Enzyme and Glucose Analysis

Frozen muscle tissue was powdered in liquid nitrogen using a mortar and pestle and 20–50 mg of tissue was homogenized in 20 volumes (20 × tissue mass) of extraction buffer (20 mM Hepes, 1 mM EDTA, and 0.1% Triton X-100, pH 7.0) using an electric homogenizer (Omni tissue homogenizer, Kennesaw, GA, United States). Sample homogenates were then centrifuged (12,000 *g*, 10 min, 4°C), and supernatants were used for enzyme assays. Enzyme activities were assayed in 96-well microplates using a Molecular Devices SpectraMax 190 spectrophotometer at assay temperatures matching those of exposure conditions at 340 nm unless stated otherwise. PK (E.C. 2.7.1.40), *LDH* (E.C. 1.1.1.27), and *HOAD* (E.C. 1.1.1.35) were assayed on fresh homogenates. Homogenates were then frozen as such at −80°C prior to the assays of *CS* (E.C. 2.3.3.1), *COX* (E.C. 1.9.3.1), and *CAT* (E.C. 1.11.1.6). Enzyme assays were performed following the protocols described in Mehdi et al. (2018). For reference, enzyme activities were also measured at assay temperatures matching both exposure temperatures (27–32°C), data shown in **Supplementary Materials** (**Supplementary Figure 1**). For glucose assay, muscle tissue samples were homogenized in two volumes of 8% perchloric acid and neutralized with 3 M K_2CO_3. Tissue glucose was measured following the standardized spectrophotometric protocol of Bergmeyer (1983) at 340 nm.

Water Chemistry

A total 100-mL water samples were collected every 7 days from each tank and later analyzed to ensure VFX concentrations were maintained within nominal levels throughout the exposure experiment. Water samples were collected a minimum of 1 h after dosing and stored immediately at −20°C until extraction. One *VFX & Temp* (Day 7) sample broke during storage and was therefore excluded from analysis. VFX samples were quantified following (Rahman et al., 2010). Briefly, 100-mL samples were spiked with 100 μL [100 μg/L] deuterated VFX. Samples were then extracted using solid-phase extraction (SPE) in Oasis HLB cartridges (6 cc, 500 mg, Waters Corporation, Milliford, MA, United States). The eluents were collected in glass tubes and evaporated under a gentle stream of nitrogen gas, and then reconstituted in 500 μL methanol and stored at −20°C until analysis. Samples were then quantified using a Sciex API 3200 QTRAP LC-MS/MS system. The method detection limit (MDL) in a 500-mL sample was 1 ng/L. Since 100-mL samples were extracted in this experiment, the detection limit was calculated to be 5 ng/L based on the original MDL.

Statistical Analysis

Data were analyzed using SigmaPlot 13.0 software (Systat Software Inc., San Jose, CA, United States). A two-way analysis of variance (ANOVA) was used to determine the main effects of VFX exposure (0–1.0 μg/L) and temperature exposure (27–32°C), as well as any interaction effects between the two stressors (VFX × temperature) on RMR, muscle enzyme activities, and glucose levels. Tukey's *post hoc* test was used to identify any significant pairwise comparisons. A one-way ANOVA was used to identify significant differences between tank replicates in VFX concentrations. Data were log or square root transformed when necessary to meet the assumptions of normality. All data are presented as untransformed means ± standard error of the mean (SEM) and significant level was set to *P*-value < 0.05 for all tests.

RESULTS

Water Chemistry and Temperature

Venlafaxine concentrations were below detection limit for the *Control* treatment (*n* = 12), 980.6 (±27.0) ng/L for the *VFX* treatment (*n* = 12), below detection limit for the *Temp* treatment (*n* = 12), and 965.2 (±59.3) ng/L for the *VFX & Temp* treatment (*n* = 11) during the exposure period (**Figure 1**). During the acclimation period, no VFX was detected in any of the tanks (*n* = 3 per treatment). Using a one-way ANOVA, no significant differences were detected between any of the tank replicates within each treatment, *P* = 0.509 for the *VFX* treatment and *P* = 0.281 for the *VFX & Temp* treatment. Similarly, water temperature in the exposure tanks was maintained at the intended temperatures throughout the entire duration of the experiment (7-day acclimation period and 21-day exposure period); *Control* treatment (27.6 ± 0.07°C), *VFX* treatment (27.1 ± 0.05°C), *Temp* treatment (32.3 ± 0.05°C), and *VFX & Temp* treatment (32.2 ± 0.06°C); actual temperature data is shown in **Supplementary Table 1**. As there were no significant differences in VFX concentrations and water temperatures between replicates within treatments, data were pooled from all three tank replicates for each treatment for statistical analysis.

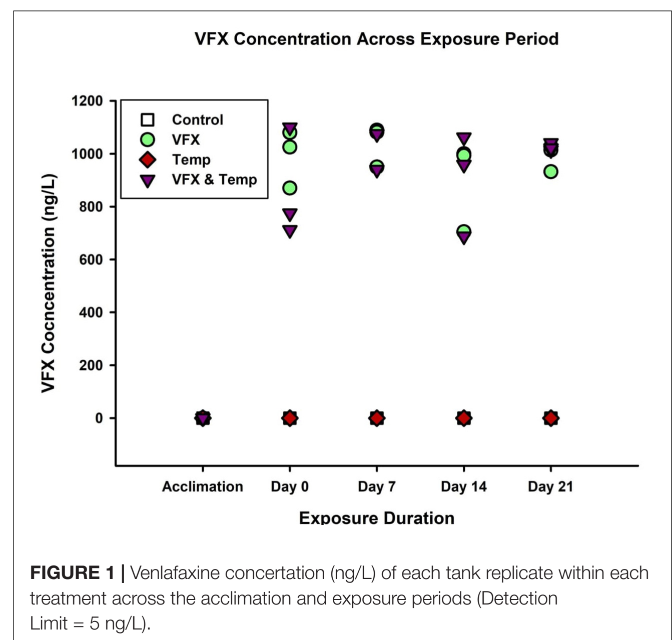

FIGURE 1 | Venlafaxine concertation (ng/L) of each tank replicate within each treatment across the acclimation and exposure periods (Detection Limit = 5 ng/L).

Routine Metabolic Rate

Routine metabolic rate was significantly higher (∼38%) in the *VFX & Temp* group compared to the *Control*. RMR also tended to be higher in the *VFX* (∼23%) and *Temp* (∼33%) groups relative to *Control*, but these differences were not statistically significant (**Figure 2**).

Muscle Glucose

Muscle glucose concentrations after the 21-day exposure period are presented in **Figure 3**. Glucose levels were significantly higher in the *VFX & Temp* group compared to the *Control* and *VFX* groups, but not the *Temp* group.

Enzyme Activities

Activities of six muscle metabolic enzymes were measured at the respective exposure temperatures per treatment (**Figure 4** and **Table 1**). PK activity was significantly higher in the *VFX & Temp* and *Temp* groups compared to the *Control* and *VFX* groups (**Figure 4A**). LDH activity was not significantly different across all treatments (**Figure 4B**). A significant temperature effect was observed in HOAD activity resulting in the *Temp* and *VFX & Temp* groups tending to be lower than the *Control* and *VFX* groups, but these pairwise comparisons were not statistically significant (**Figure 4C**). CS activity was significantly higher in the *Temp* group compared to the *Control*. CS activity was also significantly higher in the *VFX & Temp* group compared to the *VFX* group and tended (P = 0.073) to be higher than the *Control* group (**Figure 4D**). COX activity was not significantly different between treatments (**Figure 4E**). Finally, a significant interaction between VFX and temperature was demonstrated in CAT activity, where temperature induced an increase in CAT activity only when VFX was not present (**Figure 4F**).

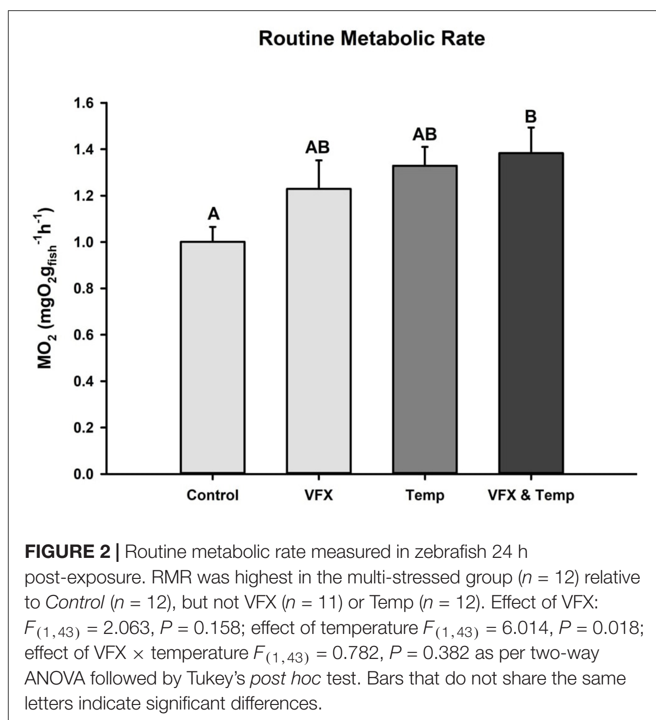

FIGURE 2 | Routine metabolic rate measured in zebrafish 24 h post-exposure. RMR was highest in the multi-stressed group (n = 12) relative to *Control* (n = 12), but not VFX (n = 11) or Temp (n = 12). Effect of VFX: $F_{(1,43)}$ = 2.063, P = 0.158; effect of temperature $F_{(1,43)}$ = 6.014, P = 0.018; effect of VFX × temperature $F_{(1,43)}$ = 0.782, P = 0.382 as per two-way ANOVA followed by Tukey's *post hoc* test. Bars that do not share the same letters indicate significant differences.

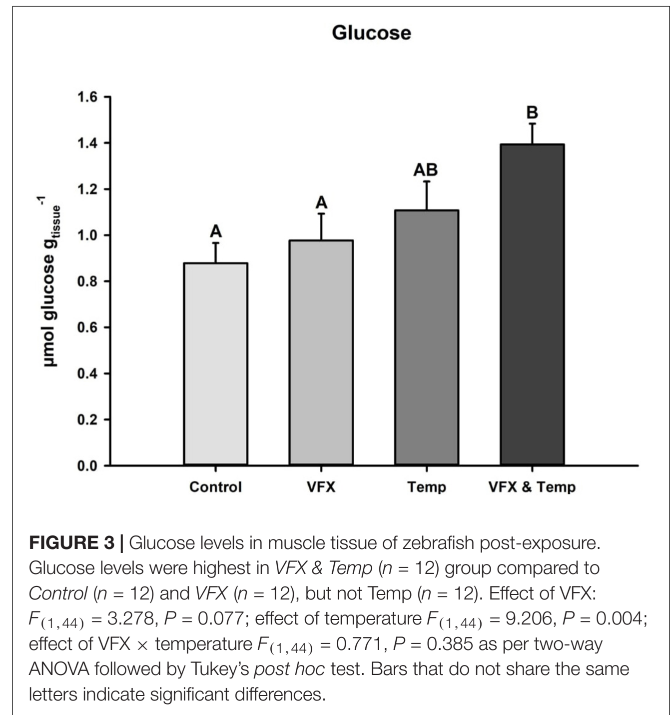

FIGURE 3 | Glucose levels in muscle tissue of zebrafish post-exposure. Glucose levels were highest in *VFX & Temp* (n = 12) group compared to *Control* (n = 12) and *VFX* (n = 12), but not Temp (n = 12). Effect of VFX: $F_{(1,44)}$ = 3.278, P = 0.077; effect of temperature $F_{(1,44)}$ = 9.206, P = 0.004; effect of VFX × temperature $F_{(1,44)}$ = 0.771, P = 0.385 as per two-way ANOVA followed by Tukey's *post hoc* test. Bars that do not share the same letters indicate significant differences.

DISCUSSION

In this study, we demonstrate that exposure to an environmentally relevant concentration of VFX in addition to a 5°C increase in water temperature can have several significant impacts on the metabolic responses of zebrafish. Most notably, VFX and temperature exposure had significant effects on RMR, various metabolic enzymes, and muscle glucose levels. These effects were most apparent when VFX and temperature were combined, however, several exceptions were observed. Studies often examine the effects of environmental perturbations using a single-stressor approach. However, this is the first study of its kind to look at the effects of both of these stressors (VFX and elevated temperature) individually and cumulatively using metabolic markers as sublethal endpoints under controlled lab conditions. At the whole-organism level, RMR tended to increase in all of the treatments relative to *Control*, but that was only significant between *VFX & Temp* and *Control*. This aligned well with our predictions, as metabolic rate is often positively correlated with temperature (Clarke and Fraser, 2004). Our results also indicate that VFX and temperature may be working cumulatively, as RMR was highest in the multi-stressed group. This is in line with other studies that have demonstrated the effects of various contaminants on metabolic rate, such as polychlorinated biphenyls, metals (McGeer et al., 2000), metals (Rajotte and Couture, 2002), and wastewater effluent (Du et al., 2018, 2019; Mehdi et al., 2018). At the tissue level, we demonstrated elevation in muscle glucose levels in the

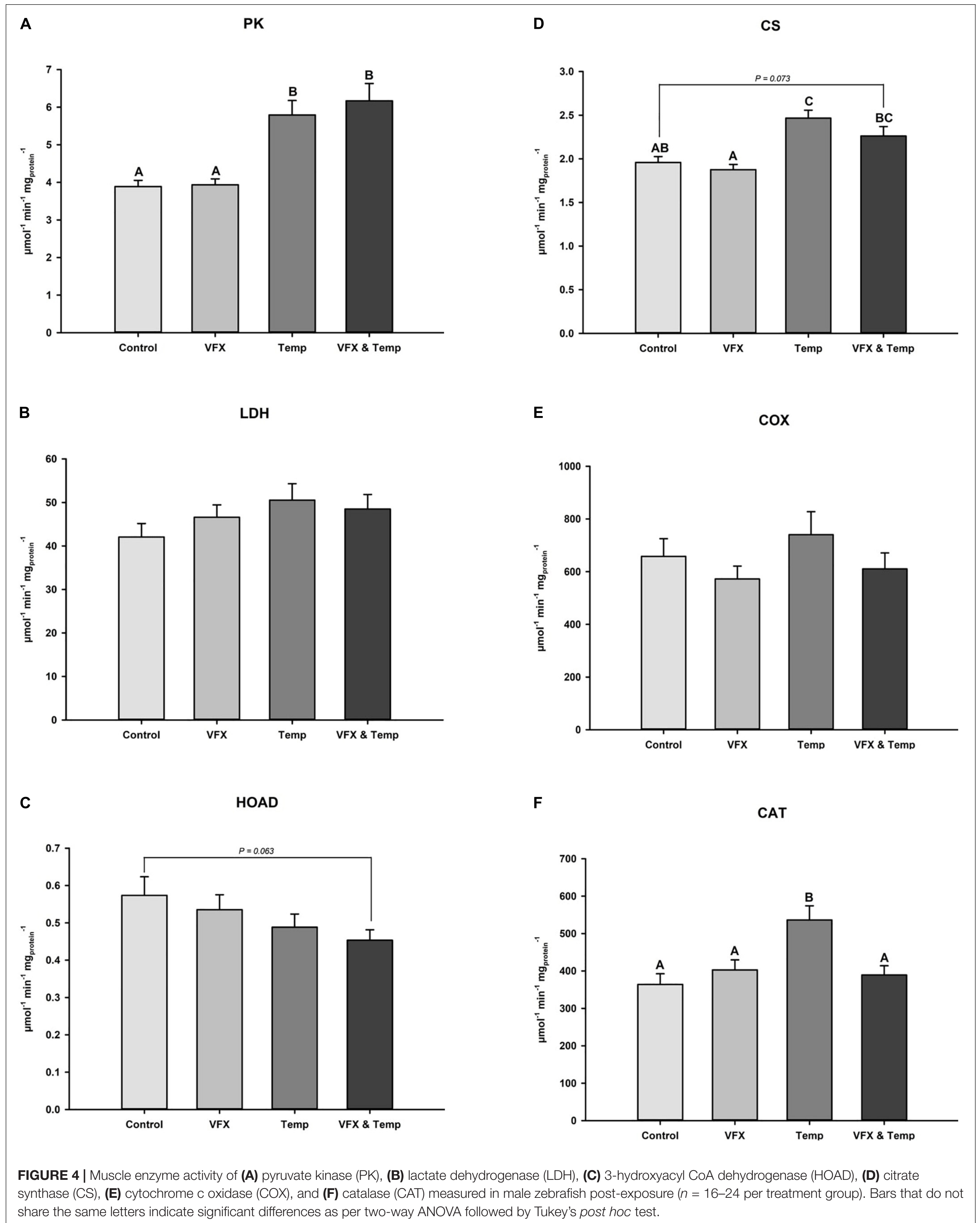

FIGURE 4 | Muscle enzyme activity of **(A)** pyruvate kinase (PK), **(B)** lactate dehydrogenase (LDH), **(C)** 3-hydroxyacyl CoA dehydrogenase (HOAD), **(D)** citrate synthase (CS), **(E)** cytochrome c oxidase (COX), and **(F)** catalase (CAT) measured in male zebrafish post-exposure (n = 16–24 per treatment group). Bars that do not share the same letters indicate significant differences as per two-way ANOVA followed by Tukey's *post hoc* test.

TABLE 1 | Fold changes in muscle enzyme activity among treatment groups relative to *Control* measured.

	Fold change relative to *Control* group				Two-way ANOVA statistics		
	Control	***VFX***	***Temp***	***VFX & Temp***	**Effect of VFX × temperature**	**Effect of VFX**	**Effect of temperature**
PK	–	1.01	1.49	1.59	$F_{(1,84)} = 0.288$; $P = 0.593$	$F_{(1,84)} = 0.470$; $P = 0.495$	$\mathbf{F_{(1,84)} = 44.908; P < 0.001}$
LDH	–	1.11	1.20	1.15	$F_{(1,84)} = 0.971$; $P = 0.327$	$F_{(1,84)} = 0.144$; $P = 0.706$	$F_{(1,84)} = 2.420$; $P = 0.124$
HOAD	–	0.93	0.85	0.79	$F_{(1,82)} = 0.002$; $P = 0.966$	$F_{(1,82)} = 0.796$; $P = 0.375$	$\mathbf{F_{(1,82)} = 4.133; P = 0.045}$
CS	–	0.96	1.26	1.15	$F_{(1,84)} = 0.550$; $P = 0.460$	$F_{(1,84)} = 3.119$; $P = 0.081$	$\mathbf{F_{(1,84)} = 29.994; P < 0.001}$
COX	–	0.87	1.12	0.93	$F_{(1,80)} = 0.105$; $P = 0.747$	$F_{(1,80)} = 2.517$; $P = 0.117$	$F_{(1,80)} = 0.790$; $P = 0.377$
CAT	–	1.11	1.47	1.07	$\mathbf{F_{(1,84)} = 9.354; P = 0.003}$	$F_{(1,84)} = 3.163$; $P = 0.079$	$\mathbf{F_{(1,84)} = 6.798; P = 0.011}$

Significant main effects and interaction effects as per two-way ANOVA are bolded.

multi-stressed group compared to the *Control*, and increased PK activity in both *Temp* and *VFX & Temp* relative to *Control* and *VFX*. HOAD activity on the other hand tended to be lower in *Temp* and *VFX & Temp* relative to *Control*. CS activity was elevated in *Temp* relative to *Control* and VFX, but a similar effect was not observed in the *VFX & Temp* group. Finally, CAT activity was significantly higher in *Temp* relative to all other treatments. Although our study did not reveal overwhelming interaction effects between our two stressors of interest (VFX and temperature), we did demonstrate that VFX and temperature can have cumulative effects on several physiological parameters. The lack of strong interaction effects has been reported in a similar study examining the effects of a psychoactive pharmaceutical, oxazepam, and temperature on the behavior of European perch (*Perca fluviatilis*; Saaristo et al., 2019). It is also important to distinguish that most of the effects observed in our study were driven by temperature rather than VFX exposure. This is not surprising, as temperature is often regarded as an "ecological master factor" that impacts many of the endpoints that our study was interested in measuring. VFX, at environmentally relevant concentrations may not be potent enough to cause strong adverse effects in metabolic responses of fishes. This should be investigated further with a dose-dependent study looking at various VFX concentrations, possibly across multiple acclimation temperatures, rather than just two.

Exposure to anthropogenic contaminants poses a metabolic cost for fishes which can be further exasperated by other biotic and abiotic stressors, such as temperature (Craig et al., 2018). In this study we observed that carbohydrate metabolism was affected by increasing water temperature and VFX exposure, as indicated by the elevated muscle glucose levels and PK activity. This was expected, as energy demand is often higher at elevated environmental temperatures, and carbohydrates are the first energy reserves to be used (Hori et al., 2006; Pörtner and Knust, 2007). However, what was more intriguing was the fact that muscle glucose levels were only higher in the group exposed to both stressors, i.e., VFX and elevated temperature, suggesting that we are in fact observing a cumulative effect from both stressors, potentially causing fish to be energy deficient. A previous study has demonstrated similar results, where female rainbow darter (*Etheostoma caeruleum*) collected downstream of municipal WWTP in the Grand River watershed, ON, Canada had higher PK activity than their female counterparts collected from an upstream site (Mehdi et al., 2018). When observing the effects of VFX and temperature on the anaerobic capacity of zebrafish, we initially predicted that LDH activity, an enzyme involved in anaerobic glycolysis would be elevated in the exposed groups, however, our results were not supportive of this hypothesis. Our results are in line with previous studies demonstrating the lack of observable effects of VFX exposure and municipal WWTP effluent exposure on LDH activity in rainbow trout liver (Ings et al., 2011; Best et al., 2014). Our results indicate that neither environmentally relevant concentrations of VFX nor a 5°C increase in water temperature have significant effects on LDH activity, thereby, not allowing us to draw concrete conclusions about the effects of our two stressors on anaerobic glycolysis.

When measuring HOAD, an enzyme involved in β-oxidation of fatty acids and is commonly used as an indicator of lipid metabolism, a modest decrease was observed in activity in the groups that were exposed to the higher temperature, especially the *VFX & Temp* group. The increase in glucose levels and the opposing trends between PK and HOAD activities indicates that fish are more reliant on carbohydrate forms of energy production rather than lipid forms at the higher exposure temperature. The decrease in HOAD activity are comparable to a previous study that examined zebrafish reared at low and high temperatures, where it was found that HOAD activity was higher in zebrafish reared at lower temperatures (Schnurr et al., 2014). The acclimation response that we observed in groups exposed to higher temperatures could be maladaptive in fishes that experience frequent temperature fluctuations. It is known that metabolic demand increases in response to increasing water temperatures in ectotherms. Therefore, fish that are not able to plastically respond to these changes may be at a disadvantage, as they are unable to increase their lipolytic activity to make up for the increased metabolic costs of higher environmental temperatures, as observed by the increase in PK activity and glucose levels in the muscle. We also measured the activity of CS, a key enzyme in the citric acid cycle, which is often used as an indicator of aerobic capacity and potentially, mitochondrial abundance in the muscle (Rajotte and Couture, 2002; Lemos et al., 2003). We observed a general increasing trend in CS activity with the onset of both stressors (VFX and temperature); however, we only saw a significant increase in the *Temp* group compared to *Control* and *VFX*. We initially predicted that the onset of both stressors would result in more aerobic phenotypes, such as the increase in aerobic capacity and mitochondrial abundance as represented by elevation in

CS activity. However, since this was only observed in the *Temp* group, it may suggest that VFX is indeed a metabolic disrupter and fish aren't capable of adaptively responding to higher acclimation temperatures by increasing their aerobic capacity. This should be investigated further by subjecting multi-stressed fish to aerobically demanding challenges, such as critical thermal tolerance and critical swimming velocity tests. Finally, we assessed the effects of VFX and temperature on oxidative stress and capacity in the muscle tissue. We measured the activity of CAT, an enzyme involved in the defense against oxidative stress from reactive oxygen species (ROS) production, and is often used as an indicator of cellular damage and environmental stress (Atli et al., 2006; Kessabi et al., 2013). The increase in CAT activity in the *Temp* group was expected, as ectothermic metabolism often increases at higher temperatures, yielding more ROS and antioxidant enzyme activities would have to increase as well to combat these harmful byproducts of mitochondrial respiration (Davidson and Schiestl, 2001; Rocha et al., 2003; Lushchak and Bagnyukova, 2006; Bagnyukova et al., 2007). The increase in CAT activity in response to elevated water temperature is considered an adaptive response, indicating higher resistance to oxidative stress (Rudneva, 1999). However, this response was not present in fish exposed to both VFX and elevated water temperature, suggesting VFX could have a deleterious effect on the antioxidant defense mechanism and mitochondrial respiration, as has been demonstrated in isolated mammalian hepatocyte studies (Deavall et al., 2012; Ahmadian et al., 2016), although we did not see any significant impacts on COX activity, implying a more direct effect on CAT activity itself, which warrants further investigation. This observed interaction effect between temperature and VFX exposure on the antioxidant defense mechanism should be studied further, especially since fishes in the wild are exposed to a myriad of energetically demanding stressors and anthropogenic contaminants such as VFX, potentially causing them to be vulnerable to oxidative stress.

CONCLUSION

In conclusion, this study aimed to explore effects of both VFX and elevated water individually and cumulatively on the metabolic physiology of zebrafish. This study also aimed to further explore how the toxicity of contaminants can change in the presence of other stressors, such as elevated temperatures. Many of the effects that were observed in our study are considered moderate, in the future, we will further investigate the costs that fish have to bare under multi-stressed conditions using fish with a narrower thermal tolerance than zebrafish, as well as using fish under different ontogenic stages. Physiological and ecological responses to multiple stressors can prove to be challenging and complex. However, multi-stressor research continues to be essential, as it aims to bridge the gap between laboratory and natural field settings, thereby, providing more accurate assessments than single-stressor research (Noyes et al., 2009; Ng et al., 2013).

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusion of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

All experimental protocols followed the guidelines of the Canadian Council on Animal Care and were approved by the animal care committee at the University of Waterloo (AUPP #15-03).

AUTHOR CONTRIBUTIONS

HM was responsible for the designing and conducting the experiments as well as writing the manuscript. LB provided help in water chemistry analysis as well as providing many of the necessary equipment for this study. PC and MS provided extensive experimental design and editorial input to make this manuscript possible.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2019.01431/full#supplementary-material

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Conflict of Interest: The 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.

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frontiers in Physiology

ORIGINAL RESEARCH
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Continuous Inking Affects the Biological and Biochemical Responses of Cuttlefish *Sepia pharaonis*

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Several marine mollusks, including cephalopods (cuttlefish, squid, and octopus) and gastropods (e.g., sea hares), can release a colored ink secretion when chased by predators or stimulated. Ink release is part of a defensive response, but the threshold for the biochemical responses caused by stimulation is unknown. The present study aimed to reveal antipredator responses of cuttlefish, such as escaping via inking and/or jetting, and to investigate its biological and biochemical responses to continuous ink release. Results showed that the behavioral responses to continuous ink release mainly manifested as blazing body pattern changes. Cuttlefish escaped from predators covered by jetting/inking and warned the potential threats by displaying a unique body pattern. Moreover, persistent inking in the presence of an overt stimulus caused uncontrollable ink release from the ink duct/anal canal (loss of control). This study first verified the characteristics of the cuttlefish ink solution, prepared a standard curve of ink solution concentrations, and fitted the relationship function between the release frequency and the released ink weight. Biological statistics indicated that cuttlefish has the ability to continuously release ink (releasing ∼90% of the ink from the ink sac) and that the individuals adapted well during the recovery period. However, re-releasing ink would result in "overexploitation" and high mortality. Hexokinase (HK), pyruvate kinase (PK), and superoxide dismutase (SOD) activities, as well as malondialdehyde (MDA) concentration increased or remained stable in different tissues after releasing ink. The expression of heat shock protein 90 and arginine kinase (AK) were upregulated by stimuli in all tissues. Biochemical changes indicated that continuous inking not only consumed considerable energy but also damaged the tissues. In summary, cuttlefish released almost 90% of their ink for active defense against predators, and it took ∼30 days for the ink sac to be refilled, but "overexploitation" resulted in serious physiological damage. These findings will be helpful to further study the defense and ink release mechanisms and to consider animal health and welfare when using cephalopods as experimental animals and for aquaculture practices.

Keywords: inking, predator–prey interactions, biochemical responses, animal care and welfare, *Sepia pharaonis*

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INTRODUCTION

Predator–prey interactions can exert strong selection pressure that affects the evolution of antipredation defense measures (Paul and Pennings, 1991; Zimmer and Ferrer, 2007; Hay, 2009). These defenses include behavioral adaptations (crypsis), body coloration (camouflage and threats), mechanical defenses (fleeing), and chemical defenses (electrical, poison, and colored secretion) (Pawlik, 1993; Hanlon and Messenger, 1996; Wyatt, 2003; Murphy, 2006; Glaudas and Winne, 2007). Prey species that may encounter various predators must be adapted for protection against various predation methods and must have defenses that affect organisms with very different sensory systems and adaptations of their own. Many marine mollusks release secretions as defense against predators, e.g., cephalopods (cuttlefish, squid, and octopus) and gastropods (sea hares) release a colored ink secretion (Hanlon and Messenger, 1996; Kasugai, 2001; Caldwell, 2005; Wood et al., 2010; Staudinger et al., 2011).

Cephalopods are prey to numerous marine vertebrates, including mammals, fish, and diving seabirds (Packard, 1972; Croxall and Prince, 1996; Smale, 1996) because they are soft bodied and lack hard protective structures such as spines and shells (Packard, 1972; Hanlon and Messenger, 1996). As an example of the high exposure of cephalopods to predators, one study of the Caribbean reef squid reported an average of seven encounters per hour (Hanlon and Messenger, 1996). Cephalopods have several types of defenses against predators similar to other species, including the ability to change color, shape, and texture, which can provide camouflage and crypsis that confuse, threaten, frighten, or bluff predators (Hanlon and Messenger, 1996; King and Adamo, 2006; Hanlon, 2007; Bush et al., 2009). Cephalopod ink is typically thought to have a defensive function through its visual effects on predators. Inking involves the release of a mass of black chemicals that can take different forms, i.e., a diffuse plume, a gelatinous mass known as a pseudomorph, or other forms and shapes (Shimek, 1983; Huffard and Caldwell, 2002; Bush and Robison, 2007).

Direct interactions with predators are often unavoidable, and inking is a visual stimulus that may act as an important defense in the emergence of danger. Cohesive ink is released as a decoy supposed to attract predators, while diffuse ink is released as a smokescreen covering the retreat (Humphries and Driver, 1970; Driver and Humphries, 1988). Sea hare ink has an adverse effect on the chemical sensory organs of predators, acting as a repulsive substance that prevents predators from attacking, an aversive substance that causes the predator to reject an inking animal once it has been taken into the mouth of the predator, or as a substance that disrupts the senses of the predator's sensory system and thus affects the predator's ability to assess or consume the animal (Sheybani et al., 2009; Nusnbaum and Derby, 2010; Wood et al., 2010). Furthermore, cephalopods also produce protean behaviors, which include unpredictable erratic escape behaviors such as jetting and inking (Hanlon and Messenger, 1996).

Particularly, changes in cephalopod behavior against predators suggest that their reaction to jetting/inking has been underestimated. Encountering a predator is a common challenge for a prey animal, and it is important to assess the biochemical changes and consequent energy stress (Sato et al., 2016). Cuttlefish releases ink from the ink sac when subjected to external stimuli (e.g., pressure) (Nithya et al., 2011). To be well prepared for danger, the ink gland cells keep producing melanin to the ink sac, the typical effector organ delegated to storage after release a large amount of ink (Palumbo et al., 2000). We observed that the pigmentation stage of embryonic development is capable of releasing ink when manipulated vigorously. Besides, cuttlefish release ink during rearing, e.g., weaning, competition for food, mating, and rapid changes in temperature and salinity (Le et al., 2014; Lee et al., 2016; Jiang et al., 2018). Furthermore, overrelease of ink results in the individual's death. However, cuttlefish are always encountering predators accompanied by inking and/or jetting in nature, but the ability of continuously releasing ink, the effect of the inking behavior on the cuttlefish itself, and the ink synthesis rate are still unknown. We investigated the effects of continuous ink release on survival, behavior, and biochemical characteristics of cuttlefish *Sepia pharaonis* and tried to elucidate the biological and biochemical responses of cephalopods to ink release, which might provide a basis for further research.

MATERIALS AND METHODS

Animals and Rearing Conditions

Sepia pharaonis eggs belonged to the second generation (F_2) of cuttlefish reared at our research facility. Experiments were conducted at the Lai Fa Aquaculture Co., Ltd. (29° 59′ N, 121° 99′ E) (Zhejiang Province, China), which specializes in aquatic technology research and application development. Posthatching, cuttlefish were reared in a cement pond (7.8 m × 3.8 m × 1.6 m, length × width × depth; area, 30 m^2), as described for the species by Jiang et al. (2018). In short, newly hatched juveniles were fed enriched live rotifers (*Artemia nauplii*) the first 3 days posthatching and then live mysids (*Hyperacanthomysis brevirostris*) twice a day (8:00 and 16:00) *ad libitum*.

After three treatments causing ink release events, the cuttlefish were cultured in the cement pond and fed frozen shrimp (white shrimp *Penaeus vannamei*) twice a day (at 7:00 and 16:00), ensuring that the cuttlefish had sufficient food supply (20% of the cuttlefish weight) at each feeding time (Domingues et al., 2004). Gentle aeration provided by air stone and airlift for the cement pool (1–1.5 m^2/piece). Natural seawater was filtered by a filter bed and ultraviolet sterilizers before it was pumped into the tank. The water quality parameters were as follows: salinity at $29 \pm 0.6‰$, temperature at 26 ± 0.9°C, pH 8.55 ± 0.42, ammonia nitrogen controlled at 0.05 ± 0.03 mg L^{-1}, and dissolved oxygen at 6.63 ± 0.21 mg L^{-1}. Temperature, salinity, and dissolved oxygen were measured daily with a YSI Pro DSS (YSI)[1], and seawater was 30% refreshed every day under the same conditions. Using natural light as a light source, a day–night cycle (12 h:12 h) was maintained during the experiment with an intensity of 200 lx. Light intensity was detected using a handheld illuminometer (Sanwa, LX2). Low light intensity was adopted to maintain low-stress levels (Koueta and Boucaud, 2003; Sykes et al., 2003).

[1] www.ysi.com

Experimental Procedure and Estimation of the Number of Animals

The total number of animals used to determine the average quantity of ink released by cuttlefish. Animals of adequate age and size (50 days old; dorsal mantle length, 5.0 ± 0.3 cm; body weight, 20.12 ± 2.65 g), and similar in size to the ones that are expected to be used during a single inking event. In addition, the weight of released ink decreased significantly with the increase in ink release frequency, the final number of individuals (dorsal length, 5.5 ± 0.3 cm; body weight, 25.36 ± 3.82 g) to be utilized in the present study resulted to be larger than 350.

At the cuttlefish, they were forced to release ink every 24 h in three ink release events (days 1-3). Each individual was placed in a foam box (58 cm × 34 cm × 26 cm, length × width × depth) containing 20 L of seawater and tested one at a time. A string net (10 cm in diameter) was placed in the foam box and simulate an attack on the cuttlefish by a predator. This resulted in a defensive behavior response to escaping and inking and/or jetting and burst swimming backward. The string net was placed directly in front of the cuttlefish while retreating for 30–60 s until it stopped swimming and showed no obvious reaction to the stimulus. The cuttlefish ink solution in the foam box was then stirred with a hand-held stirrer (GTH-100; ~2 min), and the concentration of the cuttlefish ink was measured. This allowed to calculate the weight of the released ink released. Each time the animal was dislocated from the foam box, it was temporarily housed in a plastic basket (55 cm × 18 cm, diameter × height). After three ink release events, the cuttlefish had to release ink by the same method after 5, 10, 15, 20, 25, and 30 (housing in the cement pond) and stimulated to obtain the ink after each period (n = 50) as well as to allow for the development of the ink after the continuous stimulation and the effect of re-releasing ink on the cuttlefish itself.

The ink sac was collected and weighed by dissecting the cuttlefish that had been released (dorsal mantle length, 5.4 ± 0.7 cm; weight, 23.28 ± 6.75 g, n = 50). Ink weights from the three release events were added to calculate total weight (TRW, mg) and ink weight in the ink sac (IWIS, mg) and were analyzed by descriptive statistics.

Characteristics of the Cuttlefish Ink Solution and the Concentration Standard Curve Preparation

Characteristics of the Cuttlefish Ink Solution

The fresh cuttlefish ink was diluted to a certain concentration and made uniform by stirring using a magnetic stirrer. No aggregation or sedimentation occurred at room temperature (30 ± 0.8°C) for 3 days, indicating that the cuttlefish ink solution is homogeneous and stable in a short period. The ink solution was put into the long neck funnel with a semipermeable membrane at the bottom and then transferred to the beaker full of deionized water, as shown in **Figure 1A**. A light was placed parallel to the side of the beaker to illuminate the ink solution, and a clear light path was observed (**Figure 1B**).

Preparation of the Concentration Standard Curve

1. A total of 300 mg fresh cuttlefish ink was added to a 1.5-L beaker and diluted with 1,000 mL deionized water and stirred using a magnetic stirrer (5 min) to prepare a cuttlefish ink solution of 300 mg L^{-1}.
2. According to the national standard method (Editorial Board of the State Environmental Protection Administration (SEPA), 2002), 0, 2, 4, 6, 8, 10, and 12 mL of the above 300 mg L^{-1} cuttlefish ink solution were added to separate 50-ml volumetric flasks. Deionized water was added to a volume of 50 mL and was shook well to get ink solutions of 0, 12, 24, 24, 36, 48, 60, and 72 mg L^{-1}, respectively.
3. The specific wavelength of the cuttlefish ink solution was 320 nm, determined using an ultraviolet–visible spectrophotometer (UNIC 2800 UV/VIS) at full wavelength scanning. The deionized water was used as a reference, and the absorbance values of the above-diluted solutions at 320 nm were 0, 0.135, 0.271, 0.406, 0.536, 0.671, and 0.819. The standard curve was drawn with absorbance (OD) as the X-axis and the concentration of the cuttlefish solution as the Y coordinate (**Figure 2**).

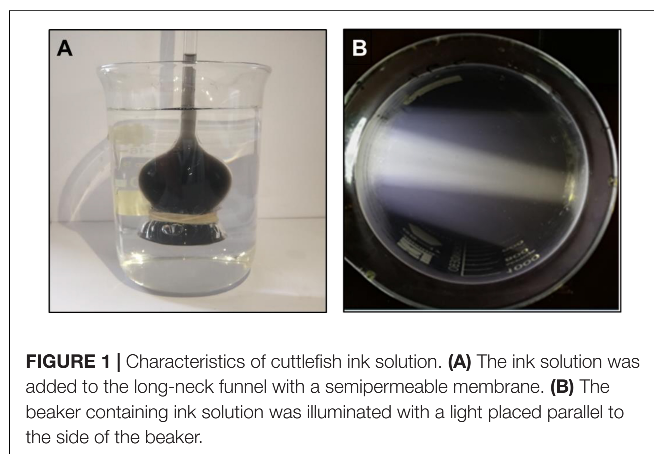

FIGURE 1 | Characteristics of cuttlefish ink solution. **(A)** The ink solution was added to the long-neck funnel with a semipermeable membrane. **(B)** The beaker containing ink solution was illuminated with a light placed parallel to the side of the beaker.

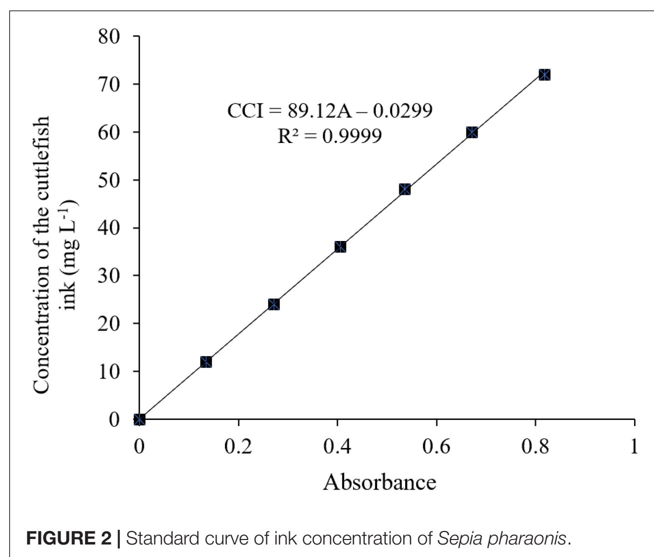

FIGURE 2 | Standard curve of ink concentration of *Sepia pharaonis*.

Behavior

Cuttlefish behavior was studied using video cameras (SONY HDR-CX450) during inking. Four behavioral elements were used to assess the effect of release ink on the cuttlefish. (1) Jet frequency (n) was calculated as the number of jets (including ink and water jetting) during a single inking treatment. Direct observations were also performed at the same time as the video recordings to count the jet frequency. (2) Body pattern changes included chromatic changes and textural, postural, and locomotor components. Cephalopods such as cuttlefishes, octopods, and squids can change their body color and postural behaviors rapidly and also exhibit a variety of visually complex appearances (Holmes, 1940; Nakajima and Ikeda, 2017). These appearances comprise a combination of chromatic, textural, postural, and locomotor components for both camouflage and communication (Neill, 1971; Hanlon and Messenger, 1988). Body pattern changes will provide a useful foundation for quantitative behavioral analyses. (3) Ink solution properties were revealed, including ink forms, mucus volume, and dispersing changes in water. Inking involves ejecting a mass of black substances that take different forms. Ink forms change with continuous release, and defensive effect can be used as an indicator to study the characteristics of the ink secretion. (4) Swimming and feeding activities were also studied. Continuous ink release consumes considerable energy, and how inking affects the ability of a cuttlefish to escape from predators and food intake can be used as an effective parameter to assess the impact of inking on the activity and feeding of the organism.

Tissue Sampling and Enzyme Activity

Tissue Sampling

As a control group, 10 cuttlefish juveniles (dorsal mantle length, 5.4 ± 0.2 cm; weight, 24.79 ± 3.48 g) were anesthetized with 5% alcohol before inking treatment. Samples of liver, gill, muscle, and brain tissues were immediately frozen in liquid nitrogen and stored at −80°C. The test group was sampled on the first (day 1), second (day 2), third (day 3), and fourth (day 8) inking treatments. Six cuttlefish juveniles were anesthetized with 5% ethanol for each treatment group, and tissues were sampled and stored as described above.

Enzyme Activity

The activities of hexokinase (HK), pyruvate kinase (PK), superoxide dismutase (SOD), and malondialdehyde (MDA) were measured with commercial assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China)[2] following the manufacturer's instructions. The assays are briefly described below.

The activities of HK (EC 2.7.1.1) and PK (EC 2.7.1.40) were determined following the procedures described by Tranulis et al. (1996) and Foster and Moon (1985), respectively. The absorbance of the samples was read at 340 nm. The enzyme activities were expressed as per milligram of total protein (specific activity). The total protein content in crude extracts was determined at 30°C using bovine serum albumin as a standard based on the Bradford (1976) method.

The activities of SOD (WST-1 method) and MDA (thiobarbituric acid method) concentration were measured at 450 and 532 nm using the xanthine oxidase and thiobarbituric acid methods according to Wang and Chen (2005) and Placer et al. (1966), respectively. The levels are expressed as units of SOD per milligram protein and nanomole of MDA per milligram protein.

Real-Time PCR Analysis of Heat Shock Protein 90 and Arginine Kinase Gene Expression Patterns

RNA Extraction and First-Strand cDNA Synthesis

Total genomic RNA was extracted from tissue (liver, gill, and, muscle) samples of juveniles *S. pharaonis* using a Trizol RNA extraction reagent (Invitrogen, Carlsbad, CA, United States), following the method of Rio et al. (2010). The concentration of RNA was measured using Nanodrop ND-1000 (Thermo Scientific, United States). Electrophoresis in 1.0% formaldehyde-denaturing agarose gel was used for assessing the quality of the RNA, and the purity of RNA was checked by measuring the ratio of OD260/OD280 (1.8–2.0). For the synthesis of first-strand complementary DNA (cDNA), 1 μg of RNA was treated with 1 U of DNase I (Sigma-Aldrich, United States). This step helps to avoid DNA contamination. The cDNA was synthesized from 1 μg of total RNA using real-time PCR kit (Takara, Japan) following the instructions for SYBR® PrimeScript™. β-Actin, the constitutively expressed housekeeping gene was used for sample normalization and as positive control. The PCR amplification of β-actin was carried out for the confirmation of cDNA synthesis.

Real-Time PCR Analysis

The fragment sequence of the heat shock protein 90 (HSP90) gene, in this experiment, was derived from the database of the *S. pharaonis* transcription constructed by this group. Arginine kinase (AK) primers were designed from this group (Si et al., 2016a,b). All primer pairs (**Table 1**) for real-time PCR were synthesized by Shanghai Sangon Biotech Co., Ltd. (Shanghai, China). The fluorescent quantitative PCR reaction solution consisted of 12.5 μL SYBR® premix Ex Taq™ (2×), 0.5 μL PCR forward primer (10 μM), 0.5 μL PCR reverse primer (10 μM), 2.0 μL RT reaction mix (cDNA solution), and 9.5 μL dH_2O. All samples were analyzed in triplicate wells using the following cycling parameters: 94°C for 5 min, followed by 40 cycles consisting of 94°C for 5 s, 60°C for 10 s, and 72°C for 15 s. The fluorescent flux was then recorded, and the reaction was continued at 72°C for 3 min. We measured the dissolution rate between 65 and 92°C. Every increase of 0.2°C was maintained for 1 s, and the fluorescent flux was recorded simultaneously. The efficiencies close to 100% were used in the $2^{-\Delta\Delta CT}$ method for relative gene expression calculation (Livak and Schmittgen, 2001). We measured the PCR efficiency by constructing a standard curve using a serial dilution of cDNA; $\Delta\Delta C_T = (C_{T,\ Target} - C_{T,\ \beta-actin})$ time x - $(C_{T,\ Target} - C_{T,\ \beta-actin})$ time 0.

[2]http://www.njjcbio.com/

TABLE 1 | Target genes and sequences of primers used to investigate messenger RNA (mRNA) levels.

Target gene	Primer	Primer sequence (5′-3′)
β-actin	Fw	TCCTGACCGAGAGAGGCTAC
	Rv	CTGCTCGAAGTCAAGAGCCA
HSP90	Fw	TCGAACTCATCCCCGATCTGA
	Rw	CTTTGTGCCGGATTTAGCGAT
AK	Fw	TTGCTGAAGTCCTTGATGCYGT
	Rw	TCATGGTRGTACCCAAGTTGC

Statistical Analysis

The results were presented as the means ± SD (n = 3). All data were subjected to one-way ANOVA. When there were significant differences, the group means were further compared using Duncan's multiple range test. $p < 0.05$ was considered to indicate statistical significance. SPSS regression curve estimation and Excel 2010 were used for the fitting function of released ink frequency and released ink weight. Analyses were performed using SPSS version 20.0 (SPSS, Chicago, IL, United States).

RESULTS

Cuttlefish Ink Solution Characteristics and Relationship Between Released Ink Frequency and Weight

There was no ink present in the beaker, and an increased height level in the long neck funnel was observed, indicating that the concentration of the ink solution was higher than that of deionized water, and ink solution could not penetrate through the semipermeable membrane (**Figure 1A**). The light pathway was visible when parallel light passed through the solution, which resulted in the Tyndall effect (**Figure 1B**). In summary, the cuttlefish ink solution was a homogeneous, stable colloidal solution. The characteristic 320-nm wavelength of the cuttlefish ink solution was obtained by ultraviolet–visible spectrophotometer (UNIC 2800 UV/VIS) full wavelength scanning. Based on the national standard method, the standard curve of concentration was CCI = 89.12 × OD - 0.0299, with R^2 = 0.9999 (**Figure 2**).

The data profile of the first three release ink events is shown in **Figure 3A**. The weight of each ink release, TRW, and IWIS were analyzed by descriptive statistics (**Table 2**). As expected, the ink weight of the first ink release event was apparently greater than that of the second event and much greater than that of the third event. According to the calculated IWIS, ∼90% of the ink from the ink sac could be continuously released. The kurtosis and skewness analysis results showed that the ink weight of the first release event and TRW exhibited deviation to the left of the mean, indicating that more data were on the right side of the mean value (André et al., 2014). The fitting function relationship between released ink frequency and weight is shown in **Figure 3B**. The exponential function is expressed as weight of released ink = 2,096.4 × $e^{-1.327}$ frequency (N), with R^2 = 0.9586.

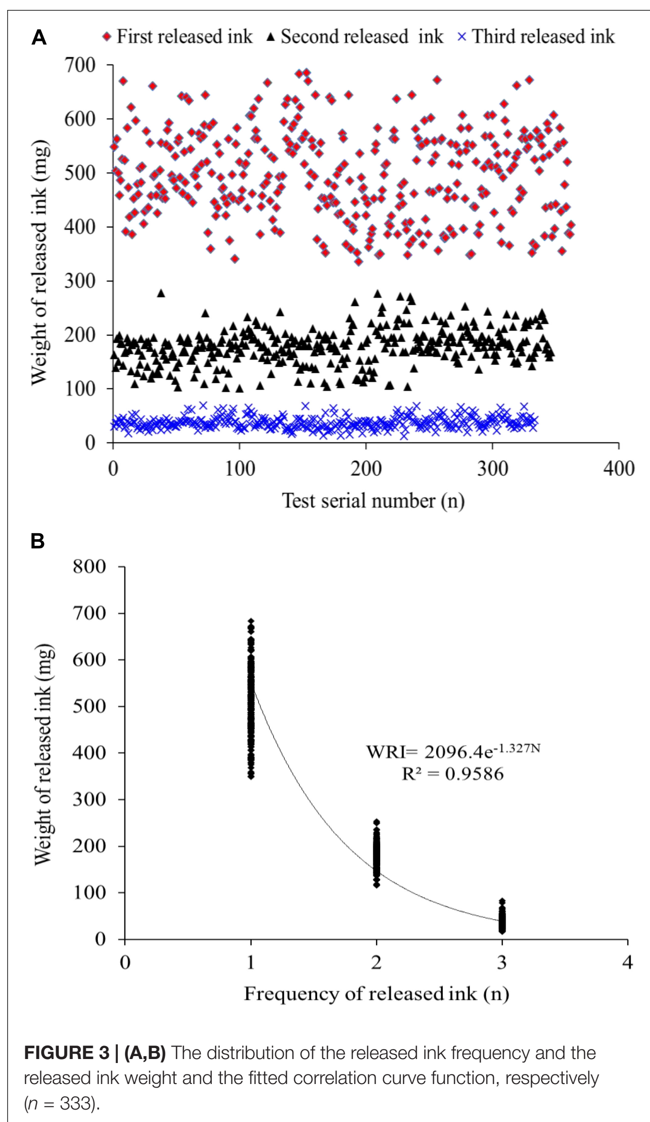

FIGURE 3 | (A,B) The distribution of the released ink frequency and the released ink weight and the fitted correlation curve function, respectively (n = 333).

Relationship Between the Released Ink Weight and Mortality at Different Times

The released ink weight dropped sharply throughout the first three inking events, being 497.98 ± 80.4, 178.34 ± 33.7, and 37.91 ± 11.02 mg, in days 1, 2, and 3, respectively, but mortalities in different groups (3.7–4.5%) were low and showed no significant differences among them (**Figure 4**). The weights of re-released ink events after culture for 5, 10, 20, and 30 days in cement pond were 163.38 ± 42.8, 355.27 ± 70.72, 438.55 ± 112.34, and 501.23 ± 77.34 mg, respectively. However, the mortalities could be as high as 100 and 98% (5 and 10 days of culture, respectively). More importantly, the cuttlefish adapted well to these changes during culture and took as long as 30 days to recover to the initial levels.

Behavior

A comparison of behavioral changes among three inking events is presented in **Table 3**. The jet frequency of the

TABLE 2 | SPSS descriptive statistics used to analyze the weight of released ink in each release event, total weight of released ink (TRW, mg, $n = 333$), and ink weight in the ink sac (IWIS, mg, $n = 50$) after three ink release events.

Items	Mean	SD	Max	Min	Kurtosis	Skewness
First ink release	497.98	80.456	685.99	336.27	−0.5583	0.0191
Second ink release	178.34	33.723	378.03	100.92	0.2962	0.0611
Third ink release	37.91	11.02	69.51	12.33	0.1798	0.5604
TRW	714.23	90.29	963.82	494.16	−1.1166	0.0191
IWIS	764.51	113.98	1108.47	549.84	0.1979	0.1052

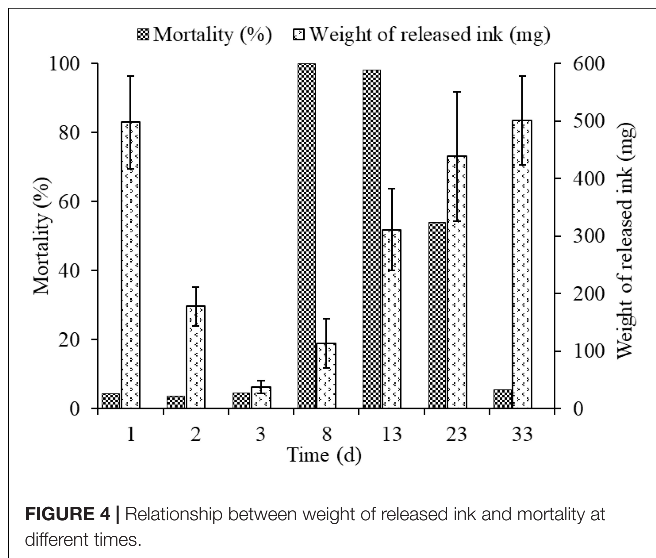

FIGURE 4 | Relationship between weight of released ink and mortality at different times.

first ink release event was significantly higher than those of the other events ($p < 0.05$), being 72 ± 11, 46 ± 7, and 28 ± 8, in the first, second, and third event, respectively. The properties of the released ink in the different events were as follows: (1) dense ink, a gelatinous-like substance with a large amount of mucus, which was not easy to disperse (**Figure 5G**); (2) dense ink, slightly diffuse plume with a small amount of mucus, easy to disperse (**Figure 5H**); and (3) smoke-like ink, almost no mucus that dispersed quickly (**Figure 5I**). Behavioral changes and feeding activity were significantly different among inking treatments (**Table 3**).

The overall responses of a cuttlefish to predators were as follows: first, the cuttlefish tried to escape at the sight of the predator (string net) (no inking) (**Figure 5A**); second, their body transformed to frighten or warn the predator (**Figure 5B**); then, the cuttlefish would flee covered by inking after the threat failed (**Figure 5C**); the cuttlefish hovered on the surface (**Figure 5D**) or sunk to the bottom and hid after continuous jetting/inking (**Figure 5E**). The body pattern changes before and after stimuli were classified as follows: the body pattern before stimuli exhibits chromatic (pale, pairs of mantle spots close to the head), textural (smooth skin), postural (flattened), and locomotor (bottom suction) features. The acts of warning or threats to the predator were expressed as a flattening of the body combined with various chromatic components such as eye spots, dark red mantle margin stripe, an iridescent mantle stripe around the fin, and an eye patch in response to a potential threat; the head and arms were flattened with exaggerated arms, the overall form was diamatic flare-like, and the pink fins were also very prominent. The animal fled covered by inking when encountering the predator, and the body pattern exhibited chromatic (pale, white square), textural (smooth skin), postural (streamlined extension), and locomotor (inking/jetting/escaping) features; the cuttlefish hovered on the surface or sunk to the bottom after releasing ink, and the body pattern exhibited chromatic (dark brown), textural (coarse skin), postural (bipod headstand/sitting), and locomotor (bottom suction/hovering) features. After the water condition changed (to fresh seawater), the body pattern exhibited chromatic (pale), textural (smooth skin), postural (flattened/curled arms), and locomotor (bottom suction) features (**Figure 5F**).

Enzyme Activity

The effects of treatment on the HK, PK, and SOD activities, as well as MDA content in the liver, gill, muscle, and brain tissues of *S. pharaonis* are shown in **Figure 6**.

Hexokinase activities were affected by ink release in all tissues ($p < 0.05$). The liver, gill, and muscle tissues showed a rapid increase in the treatment groups, becoming significantly higher than the control group. HK activities in gill and muscle tissues were not significantly different among treatment groups. However, HK activity in liver tissue after the second inking event was lower than on the first and fourth inking events.

The variation in PK activity was similar to that of HK activity. PK activities were higher in the treatment groups compared with the low values in the control group. In liver, gill, and muscle tissues PK activities were much higher after the first inking event than after the following events. PK activities were significantly higher in liver and muscle tissues when compared to those in gill tissue.

Superoxide dismutase activities in the liver, gill, and brain tissues rose with the number of treatments ($p < 0.05$). SOD activities in the liver and gill tissues were significantly higher after the fourth inking event than those after the first, second, and third events. In brain tissues, SOD activity was higher after the first and fourth inking events than after the other two events.

In gill tissue, MDA concentration did not change after treatment. Contrastingly, in the liver tissue, MDA concentration was higher after treatment than that in the control group and increased with each inking event, with the highest value after the

TABLE 3 | Behavioral changes of *Sepia pharaonis* affected by inking treatment.

Items	First ink release	Second ink release	Third ink release
Jet frequency	72 ± 11	51 ± 7	28 ± 8
Properties of ink	Dense ink, gelatinous-like,not easy to disperse	Dense ink, slightly diffuse plume, easy to disperse	Smoke-like ink, dispersed quickly
Feeding activity	Food intake decreased, feed utilization about 50–70%	Food intake reduced to half of that of the control group	No difference in food utilization
Locomotor components	Bottom suction, hovering	Bottom suction, hovering	Bottom suction
Chromatic components	Dark brown, dark arms, iridescent blue mantle margin stripe, dark ventral mantle in abdomen	Dark brown, dark arms, wide mantle edge radial bands, dark ventral mantle in abdomen	Pale, iridescent blue mantle margin stripe, pale ventral mantle in abdomen
Textural components	Coarse skin	Coarse skin	Smooth skin
Postural components	Bipod headstand/sitting	Bipod headstand/sitting	Flattened body/sitting
Eye spots	Absent	Absent	Present
Eye ring	Absent	Absent	Present
Dark square	Present	Present	Absent
Fin movement	Absent	Present	Present

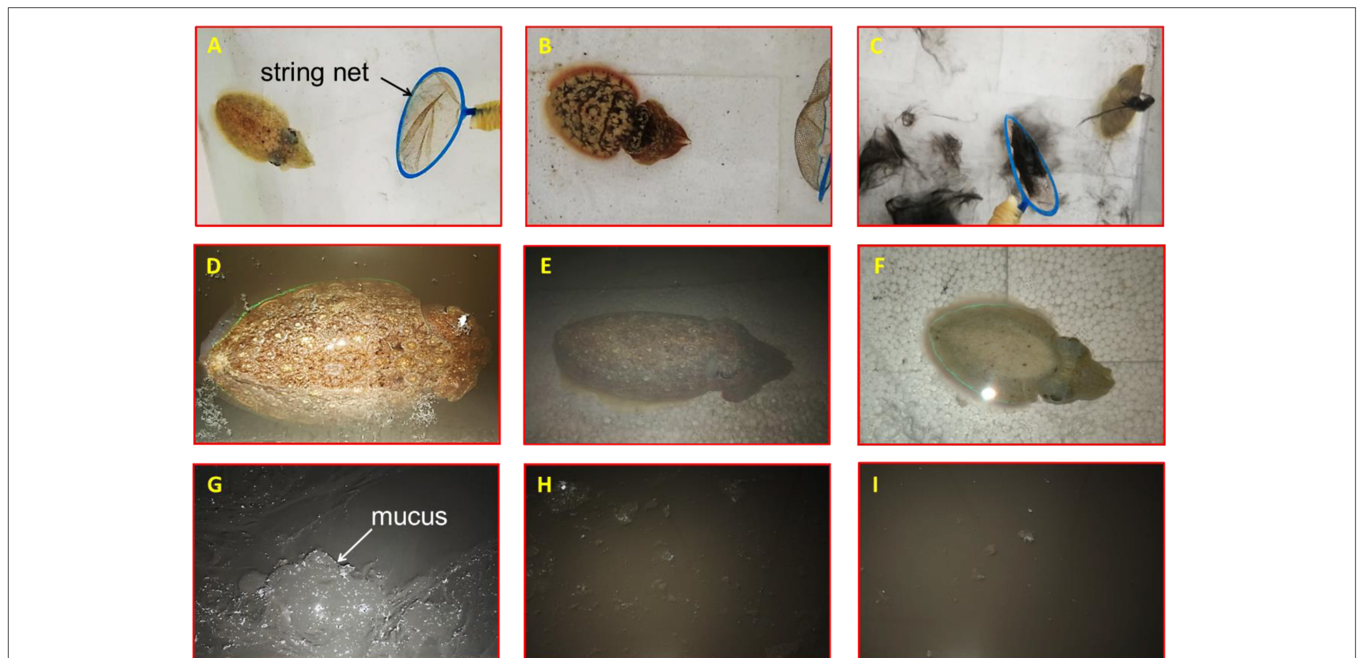

FIGURE 5 | Effect of released ink to behavioral responses of the cuttlefish *Sepia pharaonic*: **(A)** cuttlefish tried to escape at the sight of the predator; **(B)** body transformed to frighten or warn the predator; **(C)** cuttlefish fled covered by inking; **(D)** cuttlefish hovered on the surface; **(E)** cuttlefish sunk to the bottom and hid after continuous jetting/inking; **(F)** body pattern exhibited chromatic, textural, postural, and locomotor features after water condition changed. Properties of released ink: **(G)** dense ink, a gelatinous-like substance with a large amount of mucus, not easy to disperse; **(H)** dense ink, slightly diffuse plume with a small amount of mucus, easy to disperse; **(I)** smoke-like ink, almost no mucus that dispersed quickly.

fourth event. MDA concentration remained stable after treatment in the brain tissue and was higher than that of the control group.

Gene Expression

The expression of HSP90 was evaluated in liver, gill, and muscle tissues (**Figure 7**). In liver and muscle tissues, the expression of HSP90 was upregulated after the first and fourth inking events compared to that after the third event. The expression of HSP90 in gill tissues was not different between first, second, and third day of treatment but were much higher at the eighth day of treatment. The highest expression was found in the liver tissues after the fourth inking event.

The expression of AK in liver, gill, and muscle tissues were significantly ($p < 0.05$) upregulated in treatments compared to the control group. AK expression was about two times higher than that of the control group in all treatments. In the liver and muscle, AK expression was upregulated the most after the first inking event. In gill tissues, it was the highest after the first and second inking events.

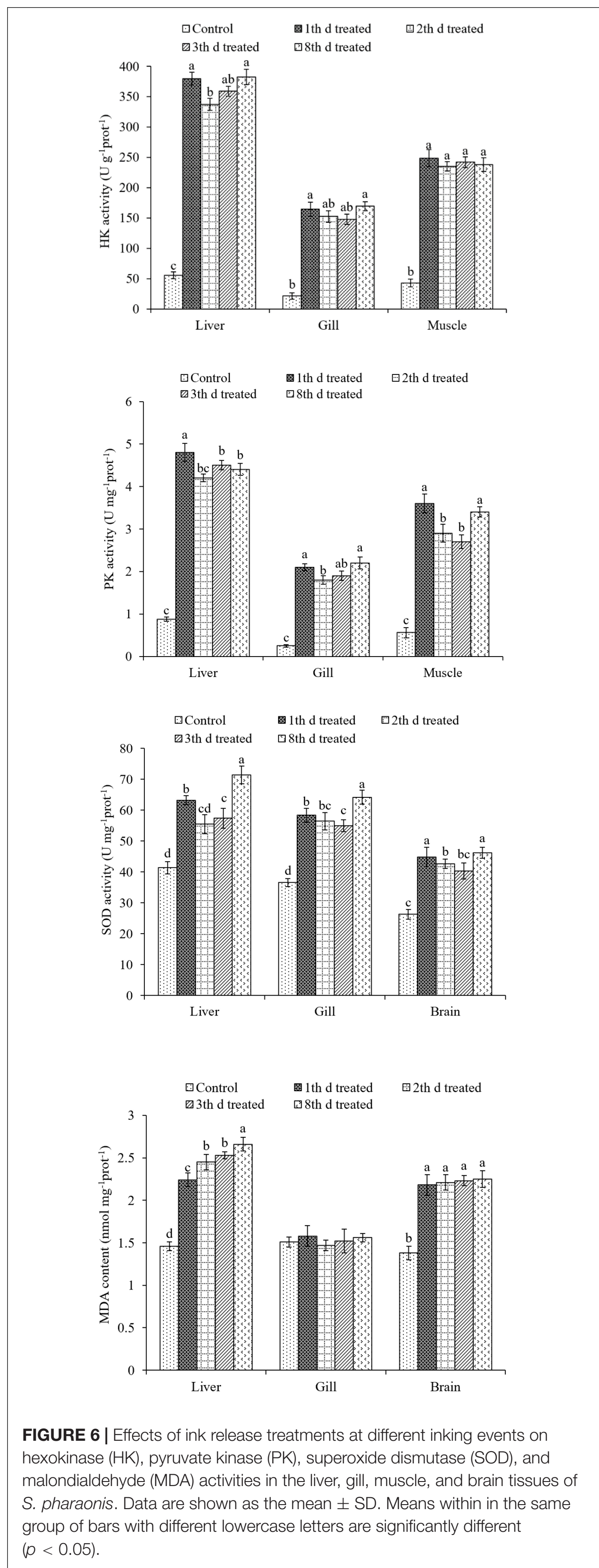

FIGURE 6 | Effects of ink release treatments at different inking events on hexokinase (HK), pyruvate kinase (PK), superoxide dismutase (SOD), and malondialdehyde (MDA) activities in the liver, gill, muscle, and brain tissues of *S. pharaonis*. Data are shown as the mean ± SD. Means within in the same group of bars with different lowercase letters are significantly different ($p < 0.05$).

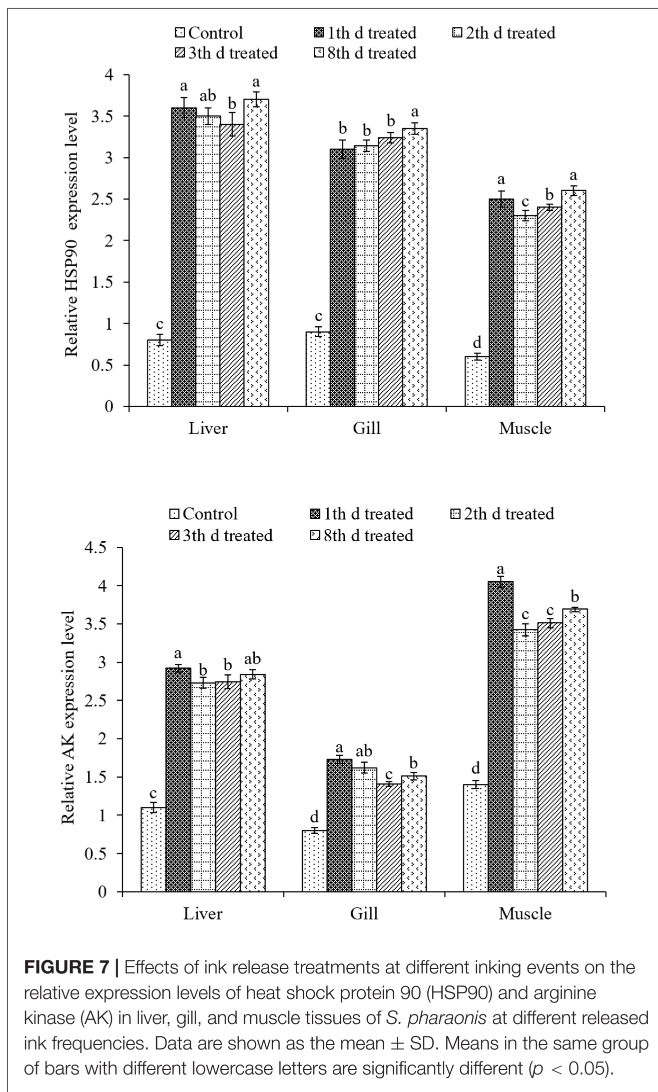

FIGURE 7 | Effects of ink release treatments at different inking events on the relative expression levels of heat shock protein 90 (HSP90) and arginine kinase (AK) in liver, gill, and muscle tissues of *S. pharaonis* at different released ink frequencies. Data are shown as the mean ± SD. Means in the same group of bars with different lowercase letters are significantly different ($p < 0.05$).

DISCUSSION

To evaluate biological and biochemical responses of cuttlefish to ink release, we started with the following two aspects: (1) verified the characteristics of the ink solution and determined the relationship between released ink frequency and weight and (2) clarified the behavioral and physiological effects of continuous inking on cuttlefish.

Predation is a constant risk for most animals. To maximize survival, preys have developed a wide repertoire of defenses from aggressive predators including behavioral displays, physical armor, and toxic chemicals (Bryan et al., 1997; Lenzi-Mattos et al., 2005). In many animal species, body patterns play an important role in predator–prey interactions, such as camouflage, aposematism, crypsis, and visual threats (Endler, 1978; Hay, 2009). Cephalopods such as cuttlefishes, squids, and octopods can not only rapidly change their body color and texture but also exhibit a variety of visually complex appearances. These body patterns include a combination of chromatic, textural,

postural, and locomotor components used for camouflage and communication (Hanlon and Messenger, 1988). This study found a series of behavioral changes in cuttlefish *S. pharaonis* while encountering predators. The cuttlefish first displayed defensive behavior against the predator and then fled but without inking or jetting. After increasing the threat proximity, cuttlefish showed a warning or threat to the predator and escapes were accompanied by ink release when an attack was imminent. Finally, the animal sank to the bottom and hid. A total of 53 chromatic and 4 uniform colors, 3 textural, 11 postural, and 9 locomotor components have been previously identified and described in detail for *S. pharaonis* (Nakajima and Ikeda, 2017). In the present study, six chromatic (eyespots, head crown, eye patch, white spots, iridescent ventral mantle, and paired mantle spots close to head), two uniform colors (pale and dark brown), two textural (smooth skin and coarse skin), five postural (flattened, streamlined extension, bipod headstand, sitting and curled arms), and five locomotor (bottom suction, inking, jetting, escaping, and hovering) components were recorded when subjected to potential predators.

To expand our understanding of ink release mechanisms of the cuttlefish *S. pharaonis*, such as continuous ink release capability and behavior, the effect of continuous jetting/inking on the organism, such as biochemical changes and the ink synthesis rate, were investigated. Cuttlefish mortalities from first to third inking events were 4.4% (16/362), 3.7% (13/346), and 4.5% (15/333) (**Figure 4**), respectively, and the weight of released ink decreased significantly along with the increase in ink release frequency. The exponential function indicated that a cuttlefish has the capability to continuously releasing ink, but the weight of released ink dropped sharply. Results showed that ∼90% of the ink in the ink sac was released (**Figure 3A** and **Table 2**). According to the jet frequency, the frequencies of the first three events were 72 ± 11, 51 ± 7, and 28 ± 8, respectively. The higher will be the jet frequency, the more energy will be consumed. Jet frequency decreased with decreasing weight of released ink and the times required to resume swimming after events 1-3 were 6 ± 1 min, 3 ± 1 min, and 3 ± 1 min, respectively. Cuttlefish food intake also decreased after ink release, and it took 2-3 days to recover feeding after three inking events. Gallardo et al. (2017) observed that continuously releasing ink may affect the digestion and utilization of prey in octopods. In our study, cuttlefish required time to "recuperate" to save ink after continuous ink release. After three inking events, 90% of the ink was released, taking ∼30 days for the ink sac to refill. However, the cuttlefish re-release of ink on the 5th and 10th day of recovery resulted in high mortality (98-100%) because continuous burst swimming consumes considerable energy, and over-exploitation probably resulted in serious physiological damage. In nature, the frequency of ink release and the weight of released ink can be adjusted in response to the stress characteristics (e.g., the strength and duration of the predator). Consequently, cuttlefish should be kept away from discomfort environments (such as handling, feeding competition, and rapid temperature and salinity changes) during culture, reducing the possible physiological damage caused by continuous inking. Furthermore, the released ink characteristics were different (i.e., gelatinous mass, slightly diffuse plume, and smoke-like ink), so the defensive effects also varied (Shimek, 1983; Bush and Robison, 2007). Therefore, a short-term ink release could act as a defensive behavior against a predator, but a "powerful" predator forces the animal to continuously release dense ink, which can also cause damage to itself.

Ink release not only requires substantial energy but also consumes considerable oxygen. The liver and gills are the main organs of the cuttlefish, accounting for 35-42 and 18-23% of their visceral mass (n = 126), respectively. In addition, liver physiology and biochemical indices can reflect the nutritional and physiological status of aquatic animals (Fontagne et al., 1998; Wang L.N. et al., 2014). Amino acids and carbohydrates are the preferred fuels for cephalopods (Storey and Storey, 1983; Agnisola et al., 1991; Hochachka, 1995; Speers-Roesch et al., 2016), and cuttlefish present well-developed glycolytic capacity in all tissues. After burst swimming, tissue metabolism was similar to that generally seen in starved cuttlefish, and carbohydrates are important fuels in cephalopod muscle (Speers-Roesch et al., 2016). HK and PK enzymes play major roles in the nutritional regulation of glycolytic pathways (Panserat et al., 2001). In this study, HK and PK activities after ink release were significantly higher than those in the control group. The liver and muscle, which fuel locomotion, had high HK and PK activities that geared them toward rapid mobilization of stored glycogen to sustain anaerobic burst swimming. However, the liver relies more on aerobic glucose, as indicated by higher HK and PK activities compared with muscle and gill tissues. Energy was drastically consumed after continuously releasing ink, resulting in death, and the mortality from re-releasing ink was 98-100% on the 5th and 10th day of culture. As the first line of defense, SOD plays pivotal roles in the elimination of reactive oxygen species (ROS) (Kim et al., 2011), and it is thought to protect muscular oxidative stress caused by exercise (Atig et al., 2012). MDA is a peroxidation product of lipids and indirectly reflects the impact degree of ROS on membrane lipid peroxidation (Liu et al., 2013). SOD activity in the treatment groups was higher than that in the control group in the liver, gill, and brain tissues. Especially after the fourth inking event, SOD activity was higher than that from other treatment groups, indicating that continuous ink release could damage the liver and brain tissues. The continuous release of dense ink leads to an increase in the production of active oxygen. To maintain the balance of oxygen free radicals *in vivo*, SOD activity increased to eliminate reactive oxygen radicals, thereby reducing the oxygen free radicals on the biofilm, resulting in an MDA content reduction in the body. Ink release induced the formation of antioxidant enzymes. However, excessive ROS can still cause an increase in MDA content. The increase in MDA levels may be due to the inability of enzymes to prevent extremely high levels of ROS from causing damage to the body (Bagnyukova et al., 2006). Moreover, MDA concentration in the liver tissue significantly increased with each inking event and was higher than that of the control group. In conclusion, liver was affected by continuous ink release.

Heat shock proteins function as molecular chaperones and play essential roles in the immunity of organisms, particularly concerning environmental stress, such as thermal, salinity, and crowding stress (Wang Q.L. et al., 2014; Xu et al., 2014; Galt et al., 2018). In this study, the expression of HSP90 was upregulated in all treatments compared to that in the control group, including liver, gill, and muscle tissues. Interestingly, the

expression of HSP90 was more upregulated in liver and gill tissues than in muscle tissues. Increased HSPs in the muscles may facilitate mitochondrial biogenesis and the folding of nuclear gene-encoded proteins into mitochondria. Various studies have revealed that mitochondrial biogenesis is an adaptive response to related stress in the muscles of fish (Tyler and Sidell, 1984; Egginton and Sidell, 1989; Orczewska et al., 2010). Moreover, HSP90 can bind to protein kinases, steroid receptors, actin, tubulin, and other substances in the cells, maintain protein structure, and deliver signals among cells (Pratt, 1997; Csermely et al., 1998). Gao et al. (2008) suggested that the stimulated increase in HSP90 expression level was one of the organisms' protective approaches against further toxicity.

Previous studies have shown that energy metabolism-related enzymes played an important role in the stress response of invertebrates such as AK (EC 2.7.3.3) (Abe et al., 2007; Yin et al., 2018), which has been found to be closely involved in adaptation to environmental stresses, e.g., pH, salinity, and heavy metal ions, in shrimp, crabs, and cuttlefish (Kinsey and Lee, 2003; Morris et al., 2005; Silvestre et al., 2006; Yin et al., 2018). In this regard, AK in marine invertebrates is distinctively found to be associated with adapting to the environmental disturbances caused by physical and chemical factors (Kinsey and Lee, 2003; Morris et al., 2005). Indeed, AK expression was upregulated in tissues, especially in muscles, which showed higher expression than other tissues. It has been generally recognized that AK plays a pivotal role in ATP buffering in invertebrates under both long-term and extreme conditions, when muscle and nerve cells require immediate and highly fluctuating energy demands via catalyzing Mg^{2+} cofactor-dependent phosphoryl transfer (Shofer et al., 1997; Voncken et al., 2013). Furthermore, expression screening and annotation of the ink sac cDNA library indicated that the main related unigenes were related to carbohydrate, amino acid, and energy metabolism, as well as cell motility (Song et al., 2012).

In summary, the study showed that cuttlefish can continuously release ink within a short period, releasing ~90% of the ink from the ink sac, but re-releasing ink during the recovery period was prone to cause exhaustion and death. Moreover, the cuttlefish adapted well to these changes during culture and took as long as 30 days to recover. Profiles of biochemical indicators indicated that continuously releasing ink damaged tissues, especially the liver tissue. Understanding the stress response to stimulus of cuttlefish is critical for optimizing production and maintaining health and welfare. Although cuttlefish can continuously release ink in to defend against predators, this defensive behavior can result in physiological damage and death. However, their intrinsic link is still not clearly understood. Therefore, further study may focus on metabolomics and the relationship between metabolites and physiological and pathological changes after ink release.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

All procedures with live animals included in this study have been approved by the Animal Research Ethics Committee of the Chinese Academy of Fishery Sciences. Research on live cephalopods is now regulated in the Member States of the European Union by the Directive 2010/63/EU. The authors are aware of the general principles stated by the Directive for the use of live cephalopod mollusks in scientific research as pointed out in several studies (Smith et al., 2013; Fiorito et al., 2015).

AUTHOR CONTRIBUTIONS

MJ, WS, and XJ conceived and designed the experiments. CZ and RY ran the experiments. JL and RP sampled animals and performed RNA extractions. MJ and WS wrote the manuscript. QH revised the manuscript. XJ provided funding and helped in discussing the results.

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Significance Assessment of *Amphora coffeaeformis* in Arsenic-Induced Hemato-Biochemical Alterations of African Catfish (*Clarias gariepinus*)

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Heavy metals have different adverse impacts on different life stages of fish species with attempts to use natural antioxidants to counteract their effects. So, the present study investigated the potential protective effects of *Amphora coffeaeformis* extract against arsenic-induced hemato-biochemical alterations in African catfish, *Clarias gariepinus*. The fish exposed to sub-lethal concentrations of arsenic; 19.2 and 38.3 mg/L (1/8 and 1/4 of 96h-LC_{50} value, 153.17 mg/L) for 15 days. The main effect of arsenic was recorded in some blood parameters such as RBC's count, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, and white blood cells. As for biochemical parameters, the main effect of arsenic was significant for alkaline phosphatase, glucose, uric acid, creatinine, albumin, globulin, and albumin/globulin. Also, the residue of arsenic in fish muscles showed significant effects. The majority of these arsenic-induced parameters were improved with dietary supplements of the diatom *A. coffeaeformis*. So, *Amphora* extract can be used as detoxification factor on fishes induced by arsenic due to its biologically active components providing protections like antioxidant, antiviral, antibacterial, and anti-inflammatory. Besides, they have excellent contents of proteins and carbohydrates which are supposed to enhance the effect of these compounds.

Keywords: arsenic, *Clarias*, *Amphora coffeaeformis*, natural product, secondary metabolite, biochemistry, detoxification

INTRODUCTION

Different heavy metals including Arsenic, Cadmium, Lead, Silver, and Mercury were found to be toxic to human beings, animals, and fishes with variability in doses and environmental factors (Govind and Madhuri, 2014). Arsenic is one of the most hazardous heavy metals released in the environment as a result of both natural and anthropogenic processes (Garelick et al., 2008). In nature, arsenic exists both in organic and inorganic forms; the inorganic form with trivalent arsenite or pentavalent arsenate are more toxic than organic forms (Oremland and Stolz, 2005). It became evident that the dispersal of arsenic-rich wastes generated by human activities leads to the water pollution and in turn, increased chronic arsenic poisoning of aquatic animals (Rahman et al., 2012)

especially fish juveniles with a reduction in survival and growth of their populations (Erickson et al., 2011). These adverse impacts may lead to the removal of entire fish populations in polluted aquatic ecosystems (Khayatzadeh and Abbasi, 2010). Consequently, accumulation of arsenic at high concentration in fishes may lead to serious health risks for humans, causing cancer and neurological disturbances (Kapaj et al., 2006; Avigliano et al., 2015). To understand the toxicity of arsenic compounds, most studies were performed in mammalian cells (García-Esquinas et al., 2013; Selvaraj et al., 2013). However, studies on arsenic toxicity to aquatic animal species, including fish, are rare. These studies stated that exposure to arsenic led to various hematological and biochemical alterations in fishes (Sayed et al., 2015a; Singh and Srivastava, 2015; Ghaffar et al., 2016). Arsenic was found to promote apoptotic and necrotic mediated cell death in fishes according to variations in arsenic concentration and exposure time (Sayed et al., 2015a). It also leads to DNA fragmentation, alteration in mitochondrial membrane potential and formation of increased reactive oxygen species (Selvaraj et al., 2013).

And because the pollution has already become very widespread, toxicity prevention is not on; and timely mitigation is the possible solution for minimization of the environmental pollutants and their impacts by using plants and algae as the most desirable mitigation technics (Ullah et al., 2015; Mahar et al., 2016; Kumar, 2018). The algae proved to be effective in the hyperaccumulation of heavy metals as well as degradation of xenobiotics (Suresh and Ravishankar, 2004). The microalgae including *Amphora*, could be a source of a diverse class of bioactive compounds, especially the carotenoids (canthaxanthin and astaxanthin), polyunsaturated fatty acids, sulfated polysaccharides, β-glucans, and vitamins E and C, which are well-documented as bioactive compounds (Lee et al., 2009; El-Sayed et al., 2018). In addition, many researchers are interested in finding any natural antioxidants having safety and effectiveness, which can be substituted for current and commercial synthetic antioxidants (Taghvaei and Jafari, 2015; Kumosani et al., 2017; Glodde et al., 2018). Benthic diatoms as microalgae were considered as natural antioxidants (Lee et al., 2008; El-Sayed et al., 2018). Diatoms such as *Amphora* possess high metal absorption capacity and heavy high multiplication rate (Anantharaj et al., 2011) and are able to activate a definite set of biochemical and physiological processes to resist the toxic action of environmental contaminants (Gaur and Rai, 2001). Such characteristics of diatom have encouraged the application of their extract in detoxification for stress-induced fishes as well as protective and antioxidant agents (Sheikhzadeh et al., 2012) in addition to their antibacterial (Choudhury et al., 2005; Manzoor et al., 2013), antiviral (Abdel-Wahab, 2018), and anti-inflammatory factors (Lauritano et al., 2016).

Marine diatoms developed on substrate could be utilized as feed supplements in enhancing the development and survival of aquaculture species (Khatoon et al., 2009). *Amphora* sp. is regularly used as primary food for larvae of highly valued and praised seafood such as *Crassostrea gigas* (Pacific oyster), *Penaeus semisulcatus* (green tiger shrimp), *Placopecten magellanicus* (sea scallop), *Crepidula onyx* (limpet), and *Haliotis* sp. (abalone) (Daume et al., 2000; Al-Maslamani et al., 2007; Chiu et al., 2007). Recent results indicated that *Amphora* supplement is promising as an alternative method to antibiotics for disease prevention in Nile tilapia culture (Ayoub et al., 2019). So, the current investigation is aimed to study the supplementation of *Amphora* extract and whether it detoxifies and protects *C. gariepinus* from arsenic exposure.

MATERIALS AND METHODS

Fish Collection

Ninety healthy fish of the Nile catfish, *Clarias gariepinus* (154.75 ± 146.9 g weight, 30.87 ± 9 cm length) were purchased from a private farm at Assiut, Egypt. Fishes immediately were transported to the Fish Biology and Pollution laboratory at the Department of Zoology, Faculty of Science, Assiut University. The experimental fishes were reared in aerated glass tanks (100 L capacity) and acclimatized for 2 weeks before being used in the experimental study. The experimental fish fed on commercial pellets (3% of fish weight) twice daily. Feces and residual food were aspirated regularly. The water temperature, pH, and dissolved oxygen concentrations were measured daily as 28.8 ± 3°C, 7.6 ± 0.34, and 3.32 ± 4.5 mg/L respectively (light cycle was 12 h light and 12 h dark).

Amphora coffeaeformis Extract Preparation

The extract of *Amphora coffeaeformis* was purchased from National Research Center, Cairo, Egypt. *Amphora* extract was sent to the Analytical Chemistry Unit at Assiut University for GC/MS analysis. The results of GC-MS analysis indicated the presence of 51 different compounds (**Table 1** and **Figure 1**). Some of these compounds were identified according to literature (Silva et al., 2014; Salahuddin et al., 2017; El-Sayed et al., 2018) to be biologically active components such as 2,6-Dimethyl-4[3H]-quinazolinone (anticancer heterocyclic compound), Neophytadiene (Terpene; antiviral activity), Phytol (Diterpene; anti-inflammatory activity) and Hexadecanoic acid (fatty acid; antioxidant). The concentrations of total protein (24.25 g/kg) and total carbohydrate (17.92 g/kg) were estimated by using UV-VIS Double Beam Labomed, Inc. PC Scanning Spectrophotometer (Model UVD-2950) while the concentration of total lipid (56.4 g/kg) was estimated by Bligh and Dyer's acidic extraction method (Bligh and Dyer, 1959).

Experimental Design

Fishes were weighed, measured and classified randomly into nine groups (10 fish/tank) according to two concentrations of arsenic (AS1, AS2), two concentrations of amphora (AM1, AM2) and their combinations (**Table 2**). Exposure was continuous for 2 weeks and water was changed daily to prevent deterioration of water quality and replenish arsenic levels. Sodium arsenate ($Na_2HAsO_4 \cdot 7H_2O$) of 98% purity was purchased from Qualikemes company, India. A stock solution of sodium arsenate was prepared and stored in clean glass bottles.

TABLE 1 | Gas chromatography mass spectrometry activity of *Amphora coffeaeformis* extract.

No.	RT (min)	Compound name	% of total	Molecular weight
1	4.238	Benzene (hydrocarbon)	1.857	78.047
2	24.615	Heptadecane (hydrocarbon)	2.393	240.282
3	4.279	Tert-amyl chloride (chlorinated hydrocarbon)	4.482	106.055
4	4.953	Bromodichloro-methane (hydrocarbon derivatives)	0.355	161.864
5	12.568	1,2-*Bis*(methoxy)-3-chloropropane (hydrocarbon derivatives)	0.159	138.045
6	23.001	Hexadecane (hydrocarbon)	0.500	226.266
7	40.778	Eicosane (hydrocarbon)	0.633	282.329
8	18.734	1,1′-Biphenyl (organic compound)	0.251	154.078
9	4.419	Trichloromethane (organic compound)	0.178	117.914
10	24.819	1H-Pyrrolo[1,2-a][1,4]diazepine-1,5(2H)-dione, hexahydro-,(S)-(9Cl) (organic compound)	1.090	168.09
11	25.653	(−)-Loliolide (organic compound)	2.067	196.11
12	27.85	Dibutyl phthalate (organic compound)	2.536	278.152
13	38.546	Octadecyl 2,2,2-trichloroethyl carbonic acid ester (organic acid)	1.338	444.196
14	30.98	Benzyl 1-naphthyl ether (organic compound)	0.651	234.104
15	36.465	2-(Tetradecyloxy)-ethanol (organic compound)	0.356	258.256
16	37.042	*Bis*(2-ethylhexyl) phthalate (organic compound)	0.861	390.277
17	37.112	1,1,3-Trichloro-2-propanone (ketone)	0.416	159.925
18	26.51	6,10,14-Trimethyl-2-pentadecanone (ketone)	1.151	268.277
19	5.427	3-Hydroxy-3-methyl-2-butanone (ketone)	0.438	102.068
20	7.654	2-Methyl-4-pentene-2-ol (alcohol)	0.415	100.089
21	26.964	3,7,11,15-Tetramethyl-2-hexadecen-1-ol (alcohol)	0.794	296.308
22	7.776	2-Hexanol (alcohol)	12.927	102.104
23	4.547	3-Penten-2-ol (alcohol)	1.881	86.073
24	9.006	Iso-valeric acid (fatty acid)	0.797	102.068
25	25.495	Tetradecanoic acid (fatty acid)	4.497	228.209
26	26.737	Pentadecanoic acid (fatty acid)	1.925	242.225
27	30.549	Oleic acid (fatty acid)	3.138	282.256
28	32.933	15-Hydroxypentadecanoic acid (fatty acid)	0.253	258.219
29	38.371	9-Octadecenoic acid (fatty acid)	0.535	504.491
30	36.855	Oleic acid (fatty acid)	1.318	282.256
31	27.996	Hexadecanoic acid (fatty acid)	13.426	256.24
32	29.948	Phytol (diterpene, anti-inflammatory activity)	1.698	296.308
33	26.445	Neophytadiene (terpene)(antiviral activity)	0.771	278.297
34	21.812	Dihydroactinidiolide (terpene)	0.314	180.115
35	24.423	8-Heptadecene (alkene)	2.357	238.266
36	26.276	(2Z)-3-Methyl-2-decene (alkene)	1.739	154.172
37	36.372	1-Nonadecene (alkene)	0.799	266.297
38	36.704	1-Docosene (alkene)	0.287	308.344
39	16.397	2-Methoxy-4-aminophenol (phenols)	0.231	139.063
40	26.638	3,5-dimethoxy-Phenol (phenols)	0.429	154.063
41	25.227	4-Methoxy-3-methyl- 6-benzofuranol (antimicrobial compound)	1.140	178.063
42	27.477	3-Isobutylhexahydropyrrolo[1,2-a]pyrazine-1,4-dione (antimalarial agent)	1.796	210.137
43	4.804	2,6-Dimethyl-4[3H]-quinazolinone (heterocyclic compound)(anticancer)	7.437	171.852
44	27.757	(+−)-15-Hexadecanolide (antioxidant)	12.529	254.225
45	44.106	Cholesterin (sterol)	0.556	386.355
46	43.401	(3.beta.,22Z)-27-Norergosta-5,22-dien-3-ol (sterol)	0.967	384.339
47	44.881	Ergosta-5,22-dien-3.beta.-ol (sterol)	0.729	398.355
48	46.887	Stigmasterol (sterol)	0.996	412.371
49	30.473	8-(2-Octylcyclopropyl)octanal (aldehyde)	0.733	280.277
50	4.646	Isoprene hydrochloride (isoprene)	0.118	117.914
51	27.605	14S,20R-Velbanamine (alkaloid)	0.372	136.125

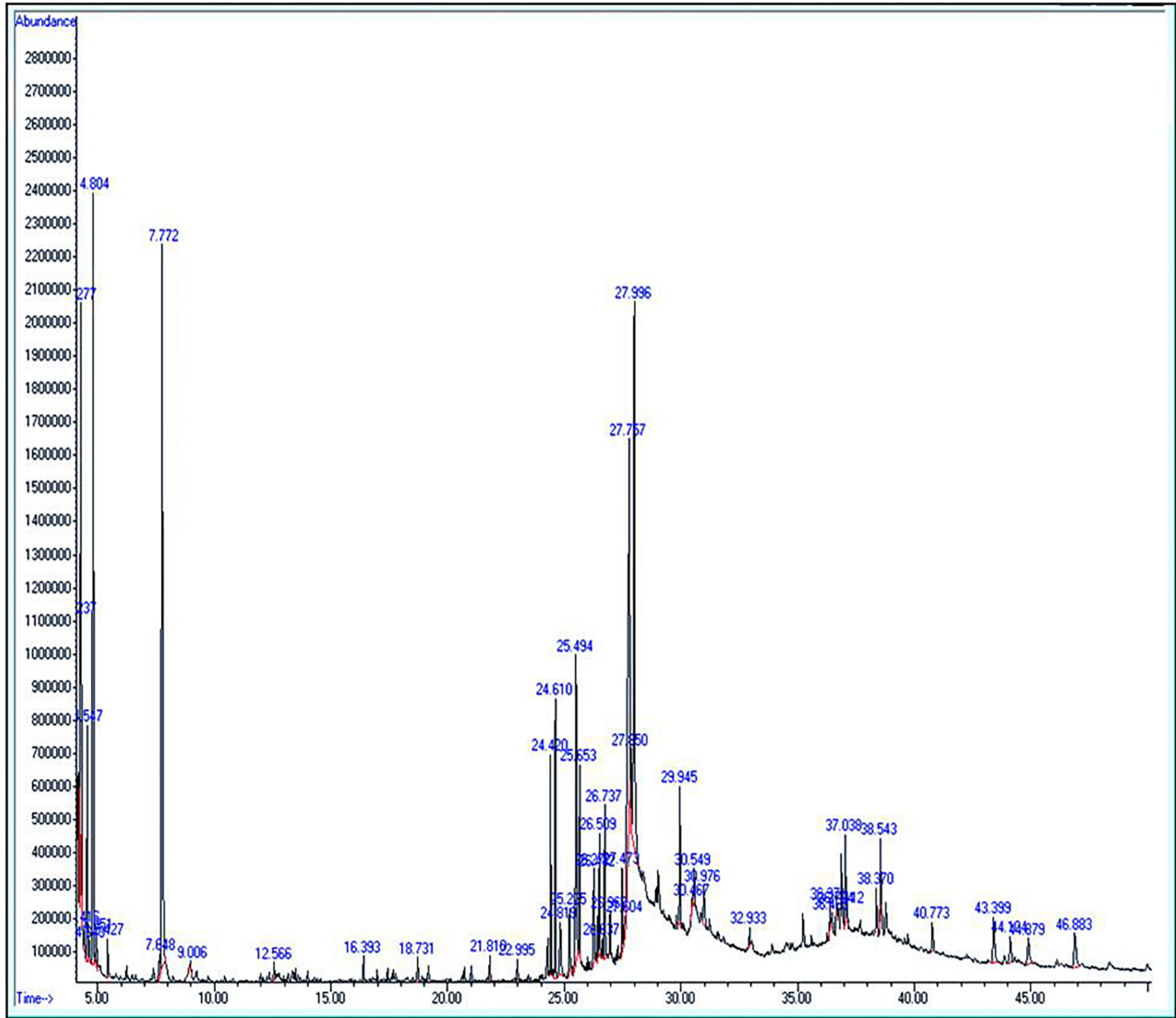

FIGURE 1 | Gas chromatography mass spectrometry profile for *Amphora coffeaeformis*.

TABLE 2 | The fish groups exposed to arsenic (AS1, AS2) concentrations, amphora (AM1, AM2) percentages and their combinations.

Treatments	Control	AS1	AS2	AM1	AM2	AS1+AM1	AS1+AM2	AS2+AM1	AS2+AM2
Arsenic (mg/L)	0	19.2	38.3	0	0	19.2	19.2	38.3	38.3
Amphora (%)	0	0	0	7	10	7	10	7	10

Three concentrations of sodium arsenate were used: zero and two sub-lethal concentrations of 19.2 and 38.3 mg/L (1/8 and 1/4 of 96h-LC_{50} value, 153.17 mg/L) (Abdel-Hameid, 2009). The arsenic solution was added to the water directly while the amphora extract was mixed with fish feed. This protocol and experimental design were reviewed and approved by the Committee of the Faculty of Science of Assiut University with respect to scientific content and compliance with applicable research.

Behavioral Assessments

The behavioral changes were recorded by observing the feeding activity, the fish equilibrium in the water beside the changes in the skin and the fins. The mortality rate was also recorded in the arsenic-exposed fishes.

Hemato-Biochemical Parameters

Two blood samples of the peripheral blood were collected from cardiac puncture. For hematological analysis, samples were freshly collected in small plastic tubes containing heparin solution (0.2 mL/mL blood) as anticoagulant. For biochemical analysis, samples were left in small plastic tubes to coagulate for 15–20 min at 4°C prior then centrifugation for 20 min at 3,000 rpm to separate serum that was used for the analysis. The red (RBCs) and white (WBCs) blood

cell counts, hematocrit (HCT), and hemoglobin (HB) were estimated by using automated technical analyzer (BCC-3000B-Dirui Company). Mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) were calculated using the formulae mentioned by Dacie and Lewis (1991). Aspartic amino transferase (AST), alanine amino transferase (ALT), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), glucose (GL), total protein (TP), albumin (AL), globulin (GLO), creatinine (CR), and uric acid (UA) were determined by kits of HUMAN Company, Germany.

Arsenic Residues

Tissue analyses were done according to Shaw et al. (2012) with minor variations. Muscle samples were weighed (approximately 1.0 g), dried (50°C for 48 h in the oven) and then digested in 5 mL concentrated nitric acid at 50°C in the oven until evaporating the nitric acid and become the mixture 1 mL approximately. The mixture was cooled, diluted to 10 mL using ultrapure deionized water and then filtered. The arsenic residues in the muscles were measured by iCAP 6200 Emission spectrometer in The Central Laboratory, Faculty of Agriculture, Assiut University.

Statistical Analysis

The means, standard errors and ranges of the parameters in concern were estimated. Levene's test of equality of error variance of the parameters was applied with a wide range of variability. So, the homogeneity of variance was assumed for raw data. The pattern of variations in the variables studies was considered on the bases of arsenic and amphora concentrations and their interaction by two-way ANOVA. Moreover, in the absence of interactions, the pattern of variations was recorded by one-way ANOVA in all treatments and control group. The Tukey-HSD test was applied for multiple comparisons. The IBM-SPSS package version 22 (Spss for Windows, 2013) was used at 0.05-level of significance. The relationships between different treatments versus the control group as root group were postulated in dendrograms using R-packages (R Core Team, 2013) and Mesquites package (Maddison and Maddison, 2018) using the hematological and biochemical characters in raw form and ANOVA-based coded form (Rae and Buckley, 2009).

RESULTS

Behavioral Changes

After exposure to arsenic, most of the fishes exhibited loss of equilibrium which was more marked with increased concentration and duration. Reduction in the feeding activity, fins hemorrhage and skin alterations were also recorded in those samples exposed to arsenic. With increased duration, arsenic- exposed fishes showed signs of tiredness and gradually lost positive rheotaxis with excessive secretion of mucus. The mortality rate (60%) was recorded in aquaria with 38.3 mg/L arsenic. The dead fishes exhibited changes and abnormality in eyes, gills, gall bladder, spleen, and liver color. On the other hand, fish groups exposed to the same doses of arsenic in combination with amphora extract did not show such abnormal behavior and did not show any mortality. Moreover, arsenic-free fishes treated with amphora extract were noticed to have a healthy status.

Hemato-Biochemical Alterations

Under treatment conditions of the present work, the uric acid was found to be significantly correlated with ALP (0.453), glucose (0.424), total protein (0.524), and globulin (0.567), whereas glucose was significantly correlated with ALP (0.575). The albumin was significantly correlated with total protein (0.554) and creatinine (0.705). The WBCs was also significantly correlated with RBCs (0.886), HB (0.875), and HCT (0.809).

The hematological and biochemical parameters of the arsenic-induced *C. gariepinus* versus treatment with *Amphora* extract are given in **Tables 3**, **4**. The arsenic main effect was significant for RBCs, HCT, MCV, MCH, and WBCs whereas that of amphora was significant for HB and WBCs. There was no significant interaction between arsenic and amphora. As regards the biochemical parameters, the arsenic main effect was significant for ALP, glucose, uric acid, creatinine, albumin, globulin, and albumin/globulin. Also, arsenic residue in muscle showed significant effects by arsenic whereas that of Amphora was significant for ALT, glucose, uric acid, creatinine, LDH, albumin, globulin, and albumin/globulin. The arsenic amphora interaction was significant for ALP, glucose, uric acid, creatinine, LDH, albumin, globulin, albumin/globulin, and arsenic residue in muscles. The main effect of amphora recorded to be significant for glucose, uric acid, creatinine, albumin, globulin, albumin/globulin, and arsenic residue in muscles. The analysis revealed that a higher amphora dose was better than a lower one in counteracting arsenic impact.

Collecting all characters studied, all the treatments were clustered against the control using all the hematological and biochemical parameters relative to their SD units (**Figure 2**). These treatments are grouped into two main groups. One of these main groups included treatments with amphora only and the arsenic treatment combined with amphora reflecting the validity of amphora extracts as antioxidants. The other main group reflects a partial protective role of *Amphora* in counteracting the arsenic impacts. Clustering of the treatments based on the ANOVA-based coding of these parameters reflected another pattern of variation (**Figure 3**).

AS1 treatment was grouped with AS1 AM1 whereas AS2 was clustered with AS2 AM2 in the main group including all treatments with amphora. AS1 AM2 represents a single cluster. Such a pattern of clustering reflects the significance of *Amphora* protective and antioxidant effects in a collective manner including all characteristics coded on the basis of ANOVA.

DISCUSSION

The toxicity of arsenic highly variable within and between different fish species with respect to factors like age, sex, dose, exposure period and its organic and inorganic forms (Hallauer et al., 2016; Mahurpawar, 2017). These findings are evident with different pollutants and different fish species

TABLE 3 | Values of blood constituent parameters of *Clarias gariepinus* exposed to arsenic, amphora and their combinations.

Treatments/ Parameters	Control	As1	As2	Am1	Am2	As1+Am1	As1+Am2	As2+Am1	As2+Am2
RBC	2.69 ± 0.179 a	2.76 ± 0.12 a	2.22 ± 0.02 a	2.25 ± 0.07 a	2.23 ± 0.06 a	2.82 ± 0.09 a	2.32 ± 0.14 a	1.87 ± 0.55 a	2.01 ± 0.3 a
(million/mm^3)	(2.36–2.94)	(2.52–2.88)	(2.18–2.26)	(2.14–2.38)	(2.16–2.34)	(2.72–3)	(2.16–2.6)	(0.93–2.84)	(1.6–2.62)
HB	11.74 ± 0.77 a	10.07 ± 0.27 ab	9.07 ± 0.27 ab	8.6 ± 0.40 ab	8.8 ± 0.5 ab	10.33 ± 0.44 ab	8.53 ± 0.66 ab	7.37 ± 1.94 b	8.07 ± 1.12 ab
(g/dL)	(10.2–12.6)	(9.8–10.6)	(8.8–9.6)	(8.2–9.4)	(8.2–9.8)	(9.8–11.2)	(7.6–9.8)	(4.1–10.8)	(6.4–10.2)
HCT	48.13 ± 1.46 a	38.13 ± 0.74 ab	32.93 ± 1.99 ab	37.8 ± 3.08 ab	34.13 ± 0.77 ab	39.87 ± 2.27 ab	32 ± 1.62 ab	27.23 ± 8.63 b	30.67 ± 6.72 ab
(%)	(46.2–51)	(37.2–39.6)	(29–35.4)	(31.8–42)	(32.6–35)	(37.4–44.4)	(30–35.2)	(12.3–42.2)	(22.6–44)
MCV	180.37 ± 10.43 a	138.87 ± 4.97 b	160.43 ± 5.08 ab	167 ± 14.47 ab	153.27 ± 4.32 ab	141.27 ± 3.48 b	137.9 ± 2.24 b	142.67 ± 5.19 b	149.3 ± 9.3 ab
(fL)	(160.4–195.6)	(130.9–148)	(152.2–169.7)	(142.3–192.4)	(148.5–161.9)	(137.2–148.2)	(135–142.3)	(132.3–148.4)	(140–167.9)
MCH	43.7 ± 1.04 a	36.57 ± 1.42 b	40.83 ± 0.86 ab	37.9 ± 0.85 b	39.47 ± 1.27 ab	36.63 ± 0.44 b	36.73 ± 1.12 b	40.41 ± 1.89 ab	40.23 ± 0.85 ab
(Pg)	(42.2–45.7)	(34–38.9)	(39.6–42.5)	(36.6–39.5)	(37.6–41.9)	(35.8–37.3)	(34.5–38)	(38–44.13)	(38.9–41.8)
MCHC	24.37 ± 1.23 a	26.4 ± 1.01 a	25.53 ± 1.23 a	23 ± 1.85 a	25.8 ± 1.25 a	25.93 ± 0.43 a	26.57 ± 0.72 a	28.47 ± 2.43 a	27.13 ± 2.02 a
(g/L)	(22.1–26.3)	(24.7–28.2)	(23.8–27.9)	(19.5–25.8)	(24–28.2)	(25.2–26.7)	(25.3–27.8)	(25.6–33.3)	(23.2–29.9)
WBC	173.93 ± 18.35 a	158 ± 17.09 ab	108.4 ± 8.13 ab	95.87 ± 6.96 ab	95.73 ± 9.37 ab	146.73 ± 11.16 ab	109.87 ± 5.27 ab	83.1 ± 31.09 b	85 ± 27.65 b
(Thousands/mm^3)	(137.6–196.6)	(130.6–189.4)	(93.2–121)	(82–103.8)	(80.8–113)	(134.2–169)	(104.2–120.4)	(47.1–145)	(54.4–140.2)

The data are presented as Means ± SE (minimum–maximum). Different letters indicate significant difference at $p < 0.05$. As1 (19.2 mg/L Arsenic), As2 (38.3 mg/L Arsenic), Am1 (7% Amphora), Am2 (10% Amphora).

including *C. gariepinus* (Sayed and Authman, 2018; Mekkawy et al., 2019) and *Oreochromis niloticus* (Mekkawy et al., 2011a; Sayed et al., 2015b). The adverse impacts of these pollutants include behavioral, hematological, and biochemical characteristics (Lavanya et al., 2011; Mekkawy et al., 2013).

In the present study, *C. gariepinus* exhibited a lot of behavioral changes (loss of equilibrium, reduction in the feeding activity, fin hemorrhages, and skin alterations) due to exposure to sub-lethal concentrations of arsenic. Similar behavioral changes were observed in arsenic-treated fishes (Dwivedi and Trivedi, 2015; Mahurpawar, 2017). Similarly, other behavioral changes were observed by Baldissarelli et al. (2012) and Mekkawy et al. (2013) after exposure to arsenic and atrazine, respectively.

The excessive secretion of mucus which was observed especially at high doses of arsenic was probably due to skin arsenic-induced irritation and hence to protect the skin. Similar findings were recorded by Singh and Banerjee (2008). Impairments of the nervous system such as reflected in the form of swimming with the sides twisted 90 degrees and loss of equilibrium were also recorded in the present study under the stress of arsenic. Such impairments were postulated by Patro (2006) and Dwivedi and Trivedi (2015). Supplementation of *Amphora* to the arsenic-treated groups of *C. gariepinus* as well as untreated groups counteracted and improved the above behavioral changes to a great extent. These findings are suggested to be due to the multiple positive role of bioactive compounds of *A. coffeaeformis* working as antioxidant, antibacterial, antiviral, anti-fungal, and anti-inflammatory (Rajput and Mishra, 2012; Salahuddin et al., 2017; El-Sayed et al., 2018).

The alterations in RBC's, HB, and HCT observed in the present study may have resulted from the disorders in hemopoietic processes due to sodium arsenate toxicity. Heath (1987) and Abo-Hegab et al. (1993) interpreted the stress-induced decrease in the hemoglobin and hematocrit values in terms of heme dilution of blood and elimination of RBCs as well as disequilibrium of the osmotic pressure inside and outside the blood cell. Similar results were observed in fish intoxicated with arsenic, pesticides, SDS, lead, and silver nanoparticles (Mekkawy et al., 2013, 2019; Amsath, 2017; Sayed et al., 2017; Sayed and Authman, 2018). Arsenic-induced changes in MCV, MCH, and WBCs of current species were evident. Some other studies reported similar results with fluctuations in some fish such as *Clarias batrachus*, and *Catla catla* (Lavanya et al., 2011; Kumar and Banerjee, 2016).

Low white blood cells of *C. gariepinus* were observed in the present study after arsenic exposure. According to Kotsanis et al. (2000) and Datta et al. (2009), the decrease in white blood cell counts during acute and sub-lethal treatment by arsenic may be attributed to the damage of the kidney, which is the primary site of hematopoiesis and/or due to inhibition of white blood cell maturation due to arsenic stress.

The liver is the major organ involved in the regulation of metabolic functions and most of the biotransformation of inorganic arsenic takes place in the liver (Kumar and Banerjee, 2016; Kumari et al., 2017). So, analysis of serum AST and ALT were widely used to demonstrate arsenic induced-hepatotoxicity (Dorcas and Solomon, 2014). The alterations of AST and ALT reported in the present study may be due to the rapid death

TABLE 4 | Values of blood constituent parameters and arsenic residue in the muscle of *Clarias gariepinus* exposed to arsenic, amphora and their combinations.

Treatments/ Parameters	Control	As1	As2	Am1	Am2	As1+Am1	As1+Am2	As2+Am1	As2+Am2
AST (U/mL)	226.5 ± 35.82 a	284.4 ± 45.17 ab	384.5 ± 33.61 b	269.5 ± 10.69 ab	256.9 ± 18.93 a	275.3 ± 6.81 ab	242.3 ± 16.04 a	247.4 ± 24.63 a	239.3 ± 12.6 a
	(155.3–269.1)	(211–366.7)	(323.6–439.6)	(250.2–286.7)	(222.8–28.82)	(267.8–288.9)	(210.6–262.4)	(205.3–290.6)	(223.5–264.2)
ALT (U/mL)	34.63 ± 3.36 a	33.57 ± 7.18 a	40.03 ± 5.32 a	24.34 ± 2.01 a	41.18 ± 1.26 a	25.26 ± 2.26 a	31.71 ± 2.46 a	28.18 ± 4.31 a	27.75 ± 2.78 a
	(28.01–38.97)	(21.3–46.16)	(29.63–47.18)	(22.27–28.36)	(39.61–43.66)	(21.12–28.92)	(27.77–36.22)	(20.1–34.81)	(22.35–31.59)
ALP (U/L)	44.28 ± 8.72 a	17.52 ± 6.82 bc	3.19 ± 1.31 c	30.09 ± 3.96 ab	23.68 ± 1.14 abc	13.91 ± 0.87 bc	17.52 ± 2.54 bc	15.94 ± 4.6 bc	27.01 ± 8.01 abc
	(30.7–60.54)	(5.23–28.77)	(1.27–5.69)	(23.47–37.17)	(21.68–25.62)	(12.36–15.36)	(12.69–21.28)	(7.29–22.94)	(16.33–42.69)
Glucose (mg/dL)	71 ± 1.53 a	54.67 ± 2.67 abc	39 ± 7.37 cd	62.33 ± 6.84 ab	54 ± 3.06 abc	45.67 ± 6.89 bcd	38.33 ± 3.18 cd	27 ± 4.51 d	49.33 ± 1.2 abc
	(69–74)	(52–60)	(25–50)	(55–76)	(50–60)	(36–59)	(33–44)	(22–36)	(47–51)
Total protein (mg/dL)	4.04 ± 0.28 a	3.64 ± 0.03 ab	2.81 ± 0.22 bc	2.57 ± 0.12 c	3.66 ± 0.02 ab	3.23 ± 0.27 abc	3.43 ± 0.33 abc	3.65 ± 0.02 ab	2.87 ± 0.22 bc
	(3.63–4.57)	(3.6–3.69)	(2.37–3.1)	(2.36–2.77)	(3.63–3.69)	(2.77–3.69)	(2.77–3.83)	(3.63–3.69)	(2.57–3.3)
Uric acid (mg/dL)	0.88 ± 0.06 a	0.41 ± 0.06 b	0.43 ± 0.04 b	0.33 ± 0.01 b	0.49 ± 0.02 b	0.46 ± 0.05 b	0.4 ± 0.01 b	0.46 ± 0.02 b	0.36 ± 0.02 b
	(0.76–0.95)	(0.33–0.52)	(0.36–0.51)	(0.31–0.35)	(0.48–0.5)	(0.36–0.52)	(0.38–0.42)	(0.43–0.49)	(0.33–0.39)
Creatinine (mg/dL)	0.51 ± 0.04 a	0.71 ± 0.04 b	0.38 ± 0.02 ac	0.27 ± 0.06 c	0.35 ± 0.01 ac	0.53 ± 0.02 ab	0.43 ± 0 ac	0.43 ± 0.07 ac	0.38 ± 0.03 ac
	(0.45–0.59)	(0.65–0.8)	(0.34–0.41)	(0.16–0.33)	(0.32–0.37)	(0.5–0.56)	(0.42–0.43)	(0.32–0.57)	(0.32–0.42)
LDH (U/L)	2966.33 ± 483.88 ab	3618.67 ± 182.47 ab	4056.67 ± 430.35 b	2655.33 ± 870.37 ab	2546 ± 240.22 ab	2653.33 ± 654.53 ab	3459.67 ± 363.13 ab	3735.67 ± 112.17 ab	1601.33 ± 357.3 a
	(2055–3704)	(3306–3938)	(3269–4751)	(1417–4334)	(2108–2936)	(1420–3650)	(2970–4169)	(3622–3960)	(1100–2293)
Albumin (mg/dL)	1.27 ± 0.13 ad	1.69 ± 0.05 b	0.96 ± 0.03 ac	1.02 ± 0.09 ac	1.1 ± 0.12 ac	1.53 ± 0.02 bd	1.21 ± 0.07 ad	1.25 ± 0 ad	0.89 ± 0.05 c
	(1.04–1.47)	(1.6–1.75)	(0.92–1.01)	(0.85–1.16)	(0.87–1.25)	(1.5–1.58)	(1.1–1.33)	(1.25–1.26)	(0.83–0.97)
Globulin (mg/dL)	2.77 ± 0.17 a	2.07 ± 0.06 bcd	1.78 ± 0.17 cd	1.55 ± 0.03 d	2.47 ± 0.11 ab	1.51 ± 0.13 d	2.28 ± 0.21 abc	2.4 ± 0.02 ab	1.96 ± 0.14 bcd
	(2.59–3.1)	(2–2.19)	(1.45–1.95)	(1.51–1.61)	(2.34–2.69)	(1.27–1.71)	(1.85–2.5)	(2.38–2.44)	(1.74–2.22)
A/G	0.46 ± 0.03 a	0.86 ± 0.03 b	0.54 ± 0.04 a	0.66 ± 0.05 a	0.45 ± 0.06 a	1.02 ± 0.09 b	0.53 ± 0.03 a	0.52 ± 0.01 a	0.45 ± 0.01 a
	(0.4–0.5)	(0.8–0.9)	(0.48–0.63)	(0.56–0.72)	(0.32–0.52)	(0.88–1.18)	(0.48–0.59)	(0.51–0.53)	(0.43–0.47)
Arsenic in muscle (mg/L)	0.01 ± 0 ab	0.02 ± 0 abd	0.052 ± 0 c	0 ± 0 a	0.01 ± 0 ab	0.03 ± 0.01 d	0.04 ± 0.01 cd	0.02 ± 0 abd	0.03 ± 0 bd
	(0–0.01)	(0.017–0.031)	(0.051–0.052)	(0–0.01)	(0–0.01)	(0.02–0.04)	(0.03–0.05)	(0.02–0.03)	(0.02–0.03)

The data are presented as Means ± SE (minimum–maximum). Different letters indicate significant difference at $p < 0.05$. As1 (19.2 mg/L Arsenic), As2 (38.3 mg/L Arsenic), Am1 (7% Amphora), Am2 (10% Amphora).

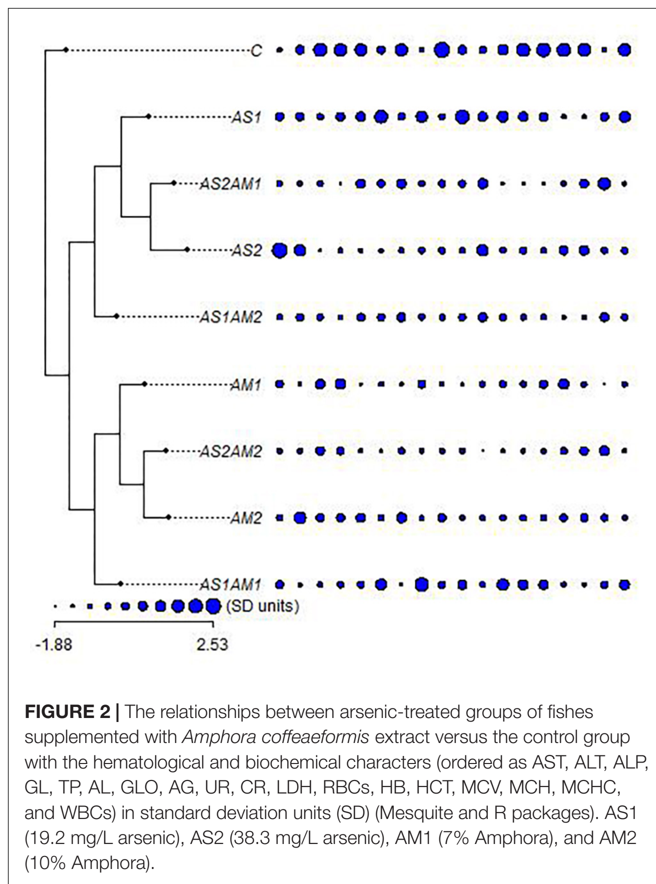

FIGURE 2 | The relationships between arsenic-treated groups of fishes supplemented with *Amphora coffeaeformis* extract versus the control group with the hematological and biochemical characters (ordered as AST, ALT, ALP, GL, TP, AL, GLO, AG, UR, CR, LDH, RBCs, HB, HCT, MCV, MCH, MCHC, and WBCs) in standard deviation units (SD) (Mesquite and R packages). AS1 (19.2 mg/L arsenic), AS2 (38.3 mg/L arsenic), AM1 (7% Amphora), and AM2 (10% Amphora).

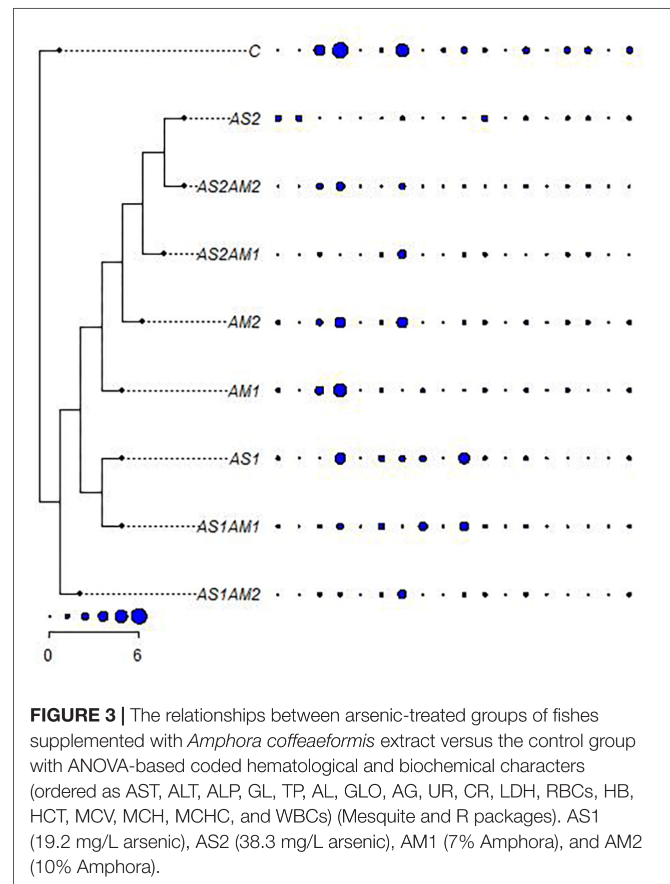

FIGURE 3 | The relationships between arsenic-treated groups of fishes supplemented with *Amphora coffeaeformis* extract versus the control group with ANOVA-based coded hematological and biochemical characters (ordered as AST, ALT, ALP, GL, TP, AL, GLO, AG, UR, CR, LDH, RBCs, HB, HCT, MCV, MCH, MCHC, and WBCs) (Mesquite and R packages). AS1 (19.2 mg/L arsenic), AS2 (38.3 mg/L arsenic), AM1 (7% Amphora), and AM2 (10% Amphora).

of numerous liver cells (extensive hepatic necrosis) which were observed in association with liver inflammation, injury, stress, and disease (Dorcas and Solomon, 2014). Similar observations were done in *C. gariepinus* and other fish species exposed to arsenic (Roy and Bhattacharya, 2006; Abdel-Hameid, 2009), 4-nonylphenol (Sayed and Soliman, 2018), cadmium (Mekkawy et al., 2011b), and atrazine (Mekkawy et al., 2013).

Different authors referred to ALP alteration in *C. gariepinus* and different fish species under stress and environmental pollution (Ezenwaji et al., 2013; Sayed and Soliman, 2017). Garima and Himanshu (2015) postulated a decrease in ALP of *C. batrachus* after exposure to arsenic trioxide due to the decrease of food intake in arsenic-exposed fishes.

Different studies reported the significance of LDH alteration under stress in fishes (Mekkawy et al., 2010; Sayed and Soliman, 2018). Moreover, it was suggested that the emergency needs of increased energy demands can be met by the lactate production for gluconeogenesis in the liver to increase LDH activity after stress (Rani et al., 2017). Altered levels of LDH were recorded in the present work and confirmed by other studies on the same species (Sayed et al., 2011; Akinrotimi et al., 2018).

In the present study, reduced glucose level was observed in arsenic-exposed fish. Similar observations have been reported by Lavanya et al. (2011) after exposure of fingerling *C. catla* to arsenic trioxide and by Garima and Himanshu (2015) after exposure of *C. batracus* to the same pollutant. Acute treatment by arsenic caused hypoxia which leads to an excess utilization of stored carbohydrates, since the glucose level decreases at such stress conditions (Garima and Himanshu, 2015; Kumari et al., 2017).

The arsenic-exposed fishes exhibited alterations in serum protein in the present study as well as in other studies (Garima and Himanshu, 2015; Singh and Srivastava, 2015). Such alterations are interpreted to be due to impaired protein synthesis produced by reactive oxygen species and free radicals (Lavanya et al., 2011; Carlson et al., 2013).

Urea and creatinine have been used as important indicators of renal health in toxicant-induced fish using a variety of both *in vivo* and *in vitro* methods (Davis and Beandt, 1994; Ajeniyi and Solomon, 2014). In the present study, the arsenic-exposed fish exhibited an increase in the creatinine at low arsenic concentrations which may be due to oxidative damage (Prusty et al., 2011), and a decrease under the high arsenic concentration which severely disturbs the metabolic processes in the kidney (Rana et al., 2018). A similar increase in creatinine level was observed by Kumari (2015) in *Oryctolagus cuniculus* exposed to arsenic. However, a decrease in creatinine level was observed by Ogamba et al. (2011) and Inyang et al. (2018) in *C. gariepinus* exposed to paraquat dichloride and lindane, respectively. Uric acid was found to decrease under arsenic stress on *C. gariepinus* of the present work and that of Ogamba et al. (2011). Different other studies revealed an increase in uric acid under arsenic stress

(Kumari, 2015) and different other pollutants (Mutlu et al., 2015; Kovacik et al., 2019).

A high level of arsenic accumulation in the muscles was observed in the present study. These findings are according to the results of Tyokumbur et al. (2014) who reported that the range of arsenic levels in the organs of *C. gariepinus* was highest in the muscles. The studies of Kim and Kang (2015) demonstrated that arsenic exposure can induce considerable arsenic accumulation in major tissues of different fish species (Al Sayegh Petkovšek et al., 2012; Gao et al., 2018).

According to Kaparapu (2018), microalgae like *Amphora*, *Chlorella*, and *Isochrysis* species are utilized as live feed for all growth stages of bivalve molluscs, for larval juvenile stages of abalone, crustaceans and some fish species, and for zooplankton used in aquaculture food webs. However, little is known regarding the role of *Amphora* extract in ameliorating the toxic damages induced by heavy metals in fishes in spite of its bioactive compounds recorded in the present work by GC-MS analysis. These compounds show effect as antioxidant, antibacterial, antiviral, anti-fungal, and anti-inflammatory (Salahuddin et al., 2017; El-Sayed et al., 2018; Munir et al., 2018).

In the present study, amphora bioactive compounds improved most of the plasma biochemical parameters for arsenic-induced fish reflecting, in turn, cytotoxic effects. So, the modulation effect of *Amphora* observed in the current study was in agreement with that of Ayoub et al. (2019) who utilized *Amphora* supplement in improvement of the lysozyme, serum protein and increased disease resistance of Nile Tilapia to *Aeromonas hydrophila* infection and El-Sayed et al. (2018) who utilized *Amphora* supplement as detoxification factor against paracetamol stress in liver tissue of rats. Moreover, the existing concentration of carbohydrates and proteins recorded in the present study may contribute to enhance the antioxidative activities of *Amphora* species as postulated by Rupérez et al. (2002) studying other diatoms.

In the present work, the main effects of arsenic and amphora and their interactions were represented by cluster analysis rooted by the control in the concept of multivariate sense for whole individuals since the treated groups may represent different populations in the environment. Although, the results of Amphora against arsenic toxicity showed positive effects, more studies are required to indicate the mechanisms of those effects.

CONCLUSION

The supplementation of amphora extracts can be used as detoxification and protective factors for *C. gariepinus* induced by arsenic due to the biologically active components of *Amphora* with antioxidant, antiviral, antibacterial, and anti-inflammatory characteristics, besides the abundant contents of proteins and carbohydrates which enhance these components.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The animal study was reviewed and approved by the Faculty of Science, Assiut University.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Conflict of Interest: The 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.

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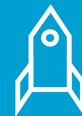

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