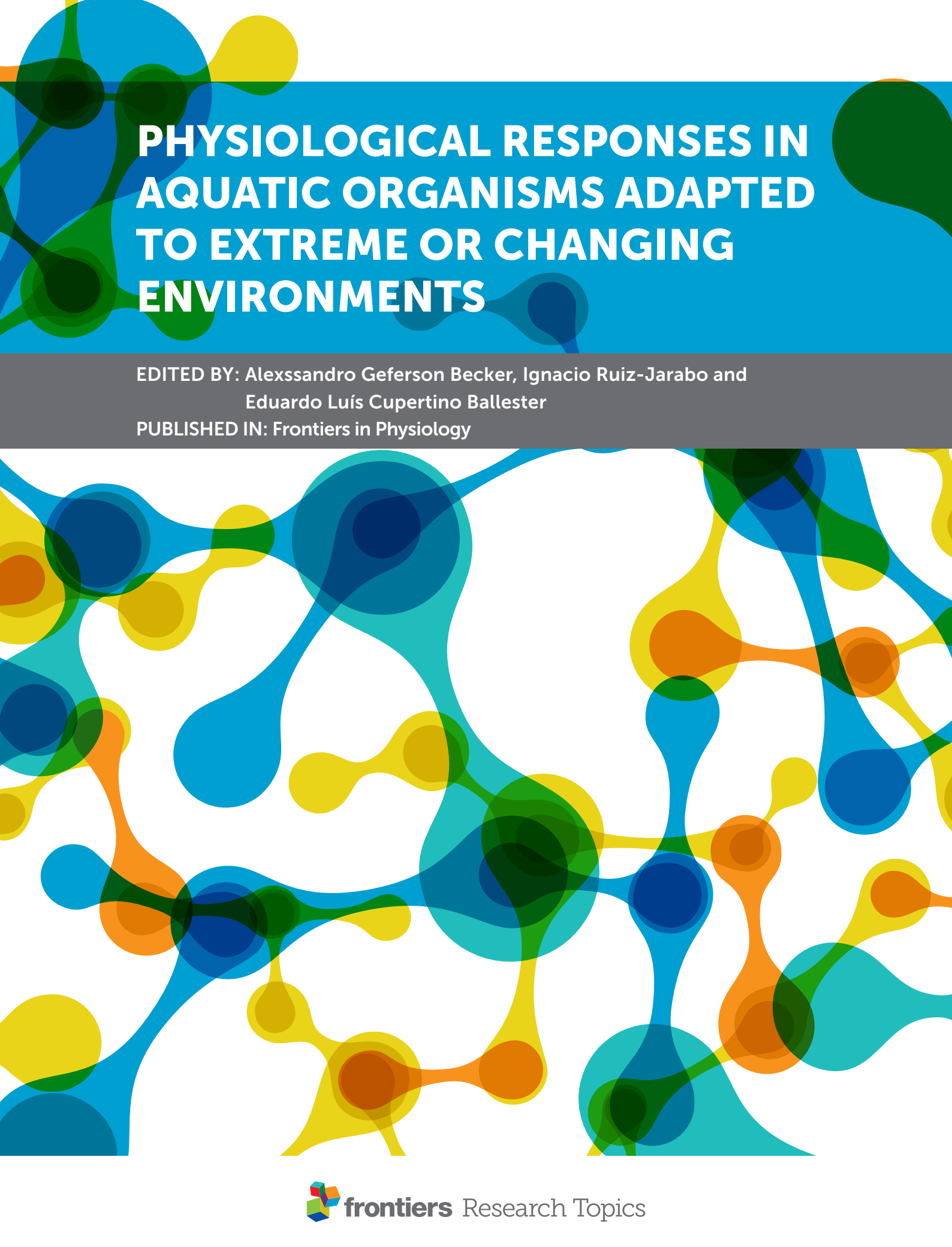




PHYSIOLOGICAL RESPONSES IN AQUATIC ORGANISMS ADAPTED TO EXTREME OR CHANGING ENVIRONMENTS

EDITED BY: Alessandro Geferson Becker, Ignacio Ruiz-Jarabo and
Eduardo Luís Cupertino Ballester
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PHYSIOLOGICAL RESPONSES IN AQUATIC ORGANISMS ADAPTED TO EXTREME OR CHANGING ENVIRONMENTS

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Diurnally Fluctuating $p\text{CO}_2$ Modifies the Physiological Responses of Coral Recruits Under Ocean Acidification

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Diurnal $p\text{CO}_2$ fluctuations have the potential to modulate the biological impact of ocean acidification (OA) on reef calcifiers, yet little is known about the physiological and biochemical responses of scleractinian corals to fluctuating carbonate chemistry under OA. Here, we exposed newly settled *Pocillopora damicornis* for 7 days to ambient $p\text{CO}_2$, steady and elevated $p\text{CO}_2$ (stable OA) and diurnally fluctuating $p\text{CO}_2$ under future OA scenario (fluctuating OA). We measured the photo-physiology, growth (lateral growth, budding and calcification), oxidative stress and activities of carbonic anhydrase (CA), Ca-ATPase and Mg-ATPase. Results showed that while OA enhanced the photochemical performance of *in hospite* symbionts, it also increased catalase activity and lipid peroxidation. Furthermore, both OA treatments altered the activities of host and symbiont CA, suggesting functional changes in the uptake of dissolved inorganic carbon (DIC) for photosynthesis and calcification. Most importantly, only the fluctuating OA treatment resulted in a slight drop in calcification with concurrent up-regulation of Ca-ATPase and Mg-ATPase, implying increased energy expenditure on calcification. Consequently, asexual budding rates decreased by 50% under fluctuating OA. These results suggest that diel $p\text{CO}_2$ oscillations could modify the physiological responses and potentially alter the energy budget of coral recruits under future OA, and that fluctuating OA is more energetically expensive for the maintenance of coral recruits than stable OA.

Keywords: ocean acidification, diurnal $p\text{CO}_2$ fluctuations, coral calcification, carbonic anhydrase, proton pump, trade-off

INTRODUCTION

Since the Industrial Revolution, about one third of human-emitted CO_2 has been absorbed by the ocean, resulting in ocean acidification (OA), a phenomenon characterized by declines in seawater pH, carbonate concentration and saturation state of calcium carbonate (CaCO_3) (Sabine et al., 2004). Relative to pre-industrial levels, average surface ocean pH has decreased by 0.1 units, and

a further reduction of 0.3–0.4 units is projected by the end of this century based on “Business as usual” scenario (Orr et al., 2005; Gattuso et al., 2015).

Ocean acidification constitutes one of the most serious threats to various marine calcifying taxa because it reduces the availability of carbonate ions that are needed to accrete CaCO_3 (Orr et al., 2005); among them reef corals, which construct and maintain complex reef framework structures, are extensively studied (Chan and Connolly, 2013; Kroeker et al., 2013). Numerous laboratory experiments have demonstrated negative yet variable effects of OA on coral skeletal growth, with a mean decline in calcification of 15% per unit decrease in aragonite saturation state (Ω_{Arg}) (Chan and Connolly, 2013). In these empirical studies, reef corals have been exposed to static pH levels consistent with open ocean projections of seawater pH declines of 0.3–0.4 units by the year 2100. However, compared with pelagic ocean environments, the carbonate chemistry on coral reef ecosystems is highly dynamic. Large daily swings in seawater pH and $p\text{CO}_2$, mainly driven by biological activities (photosynthesis and respiration), have been recorded in many reef locations around the world. During daytime, uptake of CO_2 and HCO_3^- by photosynthesis decreases seawater $p\text{CO}_2$ and elevates pH, while nighttime respiration decreases pH and increases $p\text{CO}_2$. Natural variability in carbonate chemistry is particularly characteristic of the shallow coastal reefs (Rivest et al., 2017), and pH and $p\text{CO}_2$ could vary by up to 0.7 units and 900 μatm , respectively, over a diel cycle (Santos et al., 2011; Shaw et al., 2012; Chen et al., 2015; Silverman et al., 2015). This environmental variability may greatly confound our current understanding and predictions of OA consequences on marine organisms, especially for those inhabiting high-variance ecosystems (Rivest et al., 2017). Therefore, focus is now shifting to how natural $p\text{CO}_2$ fluctuations will interact with increasing $p\text{CO}_2$ levels to affect the future performance of shallow marine organisms.

Recent evidence suggests that reef calcifiers respond very differently to constant elevated $p\text{CO}_2$ than to oscillating $p\text{CO}_2$ that simulates the daily variations in carbonate chemistry on shallow reefs. For instance, recruits of the reef coral *Seriatopora caliendrum* exposed to ecologically relevant $p\text{CO}_2$ fluctuations exhibited higher rates of calcification and survival compared to those under ambient or high $p\text{CO}_2$ (Dufault et al., 2012). Similarly, diel $p\text{CO}_2$ oscillations totally negate the inhibition of calcification by OA in corals *Acropora formosa* (Chan and Eggins, 2017) and *A. hyacinthus* (Comeau et al., 2014b), and variable $p\text{CO}_2$ partially offset the OA-induced depression in calcification by crustose coralline algae (CCA) *Porolithon onkodes* (Johnson et al., 2014). In contrast, periods of high pH in the daytime and low pH at night act additively with OA to reduce the skeletal growth of adult and juvenile coralline algae *Arthrocardia corymbosa* (Cornwall et al., 2013; Roleda et al., 2015). Another recent study demonstrated that calcification rates of the coral *Goniopora* sp. and the CCA *Hydrolithon reinboldii* exhibited limited response to both OA and extreme pH fluctuations, possibly due to strong control over carbonate chemistry within the calcifying fluid (Cornwall et al., 2018). Differential calcification responses to diurnal $p\text{CO}_2$ fluctuations

that are typical on shallow tropical reefs, together with OA, give rise to a clear need for more thorough studies of the influence of dynamic $p\text{CO}_2$ on calcification, especially under acidified seawater.

Furthermore, little is known about the biochemical mechanisms of these observed calcification responses of reef corals to future OA conditions. Symbiotic scleractinian corals are known to calcify faster in the light than in the dark, a phenomenon called “light enhanced calcification” (LEC) (Allemand et al., 2011). Photosynthesis by endosymbionts (i.e., zooxanthellae), which contributes greatly to the energy needs of coral holobiont (Muscattine et al., 1981), has been considered the primary cause of LEC. During daytime, photosynthesis consumes CO_2 and helps to maintain intracellular pH and elevated CO_3^{2-} and aragonite saturation state (Ω_{Arg}) (Gibbin et al., 2014). Together, this creates conditions favorable for calcification (McCulloch et al., 2012; DeCarlo et al., 2018). Meanwhile, release of hydroxide ions (OH^-) from photosynthesis makes the coelenteron an alkaline environment, supporting the titration of the protons (H^+) produced by calcification (Moya et al., 2008; Comeau et al., 2013a).

Carbonic anhydrase (CA), which catalyzes the inter-conversion between bicarbonate (HCO_3^-) and CO_2 , is central to carbon supply for calcification, conversion of metabolic CO_2 to prevent night acidosis, and carbon concentrating for photosynthesis by zooxanthellae (Leggat et al., 1999, 2002; Moya et al., 2008; Bertucci et al., 2013). Moreover, Ca-ATPase and Mg-ATPase are hypothesized to transport Ca^{2+} and Mg^{2+} into the extracellular calcifying fluid (ECF) and simultaneously remove H^+ from ECF in reef corals (Ip and Lim, 1991; Ip et al., 1991; Meibom et al., 2004; Zoccola et al., 2004; Allison et al., 2011), thus driving the calcification reaction toward CaCO_3 precipitation (Al-Horani et al., 2003; Allemand et al., 2011). Although studies have shown that OA could dramatically change the gene expression of CA, Ca-ATPase, and Mg-ATPase (Kaniewska et al., 2012; Vidal-Dupiol et al., 2013; Kurihara et al., 2018), the effects of elevated and fluctuating $p\text{CO}_2$ on the functions of these crucial molecules, and more importantly, the way in which they may coordinate to regulate photosynthesis and calcification under future OA remain largely unexplored.

Further, OA has been demonstrated to increase reactive oxygen species (ROS) generation and induce oxidative stress in calcifying organisms (Matozzo et al., 2013; Mangan et al., 2017; Luz et al., 2018). As the energetic costs of calcification and acid-base regulation are expected to increase as $p\text{CO}_2$ rises (Cohen and Holcomb, 2009), this may reduce the energy availability for ROS scavenging by the antioxidant system, also a high energy-demanding process. Again, current literature provides little information on the effects of seawater acidification on the oxidative stress and antioxidant functioning for scleractinian corals.

To address these critical knowledge gaps, the present study investigated the physiological and biochemical responses of the reef coral *Pocillopora damicornis* to diel $p\text{CO}_2$ fluctuations that are characteristic of their natural settings. *P. damicornis* is a widely distributed and major reef-building coral on reef flats in the Indo-Pacific region and broods symbiotic planula larvae

with maternally derived zooxanthellae (Veron, 1993). Several prior studies demonstrated that *P. damicornis* is resistant to OA, with unaffected calcification under high $p\text{CO}_2$ (Comeau et al., 2013b, 2014a, 2015). In our study site Luhuitou fringing reef, calcification by adult *P. damicornis* even responded positively to elevated $p\text{CO}_2$, suggesting the local acclimatization/adaptation to OA (Huang et al., 2014), while the post-settlement calcification and growth are highly susceptible to OA conditions (Jiang et al., 2015, 2018). Here, we extended our use of new recruits of *P. damicornis* and examined the effects of stable and fluctuating OA on a suite of physiological traits, including photochemical performance, survivorship and early development. In addition, oxidative stress and activities of CA, Ca-ATPase and Mg-ATPase putatively involved in photosynthesis and calcification were measured to illuminate the physiological changes of juvenile corals.

MATERIALS AND METHODS

Study Site and *in situ* $p\text{CO}_2$ Profiling

To quantify the pattern of diel $p\text{CO}_2$ oscillations currently experienced by corals, seawater was continuously pumped from 2 m depth on Luhuitou fringing reef into a flow-through tank on the beach. Seawater $p\text{CO}_2$ was measured *in situ* in the tank using a Picarro CRDS (Cavity Ring-Down Spectroscopy) analyzer. The accuracy of CRDS analyzer was verified by measuring certified reference gas standards, as per the manufacturer's instructions. Seawater $p\text{CO}_2$ was monitored for 7 days from 20 to 27 August 2017.

Coral Sampling and Larval Settlement

Ten mature colonies of *P. damicornis* were collected at 2 m depth on Luhuitou reef (N18°12.7', E109°28.5') by snorkeling on August 26, 2017. Colonies were transported to the Tropical Marine Biological Research Station, placed into individual 20 L tanks with flow-through seawater at ambient temperature ($28.6 \pm 0.2^\circ\text{C}$) and exposed to partially shaded sunlight (noon irradiance, ca. $300 \mu\text{mol photons m}^{-2} \text{s}^{-1}$). The outflow of each tank was passed through a cup fitted with a $180 \mu\text{m}$ mesh on the bottom to trap larvae. Larvae were collected at 08:00 on August 30, 2017 and then pooled across colonies. Groups of approximately 60 larvae were introduced into plastic petri-dishes, and settlement was induced by small chips of crustose coralline algae *Porolithon onkodes*. Twelve hours later, unsettled larvae and algal chips were discarded. Five dishes with a total of approximately 200 primary polyps were allocated to each experimental tank. The newly settled corals were reared for 7 days under three $p\text{CO}_2$ treatments as described below.

Experimental Setup

Juvenile corals were exposed to three $p\text{CO}_2$ treatments: (1) steady and ambient $p\text{CO}_2$ (Control); (2) steady and elevated $p\text{CO}_2$ (Stable OA); and (3) diurnally fluctuating and elevated $p\text{CO}_2$ (Fluctuating OA). It is important to point out that the ambient $p\text{CO}_2$ of seawater measured here was much higher than the open ocean level. However, it just reflected the current conditions

in this coastal reef ecosystem and was comparable to the values previously reported at this location (Zhang et al., 2013; Chen et al., 2015; Yan et al., 2016). The $p\text{CO}_2$ of the stable OA treatment was chosen based on the projections by the end of this century under RCP8.5 (Gattuso et al., 2015). The fluctuating OA treatment was created by superimposing the current diurnal variance onto the $p\text{CO}_2$ level predicted for 2100 (Cornwall et al., 2013; Camp et al., 2016). Limited facility precluded an additional, fluctuating treatment at ambient $p\text{CO}_2$, but this limitation did not affect the main objective of this study, i.e., comparing the physiological responses of juvenile corals to stable and oscillatory OA conditions. The experimental $p\text{CO}_2$ treatments were constructed in nine 25-L tanks, with three replicate tanks for each treatment. Each tank was filled with $0.5 \mu\text{m}$ -filtered and UV-sterilized seawater. Seawater within each tank was well mixed using submerged pumps (350 L h^{-1}) and partially (30%) changed at 20:00 every day. Each tank was covered with a transparent lid to minimize gas exchange and maintain experimental $p\text{CO}_2$ levels. The two steady regimes were established by bubbling with ambient air or elevated $p\text{CO}_2$ (1000 ppm), which was achieved by mixing air with CO_2 using a CO_2 enricher (CE100B, Ruihua, China). The fluctuating treatment was established by changing $p\text{CO}_2$ settings of the CO_2 enricher every 6 h. Preliminary high-resolution pH monitoring (every hour for 1 day) showed that this method successfully achieved step-wise and gradual decrease and increase in seawater pH, following a natural daily cycle. Mean pH of the fluctuating OA treatment was comparable to that in the stable OA treatment (7.82 vs. 7.81).

The seawater temperature in each tank was controlled independently with digital temperature controllers and titanium heaters at a targeted value of $29 \pm 0.4^\circ\text{C}$ (mean $\pm$ SD), which corresponded to the ambient and long-term mean summer temperature at the study site. Light was provided on a 12:12 h light-dark cycle between 07:00 and 19:00 h using T5 fluorescent lamps. The photosynthetically active radiation was about $200 \mu\text{mol photons m}^{-2} \text{s}^{-1}$, approximating that recorded in crevices preferred by juvenile corals at 2–3 m depths on Luhuitou reef (Lei Jiang, unpublished data). Seawater samples (100 mL) were collected from each tank at 06:00 and 18:00 every other day for the measurement of pH, salinity and total alkalinity. pH and salinity were measured with a Thermo Orion 5-star meter and the pH electrode was two-point calibrated with NBS buffers every other day. Total alkalinity (TA) was measured with an automatic titrator (AS-ALK2, Apollo, United States). The carbonate chemistry parameters were calculated using the CO2SYS program and seawater conditions for each treatment are presented in Table 1.

Chlorophyll Fluorescence Measurement

The photo-physiology of the symbionts was assessed using four distinct parameters associated with chlorophyll fluorescence. Five recruits from each replicate tank were randomly selected on the last day of the experiment, and a Diving-PAM (Pulse Amplitude Modulated) fluorometer (Walz GmbH, Germany) was used to assess the chlorophyll fluorescence parameters of *in hospite* symbionts. The fiber-optic probe was equipped with a plastic tube to ensure consistent probe orientation and

TABLE 1 | Mean ($\pm$ SD) physical and chemical parameters for each treatment.

Treatment	pH _{NBS}	Salinity (psu)	TA ($\mu\text{mol kg}^{-1}$)	DIC ($\mu\text{mol kg}^{-1}$)	pCO ₂ (μatm)	Ω_{Arag}
Control	8.11 $\pm$ 0.02	33.4 $\pm$ 0.6	2245 $\pm$ 87	1981 $\pm$ 78	508 $\pm$ 32	3.13 $\pm$ 0.2
Stable OA	7.81 $\pm$ 0.02	33.2 $\pm$ 0.4	2195 $\pm$ 81	2074 $\pm$ 78	1115 $\pm$ 77	1.70 $\pm$ 0.12
Fluct OA	7.82 $\pm$ 0.12	33.3 $\pm$ 0.6	2158 $\pm$ 126	2032 $\pm$ 146	1217 $\pm$ 621	1.77 $\pm$ 0.73

distance of 2 mm between probe and corals. Maximum and effective quantum yields (F_v/F_m and $\Delta F/F_m'$) were measured for the same batch of recruits from each tank using the equations of Genty et al. (1989): $F_v/F_m = (F_m - F_0)/F_m$; $\Delta F/F_m' = (F_m' - F_t')/F_m'$, where F_m = maximum fluorescence yield, F_0 = fluorescence yield in darkness, F_t' = fluorescence yield in actinic light and F_m' = maximum fluorescence yield in actinic light. F_v/F_m was measured at 06:00 to ensure enough time for dark adaptation and relaxation of photochemical quenching. F_v/F_m provides a measure of the maximum photochemical efficiency of photosystem II (PSII), with substantial declines indicating damage to the photosynthetic apparatus (Jones et al., 1998). $\Delta F/F_m'$ was measured at 17:00 in a light-adapted state. This ratio assesses the actual light use efficiency to drive photochemical processes (Maxwell and Johnson, 2000). The depression of $\Delta F/F_m'$ relative to F_v/F_m reflects the extent of non-photochemical quenching (NPQ), which is determined as $(F_m - F_m')/F_m'$. Finally, maximum citation pressure over PSII (Q_m) is determined from $1 - [(\Delta F/F_m' \text{ at } 17:00)/(F_v/F_m)]$, with values close to 0 indicating that most of the reaction centers are open, while values close to 1 denoting mostly closed reaction centers and photo-inhibition (Iglesias-Prieto et al., 2004).

Survival and Growth

Recruits were checked daily and the number of dead corals was recorded based on loss of polyp tissue and presence of bare skeleton. On the last day of the experiment, 15–20 recruits were randomly selected from each tank, photographed under a dissecting microscope for the growth measurements, and the number of new buds counted for each recruit. Images with a scale bar were analyzed for lateral growth using ImageJ software (National Institutes of Health). Growth was estimated as the rates of change in planar area and number of new polyps over time (Dufault et al., 2012; Jiang et al., 2018). Upon completion of the experiment, 10 recruits were sampled from each tank and analyzed for skeletal weight and ash-free tissue biomass using a Mettler-Toledo ultra-microbalance at an accuracy of $\pm 1 \mu\text{g}$ according to Anlauf et al. (2011).

Enzymatic and Oxidative Stress Assays

On the last day of the experiment, 4 groups of 15 recruits from each tank were randomly sampled using sterilized razor blades at 06:00 and 18:00. Two samples from each time were assayed for activities of Ca-ATPase and Mg-ATPase following modified protocols of Chan et al. (1986) and Prazeres et al. (2015). The remaining two samples were used to measure CA activities using the pH drift method (Weis et al., 1989). All samples were immediately snap-frozen in liquid nitrogen, transported

on dry-ice to the lab in Guangzhou and preserved at -80°C . Detailed procedures of these assays are provided in the electronic **Supplementary Material**.

In addition, two batches of 10 recruits were sampled from each tank to analyze the signs of oxidative stress, including catalase (CAT) activity and malondialdehyde (MDA) content. Up-regulation of CAT reflects an organism's capacity to detoxify ROS, and MDA is an indicator of cellular oxidative damage and lipid peroxidation. CAT was assayed using the Catalase assay kit (Beyotime, China) as per the manufacturer's instructions. MDA content was determined as the thiobarbituric acid reactive metabolites (Camejo et al., 1998) using a Lipid Peroxidation Assay Kit (Sigma-Aldrich, United States).

Data Analyses

All response data were initially tested using a nested ANOVA with tank as a random factor nested within pCO₂ treatment. However, as the tank factor was non-significant (electronic **Supplementary Material**), it was dropped from the statistical model to enhance the power of the analysis (Quinn and Keough, 2002), and the analyses were repeated using corals as independent replicates. To assess the effects of pCO₂ treatments on photo-physiology, growth, CAT activity and lipid peroxidation, one-way ANOVAs were applied with Fisher's Least Significant Difference (LSD) as planned *post hoc* multiple comparisons. The enzymatic activities were analyzed with two-way ANOVAs followed by Fisher's LSD, with pCO₂ and time as fixed effects. Further *post hoc* tests for pairwise comparison of the effect of time on the enzymatic activities at each pCO₂ treatment were performed using Student's *t*-test.

RESULTS

Environmental and Experimental Seawater pCO₂

Seawater pCO₂ at 2 m depth on Luhuitou fringing reef ranged from 215 to 1077 μatm over 7 days in summer (**Figure 1A**), averaging $528 \pm 163 \mu\text{atm}$ (mean $\pm$ SD). A prominent diel cycle was present, with the lowest value before dusk and the highest value near dawn. Mean diurnal range of seawater pCO₂ was $518 \pm 220 \mu\text{atm}$ (mean $\pm$ SD, range: 247–862 μatm).

Diurnal variations in seawater pH for each treatment are shown in **Figure 1B**. In the fluctuating OA treatment, pH decreased after 18:00 until reaching a minimum at 06:00 the next morning (**Figure 1B**). The pCO₂ values for the control, stable OA and fluctuating OA treatments were 508 ± 32 , 1115 ± 77 , and $1217 \pm 621 \mu\text{atm}$, respectively (mean $\pm$ SD, **Table 1**).

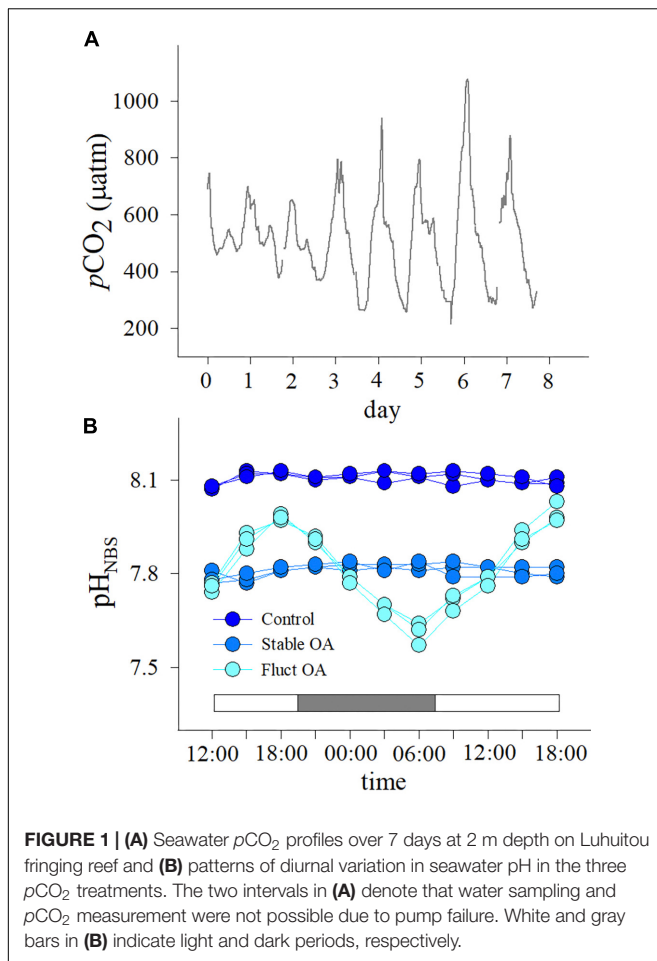


FIGURE 1 | (A) Seawater $p\text{CO}_2$ profiles over 7 days at 2 m depth on Luhuitou fringing reef and **(B)** patterns of diurnal variation in seawater pH in the three $p\text{CO}_2$ treatments. The two intervals in **(A)** denote that water sampling and $p\text{CO}_2$ measurement were not possible due to pump failure. White and gray bars in **(B)** indicate light and dark periods, respectively.

Survival and Chlorophyll Fluorescence

Coral survival rate was high and only 2 recruits, one each from the control and stable OA treatments, died during the experiment. There was no significant differences in F_v/F_m between the $p\text{CO}_2$ treatments, and mean F_v/F_m ranged from 0.488 to 0.505 ($F_{2,42} = 0.691$, $p = 0.507$; **Figure 2A**). In contrast, both the stable and fluctuating OA treatments had a significant and positive impact on $\Delta F/F_m'$ ($F_{2,42} = 15.46$, $p < 0.001$; **Figure 2A**). $\Delta F/F_m'$ was highest in the stable OA treatment but declined in the fluctuating OA treatment (**Figure 2A**). Consequently, NPQ and Q_m were significantly reduced at increased $p\text{CO}_2$ ($F_{2,42} \geq 21.14$, $p < 0.001$), with apparent trends for lower values under stable OA relative to fluctuating OA (**Figure 2B**).

Post-settlement Growth

Calcification was marginally affected by $p\text{CO}_2$ ($F_{2,87} = 3.09$, $p = 0.051$), and reduced by 10% in the fluctuating OA treatment compared to the other treatments (**Figure 2C**). Mean tissue biomass per individual ranged from 106 to 111 μg , and was similar among treatments ($F_{2,87} = 0.20$, $p = 0.818$; **Figure 2C**). Lateral growth was affected by $p\text{CO}_2$ treatments ($F_{2,155} = 3.12$, $p = 0.047$), and reduced by 8% under stable OA relative to control and fluctuating OA (**Figure 2D**). $p\text{CO}_2$ treatments significantly

influenced asexual budding ($F_{2,155} = 3.33$, $p = 0.038$), and the budding rate in the fluctuating OA treatment was only about half that in the other two treatments (**Figure 2D**).

CAT and Lipid Peroxidation

Host CAT activity was significantly elevated by OA treatments ($F_{2,15} = 4.44$, $P = 0.03$), but was similar between the stable and fluctuating OA treatments (**Figure 3A**). There was also a significant effect of $p\text{CO}_2$ treatments on MDA content in host tissue ($F_{2,15} = 6.39$, $P = 0.01$). MDA concentrations were 55 and 51% higher for corals under stable and fluctuating OA than that in the control, respectively (**Figure 3B**).

Carbonic Anhydrase

Both host and symbiont CA varied across $p\text{CO}_2$ treatments and between night and day, and treatment and time had no significant interactive effects (**Table 2**). Compared to the control, host CA was significantly elevated in the stable OA treatment and reduced in the fluctuating OA treatment (**Figure 4A**). *Post hoc* analyses showed that host CA in the fluctuating OA treatment differed significantly between light and dark conditions (Student *t*-test, $df = 10$, $t = 3.905$, $p = 0.003$). Furthermore, corals in both OA treatments exhibited significantly higher symbiont CA activities (**Figure 4B**). *Post hoc* analyses revealed that symbiont CA in the control and fluctuating OA treatments was significantly lower in the dark than that in the light (Student *t*-test, $df = 10$, $t = -4.606$, $p = 0.003$; $t = -4.885$, $p = 0.001$).

Ca-ATPase and Mg-ATPase

In general, there was a significant effect of $p\text{CO}_2$ treatments on the activities of Ca-ATPase and Mg-ATPase (**Table 2**), both of which were greatly elevated in the fluctuating OA treatment compared to the other 2 treatments. Relative to the control and stable OA treatments, Ca-ATPase activity in the fluctuating OA treatment increased by 35–43% and 78–91% during light and dark periods, respectively (**Figure 4C**). However, such pattern was less clear for Mg-ATPase activity, which was elevated in the fluctuating OA treatment by 21–41% and 25–33% in dark and light conditions, respectively. Furthermore, Ca-ATPase activity was unaffected by time (**Table 2**); by contrast, Mg-ATPase activities differed significantly between light and dark conditions (**Table 2**), largely driven by the higher nighttime Mg-ATPase activities under stable OA (Student *t*-test, $df = 10$, $t = -5.19$, $p < 0.001$; **Figure 4D**).

DISCUSSION

The present study revealed significant differences in the physiological responses of juvenile *P. damicornis* exposed to constant and fluctuating OA. Results showed that the fluctuating $p\text{CO}_2$ regime depressed the stimulatory effect of OA on photosynthetic activity. Further, corals significantly up-regulated the proton pumps and antioxidant CAT in the fluctuating OA treatment, in which calcification was only slightly reduced. However, asexual budding declined by 50% and oxidative damage still occurred under fluctuating

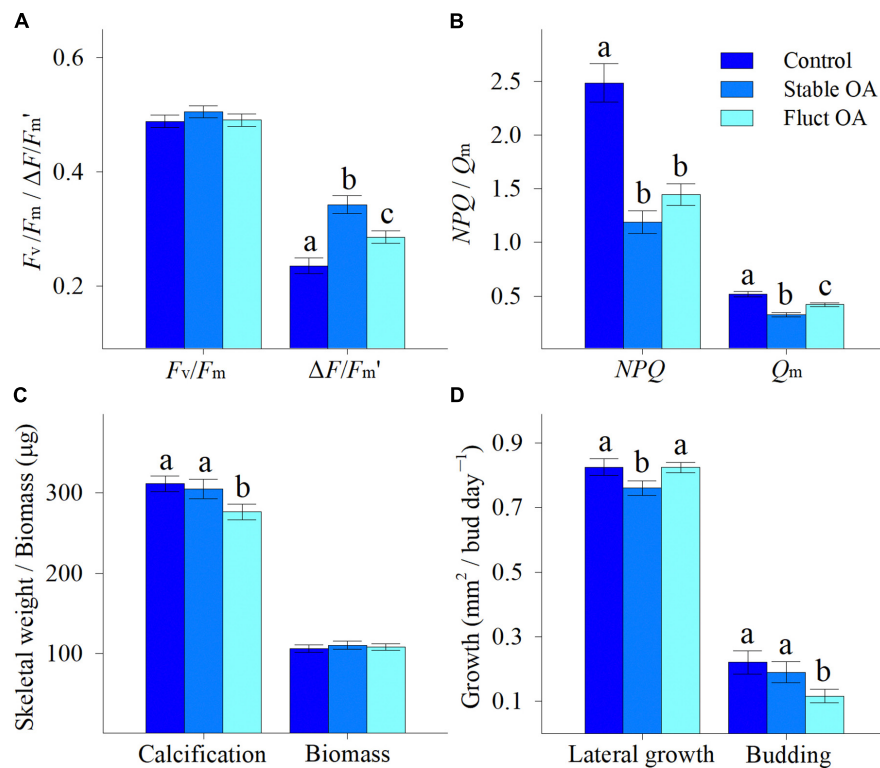


FIGURE 2 | Physiological responses of *Pocillopora damicornis* recruits following 7-day exposure to ambient pCO_2 (Control), steady-high pCO_2 (Stable OA), and fluctuating-high pCO_2 (Fluct OA). **(A)** Maximum and effective quantum yields (F_v/F_m and $\Delta F/F_m'$); **(B)** non-photochemical quenching and maximum citation pressure over PSII (NPQ and Q_m); **(C)** calcification and biomass; and **(D)** lateral growth and budding rates. Data are expressed as mean $\pm$ SE. Different letters indicate statistically significant differences in mean values among treatments.

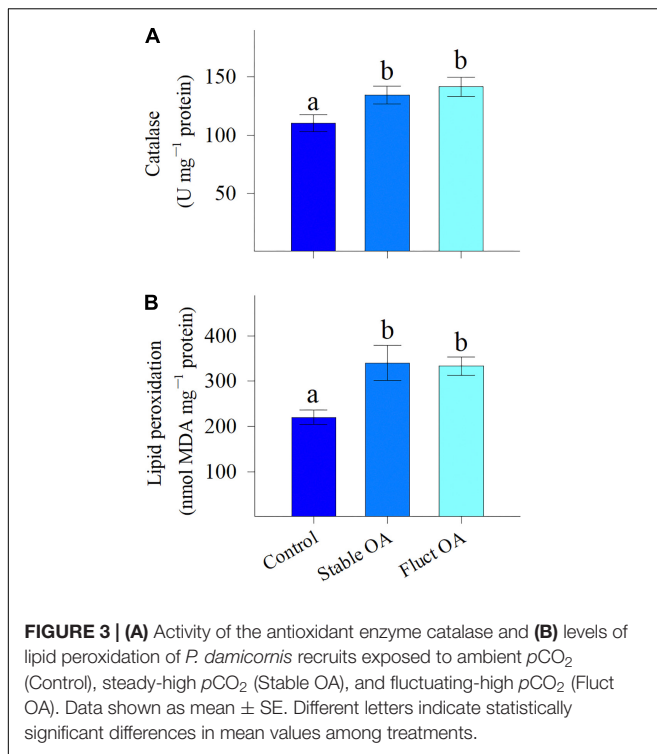
OA. Together, although the photochemical performance was enhanced by the fluctuating OA treatment, it was unable to fully compensate for the increased energy expense for coral recruits. More importantly, in the fluctuating OA treatment, corals appeared to compromise asexual reproduction and ROS detoxification to sustain skeletal growth, indicating potential trade-offs between calcification and other key physiological processes. These findings suggest that diurnal variability in pH/carbonate chemistry is likely to be an overriding factor influencing and determining the early success and recruitment of corals under future OA. Our study also highlights the importance of considering a broader spectrum of physiological traits in order to accurately and fully characterize the overall change in fitness and the possible trade-offs between different physiological functions when addressing corals' responses to environmental stress.

Boosted Photo-Physiology and Potential Involvement of Symbiont CA Under OA

Consistent with our previous findings (Jiang et al., 2015, 2018), this study showed that OA did not influence F_v/F_m , indicating that there was no photo-inhibition or damage to the photosynthetic apparatus. Further, both OA treatments greatly improved the photosynthetic efficiency of *in hospite* symbionts,

as evidenced by the elevated $\Delta F/F_m'$ and decreased NPQ and Q_m . These findings suggest that, under elevated pCO_2 , more electrons are being transported for carbon fixation and the photochemical process is more competitive for reaction centers than non-photochemical quenching. A similar photo-physiological response was observed in our previous study on *P. damicornis* recruits exposed to increased pCO_2 (Jiang et al., 2015).

Zooxanthellae possess the type II Rubisco with low CO_2 affinity, and are therefore carbon-limited under current pCO_2 levels (Leggat et al., 2002). However, CO_2 enrichment effects on photosynthesis are occasionally observed in reef corals. For example, Noonan and Fabricius (2016) showed that increased pCO_2 significantly promoted photosynthetic productivity in reef coral *S. hystrix* from central Great Barrier Reef, but not in *A. millepora*. In contrast, Strahl et al. (2015) reported that net photosynthesis of *A. millepora* and massive *Porites* spp. from volcanic CO_2 seeps in Papua New Guinea increased considerably with pCO_2 , whereas this phenomenon was not observed in *S. hystrix* and *P. damicornis*. Additionally, parabolic responses of photochemical process to pCO_2 have also been documented (Crawley et al., 2010; Castillo et al., 2014). Hence, photo-physiological responses to OA in reef corals may be species-specific and context-dependent. Moreover, different pCO_2 levels and the diverse DIC utilization modes of symbionts



(Brading et al., 2011, 2013) may contribute to these variable and contrasting results.

Corals from Luhuitou reef have periodically encountered acute $p\text{CO}_2$ levels comparable to OA projections for the end of this century. This history of exposure to dynamic and natural extremes of carbonate chemistry could facilitate plasticity and acclimatization of coral host and/or their associated symbionts to future OA. The boosted photo-physiology under OA in the present study is likely an adaptive response, and the CO_2 fertilization effect might be driven by the increased symbiont CA activities, which could accelerate inter-conversion between HCO_3^- and CO_2 and thus DIC transport to thylakoid within symbiont cells. Albeit energy-consuming, these processes would ultimately promote the accumulation of CO_2 around Ribulose biphosphate carboxylase/oxygenase (Rubisco) and support photosynthesis (Leggat et al., 1999, 2002). Intriguingly, NPQ and Q_m tended to be higher under fluctuating OA than stable OA, suggesting an increase in non-photochemical process and acidosis of the thylakoid lumen. This probably resulted from the increased energy demand of calcification (discussed below), which would sequentially reduce the availability of ATP for H^+ removal in dark reactions and trigger acidosis within the thylakoid lumen (Kanazawa and Kramer, 2002).

Oxidative Stress in Response to OA

Despite the positive photochemical response to OA, we found that both OA regimes elicited a significant and similar increase in activity of the antioxidant CAT. Nevertheless, the elevated CAT activity was not effective against the damaging ROS, and lipid peroxidation still increased under OA, as evidenced by the

TABLE 2 | Statistical results of two-way ANOVAs examining the effects of $p\text{CO}_2$ treatments and time on the activities of carbonic anhydrase (CA), Ca-ATPase and Mg-ATPase of *Pocillopora damicornis* recruits.

Variables	Source of variation	SS	df	MS	F	P
Host CA	$p\text{CO}_2$	66.08	2	33.04	20.47	< 0.001
	Time	17.37	1	17.37	10.76	0.003
	$p\text{CO}_2 * \text{Time}$	8.167	2	4.083	2.530	0.097
	Error	48.42	30	1.614		
Symbiont CA	$p\text{CO}_2$	35.43	2	17.72	11.00	< 0.001
	Time	26.64	1	26.64	16.54	< 0.001
	$p\text{CO}_2 * \text{Time}$	2.380	2	1.190	0.739	0.486
	Error	48.30	30	1.610		
Ca-ATPase	$p\text{CO}_2$	0.150	2	0.075	24.00	< 0.001
	Time	0.003	1	0.003	0.976	0.331
	$p\text{CO}_2 * \text{Time}$	0.012	2	0.006	1.912	0.165
	Error	0.094	30	0.003		
Mg-ATPase	$p\text{CO}_2$	0.123	2	0.053	6.674	0.002
	Time	0.053	1	0.061	7.685	0.015
	$p\text{CO}_2 * \text{Time}$	0.015	2	0.007	0.923	0.408
	Error	0.240	30	0.008		

Significant results are highlighted in bold.

> 50% increases in MDA contents, a specific end-product of the oxidative degradation of lipids. This supports previous reports on a range of calcifying marine invertebrates exposed to OA, including the reef coral *P. capitata* (Soriano-Santiago et al., 2013), the hydrocoral *Millepora alcicornis* (Luz et al., 2018) and the mussel *Mytilus edulis* (Mangan et al., 2017).

Reasons for this oxidative stress under OA conditions still remain unclear, particularly for corals under stable OA with considerable energetic benefits from higher photochemical activity. One parsimonious cause is that the increased photosynthetic electron flux under OA could induce the photo-reduction of oxygen, i.e., operation of the Mehler reaction at higher rates, ultimately resulting in the production of damaging hydrogen peroxide and superoxide which would subsequently diffuse into coral cytoplasm (Miyake and Yokota, 2000). ROS accumulates once the scavenging capacity of antioxidant system is exceeded, causing oxidative damage to a range of cell components, such as lipids, nucleic acids and proteins (Asada, 1999). If this is the case, the oxidative stress as measured by lipid peroxidation would have serious repercussions for the holobiont health and its capacity to cope with chronic OA exposure. Further research is clearly needed to pinpoint the exact mechanism behind the oxidative stress caused by OA in reef corals.

$p\text{CO}_2$ Regime Dictates Coral Calcification and Host CA Function Under OA

Paradoxically, while stable OA reduced lateral growth, it did not affect calcification, suggesting that linear extension and calcification may be decoupled in newly settled corals under OA. The lack of response in early development to static OA contrasts with prior work reporting that steady declines in pH

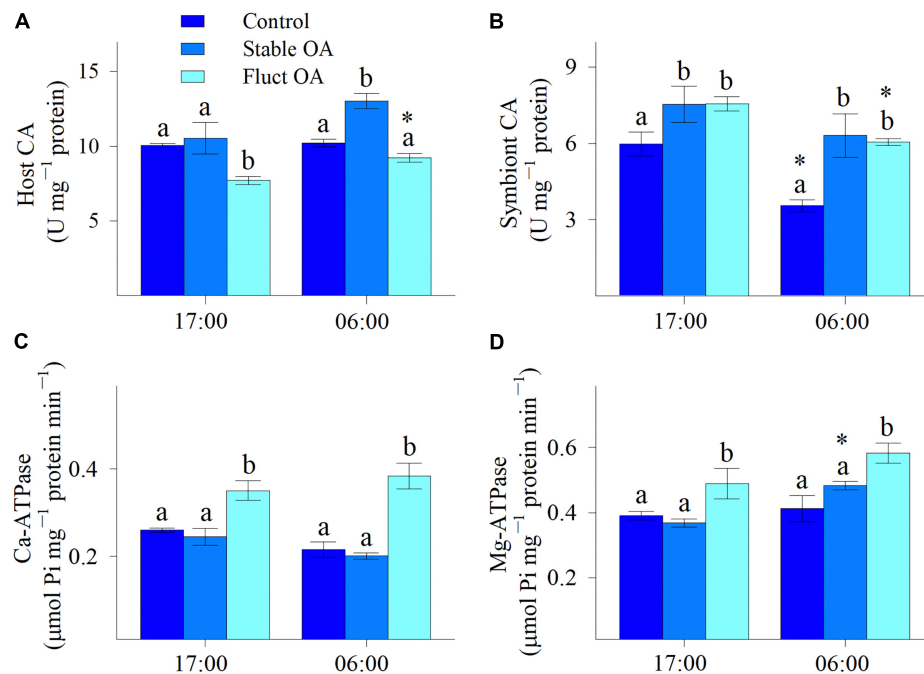


FIGURE 4 | Enzymatic activities of *P. damicornis* recruits exposed to ambient $p\text{CO}_2$ (Control), steady-high $p\text{CO}_2$ (Stable OA), and fluctuating-high $p\text{CO}_2$ (Fluct OA). (A) Host CA; (B) symbiont CA; (C) Ca-ATPase; and (D) Mg-ATPase. Data shown as mean $\pm$ SE. Different letters indicate statistically significant differences in mean values among treatments, while asterisks denote significantly different means between night and day within each treatment.

considerably reduced linear growth and calcification of coral recruits (Albright et al., 2010; Jiang et al., 2015, 2018). The most plausible explanation for this discrepancy is the short exposure duration in this study, during which energy reserves might be adequate to sustain calcification. Unexpectedly, calcification was more adversely affected by fluctuating OA than stable OA, which is in strong contrast to prior studies demonstrating either no, partial or complete mitigation of negative OA effects on calcification in adult corals by diel $p\text{CO}_2$ oscillations (Comeau et al., 2014b; Chan and Eggins, 2017; Cornwall et al., 2018). Furthermore, Dufault et al. (2012) reported that calcification by new recruits of *S. caliendrum* from Hobihu, Taiwan responded positively to diurnally fluctuating $p\text{CO}_2$. It should be noted that the seawater $p\text{CO}_2$ range (365–515 μatm) at the study site of Dufault et al. (2012) was narrower than that at our location (215–1077 μatm). Moreover, OA treatments were more extreme in this study and the nighttime $p\text{CO}_2$ of the fluctuating OA treatment in this study was much higher than that of Dufault et al. (2012). Collectively, these large differences between $p\text{CO}_2$ histories, treatment conditions and also life stages likely account for this magnitude of $p\text{CO}_2$ fluctuations influence on coral skeletal growth.

The greater sensitivity of calcification by coral recruits to fluctuating OA than to stable OA can be attributed to at least three non-exclusive reasons. Firstly, the higher photosynthetic activity under stable OA could better promote and fuel calcification, either through direct energy supply for calcification or through the generation of OH^- which help neutralize the H^+ released from ECF, and thus facilitate a higher pH gradient for

CaCO_3 precipitation (Jokiel, 2011; Comeau et al., 2013a; Gibbin et al., 2014). Secondly, in light of the comparable tissue biomass and the decreased lateral growth in the stable OA treatment, area-normalized biomass was higher in stable OA than fluctuating OA. Hence, the thicker tissue layer of recruits under stable OA could create a better separation between ECF and low- Ω_{Arg} ambient seawater and improve corals' capacity to modulate the internal chemical microenvironment and buffer external acidification (Krief et al., 2010). Finally, it has been proposed that the nighttime storage of DIC mediated by host CA could stimulate daytime calcification of corals under diurnally fluctuating $p\text{CO}_2$ (Dufault et al., 2012). However, our data did not support this hypothesis. Instead, host CA under fluctuating OA was unaffected at night but reduced in light conditions, while host CA under stable OA was significantly up-regulated during the night. Although our study did not distinguish between the proportions of host CA utilized in calcification and photosynthesis, the up-regulation of Ca-ATPase under fluctuating OA (discussed below) most likely points to an increase in host CA functioning in calcification, particularly given that DIC assimilation and Ca^{2+} pumping are tightly coupled in calcification (Tanbutté et al., 1996; Furla et al., 2000; Marshall and Clode, 2003). In other words, the declined host CA activity under fluctuating OA could simply reflect the reduced CA function in DIC uptake for photosynthesis.

This presumption is further evidenced by the increased H^+ pumping activities under fluctuating OA (discussed below) which would produce more CO_2 that can be recycled for photosynthesis (Furla et al., 2000; Moya et al., 2008). In this case, DIC uptake by host CA from the external seawater for photosynthesis may be

reduced to save energy. On the other hand, the increased host CA in the stable OA treatment at night suggests that more DIC could be transported into holobiont (Moya et al., 2008; Bertucci et al., 2013), and this situation would alleviate the daytime DIC competition between calcification and photosynthesis (Furla et al., 2000), further corroborating the unaltered calcification and higher photochemical efficiency under stable OA. It can be therefore concluded that CA activities within coral holobiont are potentially mediated by both environmental $p\text{CO}_2$ and associated changes in other cellular functions.

Proton Pumping and the Trade-Off Between Calcification and Polyp Budding

Interestingly, Ca- and Mg-ATPase activities (i.e., H^+ , Ca^{2+} , and Mg^{2+} pumping) were unaffected in the stable OA treatment, in which calcification was maintained. Also, by using geochemical proxies to assess the chemical profiles within ECF in response to OA, Cornwall et al. (2018) found no change in Ca^{2+} pumping and calcification rates for the coral *Goniopora* sp. and coralline algae *H. reinboldii* under seawater acidification. To a large extent, the unresponsiveness of Ca- and Mg-ATPases to stable OA here could be ascribed to the enhanced photosynthetic performance, which will produce more OH^- for the titration of H^+ from ECF and thus make H^+ export easier (Jokiel, 2011; Comeau et al., 2013a; Yuan et al., 2018).

Studies of the role of Ca- and Mg-ATPases in corals' response to OA have yielded inconsistent results. For instance, Vidal-Dupiol et al. (2013) found that the gene coding for Ca-ATPase was upregulated at pH 7.8 and 7.4 but was down-regulated at pH 7.2 in the coral *P. damicornis*, while Kurihara et al. (2018) reported that gene expression of Ca-ATPase in *A. digitifera* did not change in response to high $p\text{CO}_2$. Direct comparisons between these findings and our results are challenging because gene expression patterns may not reflect the exact content and function of proteins, due to post-translational modifications. On the other hand, de Barros Marangoni et al. (2017) observed a 1.6-fold increase in Ca-ATPase activity in the hydrocoral *M. alcinoris* exposed to acidified seawater (pH < 7.5) for 30 days but not for 16 days; likewise, Prazeres et al. (2015) found that Ca- and Mg-ATPase activities of benthic foraminifera *Amphistegina lessonii* significantly increased (+50%) after 30 days at pH 7.6, while the 15-days exposure exerted no effect. However, another resistant species *Marginopora vertebralis* exhibited unaltered Ca-ATPase activity and a 30% inhibition of Mg-ATPase activity following 30-days exposure to lowered pH (Prazeres et al., 2015). Taken together, treatment duration, together with species-specificity, appears to largely influence the response of these crucial enzymes to OA.

Notably, fluctuating OA elicited significant increases in activities of Ca- and Mg-ATPases, and this means that H^+ pumping was highly activated to maintain pH within the ECF (pH_{CF}). As suggested by Comeau et al. (2018), seawater pH and DIC independently control the pH_{CF} of multiple calcifying species, including *P. damicornis*, and pH_{CF} declined with both decreasing seawater pH and increasing seawater DIC. Therefore,

when seawater pH was lowest and [DIC] was highest at night in the fluctuating OA treatment, Ca- and Mg-ATPase activities were up-regulated to elevate pH_{CF} and Ω_{Arg} , that is, to create a favorable physiochemical microenvironment for calcification (McCulloch et al., 2012; Cai et al., 2016). Nevertheless, given the up-regulation of H^+ pumps under fluctuating OA, more metabolic CO_2 will be generated by mitochondria, further exacerbating the nighttime acidosis of calciblastic cells and thus making H^+ removal more difficult and skeleton more prone to dissolution (Furla et al., 2000; Moya et al., 2008; Jokiel, 2011). Surprisingly, activities of Ca- and Mg-ATPases in the fluctuating OA treatment also greatly increased during the daytime when seawater was $[\text{H}^+]$ lowest. The daytime up-regulation of H^+ pumping under fluctuating OA may simply act as a strategy to promote light calcification and offset the possible night dissolution, thus maintaining overall calcification performance.

Additionally, the enhanced Ca- and Mg-ATPase activities indicate that more Ca^{2+} and Mg^{2+} would be delivered into ECF. It has been recently demonstrated that the ability to increase ECF $[\text{Ca}^{2+}]$ is a key mechanism enabling OA resistance in the reef coral *P. damicornis* (DeCarlo et al., 2018). Furthermore, Mg^{2+} is an important and basic component of center of calcification which controls the nucleation and serves as the basis for the growth of fibrous aragonite crystals (Meibom et al., 2004; Shapiro et al., 2018). The active $[\text{Mg}^{2+}]$ elevation would be, therefore, advantageous for calcification under fluctuating OA. However, the stronger biological control of pH, $[\text{Ca}^{2+}]$ and $[\text{Mg}^{2+}]$ within ECF was still paralleled by a 10% decline in calcification, suggesting that the negative effects of fluctuating OA on skeletal growth could not be fully counteracted by active H^+ pumping. Moreover, this appeared to come at a cost on asexual reproduction, with a 50% decline in polyp budding, which is extremely energetically expensive for a newly settled coral (Graham et al., 2013). Thus, the increased cost associated with maintaining calcification under fluctuating OA may severely compromise energy investment in asexual budding. These results, together with our previous findings (Jiang et al., 2015, 2018), reaffirm the notion that energy is preferentially allocated to skeletal growth over asexual reproduction in juvenile corals when subjected to OA. Therefore, the measurement of calcification alone may be inadequate to detect the physiological maladaptation and overall changes in fitness of coral recruits under stressful conditions.

CONCLUSION

Overall, this study demonstrated significant impacts of $p\text{CO}_2$ fluctuations on the physiological and biochemical properties of juvenile *P. damicornis* in response to OA, and forged the links between coral physiology and the functions of CA, Ca-ATPase, and Mg-ATPase under different $p\text{CO}_2$ regimes. Crucially, the boosted photosynthetic activity under fluctuating OA was insufficient to satisfy the increased energy demand for calcification, potentially causing disproportional energy allocation and arresting other key physiological processes, such as asexual budding and antioxidant system. Evidently,

fluctuating OA is more energetically costly than stable OA for the maintenance of newly settled corals. Since diel $p\text{CO}_2$ oscillations are expected to become more pronounced (Shaw et al., 2013), future OA conditions are likely to be more detrimental to the post-settlement development and early success of corals than predicted, especially in highly dynamic coastal reefs.

DATA AVAILABILITY STATEMENT

All datasets associated with this study are included in the manuscript and the **Supplementary Files**.

AUTHOR CONTRIBUTIONS

LJ and HH conceived and designed the study. LJ conducted the experiments and performed the laboratory analyses. LJ analyzed the data and drafted the manuscript. Y-JG, FZ, Y-YZ, LM, X-CY, X-ML, G-WZ, M-LG, LC, J-SL, P-YQ, and HH contributed to lab analysis and interpretation of the results. All authors commented on the draft and gave final consent for publication.

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Response of the Chinese Soft-Shelled Turtle to Acute Heat Stress: Insights From the Systematic Antioxidant Defense

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Understanding the responses of animals to acute heat stress can help to reveal and predict the effect of more frequent extreme hot weather episodes on animal populations and ecosystems in the context of global climate change. Antioxidant defenses can help to protect animals against oxidative stress caused by intense temperature variation. In the present study, systematic antioxidant responses to acute heat stress ($\Delta 15^{\circ}\text{C}$ and maintained for 12 h) and subsequent recovery were assessed by evaluating gene transcript levels and relative enzyme activities in tissues of *Pelodiscus sinensis*, a subtropical freshwater turtle. Targets included nuclear factor erythroid 2-related factor 2 (Nrf2, the upstream transcription factor), antioxidant enzymes, and the glutathione (GSH) and ascorbic acid (AA) systems. Results showed three main patterns of expression change among antioxidant genes: (1) gene expression of Mn-superoxide dismutase (Mn-SOD), glutathione peroxidase 4 (GPx 4), and catalase (CAT) increased in response to heat stress or recovery in the liver; (2) transcripts of most genes did not change in brain, liver, and kidney of *P. sinensis*; and (3) expression of several GST isoforms were affected by heat stress or recovery in brain and kidney. However, relative enzyme activities involved in antioxidant defense were little affected by acute heat stress and recovery, indicating a relatively conservative antioxidant response in *P. sinensis*. Furthermore, results for malondialdehyde (MDA) levels indicated that acute heat stress and recovery did not cause a net increase in oxidative damage in turtle tissues and, in particular, MDA levels in spleen decreased along with increased splenic ascorbic acid concentration. Overall, the present study revealed a conservative antioxidant response in *P. sinensis*, which may be indicative of a high basal stress tolerance and relate with adaptation to climate change in freshwater turtles.

Keywords: acute temperature elevation, antioxidant defense, ascorbic acid, freshwater turtle, glutathione

INTRODUCTION

An increase in the intensity and frequency of extreme weather is predicted to occur as the result of global climate change (IPCC, 2014). Extreme weather can acutely and significantly affect behavior, growth, and physiology of animals and in some cases cause mortality (McKechnie and Wolf, 2010; Tinsley et al., 2015). Many species have adapted over evolutionary time to seasonal changes in weather (Mousseau and Roff, 1989; Hoffmann and Sgrò, 2011). However, many extreme weather events that are associated with rapid temperature elevation or drastic temperature variation can directly affect survival and physiological performance, such as energy metabolism or immunity, of animals (Gillooly et al., 2001; Zhang et al., 2015). Therefore, it is crucial to understand the effects of acute heat stress caused by extreme hot weather on animals.

There are typically three options for animals responding to changing environmental conditions: (1) move to a different geographic habitat or range (Hahn et al., 2008), (2) physiological responses/adaptations (Breuner and Hahn, 2003), or (3) death. Extreme weather change usually happens very quickly with no prior signaling and, thus, a rapid physiological response generally takes priority, especially in those animals with low mobility. Antioxidant defense, which includes antioxidant enzymes and low molecular weight oxyradical scavengers, is an important physiological component of the stress response (Hermes-Lima et al., 2001). Antioxidant defenses work to clear overproduction of reactive oxygen species (ROS), which are often by-products of disrupted mitochondrial respiration and can be induced by multiple environmental stresses, including temperature variation, hypoxia, UV radiation or exposures to toxic chemicals or metals (Leveelahti et al., 2014; Szekeres et al., 2014; Ibrahim, 2015). Antioxidant enzymes, including superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx), catalyze reactions to destroy ROS (Hermes-Lima et al., 2001). In addition, low molecular weight scavengers, such as glutathione (GSH) or ascorbic acid (AA), also play important roles as reductants in eliminating ROS (Meister and Anderson, 1983; Rice et al., 1995). Insufficient antioxidant defenses result in accumulation of oxidative damage on biomacromolecules, including DNA, protein and lipid, that can lead to final death. Therefore, antioxidant responses to adverse stress can affect the survival of animals and be related to fitness (David et al., 2010; Nicola et al., 2011). Among ectothermic or endothermic vertebrates, numerous studies have revealed enhanced antioxidant defenses during or following environmental stress, indicating that antioxidant defenses are an essential part of stress tolerance and that they are induced in many situations as a preparation for oxidative stress (Lushchak and Bagnyukova, 2006; Schülke et al., 2012; Oliveira et al., 2018).

Turtles are important ectotherms in freshwater systems, where they can experience seasonal or episodic thermal stress. However, there is little information about antioxidant responses by freshwater turtles to acute heat stress. Studies have shown that strong antioxidant defenses benefit the stress tolerance of freshwater turtles when they exposed to environmental stresses, including acute temperature reduction, supercooling, freezing, or

hypoxia (Churchill et al., 1997; Willmore and Storey, 1997a,b; Zhang et al., 2017b). Among these studies of freshwater turtles, a relatively conservative antioxidant response is often indicated by a mild mRNA transcript response and generally stable enzyme activities. However, several questions concerning antioxidant regulation in the heat stress response of freshwater turtles are unanswered. Stresses employed in previous studies, including low temperature, freezing or hypoxia, can be considered as being energy limited or causing metabolic inhibition. However, acute heat stress will clearly elevate the metabolic rate of ectotherms, with consequences for the antioxidant response that are unknown. Therefore, analysis of the systemic antioxidant response to acute heat stress is essential for understanding the stress response strategy of freshwater turtles and revealing their physiological capacity to respond to extreme hot weather.

In the present study, we examined the systematic antioxidant responses by the Chinese soft-shelled turtle, *Pelodiscus sinensis* to acute heat stress ($\Delta 15^{\circ}\text{C}$ in less than 5 min and maintained for 12 h) and following recovery. This species has previously been shown to have good cold tolerance as well as resilience (Zhang et al., 2017b, 2018). We measured changes in the expression levels of nuclear factor erythroid2-related factor 2 (Nrf2), a principal regulator of many antioxidant genes, the mRNA transcript responses by multiple antioxidant enzyme genes (e.g., SOD, CAT, and GPx) and the relative activities of antioxidant enzymes in the brain, liver, and kidney of *P. sinensis*. Changes in mRNA transcript and relative enzyme activities of GSH related enzymes, including glutathione synthetase (GS), glutathione reductase (GR), and glutathione-S-transferase (GST), were also measured in these three tissues as well as L-gulonolactone oxidase (GLO), the key enzyme that regulates AA synthase in the kidney. Levels of AA and the lipid peroxidation biomarker, malondialdehyde (MDA), were also quantified in brain, liver, spleen, kidney, and plasma of *P. sinensis*. MDA was selected because *P. sinensis* is rich in unsaturated fatty acid and MDA was sensitive to acute temperature variation in our previous studies (Huang et al., 2005; Zhang et al., 2017b; Chen et al., 2018). Based on the previous findings in antioxidant strategy of the turtles, we predicted the use of a conservative antioxidant response, composed of small changes in transcript levels and relatively stable enzyme activities, in *P. sinensis* in response to acute heat stress and recovery thereafter.

MATERIALS AND METHODS

Ethics Statement

The experiments were conducted according to the standards of the Ethics and Animal Welfare Committee (EAWC) of Beijing Normal University (Approval No. CLS-EAW-2014-010).

Animal Treatment

The optimum growth temperature for *P. sinensis* ranges from 25 to 32°C. When temperature drops below 15°C, the turtles reduce their physiological activities and prepare for hibernation (Zhang et al., 2017a; Chen et al., 2018); whereas when water temperature rises above 35°C, they enter torpor and die quickly

(Niu et al., 1998). Periods of extreme hot weather normally occur in summer, during which the ambient temperature allows fully functional physiological performance of *P. sinensis*, but the higher end of thermal fluctuation may push the turtles beyond their survival limits. Hence in order to investigate the maximum antioxidant response in the turtle's functional thermal range, we decided to set the acclimation temperature at 18°C, and the heat stress temperature at 33°C.

Acclimation

Juvenile turtles, *P. sinensis* ($n = 48$, mean = 107.1 ± 1.6 g) were purchased from a turtle hatchery (Yutian County, Hebei Province, China) and reared in the laboratory at $18 \pm 1^\circ\text{C}$ room and water temperatures for 3 weeks. The photoperiod was 12L/12D and the turtles were fed daily with commercial standard diets (Hebei Haitai Tech. Ltd., Shijiazhuang, China).

Acute Heat Stress

After acclimation, turtles were fasted for 48 h with water temperature maintained at $18 \pm 1^\circ\text{C}$. A random selection of animals ($n = 16$) were then sacrificed as the control group. Water temperature was then raised acutely to 33°C in less than 5 min and maintained for 12 h. This 12 h time of high temperature stress was selected because it is a typical length of environmental stress applied in similar studies and is also probably the longest time that turtles would experience heat stress in their natural habitat considering diurnal patterns of environmental temperature variation. At the end of the 33°C heat treatment, 16 individuals were sacrificed randomly and defined as the heat stress group ($n = 16$, $\Delta 15^\circ\text{C}$, 12 h). Then water temperature was dropped back to 18°C and maintained

for 24 h to allow animals to recover. The remaining turtles, defined as the recovery group, were then sacrificed ($n = 16$). All turtles were sacrificed by quick decapitation (the typical mode of euthanasia in turtle studies) (Willmore and Storey, 1997a,b; Baker et al., 2007). Blood samples were collected from the neck-chest fracture section into pre-heparin sodium treated EP tubes and then centrifuged at 5500 g for 10 min. Plasma was removed into new tubes and then frozen. The brain, liver, kidney, and spleen were quickly excised, frozen in liquid nitrogen and transferred to -80°C for storage. For analysis of the antioxidant enzyme system, transcript levels and enzyme activities of SOD, CAT, and GPx were measured. For the GSH system and AA system, transcript levels and enzyme activities of GS, GR, GST, and GLO were measured. AA and MDA concentrations in tissues were also measured. Within each group, half of the turtles ($n = 8$) were employed to detect changes in the antioxidant enzyme system and the other half were used to assess the changes in GSH and AA systems.

Total RNA Extraction and RT-PCR

The protocol was the same as in our previous studies (Chen et al., 2015; Zhang et al., 2017a). Total RNA was extracted from all tissues (30–100 mg) using a combination of Trizol reagent (Takara, Japan) and the Nucleospin RNA II kit (Macherey-Nagel, Germany) following the product manual. After RNA extraction, the quality and quantity of RNA ($A_{260}/A_{280} > 1.9$, concentration: $0.2\text{--}0.8 \mu\text{g}/\mu\text{L}$) were determined using agarose gel electrophoresis and a NanoDrop 2000 spectrophotometer (Thermo, United States).

Reverse transcription was conducted with PrimerScript II first strand cDNA synthesis kit (Takara, Japan). A $2 \mu\text{g}$ amount

TABLE 1 | Gene abbreviation, GenBank accession number, forward and reverse primer sequences, and real-time PCR efficiency for the target genes analyzed.

Gene abbreviation	Accession number	Forward primer	Reverse primer	PCR efficiency
<i>EF-1α</i>	NM_001286922.1	CCATCGTTGACATGGTCCCA	ACTTTGTGACCTTGCCAGCT	1.98 ± 0.04
<i>GAPDH</i>	NM_001286927.1	TTCATGGCACTGTCAAGGCT	GGTTGACGCCCATCACAAAC	1.91 ± 0.01
<i>Nrf2</i>	JX470526	GCAGCATCTTCCTTGTCTCTAA	AGTTAGCTTCCTTGCCCTGTCAAA	1.99 ± 0.03
<i>Cu/ZnSOD</i>	JX470524	TGCAGGTGCTCACTTCAATCC	CAACATGCCTCTCTTGATCTTG	2.05 ± 0.02
<i>MnSOD</i>	JX470525	GCCATCAAGCGTGATTTTCG	CTGATACTGCTGTCAGCTTCTCCTT	1.96 ± 0.02
<i>CAT</i>	JX452102	GCAGCGCTTCAATAGTGCAA	GTTTCATCTTCTTCAGCAGCTTTGG	2.06 ± 0.01
<i>GPx1</i>	KC357250	GCTTCCCGTGCAACCAAGTT	CGGCCGACGTAACCTGA	2.04 ± 0.03
<i>GPx3</i>	JX470527	AACCAGTTTGGCAAGCAAGAG	CGGGCCGACGATATTTTC	2.04 ± 0.00
<i>GPx4</i>	JX470528	GCCTCGTCTGCATCATCGT	GTGTAGTTCACGGCCGTCTTG	1.93 ± 0.01
<i>GS1</i>	XM_006116645.1	TTCTGGAACGTGCTCTTG	CCAAACTTCCCTTCGGAAA	1.98 ± 0.02
<i>GS2</i>	XM_006116646.1	AAGCACCTCTTTGAGCAAG	CCCAAGAAGACCGTCTGCA	1.99 ± 0.02
<i>GR</i>	XM_006125372.1	ACGTTGACTGTCTGCTGTGG	TGCAACTGGAGTCAGGAGTG	1.97 ± 0.01
<i>GST1</i>	XM_006130271.1	AGATACAGCGACGTTTGGCA	GCCCCAACGAAGATCCTGAA	1.96 ± 0.02
<i>GST3</i>	XM_006139372.1	ACCCTGAACACGGCCATATC	AGTATGAGTGCCGCTGAACC	1.94 ± 0.01
<i>GSTK1</i>	XM_006111374.1	ACAGCCGTGGATATGACACA	TCAGGCGATTCTTCACCTCT	1.98 ± 0.01
<i>GSTP1</i>	XM_006110953.1	ACGGAAACGTAACCCCTGTACC	TAGATGAGCTGGGCGTATTTG	1.91 ± 0.01
<i>GSTC</i>	XM_006119312.1	TGGCAACTGACATGGTGATT	CCAGCCCCATACAGTGTTTT	1.97 ± 0.02
<i>GSTZ1_X1</i>	XM_006134809.1	ACTGGCAGCTTTAAGAAACCT	TACTAAGGCACCACTCATCT	1.95 ± 0.01
<i>GSTZ1_X2</i>	XM_006134810.1	CCACCCAGCTGAGTGAAAA	AAGAACCGGCTTTCCAGAGG	2.01 ± 0.03
<i>GLO</i>	HQ619721.1	TATCAGGACCACACCGACAA	AGAGCAGGAACCTCCAGCAGA	2.04 ± 0.01

of total RNA template was used for each 30 μ L reaction. The cDNA was then diluted six times and stored at -20°C for use in real-time PCR.

Real-Time PCR for Measurement of Gene Expression

Primer sequences, gene accession numbers and PCR efficiencies for real-time PCR are shown in **Table 1**. The PCR efficiency for each pair of primers was calculated in accordance with our previous study (Zhang et al., 2017a).

Real-time PCR was conducted on a 7500 real-time PCR system (Applied Biosystems, United States). The reaction condition included 10 μ L of $2 \times$ SYBR Green PCR Master Mix (Applied Biosystems, United States), 3 μ L cDNA template, 20 μ M each of forward and reverse primers and 6.5 μ L Milli-Q H_2O . Elongation factor 1 alpha 1 (EF-1 α) was selected as the control gene for brain and liver whereas glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was selected for kidney. The choice of different control genes for different tissues was based on prior testing of several putative control genes that revealed genes whose expression of (EF-1 α or GAPDH) remained constant in a given tissue across all three experimental conditions. The relative expression of each

gene was calculated in accordance with the $2^{-\Delta\Delta\text{CT}}$ method (Schmittgen and Livak, 2008).

Enzyme, Protein, and AA Assays

All enzyme activities were measured using Diagnostic Reagent Kits (Nanjing Jiancheng, China) except for GPx and GLO. The former was measured using a kit from Beyotime company (China), and the latter was determined using high-performance liquid chromatography (HPLC). Tissue samples were homogenized in a ninefold volume of ice-cold physiological saline (0.68% NaCl) and then activities were measured according to product manuals. Detailed methods for activity assays were described in our previous studies (Zhang et al., 2017a,c). Briefly, SOD activity was measured with a cytochrome *c* reduction inhibition reaction using the xanthine–xanthine oxidase system, indicated by an absorption value at 550 nm. The rate of H_2O_2 decomposition per 1 mg protein was used to measure CAT activity. GPx activity was measured by determining the coupled oxidation rate of NADPH during glutathione reductase recycling of oxidized glutathione from GPx regulated reduction of *t*-butyl peroxide. GR activity was determined by monitoring the NADPH-dependent oxidation of GSSG via the change in absorption value at

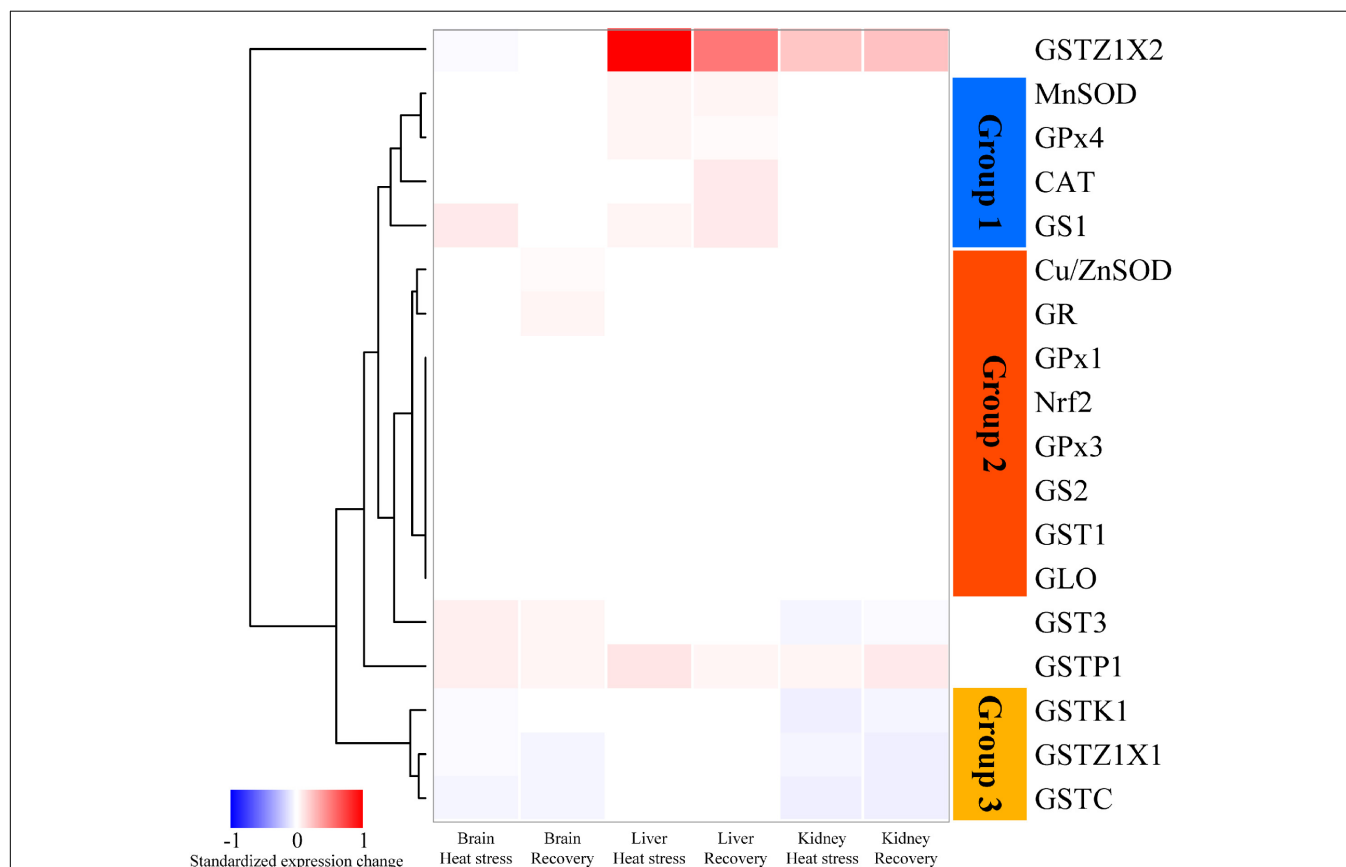


FIGURE 1 | Heatmap clustering of antioxidant gene expression in brain, liver, and kidney of *P. sinensis*. The color scale indicates the standardized expression change in response to heat stress and recovery. The red indicated the upregulated value and the blue indicated the downregulated one. GLO only showed the data in the kidney because it only works specifically in the kidney.

340 nm. GST activity was measured by its conjugation with 3,4-dichloronitrobenzene. MDA concentration was determined via the reaction of MDA and 2-thiobarbituric acid. All these enzyme activities were measured at 25°C, a common temperature for measuring enzyme activity in reptiles.

The assays for renal GLO activity and AA concentration were as described in a previous study (Chen et al., 2015). Briefly, GLO activity was analyzed as the AA concentration synthesized per gram tissue per hour. Total AA concentration (includes ascorbate and dehydroascorbate) was measured by the HPLC method and with an AA standard (10–20 µg AA mL⁻¹).

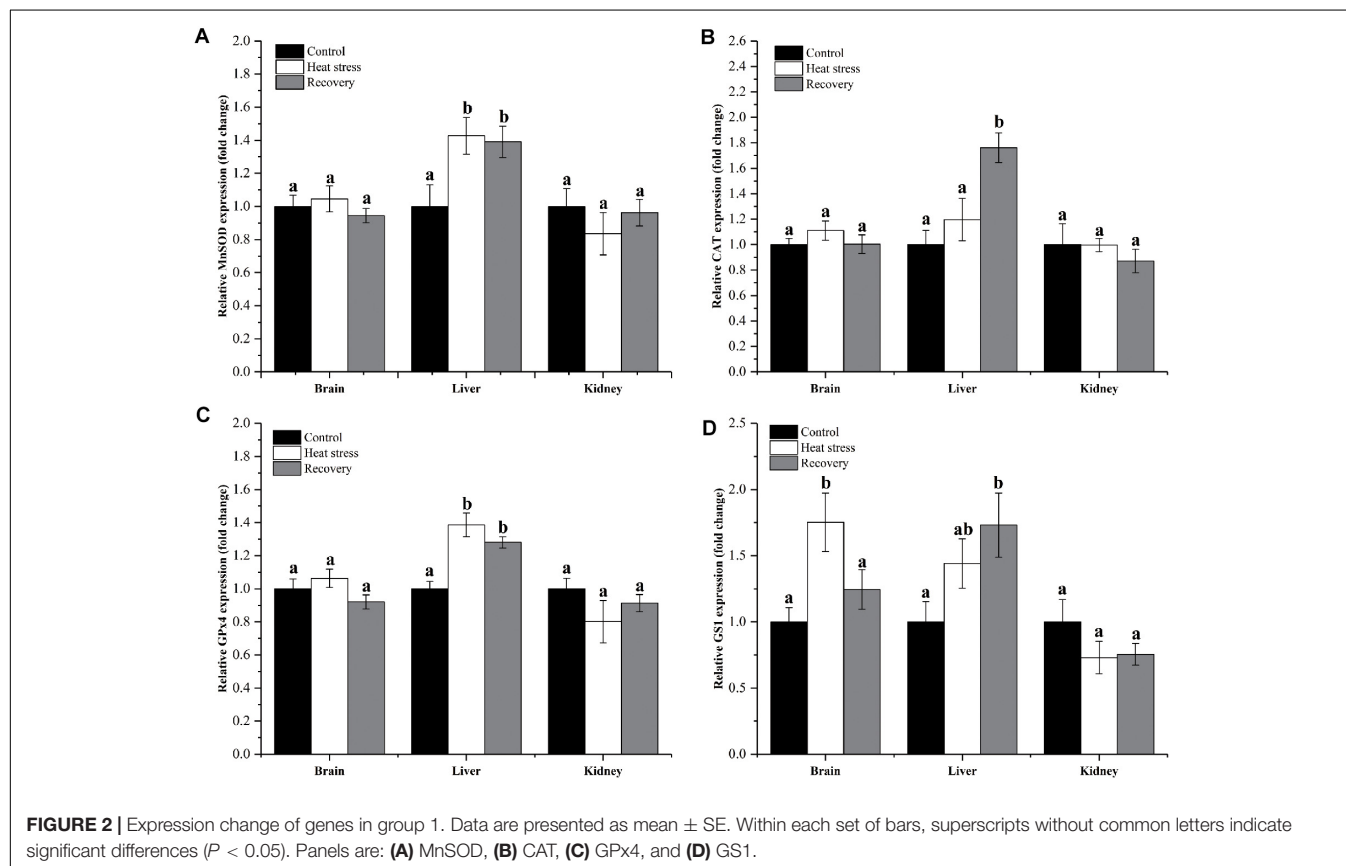
Statistics

A hierarchical clustering algorithm was generated with all standardized data according to a Euclidean method. Gene expression data were standardized to the fold change and then mapped to the interval (−1, 1), wherein the downregulated genes have a negative value and the upregulated genes have a positive value, for clustering analysis. The clustering analysis and the heatmap were conducted with R v. 3.5.0 (Development Core Team, 2018). All data are presented as mean ± SE with $P < 0.05$ accepted as a significant difference. All data were checked for normality and homogeneity of variance. One-way analysis of variance (ANOVA) followed by a Duncan *post hoc* test was employed for between-group comparison if normality and homogeneity of variance were both appropriate; otherwise a Kruskal–Wallis test followed by Mann–Whitney *U post hoc*

test were used (Dytham, 2011). ANOVA analysis was conducted using SPSS package (v. 19, SPSS Inc., United States) and all graphs were made using Origin software (v. 9.0, OriginLab Corporation, United States) except the heatmap.

RESULTS

Figure 1 shows a heatmap illustrating the relative changes in expression patterns by Nrf2 and multiple antioxidant enzymes measured in brain, liver and kidney of turtles exposed to heat stress (12 h at 33°C) and recovery (24 h back at 18°C), as compared with controls (18°C acclimated). The data clustered into three groups. In Group 1 (blue bar), genes including MnSOD, CAT and GPx4, mainly showed significant upregulation of expression in response to acute heat stress and/or recovery in the liver (**Figure 1**). Group 2 genes (orange bar) include Nrf2 and several antioxidant genes (Cu/ZnSOD, GPx1, GPx3, GS2, GR, GST1, and GLO). These showed relatively conservative gene expression responses (minor or not significantly different) to acute stress and subsequent recovery (**Figure 1**). Group 3 showed genes whose expression was mainly depressed by acute heat stress and following recovery in brain or kidney; these include three GST isoforms (**Figure 1**). In addition, three GST isoforms, GSTZ1X2, GST3, and GSTP1, also showed unique patterns of change (**Figure 1**).



Expression changes of genes clustered in group 1 are shown in **Figure 2**. MnSOD and GPx4 mRNAs increased significantly by about 1.4-fold during acute heat stress and recovery in the liver, but not in the other two tissues (MnSOD: $P_{\text{brain}} = 0.551$,

$P_{\text{liver}} = 0.025$, and $P_{\text{kidney}} = 0.534$; GPx4: $P_{\text{brain}} = 0.184$, $P_{\text{liver}} < 0.001$, and $P_{\text{kidney}} = 0.297$; **Figures 2A,C**). CAT mRNA transcripts increased only during the recovery period in the liver, by approximately 1.8-fold ($P_{\text{brain}} = 0.371$, $P_{\text{liver}} = 0.002$, and

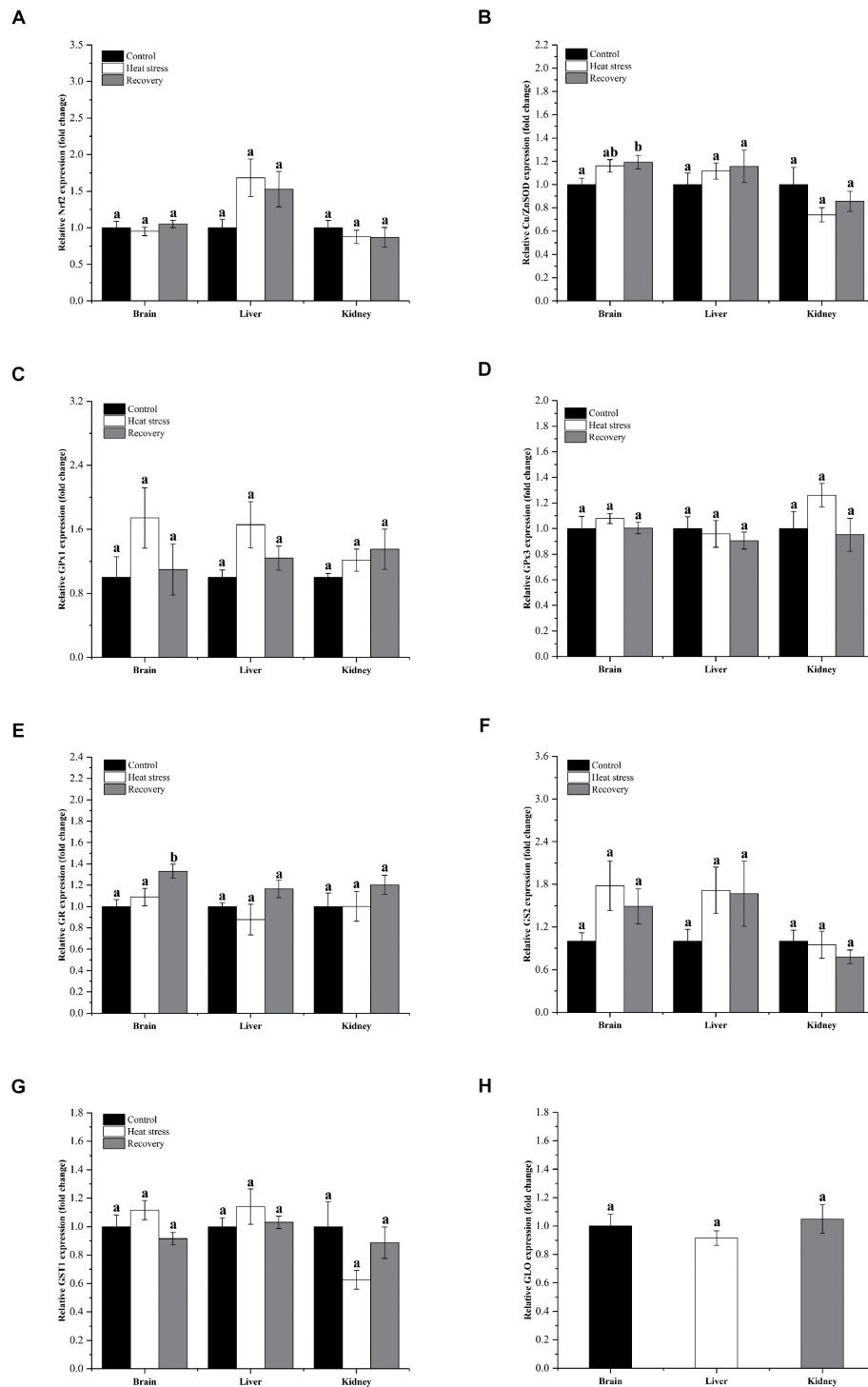


FIGURE 3 | Relatively conserved gene expression in group 1. Data are presented as mean $\pm$ SE. Within each set of bars, superscripts without common letters indicate significant differences ($P < 0.05$). Panels are: **(A)** Nrf2, **(B)** Cu/ZnSOD, **(C)** GPx1, **(D)** GPx3, **(E)** GR, **(F)** GS2, **(G)** GST1, and **(H)** GLO.

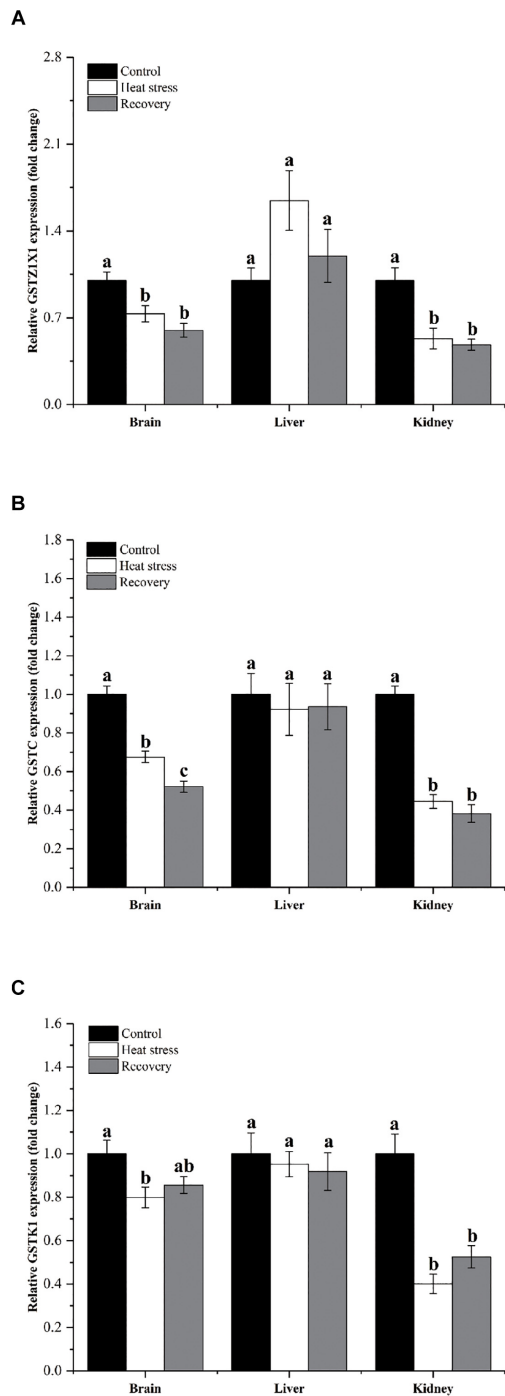


FIGURE 4 | Expression change of genes in group 3. Data are presented as mean $\pm$ SE. Within each set of bars, superscripts without common letters indicated significant difference ($P < 0.05$). Panels are: **(A)** GSTZ1X1, **(B)** GSTC, and **(C)** GSTK1.

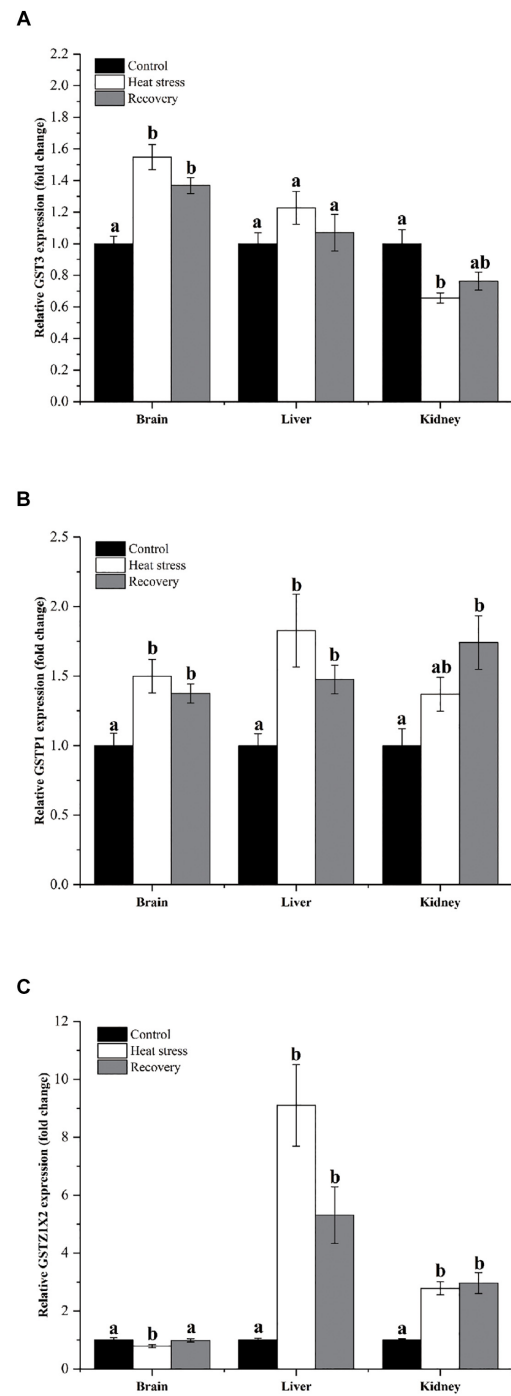


FIGURE 5 | Expression changes by three GST isoforms with unique patterns. Data are presented as mean $\pm$ SE. Within each set of bars, superscripts without common letters indicated significant differences ($P < 0.05$). Panels are: **(A)** GST3, **(B)** GSTP1, and **(C)** GSTZ1X2.

$P_{\text{kidney}} = 0.527$; **Figure 2B**). Expression of GS1 increased during acute heat stress in the brain and during the recovery period in the liver (both ~ 1.75 -fold) ($P_{\text{brain}} = 0.014$, $P_{\text{liver}} = 0.044$, and $P_{\text{kidney}} = 0.281$; **Figure 2D**).

Figure 3 shows detailed expression changes of genes clustered to group 2. Transcript levels of all these genes remained stable during acute heat stress and recovery in all three tissues except for Cu/ZnSOD and GR (all P values are given in

Supplementary Table S1). Both Cu/ZnSOD and GR mRNA levels increased significantly during recovery in the brain of *P. sinensis* (Cu/ZnSOD: $P_{\text{brain}} = 0.046$, GR: $P_{\text{brain}} = 0.011$; **Figures 2B,F**). In addition, GLO expression in kidney did not change over the experimental course, and can also be classified into group 2 ($P = 0.725$; **Figure 3H**).

Figure 4 shows the expression changes of three GST isoforms. GSTZ1X1 mRNA decreased during acute heat stress and following recovery in the brain and kidney, but not in the liver ($P_{\text{brain}} = 0.001$, $P_{\text{liver}} = 0.071$, and $P_{\text{kidney}} = 0.006$; **Figure 4A**). GSTC showed the same pattern in all three tissues ($P_{\text{brain}} < 0.001$, $P_{\text{liver}} = 0.891$, and $P_{\text{kidney}} < 0.001$; **Figure 4B**). GSTK1 mRNA decreased by about 20% during acute heat stress in the brain and was reduced by 50–60% during both acute heat stress and following recovery in the kidney ($P_{\text{brain}} = 0.029$, $P_{\text{liver}} = 0.777$, and $P_{\text{kidney}} < 0.001$; **Figure 4C**).

Expression changes of three other GSH isoforms showed unique patterns of change, as shown in **Figure 5**. GST3 mRNA increased by about 1.4- to 1.5-fold during acute heat stress and recovery in the brain, but decreased during acute heat stress in the kidney ($P_{\text{brain}} < 0.001$, $P_{\text{liver}} = 0.247$, and $P_{\text{kidney}} = 0.007$; **Figure 5A**). GSTP1 showed increased mRNA during acute heat stress and recovery in the brain (1.5-fold) and liver (1.8-fold), whereas transcript levels increased significantly in kidney (1.70-fold) during the recovery period ($P_{\text{brain}} = 0.004$, $P_{\text{liver}} = 0.003$, and $P_{\text{kidney}} = 0.008$; **Figure 5B**). GSTZ1X2 expression was depressed during acute heat stress in the brain but was strongly elevated during both stress and recovery in the liver (nine and fivefold) and kidney (~threefold) ($P_{\text{brain}} = 0.048$, $P_{\text{liver}} < 0.001$, and $P_{\text{kidney}} = 0.001$; **Figure 5C**).

Activities of six antioxidant enzymes are shown in **Figure 6**. Except for GPx and GR, enzyme activities did not change throughout the experiment in any tissue (all P values in **Supplementary Table S1**). Hepatic GPx activity decreased by ~50% during acute heat stress but renal GPx activity increased ~25% in the same period ($P_{\text{brain}} = 0.796$, $P_{\text{liver}} = 0.010$, and $P_{\text{kidney}} = 0.04$; **Figure 6C**). GR activity increased in the brain during acute heat stress and following recovery by ~45% ($P_{\text{brain}} = 0.021$, $P_{\text{liver}} = 0.330$, and $P_{\text{kidney}} = 0.934$; **Figure 6D**).

Figure 7 shows the changes in AA and MDA concentrations in four tissues and plasma of turtles. AA concentrations in the spleen significantly increased (fourfold over controls) during the recovery period ($P = 0.011$; **Figure 7A**). However, AA concentrations did not change significantly in other tissues during stress or recovery ($P_{\text{brain}} = 0.082$, $P_{\text{liver}} = 0.903$, and $P_{\text{kidney}} = 0.06$; **Figure 7A**) or in plasma ($P_{\text{plasma}} = 0.376$; **Figure 7B**). MDA concentration decreased by about 20% in the spleen of *P. sinensis* during both acute heat stress and recovery periods ($P = 0.022$; **Figure 7C**). In other tissues and plasma, MDA concentration did not change ($P_{\text{brain}} = 0.324$, $P_{\text{liver}} = 0.437$, $P_{\text{kidney}} = 0.104$, and $P_{\text{plasma}} = 0.135$; **Figures 7C,D**).

DISCUSSION

Acute heat stress usually links with intensely elevated metabolic rate in ectotherms, leading to greater oxygen consumption as well

as increased ROS production. Enhanced antioxidant defenses induced by acute heat stress have been documented in several ectotherm groups, including snails, fish, and insects (Lushchak and Bagnyukova, 2006; Yang et al., 2010; Troschinski et al., 2014). In the present study, *P. sinensis* showed a relatively conservative antioxidant response to acute and intense heat stress at 33°C followed by 24 h recovery, which suggests a well-balanced performance to an extreme environmental stress.

Activation of Nrf2 expression has been observed in other animal species exposed to heat stress (Wang et al., 2015; Zachut et al., 2017). However, the current data for *P. sinensis*, indicates that the activation of Nrf2 at the transcript level may be unnecessary to the antioxidant response to acute heat stress. Our previous study found that expression of Nrf2 can be activated during the arousal (rewarming) period of hibernating *P. sinensis* hatchlings (Zhang et al., 2017a). It seemed that, after substantial development and growth, the constitutive expression of Nrf2 in the juvenile *P. sinensis* used in the present study appeared to be sufficient to mediate the response to temperature elevation without the need for further gene up-regulation, indicating a conservative response to acute heat stress in *P. sinensis* juveniles. In addition, an alternative yet non-conclusive explanation could be that other mechanisms enable longer half-life of Nrf2 protein in the cell/nucleus may also involve during the process, hence render an increase in Nrf2 mRNA expression is unneeded (Nguyen et al., 2009).

Antioxidant enzymes, including SOD, CAT, and GPx, make up the most important line of defense against oxidative stress in tissues. SOD reduces superoxide radicals to form hydrogen peroxide and both CAT and GPx function in clearing hydrogen peroxide (or other peroxyradicals) (Hermes-Lima and Zenteno-Savín, 2002; Ishii et al., 2002). In the present study, the slight increase in Cu/ZnSOD mRNA in the brain of *P. sinensis* during the recovery period indicated that there may be an increased oxidative stress at this time that triggered increased SOD expression. However, in the liver of *P. sinensis*, our results showed enhanced expression of several antioxidant enzymes during heat stress or following recovery, including MnSOD, CAT, and GPx4. MnSOD is an important isoform of SOD located in the mitochondria and its up-regulation indicates a potential oxidative stress in these organelles during acute heat stress (Jin et al., 2005). Such oxidative stress may be derived from increased superoxide production caused by an enhanced mitochondrial respiratory rate accompanying the rising metabolic rate that results from the elevated ambient temperature. In turn, oxidative stress may damage cell membrane lipids and stimulate increased GPx4 gene expression, whose protein product is known to provide special protection for organelle membrane systems (Brigeliusflohé and Maiorino, 2013). These effects lasted to the recovery period wherein an up-regulation of CAT expression was observed. Interestingly, our previous study found that increased CAT expression occurred in the liver of *P. sinensis* during the recovery period from acute cold stress (Chen et al., 2019). This indicates that activation of CAT is important for *P. sinensis* during the recovery process from intense temperature fluctuation although it did not appear to be sensitive to the temperature stress

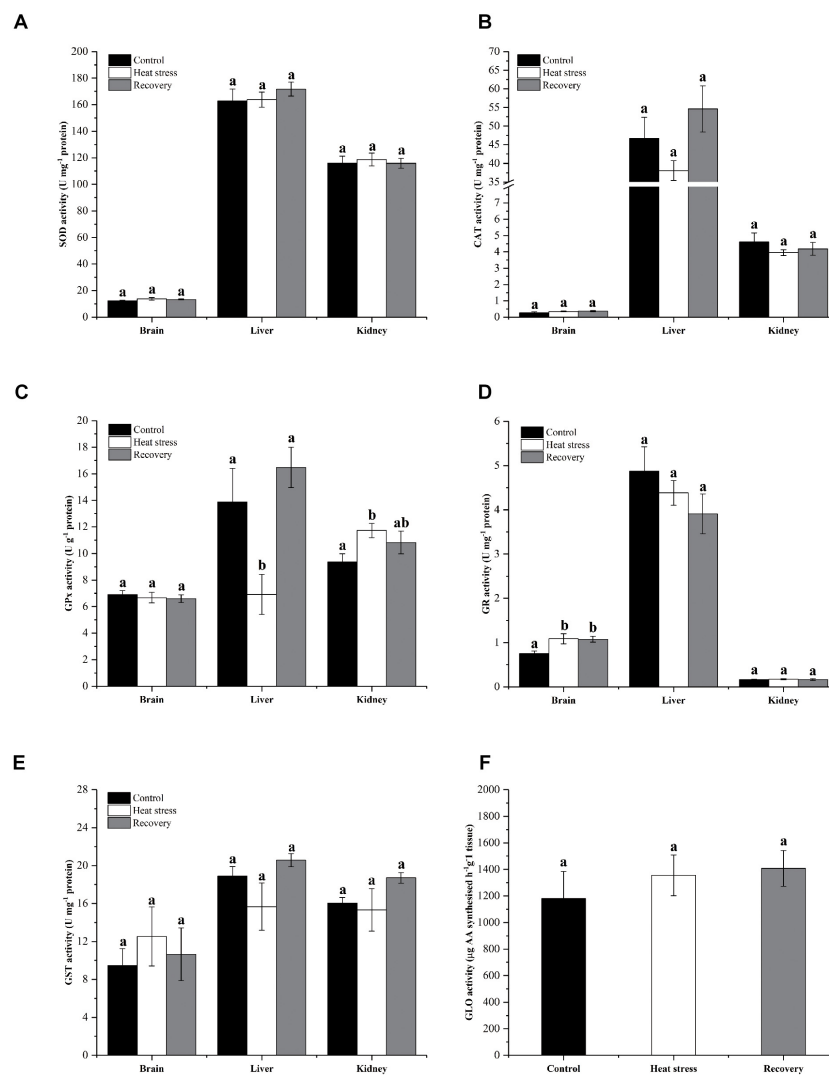


FIGURE 6 | Activities of antioxidant enzymes. Data are presented as mean $\pm$ SE. Within each set of bars, superscripts without common letters indicated significant difference ($P < 0.05$). Panels are (A) SOD activity (U mg^{-1} protein), (B) CAT activity (U mg^{-1} protein), (C) GPx activity (U g^{-1} protein), (D) GR activity (U mg^{-1} protein), (E) GST activity (U mg^{-1} protein), and (F) nephric GLO activity ($\mu\text{g AA synthesized h}^{-1} \text{g}^{-1}$ tissue).

itself. It is also worth noting that the isoform of SOD or GPx which responded to acute heat stress showed lower constitutive expression levels in the tissues of *P. sinensis* than other isoforms, which was also observed in our previous studies (Zhang et al., 2017a). This may indicate that *P. sinensis* regulates isoforms of antioxidant enzyme genes with normally low expression levels while maintaining those with more dominant expression at stable levels in response to acute heat stress or recovery. We also observed that the changes in antioxidant gene expression did not result in a corresponding change of enzyme activities in tissues of *P. sinensis*, which may due to post-transcriptional regulation and was also reported in our previous studies (Zhang et al., 2017a; Chen et al., 2019). In other animals, increased activities of SOD or CAT were observed during heat stress. For example, heat stress ($\Delta 14^{\circ}\text{C}$ for 12 h) increased SOD activity by fourfold in the brain and liver in goldfish *Carassius*

auratus (Lushchak and Bagnyukova, 2006). Similarly, heat stress ($\Delta 6^{\circ}\text{C}$ for 12 h) induced increased CAT activity in the fish *Onychostoma macrolepis* (Yu et al., 2017). Considering the intensity of the acute heat stress used in the present study ($\Delta 15^{\circ}\text{C}$ in less than 5 min and maintained for 12 h), these stable enzyme activities in tissues of *P. sinensis* may indicate a conservative antioxidant enzyme response to acute heat stress or recovery. Analysis of GPx activity, however, showed a different response that compared with the other two antioxidant enzymes. We propose that the decreased in hepatic GPx activity may due to a reduced need for GPx because it shares a similar antioxidant function with CAT. In addition, since GPx needs GSH as a substrate, its activity might be limited if thermal stress impaired glutathione metabolism. In the kidney, the increased GPx activity during heat stress may be related to enhanced metabolic capacity with rising temperature. We have reported

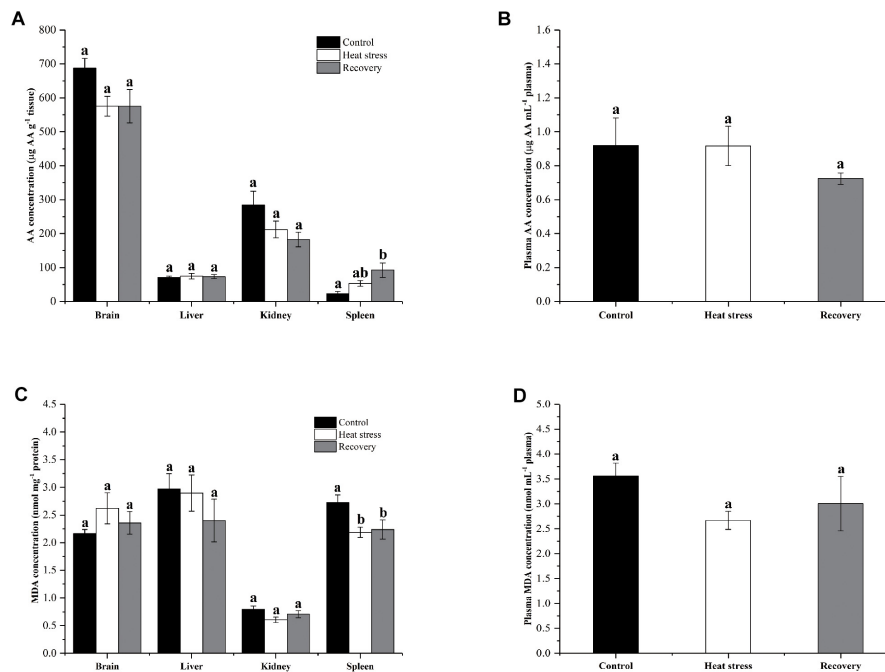


FIGURE 7 | Change in AA and MDA concentrations in tissues and plasma of *P. sinensis*. Data are presented as mean $\pm$ SE. Within each set of bars, superscripts without common letters indicated significant difference ($P < 0.05$). Panels are (A) AA concentrations in tissues ($\mu\text{g AA g}^{-1}$ tissue), (B) AA concentration in plasma ($\mu\text{g AA g}^{-1}$ mL $^{-1}$ plasma), (C) MDA concentration in tissues (nmol mg $^{-1}$ protein), and (D) MDA concentration in plasma (nmol mL $^{-1}$ plasma).

that nephric GPx activity can be depressed by acute cold stress in *P. sinensis* (Chen et al., 2019), and thus, nephric GPx activity of *P. sinensis* is more sensitive to temperature fluctuations than other enzymes.

Glutathione and AA are important antioxidant scavengers in animals, especially in freshwater turtles (Chen et al., 2015; Zhang et al., 2017c). GSH works as the essential substrate of GPx or GST and is synthesized *de novo* by GS or replenished to its reduced form by GR (Saez et al., 1990). It was noteworthy that a potential need for greater GSH content can be inferred in the brain of *P. sinensis* from enhanced GS1 expression and GR activity during acute heat stress as well as increased transcription and activity levels of GR during recovery. This may help to maintain the redox balance in brain of *P. sinensis* during acute heat stress and recovery. By contrast, in the liver, our treatments mainly affected the expression of GS1, an GS isoform with relatively lower constitutive expression levels than GS2 in tissues of *P. sinensis* (Zhang et al., 2017c). In the GSH system, the most impressive change in pattern was observed for GST isoforms, an important family of detoxifying enzymes. Since GSTP1 was highly inducible by acute heat stress and recovery in all three tissues, it may prove be useful as a biomarker of heat stress in *P. sinensis*. The expression levels of different GST isoforms may compensate for each other to some extent, which then resulted in an overall stable GST activity in tissues of *P. sinensis*. Similar results were observed in hibernating *P. sinensis* hatchlings (Zhang et al., 2017c). It seems that net GST activity is stable in the stress response of *P. sinensis* despite of fluctuations in transcript levels of its different isozymes.

Acute heat stress and recovery caused increased splenic AA concentration along with decreased MDA levels, which was also observed in kidney of *P. sinensis* in arousal (rewarming) from hibernation (Chen et al., 2018). Both of these results indicate an essential protective role of AA for *P. sinensis* tissues, especially during the recovery period from stress. Meanwhile, our previous study also indicated that spleen is sensitive to intense or prolonged oxidative stress (Zhang et al., 2019). Thus, we proposed that the spleen requires extra antioxidant protection, in the form of increased AA concentrations, during acute heat stress and recovery. This may be of the results of limited AA storage capacity of this small-sized tissue or dealing with the needs of the hematological function of spleen, which could be important in meeting metabolic demands during acute heat stress. When AA replenishment is limited or cannot meet the needs of the spleen under stress conditions, *P. sinensis* may be at risk of oxidative damage to the spleen (Chen et al., 2018). Therefore, one key adjustment needed for *P. sinensis* to endure acute heat stress or frequent extreme weather may be to maintain an effective and sufficient AA supply for important tissues, such as spleen.

From a systematic view, our results suggest a relatively conservative antioxidant response strategy and also a good tolerance of acute heat stress of *P. sinensis*. In this response strategy, the turtle maintains high constitutive expression and activity levels of most antioxidant enzymes in important tissues when being exposed to environmental stress. Meanwhile, in the liver, the most important metabolic tissue, the isoforms of antioxidant enzymes with relatively lower constitutive expression levels responded to temperature elevation. Another important

process is the rapid regulation of AA storage in tissues (especially spleen) to meet the extra need of antioxidant defense. Interestingly, a similar strategy was also found in *P. sinensis* dealing with cold stress and recovery (Chen et al., 2019). Together these two studies indicate a good adaptive capacity of the antioxidant defense system in this subtropical freshwater turtle to acute temperature fluctuation. The finding that these turtles rely on stable/conservative and basal constitutive antioxidant defenses to endure environmental stress, i.e., acute heat stress in this case, was also congruent with the strategy identified in similar studies on other turtle species under various stress contexts (Willmore and Storey, 1997a; Baker et al., 2007). These all suggested a unique but shared stress response strategy in turtles as comparing with other animals. The stable and conservative antioxidant response allows *P. sinensis* to endure the heat stress employed here but it also suggested that this conservative antioxidant response may not always be able to meet the need for antioxidant protection under more severe or prolonged stress conditions. In particular, the AA replenishment capacity in the spleen may affect the destiny of *P. sinensis* during acute heat stress, in the content of more frequent extreme hot weather.

In conclusion, this is the first study to assess the antioxidant response to acute heat stress in a freshwater turtle species. A novel systemic antioxidant response was observed in tissues of *P. sinensis* that was different from those seen in other animal groups. We can summarize three main changing patterns of antioxidant genes in *P. sinensis* during acute heat stress and recovery: (1) Acute heat stress or recovery caused increased gene expression of several isoforms of antioxidant enzyme genes in the liver of *P. sinensis*; notably, these were isozymes that showed relatively low constitutive expression levels as compared with than other isoforms in the same gene family; (2) Expression of most antioxidant genes and Nrf2 did not change in brain, liver and kidney of *P. sinensis* throughout; (3) Acute heat stress and recovery affected the expression of GST genes in the brain and kidney of *P. sinensis*. Overall, antioxidant enzyme activities remained stable and no oxidative damage was observed, as

indicated by MDA levels, in tissues of *P. sinensis*. The data also indicate that the spleen needs extra protection from AA to endure acute heat stress and recovery periods. Our study revealed a relatively conservative antioxidant defense system, which can allow *P. sinensis* to endure intense temperature elevation, from 18 to 33°C with no oxidative damage.

ETHICS STATEMENT

The experiments were conducted according to the standards of the Ethics and Animal Welfare Committee (EAWC) of Beijing Normal University (Approval No. CLS-EAW-2014-010).

AUTHOR CONTRIBUTIONS

BC and CN conceived the study. WZ, BC, LY, and HJ collected the data. WZ and BC analyzed the data. WZ wrote the initial draft of the manuscript. BC, CN, and KS revised the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fphys.2019.00710/full#supplementary-material>

TABLE S1 | *P* values and statistics for all parameters in tissues of *P. sinensis* during acute heat stress and recovery periods.

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Short- and Medium-Term Exposure to Ocean Acidification Reduces Olfactory Sensitivity in Gilthead Seabream

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The effects of ocean acidification on fish are only partially understood. Studies on olfaction are mostly limited to behavioral alterations of coral reef fish; studies on temperate species and/or with economic importance are scarce. The current study evaluated the effects of short- and medium-term exposure to ocean acidification on the olfactory system of gilthead seabream (*Sparus aurata*), and attempted to explain observed differences in sensitivity by changes in the protonation state of amino acid odorants. Short-term exposure to elevated PCO_2 decreased olfactory sensitivity to some odorants, such as L-serine, L-leucine, L-arginine, L-glutamate, and conspecific intestinal fluid, but not to others, such as L-glutamine and conspecific bile fluid. Seabream were unable to compensate for high PCO_2 levels in the medium term; after 4 weeks exposure to high PCO_2 , the olfactory sensitivity remained lower in elevated PCO_2 water. The decrease in olfactory sensitivity in high PCO_2 water could be partly attributed to changes in the protonation state of the odorants and/or their receptor(s); we illustrate how protonation due to reduced pH causes changes in the charge distribution of odorant molecules, an essential component for ligand-receptor interaction. However, there are other mechanisms involved. At a histological level, the olfactory epithelium contained higher densities of mucus cells in fish kept in high CO_2 water, and a shift in pH of the mucus they produced to more neutral. These differences suggest a physiological response of the olfactory epithelium to lower pH and/or high CO_2 levels, but an inability to fully counteract the effects of acidification on olfactory sensitivity. Therefore, the current study provides evidence for a direct, medium term, global effect of ocean acidification on olfactory sensitivity in fish, and possibly other marine organisms, and suggests a partial explanatory mechanism.

Keywords: olfaction, ocean acidification, fish, amino acid, receptor, olfactory epithelium, carbon dioxide, protonation

INTRODUCTION

Since the Industrial Revolution, atmospheric carbon dioxide (CO_2) levels have been steadily increasing and the oceans of the world act as a sink for much of this. As atmospheric CO_2 has increased from pre-industrial (about 280 $\mu\text{atm CO}_2$) to present day values (about 390 $\mu\text{atm CO}_2$) (Doney et al., 2009), this has led to a fall in oceanic pH of 0.1 unit, with a further fall of 0.77 units predicted by the year 2300 (Doney et al., 2009). Global mean oceanic CO_2 levels are expected to reach 1,000 μatm by the year 2100, and 1900 $\mu\text{atm CO}_2$ by 2300 (Caldeira and Wickett, 2003). Studies prior to 2009 suggested that fish, as effective acid-base regulators, were not particularly sensitive to changes in PCO_2 . However, more recent studies have documented notable impacts on neurosensory and behavioral endpoints in fish at PCO_2 levels predicted to occur before the end of the 21st century (reviewed in: Heuer and Grosell, 2014; Clements and Hunt, 2015; Ashur et al., 2017). Consistent disturbances have been noted across a range of sensory systems, including olfaction (Dixon et al., 2010; Cripps et al., 2011; Devine et al., 2012a,b), hearing (Simpson et al., 2011), and vision (Chung et al., 2014), and have also been implicated in processes linked to general cognitive function including changes in lateralization (Domenici et al., 2012) and learning (Ferrari et al., 2012). The implications of these disturbances are expected to be substantial and include changes to dispersal, recruitment, connectivity, social interactions, predator-prey dynamics, population replenishment, biodiversity, habitat preference, and settlement timing; all of which could drastically affect population and ecosystem dynamics (Heuer and Grosell, 2014).

Evidence suggests that predicted end-of-the-century PCO_2 levels will have adverse effects on the olfactory-mediated behavior of most fish species, including reef species (Dixon et al., 2010; Munday et al., 2016a,b), temperate species (Jutfelt et al., 2013), and sharks (Green and Jutfelt, 2014; Dixon et al., 2015). For example, clownfish larvae exposed to 1,000 $\mu\text{atm CO}_2$ did not respond appropriately to the odor of predatory fishes (Dixon et al., 2010); moreover, juvenile damselfish exposed to increased PCO_2 and then released into the wild suffered an 8-fold increase in predation (Munday et al., 2010). In both studies, control juveniles avoided predator odors, whereas CO_2 -treated juveniles were attracted to them (Dixon et al., 2010; Munday et al., 2010), suggesting that mal-adaptive behavioral responses may result from altered sensory processing by the central nervous system. An important step in understanding the basis for neurosensory disruption in fish was reported by Nilsson and colleagues in 2012, who demonstrated that the GABA and GABA_A receptors were associated with disruptions in olfaction and lateralization during CO_2 exposure (Nilsson et al., 2012). The addition of gabazine, a GABA_A receptor antagonist, was found to re-establish adaptive olfactory-mediated behavior and lateralization in CO_2 -exposed fish (Nilsson et al., 2012). Since then, other studies have demonstrated the ability of gabazine to restore much of the high PCO_2 related altered behaviors (Chivers et al., 2014; Hamilton et al., 2014; Lai et al., 2015).

To date, disruption in brain neurotransmitter function has been proposed as the main mechanism involved in the behavioral effects of high CO_2 in fish (critically reviewed by Tresguerres and Hamilton, 2017). However, the direct effect of high PCO_2 /low pH on the olfactory system may have been underestimated. Recently, acute exposure to acidified seawater was shown to directly reduce olfactory sensitivity of the sea bass (*Dicentrarchus labrax*) to some odorants (Porteus et al., 2018); however, the mechanism remains unknown. In addition, neither medium- or long-term effects on sensitivity were not investigated, nor whether the olfactory system was able to respond at the structural level and counteract the effects of acidification. Assessing the occurrence of morphological and/or physiological alterations in response to increased PCO_2 is crucial to predict the effects of ocean acidification in fish. Therefore, the main objective of the current work was to evaluate the effects of both acute and longer-term exposure to low pH/high PCO_2 on the olfactory sensitivity of the gilthead bream (*Sparus aurata*), an economically important species. The observed effects on olfactory sensitivity were considered in the context of predicted effects of pH change on the protonation of odorant molecules. Finally, we evaluated whether exposure to high PCO_2 provoked structural changes in the olfactory epithelium that might be indicative of adaptation of the olfactory epithelium to lower water pH.

MATERIALS AND METHODS

Fish Maintenance

Animal maintenance and experimentation were carried out in certified experimental facilities and followed Portuguese national legislation (DL 113/2013) under a “group-1” license by the Veterinary General Directorate, Ministry of Agriculture, Rural Development and Fisheries of Portugal. Gilthead seabream (*S. aurata*: 180 $\pm$ 7 g; 22.8 $\pm$ 0.6 cm), hereafter “seabream,” were obtained from a commercial supplier (Maresa-Mariscos de Esteros, SA, Huelva, Spain) and maintained in 1,000 L tanks with continuously running natural seawater, under natural photoperiod and temperature, and fed with commercial pellets (Sparos, Olhão, Portugal). For experimental purposes, seabream were randomly distributed into six 100 L tanks in an open circuit system kept at natural temperature and photoperiod. Three tanks were kept in control conditions (“control fish”), while the other three were kept at low pH by bubbling CO_2 gas (see below) into the water and maintained for 4–8 weeks before electrophysiology experiments (“high CO_2 fish”). No mortality was observed during this time.

Seawater Chemistry

Seawater was pumped from the ocean into two (2,000 L) header tanks, where it was aerated with ambient air (control) or CO_2 to achieve the desired pH (elevated- CO_2 treatment). The PCO_2 of the CO_2 treatment header tank was maintained at the target value of 1,000 μatm using a pH probe connected to an internal controller (EXAxt PH450G, Yokogawa Iberia, Portugal).

Control and high CO₂ seawater was supplied to 100 L tanks at 2 L min⁻¹. Seawater pH (Orion star A221, Thermo Scientific, Portugal), temperature (Orion star A221, Thermo Scientific, Portugal), and salinity (WTW, cond3310, Spain) were recorded daily in each tank. Water samples were analyzed for total alkalinity twice a week by Gran titration (DL15 titrator, Mettler Toledo, Portugal) using a certified acid titrant (0.1 M HCl, Fluka Analytical, Sigma-Aldrich). Carbonate chemistry parameters (Table 1) were calculated in CO2SYS (Pierrot et al., 2006) using the constants K1 and K2 from Mehrbach et al. (1973) refit by Dickson and Millero (1987) and Dickson (1990) for KHSO₄.

Selection of Odorants

Odorant, in the context of the current study, can be defined as any chemical detected by the olfactory system which, in vertebrates, is the chemosensory system associated with cranial nerve I (discussed in Mollo et al., 2014, 2017). The effect of acute (minutes) and medium-term (4 weeks) exposure to high PCO₂ on olfactory sensitivity was tested for amino acids, bile fluid, and intestinal fluid. Olfactory sensitivity to amino acids in fish is believed to be mainly involved in food detection (Hara, 1994; Velez et al., 2007). Bile fluid and intestinal fluid are known to be potent olfactory stimuli in fish, possibly involved in chemically-mediated interactions both within and between species (Velez et al., 2009; Huertas et al., 2010; Buchinger et al., 2014; Fatsini et al., 2017; Hubbard et al., 2017). Therefore, we tested the effect of elevated CO₂/low pH on the olfactory responses of seabream to conspecific bile, intestinal fluid, and a range of amino acids in fish maintained under control conditions (pH nominally 8.2; PCO₂ nominally 350 µatm) and high CO₂ conditions (pH nominally 7.7, PCO₂ nominally 1,000 µatm) for 4–8 weeks.

Collection of Body Fluids

Body fluids (bile and intestinal fluid) were collected from five seabream (144 ± 16 g; 18.7 ± 1.0 cm; all males) kept in normal seawater. Fish were anesthetized with 2-phenoxy ethanol (Sigma-Aldrich, Portugal; 2.0 ml L⁻¹) and quickly sacrificed by decapitation. Bile fluid was taken directly from the gall bladder and the intestinal fluid was extracted from the posterior 10 cm of intestine. Samples were pooled, diluted in distilled water (1:2), mixed thoroughly, centrifuged, aliquoted, and frozen (−20°C) until use.

Olfactory Nerve Recording

Seabream were anesthetized in aerated natural seawater containing 300 mg L⁻¹ MS222 (ethyl-3-aminobenzoate methanesulfonate

salt, Sigma-Aldrich, Portugal) until the response to a tail pinch had stopped; an intramuscular injection of the neuromuscular blocker gallamine triethiodide (Sigma-Aldrich, Portugal; 10 mg/kg in 0.9% NaCl) was then given. Fish were then placed in a padded V-support and the gills flushed with aerated seawater containing 150 mg l⁻¹ MS222. The pH of the water flushing the gills was around 8.2 for control fish and 7.7 for fish kept in high CO₂. Therefore, for control fish, only the olfactory epithelium experienced high CO₂ conditions during electrophysiology experiments; water chemistry parameters during experiments are summarized in Table 2.

The olfactory rosette was exposed by cutting the skin and connective tissue overlying the nasal cavity. The nostril was constantly irrigated with filtered sea water (without anesthetic) under gravity (flow rate: 6 ml min⁻¹) via a glass tube. Test solutions were delivered to the tube irrigating the nasal cavity via a computer-operated three-way solenoid valve for 4 s. The olfactory nerves were exposed by removal of the skin, connective tissue, and overlying bone (Hubbard et al., 2000, 2003a). Charcoal-filtered sea water was used to make up the odorants solutions and to irrigate the olfactory rosette during experiments. This water was either bubbled with air (control) or CO₂ (low pH) until the desired pH_{NBS} was reached. Amino acid solutions were prepared from frozen aliquots of 10⁻² M; all stimuli were diluted in charcoal-filtered sea water (either control or high CO₂ as appropriate) immediately prior to use. The order in which odorants were given was randomized, but each odorant was always given from the lowest to the highest concentration.

The electrodes were placed in the olfactory nerve in a position that gave maximal response to 10⁻³ M L-serine. Olfactory nerve activity was recorded using tungsten micro-electrodes (0.1 MΩ, World Precision Instruments, UK) as previously described (Hubbard et al., 2000). Fish were connected to earth via a copper wire inserted in the flank. The raw signal was amplified (x20,000; AC pre-amplifier, Neurolog NL104; Digitimer Ltd., Welwyn Garden City, UK), filtered (high pass: 200 Hz, low pass: 3,000 Hz; Neurolog NL125, Digitimer Ltd.) and integrated (time constant 1 s; Neurolog NL703, Digitimer Ltd.). Raw and integrated signals were digitized (Digidata 1440A, Molecular Devices, San Jose, CA, USA) and recorded on a PC running AxoScope™ software (version 10.6, Molecular Devices).

TABLE 1 | Water chemistry parameters for control and elevated CO₂ tanks.

Parameter	Control CO ₂	High CO ₂
pH _{NBS}	8.184 ± 0.004	7.813 ± 0.001
Temperature (°C)	17.0 ± 0.2	18.4 ± 0.3
Salinity (ppt)	34.6 ± 0.02	34.6 ± 0.02
Total Alkalinity (µmol/kg SW)	2,511 ± 15	2,531 ± 21
pCO ₂ (µatm)	349 ± 19	1,039 ± 49

Data are given as mean ± SEM. The slight difference in temperature was caused by different exposure to the sun of the header tanks (temperatures were measured at 14:30).

TABLE 2 | Water chemistry parameters for control and elevated CO₂ fish in the electrophysiology set up.

	Control fish		High CO ₂ fish	
	Control water	High CO ₂ water	Control water	High CO ₂ water
pH _{NBS}	8.20 ± 0.01	7.70 ± 0.01	8.19 ± 0.01	7.72 ± 0.01
Temperature	22.0 ± 0.2	22.0 ± 0.2	20.4 ± 0.3	20.4 ± 0.3
Salinity	34 ± 0.2	34 ± 0.2	34 ± 0.2	34 ± 0.2
Total alkalinity (µmol/kg SW)	2,562 ± 32	2,575 ± 29	2,648 ± 15	2,655 ± 17
pCO ₂	296 ± 12	1,150 ± 23	318 ± 9	1,127 ± 23

Data are given as mean ± SEM.

All integrated response amplitudes were normalized to the amplitude of the integrated response to 10^{-3} M L-serine (the “standard”). Responses to the standard were recorded regularly at the beginning and end of each group of samples (every three to five samples) throughout the recording session. Each stimulus was applied for 4 s, with at least 1 min between odorants to allow complete recovery of the receptors.

Calculation of Amino Acid Protonation State

Odorant molecules, such as amino acids, can be subjected to ionization processes depending on the pH of their surroundings. Amino acids possess two or more ionizable functional groups that are de-protonated at different pH levels. The pK_a value of each ionizable group of a molecule represents the pH at which 50% of the molecules in solution are protonated at this group and 50% remain unchanged. The Henderson-Hasselbalch equation relates pH and the group-specific pK_a values for each amino acid, and therefore calculates the abundance of different protonation states across the entire pH range of interest (for details, see Po and Senozan, 2001, and references therein). At any given pH, a mixture of different protonation states can be present. For the current study, we assumed that the amino acid forms with deprotonated head group to be the form binding to the olfactory receptors. We, therefore, calculated the concentration of this form (= effective concentrations) of different amino acids used in the experiments for the control (pH 8.2) and treatment (pH 7.7) conditions based on the concentration used during the bioassay and the group-specific pK_a values for each amino acid obtained from the CRC Handbook of Chemistry and Physics (Lide, 2004).

Comparison of pH-Induced Changes in Amino Acid Protonation State to Olfactory Potency

For direct comparison of the change in protonation state and the olfactory response for different concentrations and pH conditions, the respective effective stimulus concentrations were inserted into the linear regression equation/Hill plot obtained from the integrated nerve response at a given pH (see “Data Analysis” for details). The resulting points were plotted in the same figure to visualize the extent of change caused by stimulus protonation vs. the extent of change observed for the nerve response.

Conformers and Charge Distribution of Different Protonation States by Quantum Chemical Calculations

We used quantum chemical calculations to obtain the energetically most favorable model conformers for the two protonation states of interest and assess differences in their molecular electrostatic potential (MEP), which describes the charge distribution around the molecule. Starting from the structures published by Rak et al. (2001), the protonation state models of L-arginine were optimized using the PBE0 exchange correlation functional

(Adamo and Barone, 1999) with a pc-2 basis set (Jensen, 2001, 2002a,b) and water as implicit solvent using COSMO (Klamt and Schüürmann, 1993) implemented in the ORCA suite of programs (Neese, 2012) (Version 3.0.0). We used the RIJ-COSX approximation (Neese et al., 2009) with a def2-TZVPP/J auxiliary basis set (Weigend and Ahlrichs, 2005) and included D3 dispersion corrections following Grimme et al. (2010, 2011). The VeryTightSCF and TightOpt criteria implemented in ORCA were used to stop the SCF gradient and the optimization at a total energy change of $<10^{-8} E_h$, respectively. Full optimization was confirmed by ensuring that there were no negative numerical frequencies for each conformer. The calculation of the molecular electrostatic potential (MEP) was performed with the GAMESS program (vJan122009R1) using the PBE exchange correlation functional (Perdew et al., 1996) in conjunction with a STO-3G basis set (Hehre et al., 1969; Collins et al., 1976). Three-dimensional electron density iso-surface was visualized with 100 grid points, a medium grid size, and a contour value of $0.03 e \cdot a_0^{-3}$ using the MacMolPlt program (Bode and Gordon, 1998) (v7.5141). The density iso-surface was colored according to the MEP with a maximum value of $0.25 E_h \cdot e^{-1}$ and the RGB color scheme with red representing positive, green neutral, and blue negative charge.

Histology

Seabream were killed rapidly using an anesthetic overdose (500 mg/l MS222). Olfactory rosettes were carefully dissected from 12 fish (six controls, and six in high CO_2 water for at least 4 weeks) under a stereo-microscope. Olfactory epithelia were fixed in Bouin's solution (Sigma, Portugal) for 20 h and washed with 70% ethanol. Tissues were then embedded in paraffin and sectioned longitudinally (5 μ m sections). Serial sections of the tissues were collected until the complete olfactory organ was visible (including the central raphe). The dewaxed and rehydrated sections were stained with Mayer's hematoxylin/eosin and neutral and acidic mucus was identified using a combined periodic acid-Schiff (PAS)-Alcian blue stain. Slides were examined under a microscope (Leica DM 2000, Famacilção, Portugal) and photographic images were obtained using a digital camera (Leica DFC 480, IM50-software). For analysis, we focused on the three central lamellae (lamellae 7, 8, and 9) of the olfactory rosette. The ratio between nonsensory vs. sensory epithelium within an olfactory lamella was assessed by dividing the length of the apical nonsensory epithelium by the length of the olfactory lamella (from the top to the central raphe). Images were analyzed using the software Fiji Image J. Statistically significant differences between the ratio of nonsensory vs. sensory epithelium in control animals and experimental animals was assessed using Student's *t* test (Prism 6). The goblet (mucus producing) cells in the apical surface of the lamellae were quantified using Fiji Image J.

Data and Statistical Analysis

All statistical analyses of electrophysiological results were carried out on normalized data. To determine whether there were effects of tank pH (i.e., control vs. high CO_2), odorant pH and concentration, an ANOVA analysis was performed

(SPSS statistics). Factors that showed an effect were further analyzed by linear regression or fit to a three-parameter Hill plot.

Olfactory nerve responses to L-serine, L-leucine, L-arginine, bile fluid, and intestinal fluid were analyzed by linear regression; comparison between nerve responses at different tank, and odorant pH was assessed by linear regression of log-transformed data (Hubbard et al., 2003b) and comparing both the slopes and elevations (Prism 6). The slope of each curve indicates how a given increase in odorant concentration evokes a higher amplitude response; this depends on the binding affinity between ligand (odorant in this case) and receptor. Detection thresholds, which also depend on ligand-receptor affinity, were determined for each independent experiment, from the intercept with the x -axis of linear regression fit to individual concentration-response curves for each odorant and each treatment (Prism 6). Differences between detection thresholds were tested using Student's t test for paired data (log). Responses to L-glutamine and L-glutamic acid were described by a three-parameter Hill curve as previously described (Hubbard et al., 2000). EC_{50} and E_{max} were calculated for each fish. These data were then compared using Student's t -test (Prism 6).

To evaluate whether normalized data could be used to compare olfactory responses between control and high CO_2 fish, we first compared raw integrated responses to L-serine at pH 8.2 in control and high CO_2 fish. There were no differences in the slope ($p = 0.71$) or elevation ($p = 0.88$) of the concentration-response curves to L-serine in control and experimental animals (data not shown); therefore, we compared normalized olfactory nerve responses in control and experimental fish. A significance level of $p < 0.05$ was used throughout.

RESULTS

For ANOVA analysis, the factors analyzed were the effects of tank pH (i.e., the pH at which the fish were kept: pH 7.7 or 8.2), odorant pH (i.e., the pH of the odorant solutions delivered to the olfactory epithelium: pH 7.7 or 8.2) and odorant concentration (10^{-3} to 10^{-6} M for the amino acids). Results are summarized in Table 3. While tank and odorant pH only showed significant effects for some of the odorants, the concentration always had a significant effect. Factors that showed an effect were further analyzed by linear regression or fit to a Hill three-parameter concentration-response curve.

Effects of Odorant pH on Olfactory Sensitivity in Control Fish

In control seabream (kept at pH 8.2 prior to electrophysiological recording), the elevation of the concentration-response curves to L-serine ($F_{45} = 9.363$, $p < 0.01$), L-leucine ($F_{53} = 25.225$, $p < 0.0001$), and L-arginine ($F_{52} = 0.1736$, $p = 0.68$) tested at pH 8.2 was significantly higher than at pH 7.7. However, there were no differences between the slope of the concentration-response curves at pH 8.2 and 7.7 (Figures 1A–C). In addition, exposure to low pH water significantly increased the threshold of detection (Figure 1F)

TABLE 3 | ANOVA analysis (Univariate analysis of variance, SPSS) for factors: tank pH, odorant pH, and odorant concentration on the detection of different odorants.

Odorant	Tank pH	Odorant pH	Odorant concentration
L-serine	$F_{95} = 0.943$, $p = 0.335$	$F_{95} = 24.316$, $p < 0.0001$	$F_{95} = 105.056$, $p < 0.0001$
L-leucine	$F_{103} = 2.347$, $p = 0.996$	$F_{103} = 39.496$, $p < 0.0001$	$F_{103} = 38.899$, $p < 0.0001$
L-arginine	$F_{103} = 0.495$, $p = 0.110$	$F_{103} = 16.278$, $p < 0.0001$	$F_{103} = 70.548$, $p < 0.0001$
L-glutamine	$F_{95} = 3.225$, $p = 0.067$	$F_{95} = 0.835$, $p = 0.438$	$F_{95} = 53.78$, $p < 0.0001$
L-glutamate	$F_{95} = 4.775$, $p = 0.002$	$F_{95} = 29.08$, $p < 0.0001$	$F_{95} = 158.742$, $p < 0.0001$
Bile fluid	$F_{95} = 4.973$, $p = 0.09$	$F_{95} = 0.786$, $p = 0.46$	$F_{95} = 83.962$, $p < 0.0001$
Intestinal fluid	$F_{95} = 4.276$, $p = 0.004$	$F_{95} = 5.518$, $p = 0.06$	$F_{95} = 59.682$, $p < 0.0001$

Factors that have significant effects are mentioned in bold.

for L-serine ($T_5 = 3.170$, $p < 0.05$), L-leucine ($T_6 = 2.848$, $p < 0.05$), and L-arginine ($T_6 = 2.862$, $p < 0.05$).

In contrast to L-serine, L-arginine, and L-leucine, the concentration-response curves of L-glutamine and L-glutamate did not fit linear regression well; the best-fitting model was the three-parameter Hill curve (Figures 1D,E). Olfactory nerve responses of control fish to L-glutamine at pH 8.2 and 7.7 were statistically equivalent (Figure 1D). There was no difference ($T_5 = 2.205$, $p = 0.08$) between the EC_{50} for L-glutamate of control fish tested at pH 8.2 (1.142×10^{-4} M) and pH 7.7 (3.89×10^{-4} M), but the maximum response (E_{max}) was significantly ($T_5 = 2.886$, $p < 0.05$) higher at odorant pH 8.2 (0.88 ± 0.05) than at pH 7.7 (0.78 ± 0.05 ; Figure 1E).

For bile fluid, there were no differences in slope ($F_{36} = 1.419$, $p = 0.24$) or elevation ($F_{37} = 0.022$, $p = 0.83$) at pH 8.2 and 7.7 (Figure 2A). For intestinal fluid, the slope of the curves at pH 8.2 and 7.7 was not different ($F_{44} = 0.072$, $p = 0.79$); however, the elevation of the curves was statistically higher ($F_{45} = 6.374$, $p < 0.05$) at pH 8.2 than 7.7 (Figure 2B).

Effect of Odorant pH on Olfactory Sensitivity in High CO_2 Fish

As for controls, in high CO_2 fish (kept at pH 7.7 for at least 4 weeks prior to electrophysiological recording) the elevation of the concentration-response curves to L-serine ($F_{45} = 42.156$, $p < 0.0001$), L-leucine ($F_{45} = 46.971$, $p < 0.0001$), and L-arginine ($F_{43} = 9.888$, $p < 0.01$) tested at pH 8.2 was significantly higher than at pH 7.7 (Figures 3A–C). However, there were no differences between the slopes of the concentration-response curves at pH 8.2 and 7.7 (Figures 3A–C). In addition, exposure to low pH water significantly increased the threshold of detection (Figure 3F) to L-serine ($T_5 = 3.464$, $p < 0.05$) and L-leucine ($T_5 = 3.084$, $p < 0.05$) but not to L-arginine ($T_{10} = 1.578$, $p = 0.07$).

As in the case of control fish, in the high CO_2 animals the best-fitting model for the concentration-response curve to

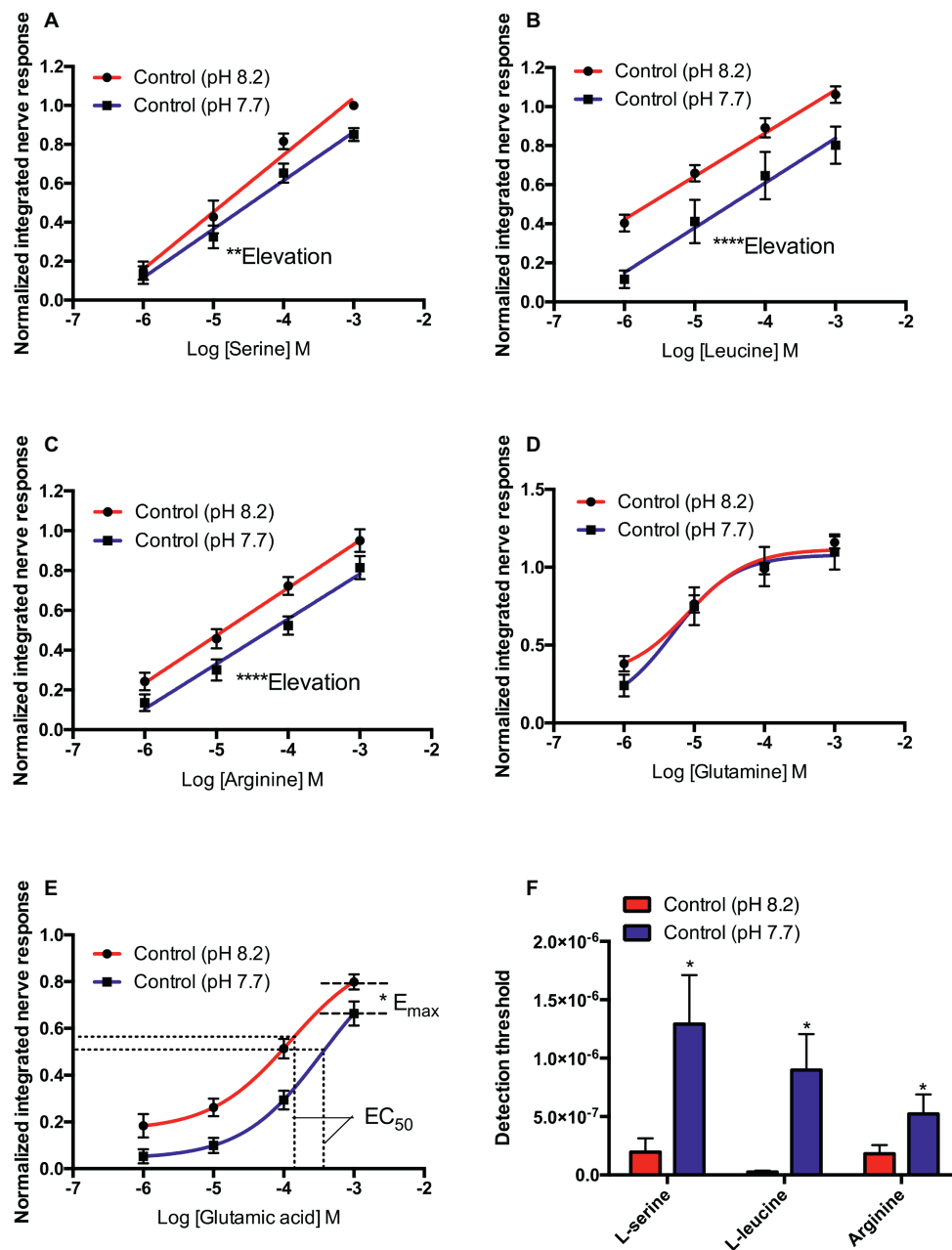


FIGURE 1 | Normalized olfactory nerve responses of control fish (kept at pH 8.2) to (A) L-serine, (B) L-leucine, (C) L-arginine, (D) L-glutamine, and (E) L-glutamic acid; under control (red: odorant pH 8.2) and elevated PCO_2 conditions (blue: odorant pH 7.7). (F) Effects of acute exposure to elevated PCO_2 in the olfactory detection threshold of L-serine, L-leucine, and L-arginine. Values are shown as mean $\pm$ S.E.M. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; $n = 6$.

L-glutamine and L-glutamate was the three-parameter Hill curve (Figures 3D,E). Sensitivity of high CO_2 fish to L-glutamine and L-glutamate at pH 8.2 and 7.7 were statistically equivalent; there were no differences between the EC_{50} or the E_{max} for these odorants (Figures 3D,E).

The olfactory nerve responses of high CO_2 fish to bile fluid and intestinal fluid at pH 8.2 and 7.7 were statistically equivalent; there were no differences in the slope or elevation of the concentration-response curves (Figure 4).

Short-Term (Acute) vs. Medium-Term Effects

When tested at odorant pH 8.2 or 7.7, responses to L-serine, L-leucine, L-arginine, L-glutamine, and bile fluid were similar in control and high CO_2 fish; that is, medium-term exposure to high CO_2 did not influence the effects of acute changes in odorant pH. As there were significant effects of medium-term exposure to high PCO_2 on the olfactory response to L-glutamic acid and intestinal fluid (Table 3), we compared responses of control and high CO_2 fish at the

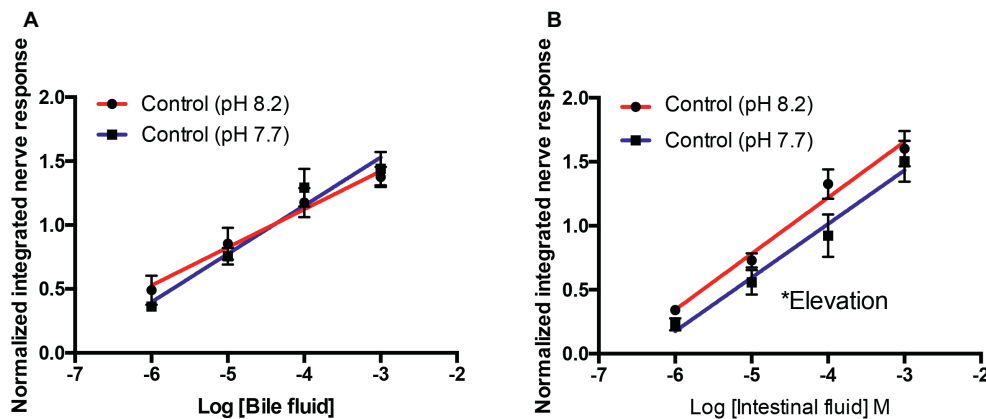


FIGURE 2 | Normalized olfactory nerve responses of control fish (kept at pH 8.2) to conspecific bile fluid (A) and intestinal fluid (B) with odorant pH 8.2 (red) and pH 7.7 (blue). Values are represented as mean $\pm$ S.E.M; * $p < 0.05$, $n = 6$.

same odorant pH. Comparing sensitivity to L-glutamic acid between control and high CO_2 fish at odorant pH 8.2 showed that there were no significant differences between EC_{50} ($T_9 = 0.543$, $p = 0.60$) or E_{max} ($T_9 = 1.814$, $p = 0.10$) (Figure 5A). At odorant pH 7.7, however, the EC_{50} of control fish was significantly ($T_{10} = 2.357$, $p < 0.05$) higher than that of high CO_2 fish. There was no difference between the E_{max} ($T_{10} = 1.317$, $p = 0.22$) of control and high CO_2 fish (Figure 5B).

For intestinal fluid at odorant pH 8.2, differences between the slopes of control and high CO_2 fish were highly significant ($F_{44} = 8.15$, $p < 0.01$); it was therefore not possible to compare the intercepts (Figure 5C). For odorant pH 7.7, there was no differences in the slope ($F_{44} = 1.70$, $p = 0.20$) of control and high CO_2 fish; however, the elevation was significantly different ($F_{45} = 4.51$, $p < 0.05$); the controls were more sensitive than the high CO_2 fish (Figure 5D).

Comparison of pH-Induced Effects on Protonation State and Olfactory Sensitivity

The effect of high CO_2 water on the olfactory sensitivity was reversed by exposing the olfactory epithelium to normal seawater for 10 min, with the exception of the decrease in amplitude of response to intestinal fluid seen in high CO_2 fish. The transient nature of the observed changes in olfactory sensitivity at low pH, and that the extent of the changes depended on the odorant, raised the hypothesis that the mechanism involved is related to pH-driven changes in protonation of odorants and/or receptor(s), due to the reduction in water pH. To test this hypothesis, and understand the real impact of pH on olfactory sensitivity, the proportion of each protonation state over the pH range was calculated based on the pK_a using the Henderson-Hasselbalch equation. Assuming that the protonation state of the ligand (odorant) and/or the binding site of its receptor affect(s) binding affinity (reviewed in Strasser et al., 2017), we calculated the stimulus concentrations at different states of protonation (Table 4).

These values were introduced into the linear regression or three-parameter Hill equation obtained experimentally from the response to odorants at a pH 8.2. The resulting points were plotted to simulate the extent of change caused by stimulus protonation state and compared with the extent of change observed for the olfactory nerve response at pH 7.7.

For all amino acids tested, a reduction in pH caused a reduction in the concentration of non-protonated ligand. The concentrations of active ligand at pH 7.7 were 32–35% lower than at pH 8.2 (Table 4). For L-serine, L-leucine, and L-arginine, the reduction in the olfactory response recorded was similar to that predicted by the theoretical protonation model (Figures 6A–C). However, for L-glutamine, a decrease in olfactory sensitivity was predicted (Figure 6D) but there was little observed difference between pH 8.2 and 7.7. Finally, for L-glutamic acid the decrease in the olfactory nerve response was lower than predicted by the protonation model (Figure 6E).

It is well-known that pH affects the protonation state of amino acids. This protonation changes the overall molecular charge and the charge distribution, which could affect interaction of the odorant with its receptor. To assess this, quantum chemical calculations were performed to obtain the energetically most favorable conformers and then the charge distribution for the two relevant protonation states of L-arginine were obtained (Figure 7) as an example. While the positive charge (red) of the side chain remains, the distribution of the negative charge (blue) around the head group shifts with protonation of the amino group.

Histology

The olfactory epithelia of fish kept for 4–8 weeks in water of pH 7.7 showed pronounced morphological changes, including an increase in the area of the non-sensory epithelium, and an increase in the number of mucus cells. The oval olfactory rosette (OR) consists of 32–38 lamellae (OL) that radiate from a median raphe (MR). There were no marked differences in the gross anatomy of the olfactory organ between fish kept

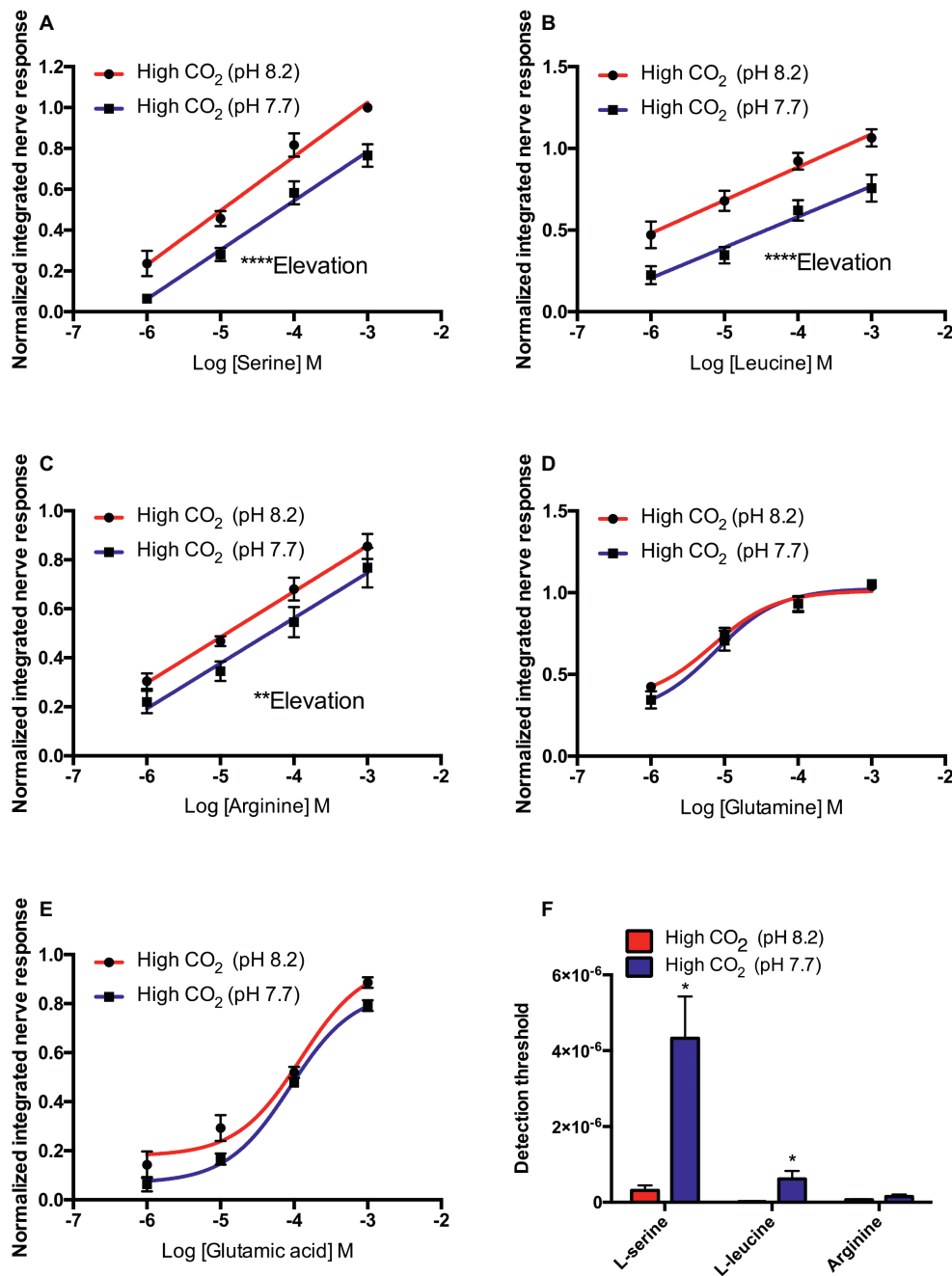


FIGURE 3 | Normalized olfactory nerve responses of high CO₂ fish (kept at pH 7.7) to (A) L-serine, (B) L-leucine, (C) L-arginine, (D) L-glutamine, and (E) L-glutamic acid; under odorant pH 8.2 (red) and odorant pH 7.7 (blue) conditions. (F) Effects of exposure to elevated PCO₂ in the olfactory detection threshold of L-serine, L-leucine, and L-arginine. Values are shown as mean ± S.E.M. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; $n = 6$.

in control and high CO₂ water. Each lamella has two layers of epithelium that enclose the central core (CC). The central core is separated from the epithelium by the basement membrane (BM). The non-sensory area consists of a stratified epithelium covering the outer margins of the lamella (NSE). The sensory area consists of pseudostratified epithelium covering lateral sides of the lamella (SE) (Figures 8C,D).

In high CO₂ fish (pH 7.7), the ratio between non-sensory and sensory epithelium (0.34 ± 0.02) is significantly higher ($T_8 = 5.583$, $p < 0.001$) than in controls (0.21 ± 0.01). Furthermore, the number of mucus cells *per* lamellae was significantly higher ($T_8 = 3.786$, $p < 0.01$) in high CO₂ fish (21.6 ± 1.7) than controls (11.8 ± 2.0) (Figure 8). Interestingly, mucus cells are present only in the nonsensory epithelium; the increased number

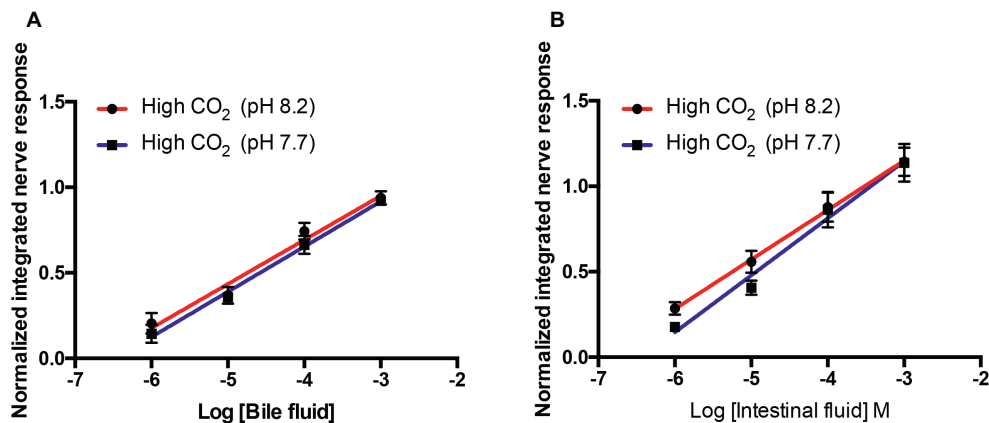


FIGURE 4 | Normalized olfactory nerve responses of high CO₂ fish (kept at pH 7.7) to conspecific (A) bile fluid and (B) intestinal fluid with odorant pH 8.2 (red) and pH 7.7 (blue). Values are shown as mean $\pm$ S.E.M; $n = 6$.

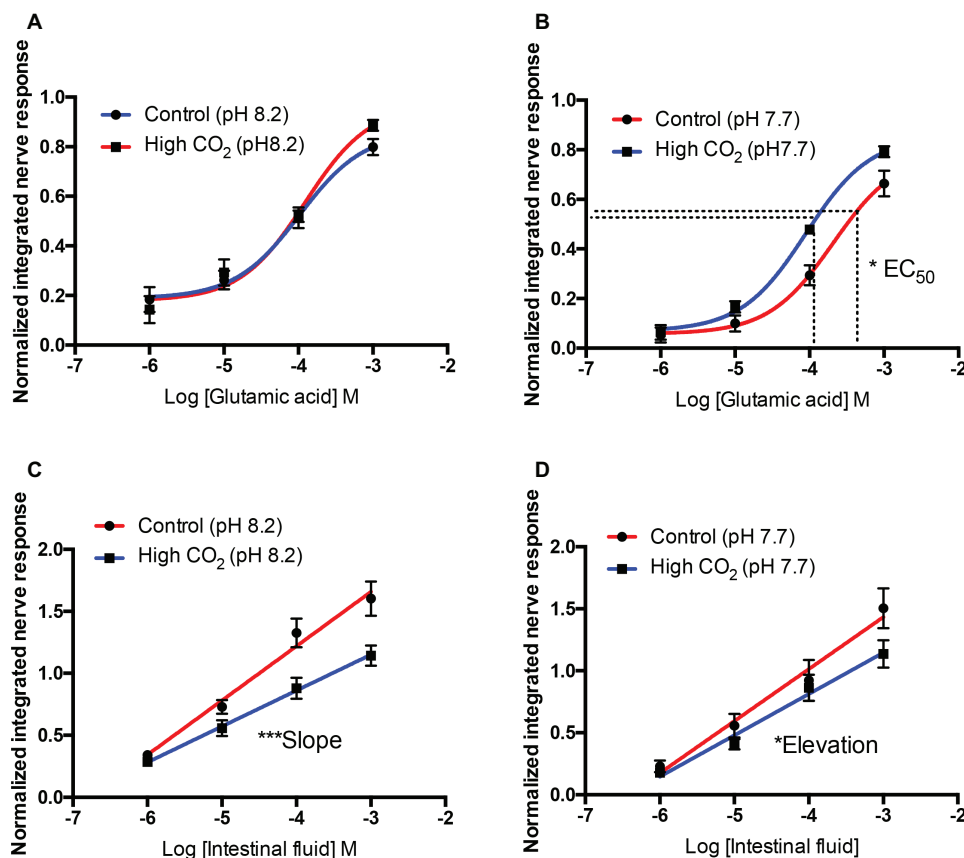


FIGURE 5 | Normalized olfactory nerve responses of control and high CO₂ fish to L-glutamate (A,B) and intestinal fluid (C,D) with the nostril conditioned with water pH 8.2 (A,C) and pH 7.7 (B,D). Values are shown as mean $\pm$ S.E.M. * $p < 0.05$; *** $p < 0.001$; $n = 6$.

of these cells in high CO₂ seabream may be related to the augmented area of non-sensory epithelium. In addition, to the higher number of mucus cells, the color of these cells, when examined for neutral and acidic mucus by combined Periodic

Acid-Schiff (PAS)-Alcian blue staining, was deep pink in high CO₂ fish and colorless or light pink in controls (Figure 8); these differences suggest a shift in pH of the mucus from acidic to more neutral.

TABLE 4 | Calculated effective stimulus concentrations during bioassays at pH 8.2 and 7.7 as well as the respective change in stimulus concentration in percentage between pH-conditions.

Amino acid	Stimulus concentration used during bioassay (M)	Effective stimulus concentration at pH 8.2 (M)	Effective stimulus concentration at pH 7.7 (M)	Change in effective stimulus concentration
L-Serine	10 ⁻⁶ to 10 ⁻³ M	8.90 × 10 ⁻⁸ to 10 ⁻⁵	3.00 × 10 ⁻⁸ to 10 ⁻⁵	-33.7%
L-Leucine	10 ⁻⁶ to 10 ⁻³ M	4.00 × 10 ⁻⁸ to 10 ⁻⁵	1.30 × 10 ⁻⁸ to 10 ⁻⁵	-32.5%
L-Arginine	10 ⁻⁶ to 10 ⁻³ M	13.7 × 10 ⁻⁸ to 10 ⁻⁵	4.77 × 10 ⁻⁸ to 10 ⁻⁵	-34.8%
L-Glutamine	10 ⁻⁶ to 10 ⁻³ M	10.5 × 10 ⁻⁸ to 10 ⁻⁵	3.58 × 10 ⁻⁸ to 10 ⁻⁵	-34.1%
L-Glutamate	10 ⁻⁶ to 10 ⁻³ M	5.10 × 10 ⁻⁸ to 10 ⁻⁵	1.67 × 10 ⁻⁸ to 10 ⁻⁵	-32.7%

Concentrations were tested in steps of 10⁻¹ within the range indicated.

DISCUSSION

The current study shows that, in the seabream, increased oceanic PCO₂ and/or reduction of pH predicted for the end of the century cause(s) a significant reduction of olfactory sensitivity to some, but not all, odorants. Significantly, this reduction persisted in seabream kept in high PCO₂ water for several weeks; fish were unable to compensate. The effect of exposure to high CO₂/low pH was complex and varied with the odorant type. The change in olfactory sensitivity can be partly – but not entirely – explained by a shift in the protonation state of the odorant molecule. Moreover, the current study shows that after several weeks exposure to high PCO₂ there were morphological changes in the olfactory epithelium indicative that exposure to high CO₂/low pH evokes a structural re-organization.

Overall, acute exposure of the olfactory epithelium to high CO₂/low pH water reduced the olfactory response for five out of seven odorants tested, and increased the detection threshold in three out of those five, similar to previously reported in bass (Porteus et al., 2018). Importantly, however, the current study also showed that this effect persisted, even after 4 weeks exposure to acidified water. There was an increase in the threshold of detection and a decrease in the amplitude of responses to L-serine, L-leucine and L-arginine, but not to bile fluid. This means that the fish would have to be closer to an odor source in order to detect it. This would have obvious repercussions in food-search and predator-avoidance behaviors, but may also help explain disrupted olfactory-guided homing and/or migration (Munday et al., 2009; Devine et al., 2012a). There were differences in the amplitude of response to intestinal fluid, but the detection threshold did not change. As intestinal fluid is likely a mixture of different odorants, this may reflect a reduction in all or just some; if the latter, then this may imply a qualitative effect on olfactory sensitivity, as well as a quantitative effect. When tested at odorant pH 8.2 or 7.7, responses to L-serine, L-leucine, L-arginine, L-glutamine, and bile fluid were similar in control and high CO₂ fish. This suggests that medium term exposure to high CO₂ does not cause changes in the olfactory sensitivity to these odorants *per se*; rather, the seabream is unable to compensate for the effect of low pH/high PCO₂ on acute sensitivity. However, at odorant pH 7.7, olfactory sensitivity to glutamate was higher in high CO₂ fish than in controls, suggesting that, in this specific case, the sensitivity to L-glutamate can somehow acclimate to high CO₂/low pH. This may involve changes in the expression and/or type of receptor(s) in the olfactory

epithelium; downregulation, but not upregulation of olfactory receptors was seen in the bass (Porteus et al., 2018), whereas no such changes were seen in the salmon (Williams et al., 2019). In contrast, the sensitivity to intestinal fluid when tested in seawater at pH 8.2, was higher in control fish than in high CO₂ fish. This suggests that medium-term exposure to CO₂ impairs the olfactory detection of intestinal fluid and, unlike the other stimuli tested, this was not reversible by bathing the olfactory epithelium with normal seawater. Again, this is likely to reflect a change in receptor expression. Thus, the effect of increased PCO₂ on olfactory sensitivity varies with the odorant type.

The sensitivity to some odorants decreased as soon as the olfactory epithelium was exposed to high CO₂ water; this effect occurred in both control and high CO₂ fish and was reversed by bathing the epithelium with seawater at pH 8.2. We propose that the effect can be directly linked to the effects of pH on the odorant molecules and/or their receptors in fish as well as crabs (Roggatz et al., 2016). We show the changes in charge distribution can alter the conformation of protonated L-arginine, for example. Changes in protonation state are reversible and much more rapid (<micro-seconds) than any physiological and/or behavioral change which may take several days (for example, see Munday et al., 2009, 2010; Hamilton et al., 2014). Olfactory sensitivity in vertebrates, including fish, is mediated by G-protein coupled receptors (GPCRs) (Buck and Axel, 1991; Ngai et al., 1993). Changes in pH have been shown to alter GPCR-ligand affinity *in vitro* (Ruan et al., 2009), and charged amino acid residues in the receptor play multiple roles in receptor-ligand interaction (Strasser et al., 2017).

Another effect of medium term exposure to high CO₂ water was the reduction of olfactory sensitivity to odorants in intestinal fluid. This may be due to qualitative and/or quantitative changes in the olfactory epithelium, as observed in the present study, which we hypothesize may cause a change in the expression of some odorant receptors (as in the bass; Porteus et al., 2018). Finally, in the case of L-glutamate, the decrease in olfactory sensitivity in high CO₂ water was lower in animals chronically exposed than in those acutely exposed. This may show a degree of adaptation to high PCO₂ and may be a result of morphological re-organization and/or changes in olfactory receptor expression. Further studies are required to evaluate whether exposure to high CO₂ water induces changes in gene expression in the olfactory epithelium. Nonetheless, it should be noted that it is difficult to separate the direct effect of increased PCO₂ and the consequent reduction in pH (Ou et al., 2015). A recent study in coho salmon (Williams et al., 2019), while showing that

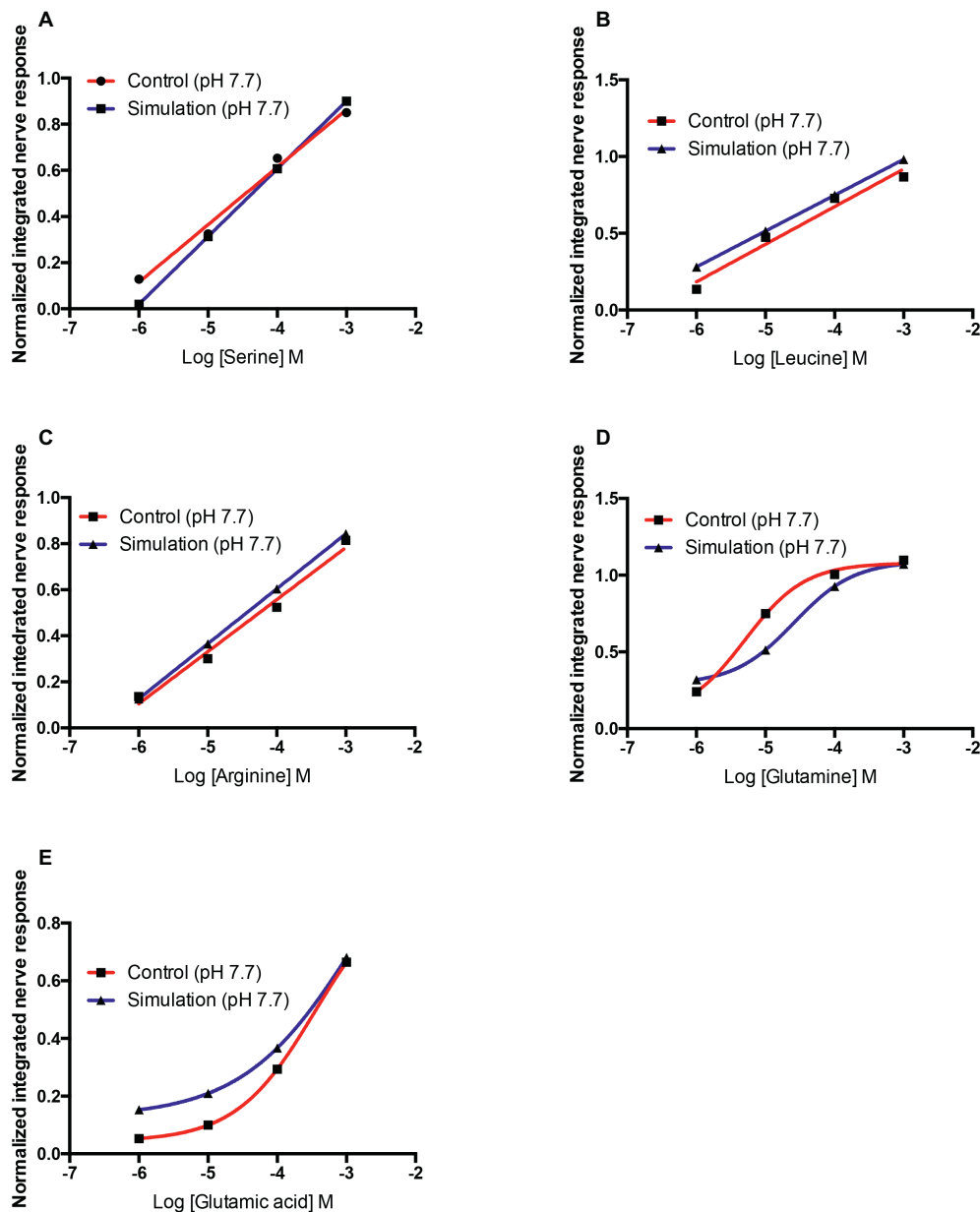


FIGURE 6 | Normalized olfactory nerve responses of control fish (red) with the nostril conditioned with water pH 7.7 and expected response (blue), based on the effective odorant concentration at pH 7.7 and the linear regression equation or three-parameter Hill equation fit **(A)** L-serine, **(B)** L-leucine, **(C)** L-arginine, **(D)** L-glutamine, and **(E)** L-glutamic acid. Values are shown as mean $\pm$ S.E.M.

increased PCO_2 affects both olfactory-mediated behavior and gene expression in the olfactory system, suggested that olfactory sensitivity is unaffected by increased PCO_2 /low pH at the level of the olfactory epithelium. However, this study used a different experimental approach (i.e., olfactory responses at different PCO_2 /pH levels were not compared in the same fish), and the high concentration (10^{-2} M) of odorants used, coupled with the lower sensitivity of the electro-olfactogram (EOG) when used in seawater (Silver et al., 1976; Hubbard et al., 2011) makes this negative finding difficult to interpret.

Does Exposure to High PCO_2 Modify the Olfactory Epithelium Organization?

Medium term (4 weeks) exposure to high CO_2 induced an increase in the ratio between nonsensory vs. sensory epithelium and an augmentation in the number of mucus cell per lamellae. Furthermore, there was a shift in pH of the mucus from acidic to more neutral. This suggests that high CO_2 seawater evokes modification of the epithelium and causes increased proliferation of neutral mucus-producing goblet cells to reinforce the outer extrinsic barrier. This may be an adaptive response, as the

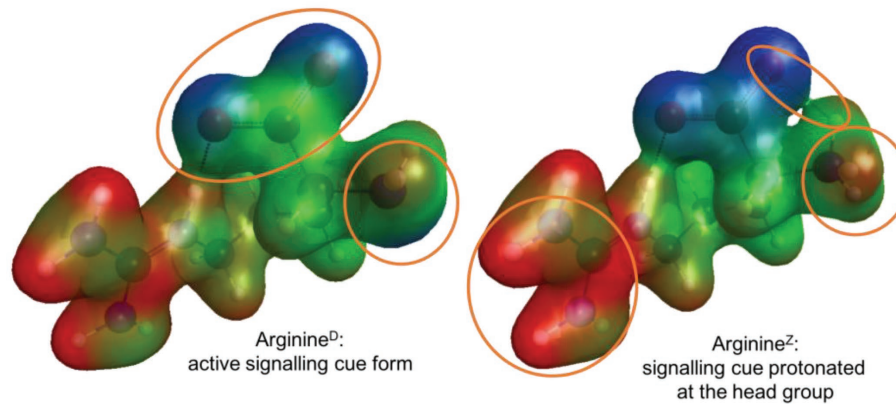


FIGURE 7 | Active (Arginine^D, left) and protonated (Arginine^Z, right) conformers of L-arginine with their molecular electrostatic potential mapped onto an iso-electron density surface. Negative charge is colored in blue, neutral in green, and positive charge in red. The orange circles highlight differences in the charge distribution between the two conformer models.

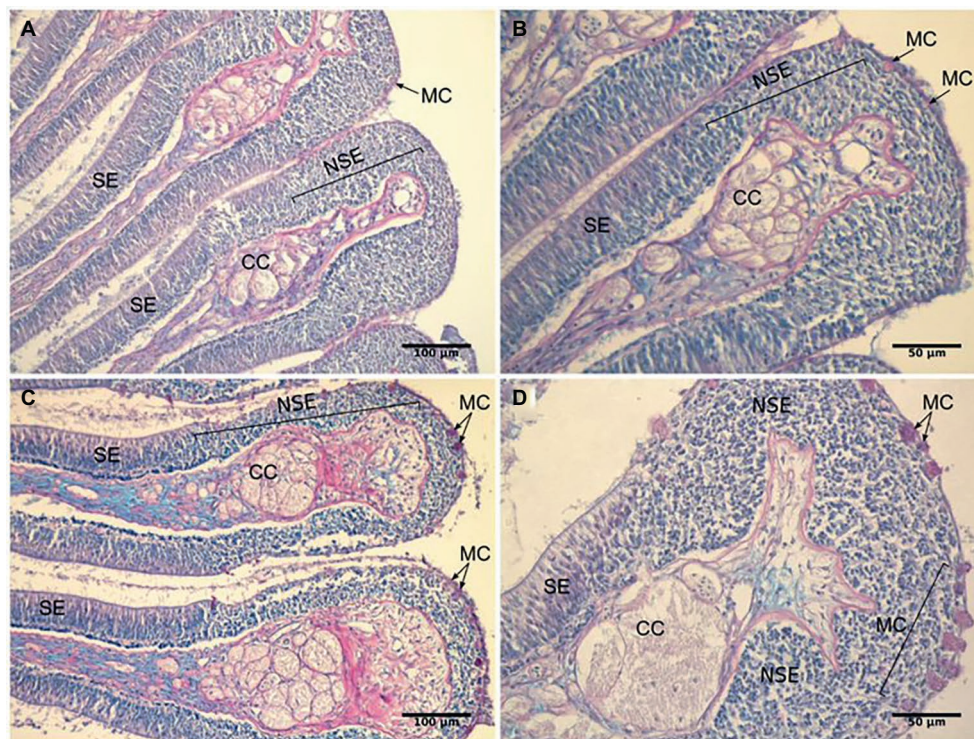


FIGURE 8 | Representative histological sections of the olfactory lamellae of the gilthead seabream stained with periodic acid-Schiff/Alcian blue to show the effects of exposure to high PCO_2 /low pH on tissue morphology. (A,B) control fish; (C,D) high CO_2 fish. CC, Central core; MC, Mucus cell; NSE, non-sensory epithelium; SE, sensory epithelium.

mucus will protect the underlying epithelial cells, including the olfactory receptor neurones, but the charge, viscosity and the thickness of the layer may also reduce the odorant/receptor interaction and thus lower sensitivity (Rygg et al., 2013; Corfield, 2015). However, the olfactory sensitivity of seabream exposed for 4 weeks to high PCO_2 was similar to that of controls, when tested at the same pH. The enlarged area of non-sensory

epithelium might also represent an adaptation to high CO_2 ; an augmented area of non-sensory epithelium positioned in the upper and outer margin of the olfactory lamellae results in a localization of the sensory area deeper in the olfactory lamellae, and as a consequence it is less exposed. On the other hand, it could represent impairment of olfactory epithelium formation, growth and/or renewal, and ultimately it might contribute to

decreased olfactory sensitivity given that the sensory area is reduced. Initial studies clearly indicate changes in gene expression induced by ocean acidification in the olfactory system (Porteus et al., 2018; Williams et al., 2019). Further studies are required to evaluate the mucin forms expressed, the factors driving the increased number of goblet cells, necrosis and/or apoptosis of the epithelium and changes in the number, type and distribution of olfactory receptor neurones and/or receptors and ion pumps and channels within the olfactory epithelium.

Does Seawater pH Change the Protonation of Odorant Molecules and Thereby Modify Olfactory Sensitivity?

The transient nature of the observed changes in olfactory sensitivity raised the hypothesis it is due to changes in the protonation state of odorant and/or receptor, due to changes in water pH, as previously suggested for the crab pheromone (Roggatz et al., 2016). Fish odorants are mostly low molecular weight organic molecules with high water solubility (Laberge and Hara, 2004), such as amines (Rolen et al., 2003), amino acids, nucleotides, nucleosides and organic acids (reviewed in Hara, 1994). Amino acids contain both functional groups attached to the same carbon (α -carbon) and the various alpha amino acids differ in the side chain attached to the α -carbon. Amino acid detection by the olfactory epithelium probably involves different receptors that are relatively ligand-specific and for activation require specific molecular features (Friedrich and Korsching, 1997, 1998; Fuss and Korsching, 2001). Our hypothesis is that change of pH in future oceans could lead to changes in protonation states of amino acids (and other odorants) which, in turn, may lead to significant alterations in their structure and affinity for their receptor(s). Herein, we determined that the difference in pH from 8.1 to 7.7 would reduce the concentration of non-protonated amino acids by up to 35%. This coincided with a reduction in olfactory response, except for L-glutamine. This may be because, for L-glutamine, the binding site of the receptor is not dependent on the protonation state of the ligand. Furthermore, for L-glutamic acid, reductions in olfactory responses were greater than predicted by the change in protonated/non protonated ratio. This could be explained by the occurrence of changes of charge distribution within the binding site of the receptor (Tierney and Atema, 1988; Strasser et al., 2017); thus, conformational changes in both odorant and receptor may contribute to the decrease in olfactory sensitivity. However, other mechanisms – such as effects on the transduction pathways (Rong et al., 2018) – may also play a role.

In conclusion, the current study shows that predicted future oceanic pH will influence fish olfactory sensitivity by acting directly on the olfactory epithelium. Exposure to high CO₂ water decreased olfactory nerve responses to most of the odorants tested. Furthermore, seabream were unable to compensate for high CO₂ levels and, even after 4 weeks exposure to high, the olfactory sensitivity remained reduced. Our hypothesis is that changes of charge, structure and consequently function of odorant molecules and/or their receptors due to pH change reduces receptor-ligand affinity, as has previously been shown in crabs (Roggatz et al., 2016). Thus, we propose that this is a purely

physicochemical issue and explains why fish are unable to compensate. However, changes in the protonation state of odorants is not the only mechanism involved in olfactory disruption due to high CO₂ water; long exposure of seabream led to morphological changes in the olfactory epithelium and diminution of olfactory sensitivity to intestinal fluid. Importantly, the protonation effect and the modification of epithelial structure presumably act in combination with the reported alterations in brain neurotransmitter function (Nilsson et al., 2012) and suggest the increase in CO₂ in seawater acts at multiple levels in sensory systems and will give rise to complex and diverse outcomes (Kelley et al., 2018). Further studies are required to evaluate whether changes in olfactory receptor expression or modifications in the olfactory epithelium are contributing to the long term effects of CO₂ on the olfactory sensitivity of seabream and, probably, other fish.

DATA AVAILABILITY

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

ETHICS STATEMENT

Animal maintenance and experimentation was carried out in certified experimental facilities and followed Portuguese national legislation (DL 113/2013) under a “group-1” license by the Veterinary General Directorate, Ministry of Agriculture, Rural Development and Fisheries of Portugal.

AUTHOR CONTRIBUTIONS

ZV, JH, and PH contributed to conception and design of the study. ZV and PH designed and carried out the electrophysiology experiments. CR and DB designed and carried out the chemical modeling. ZV did the histology. ZV, CR, and PH wrote the first draft of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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Tolerance, Growth, and Physiological Responses of the Juvenile Razor Clam (*Sinonovacula constricta*) to Environmental Ca^{2+} and Mg^{2+} Concentrations

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To facilitate transplanting razor clam (*Sinonovacula constricta*) populations to inland saline-alkaline waters (ISWs), we evaluated the tolerance of juvenile *S. constricta* (JSC) to Ca^{2+} and Mg^{2+} concentrations, and determined the effects of these ions on JSC growth and physiological parameters. After 30 days stress, the tolerable ranges of JSC to Ca^{2+} and Mg^{2+} were determined to be $0.19 \text{ mmol}\cdot\text{L}^{-1}$ – $19.46 \text{ mmol}\cdot\text{L}^{-1}$ and $0 \text{ mmol}\cdot\text{L}^{-1}$ – $29.54 \text{ mmol}\cdot\text{L}^{-1}$, respectively. The concentrations of Ca^{2+} (less than $0.65 \text{ mmol}\cdot\text{L}^{-1}$ or more than $3.24 \text{ mmol}\cdot\text{L}^{-1}$) and Mg^{2+} (less than $0.37 \text{ mmol}\cdot\text{L}^{-1}$ or more than $14.17 \text{ mmol}\cdot\text{L}^{-1}$) significantly inhibit JSC growth. Physiological enzyme activity no significant response when the concentrations range of Ca^{2+} and Mg^{2+} are $0.93 \text{ mmol}\cdot\text{L}^{-1}$ – $6.49 \text{ mmol}\cdot\text{L}^{-1}$ and $0.37 \text{ mmol}\cdot\text{L}^{-1}$ – $14.77 \text{ mmol}\cdot\text{L}^{-1}$, respectively. For transplantation practice, these data indicate that only high concentrations of Ca^{2+} (3.24 – $6.825 \text{ mmol}\cdot\text{L}^{-1}$) and Mg^{2+} (14.77 – $33.69 \text{ mmol}\cdot\text{L}^{-1}$) in target inland saline-alkaline water had significantly impact on growth and physiological response. In addition, present study suggests that the increase in Ca^{2+} and Mg^{2+} ion concentrations caused by ocean acidification will not affect the survival, growth and physiology of *S. constricta*. Current research suggests that *S. constricta* can adapt to extreme changes in the marine environment (Ca^{2+} and Mg^{2+}) and may be an excellent candidate for inland saline-alkaline water transplantation practice.

Keywords: *Sinonovacula constricta*, Ca^{2+} and Mg^{2+} ions, survive, growth, physiology

INTRODUCTION

Saline-alkali soil and inland saline-alkaline waters (ISWs) exist in more than 100 countries (Shi, 2009). In China, there are approximately 1 million km^2 of saline-alkaline soil and approximately 0.3 million km^2 of ISW (He et al., 2010). Saline water resources have long been used for agriculture, but traditional terrestrial crops are not salt-tolerant and cannot be irrigated with salt water. Therefore,

ISWs are often low-yielding land (Qadir et al., 2001; Wang et al., 2011). The use of ISWs to culture economically important marine organisms represents an effective means to use inland saline-alkali resources, such as the most successful species *Litopenaeus vannamei* (Roy et al., 2007, 2010). Inland aquaculture of economically important marine organisms worldwide is mainly concentrated in ISW areas, and is practiced in several countries. However, there are few reports on marine mollusc transplanting in ISWs; these reports only describe aquaculture of *Mytilus edulis* (Dinh and Fotedar, 2016), *Ruditapes philippinarum* (Hiele et al., 2014), *Haliotis laevigata* (Doupé et al., 2003), *Trochus niloticus* (Lee, 1997), and *Crassostrea gigas* and *Saccostrea glomerata* (Ingram et al., 2015).

In China, some ISWs have been used for aquaculture, but most of them have historically been in a state of disuse (Lv et al., 2012). According to incomplete statistics, more than 33 economically important organisms are cultured in ISWs in China (mainly freshwater fish) (Zhang and Zhang, 1999). However, the practice of aquaculture of economically important marine mollusc in ISW has only been attempted with *Cyclaina sinensis* (Lin et al., 2012) in China; this venture ultimately did not succeed.

The ion composition of ISW is quite different from that of seawater. Monovalent and divalent cations and their interactions have an important influence on the physiology of organisms (Duchen, 2000). All of the main ionic components in ISW (Ca^{2+} , Mg^{2+} , Na^+ , K^+ , SO_4^{2-} , HCO_3^- , OH^- , Cl^- , Ca^{2+} : Mg^{2+} , Na^+ : K^+ , SO_4^{2-} : Cl^-) are factors that affect the survival and growth of aquatic animals (Hadfield et al., 2012; Hiele et al., 2014; Raizada et al., 2015). Ca^{2+} is a crucial element of the outer shell of marine crustaceans and mollusc, is involved in muscle activity, shell formation, neurotransmission, and osmotic regulation, and plays an important role in the growth of marine invertebrates such as crustaceans and mollusc (Wilbur and Yonge, 2013; Yang, 2011). Mg^{2+} is found in the active region of many enzymes, it is a metabolic cofactor, and is an important participant in the active transport of substances involved in maintenance of the cell membrane. Studies have shown that Mg^{2+} has an important effect on the survival, growth, and osmotic pressure of marine crustaceans (Mantel and Farmer, 1983). The imbalance of ionic components in ISW tends to affect aquaculture practices more than salinity, as demonstrated in *L. vannamei* by Saoud et al. (2003). In addition, it has been reported that the high content of Mg^{2+} , Ca^{2+} , and K^+ , and low $\text{Ca}^{2+}/\text{Mg}^{2+}$ and Na^+/K^+ ratios in ISW affect the survival of cultured *L. vannamei*.

Many marine invertebrates are osmotic conformers: the osmotic concentration (and usually the ionic composition) of the extracellular fluid is similar to that of the ambient seawater (Larsen et al., 2014). Thus, in aquatic mollusc can be found the stenohaline, euryhaline or oligohaline osmotic conformers, and, also the freshwater bivalves (Deaton, 2008). Most of the intertidal zone bivalve belongs to the euryhaline osmotic conformers, and usually their conformational floor are reported to be between 0.7 ppt and 3.4 ppt (Otto and Pierce, 1981; Deaton, 1992, 2008). There is also volume regulation in marine mollusc, the process that returns the cytoplasmic and extracellular fluid compartments to osmotic equilibrium with the ambient medium (Smith and Pierce, 1987; Deaton, 1992; Larsen et al., 2014). The capacity

for volume regulation in marine osmotic conformers varies from limited to high (some molluscs) (Virkar, 1966; Pierce, 1971; Gainey, 1978). Although the study of osmotic and ionic regulation in bivalve is not sufficient, Ca^{2+} and Mg^{2+} play an important role in this system.

The Ca^{2+} and Mg^{2+} ion contents vary widely in ISWs in different regions of China. For example, the Ca^{2+} content of ISWs in Haolebaojiao (Wu Shen banner, Ordos City, the Inner Mongolia Autonomous Region, China) is only $0.199 \text{ mmol}\cdot\text{L}^{-1}$ of seawater (Tian, 2000). In contrast, the Ca^{2+} content of Bosten Lake is $5.61 \text{ mmol}\cdot\text{L}^{-1}$ that of seawater with the same salinity (Shi, 1989). Shi (2009) found that the Mg^{2+} content in ISW in the middle reaches of the Yellow River in China was $2.33\text{--}8.15 \text{ mmol}\cdot\text{L}^{-1}$. Therefore, studying the effects of Ca^{2+} and Mg^{2+} on marine invertebrates can help facilitate transplanting marine invertebrates in China ISW.

Sinonovacula constricta is a eurythermal and euryhaline marine mollusc that is distributed in estuary and intertidal zones (Lin and Wu, 1990). *S. constricta* feeds on microalgae, and fixes calcium ions in its aquatic environment during the formation of its shell, which can contribute to the improvement of an ISW environment. Peng et al. (2018) reported that *S. constricta* tolerate high pH and carbonate alkalinity under long-term stress, and suggested that *S. constricta* is a potentially good species for culture in ISW. However, the effects of different concentrations of Ca^{2+} and Mg^{2+} on the survival, growth, and physiological responses of *S. constricta* have not been reported. In addition, ocean acidification and climate warming also directly affect the concentration of Ca^{2+} and Mg^{2+} ions in the ocean (Orr et al., 2005). Therefore, understanding the critical tolerances and physiological responses of *S. constricta* to Ca^{2+} and Mg^{2+} stress is crucial for the development of optimal ISW culture conditions and ocean environments changing. Thus, this study investigated the effects of Ca^{2+} and Mg^{2+} on the survival, growth, and physiological responses of juvenile *S. constricta* (JSC), and the possibility of this species as a potential candidate for transplantation to ISWs, providing a theoretical basis for this process.

MATERIALS AND METHODS

Experimental animals were treated according to the guidelines of the Institutional Animal Care and Use Committee of Shanghai Ocean University (IACUC-SHOU), Shanghai, China, under examination and approval number SHOU-DW-2018-014. In this tests the animals were artificially propagated as juveniles.

Healthy JSCs (20 days old) were obtained commercially (Zhejiang Sanmen Donghang). Before experimentation, all JSCs were kept in at 8 ppt seawater for 3 days, then were kept at 6 ppt artificial seawater (Ca^{2+} : $1.71 \text{ mmol}\cdot\text{L}^{-1}$, Mg^{2+} : $9.23 \text{ mmol}\cdot\text{L}^{-1}$) for 4 days. In these conditions the total salinity of water is set at 6 ppt, since the salinity of ISW (carbonate type) in China is around 6 ppt (Yao et al., 2010). Artificial seawater was formulated using chemical reagents (AR degree, Sangon Biotech) and distilled water according to the Artificial Seawater Recipe (Chu) in Table 1A (Harvey, 1957; Yao et al., 2010).

Approximately 10,000 specimens of JSCs were obtained before experiments, and then 270 JSCs were randomly selected to measure shell length (0.2102 ± 0.058 cm) and body weight (0.000892 ± 0.00015 g). Water temperature in experiments was maintained between 20 to 22°C .

Long-Term Stress Study

Juvenile *S. constrictas* were divided into 29 groups for long-term stress testing (Table 1B), consisting of a control group (CK), 14 groups with different concentrations of Mg^{2+} (DCMg: G1–G14), and 14 groups with different concentrations of Ca^{2+} (DCCa: G1–G14). Each of the 29 groups had 3 parallel trials. For each parallel stress trial, 90 JSCs were cultured in an 11 cm diameter round plate for a month. 150 mL test water (with the appropriate salinity and Ca^{2+} or Mg^{2+} concentration) was added to the round plate, and the test water was changed once every single day. Feed-water was prepared as follows: test water was used to replace the culture water of *Chaetoceros calcitrans* by centrifugation, and the concentration of *C. calcitrans* was adjusted to 400–480 cells μL^{-1} in test water. For every single feeding, 15 mL feed-water was used to replace 15 mL of test water. JSCs were fed thrice a day.

Live JSCs were counted at day 0 (0-day) and at day 30 (30-day). At 0-day, 36 JSCs (6 repeats: 6 JSCs per repeat) were selected to determine the activities of the following enzymes: $\text{Na}^{+}/\text{K}^{+}$ -ATPase (NKA), aspartate aminotransferase (AST), superoxide dismutase (SOD), acetylcholinesterase (AChE), and lysozyme (LZM). At 30-day, live JSCs were used to measure shell length, body weight, and enzymes activity (NKA, SOD, AST, AChE, and LZM). Nine JSCs (representing three separate tests with three JSCs in each group) were used to measure enzyme activities. Briefly, after shell removal, JSC tissue was homogenized

in normal saline water in a ratio of 1 mg: 9 μL . After homogenization, the total protein content of the sample was measured by Coomassie Brilliant Blue Total Protein Assay kit (Nanjing Jiancheng Bioengineering Institute, China), according to the manufacturer's instructions. NKA, AST, SOD, AChE, and LZM enzyme activities were measured by corresponding kits from Nanjing Jiancheng Bioengineering Institute Ltd., as previously described (Peng et al., 2018).

$\text{Na}^{+}/\text{K}^{+}$ -ATPase activity was measured by Pi released from ATP, in the incubation solution containing: 100 mM NaCl, 20 mM KCl, 4 mM MgCl_2 , 1 mM EGTA, 0.2 mM 2-methyl-8-(phenylmethoxy)imidazol(1,2- α) pyridine-3-acetonitrile 40 mM Tris-HCl (pH 7.4) and 3 mM Na_2ATP . The incubation time with substrate was 10 min. Pi concentration was calculated from the regression line based on standard Pi solutions. The units of NKA activity are expressed as U ($\mu\text{mol Pi mg prot}^{-1} \text{h}^{-1}$). AST can transfer amino and keto groups between alpha ketoglutarate and aspartic acid, then generate glutamic acid, and oxaloacetate. Oxaloacetate can be decarboxylated to pyruvic acid by itself during the reaction. Pyruvic acid reacts with 2,4-Dinitrophenylhydrazine to form 2,4-Dinitrophenylhydrazone, which is reddish brown in an alkaline solution. After measuring the OD, the standard curve was checked to obtain AST activity, which is expressed as U ($\mu\text{mol Pyruvic acid mg prot}^{-1} \text{min}^{-1}$). SOD activity was tested using SOD assay kit by WST-1 method. The WST-1 method depends on produces a water-soluble formazan dye upon reduction with the superoxide anion. The rate of the reduction of WST-1 with O_2^{-} are linearly related to the xanthine oxidase activity, and this reduction is inhibited by SOD. Therefore, the 50% inhibition activity of SOD can be determined by the colorimetric method. The SOD activity is expressed as units per mg of protein (U mg prot^{-1}), where the one U represents the amount of enzyme required to achieve a SOD inhibition rate of 50%. AChE hydrolyzes acetylcholine to produce choline and acetic acid. Choline can react with sulphydryl chromogenic reagent to form Sym-Trinitrobenzene (yellow compound), after measuring the OD, the standard curve was checked to obtain AChE activity, which is expressed as units per mg of protein (U mg prot^{-1}), where the one U represents at 37°C for 6 min of reaction conditions, the amount of enzyme required to hydrolyze 1 mmol of acetylcholine. The activity of LZM was detected using a turbidimetric method, which light transmittance of the bacterial turbid solution. Lysozyme can lysis bacteria by hydrolyze peptidoglycan on the bacterial wall to increase the light transmission of the bacterial turbid solution. After measuring the light transmission, the standard curve was checked to obtain LZM activity, which is expressed as units per mg of protein (U mg prot^{-1}), where the one U equivalent to the activity capacity of standard LZM. All data of enzyme activities are ultimately represented as a ratio of 30-day to 0-day.

Acute Stress Study

Within 3 days of the start of the long-term stress study, we found that all JSCs died in the following groups: DCMg-G10, DCMg-G11, DCMg-G12, DCMg-G13, DCMg-G14, DCCa-G13, and DCCa-G14. To more fully explain the physiological effects of Ca^{2+} and Mg^{2+} on JSCs, we designed and conducted an

TABLE 1A | Artificial seawater recipe (Chu).

Reagents	Dosage ($\text{mg}\cdot\text{L}^{-1}$)	Dosage ($\text{mmol}\cdot\text{L}^{-1}$)
NaCl	4,143	70.89322382
MgCl_2	879	9.232223506
Na_2SO_4	691.2352941	4.866483344
CaCl_2	190.0305882	1.712303012
KCl	127.7647059	1.713812285
NaHCO_3	33.88235294	0.403313331
KBr	16.94117647	0.142360435
H_2BO_3	4.588235294	0.075451986
$\text{Al}_2(\text{SO}_4)_3$	0.529411765	0.001547336
$\text{BaCl}_2\cdot 2\text{H}_2\text{O}$	0.015882353	6.50303E-05
LiNO_3	0.176470588	0.002559771
SrCl_2	4.235294118	0.026717727
NaF	0.529411765	0.012605642
NaNO_3	8.823529412	0.103806228
Na_2HPO_4	0.882352941	0.006214891
$\text{Na}_2\text{SiO}_3\cdot 9\text{H}_2\text{O}$	1.764705882	0.006211894
MnCl_2	0.035294118	0.000280473
$\text{FeC}_6\text{H}_5\text{O}_7\cdot 3\text{H}_2\text{O}$	0.095294118	0.000318875
$\text{CuSO}_4\cdot 5\text{H}_2\text{O}$	0.007058824	2.82799E-05
$\text{ZuSO}_4\cdot 7\text{H}_2\text{O}$	0.001764706	6.13939E-06
$(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}\cdot 4\text{H}_2\text{O}$	0.004411765	3.5702E-06

TABLE 1B | Design of different Ca²⁺ and Mg²⁺ concentrations for the long-term stress test.

Treatments	CK ^{ab}	DCMg-G1 ^b	DCMg-G2	DCMg-G3	DCMg-G4	DCMg-G5	DCMg-G6	DCMg-G7	DCMg-G8	DCMg-G9	DCMg-G10 ^b	DCMg-G11	DCMg-G12	DCMg-G13	DCMg-G14
Ca ²⁺ (mmol·L ⁻¹)	1.71	1.71	1.71	1.71	1.71	1.71	1.71	1.71	1.71	1.71	1.71	1.71	1.71	1.71	1.71
Mg ²⁺ (mmol·L ⁻¹)	9.23	0	0.046	0.092	0.18	0.37	1.85	3.69	14.77	29.54	73.86	147.72	211.02	295.43	379.84
Ca ²⁺ /Mg ²⁺ ratio	0.19	-	37.13	18.57	9.28	4.64	0.93	0.46	0.12	0.058	0.023	0.012	0.0081	0.0058	0.0035
Treatments	-	DCCa-G1 ^b	DCCa-G2	DCCa-G3	DCCa-G4	DCCa-G5	DCCa-G6	DCCa-G7	DCCa-G8	DCCa-G9	DCCa-G10	DCCa-G11	DCCa-G12	DCCa-G13 ^b	DCCa-G14
Mg ²⁺ (mmol·L ⁻¹)	-	9.23	9.23	9.23	9.23	9.23	9.23	9.23	9.23	9.23	9.23	9.23	9.23	9.23	9.23
Ca ²⁺ (mmol·L ⁻¹)	-	0	0.093	0.19	0.28	0.65	0.93	2.32	3.24	6.49	19.46	51.89	103.78	207.55	415.1
Ca ²⁺ /Mg ²⁺ ratio	-	-	0.01	0.02	0.03	0.07	0.1	0.25	0.35	0.7	2.11	5.62	11.24	22.48	44.96

^aOnly one number of control group (CK) be set in long-term stress study. ^bThe groups were used in acute stress study.

acute stress study. The CK, DCMg-G1, DCMg-G10, DCCa-G1, and DCCa-G13 were used in the acute stress experiment. In this experiment, the animals were exposed to the same conditions used in the long-term stress tests, except that the clams were not fed algae. At 0-h, 12-h, 24-h, and 48-h, ten JSCs from each group were used to measure oxygen consumption, then the hemolymph of JSCs was collected, as previously reported (Peng et al., 2017), to separate haemocytes and to measure metabolic and phagocytic activities.

The method used for measuring oxygen consumption rate was previously reported (Peng et al., 2019). After hemolymph collection, 20 μL of JSC haemocyte suspension solution was diluted with TBS to 100 μL. Then 10 μL of 1/10 diluted carboxylate-modified microspheres (diameter 1 μm, yellow-green fluorescent, FluoSpheres®, Invitrogen) was added, and samples were incubated in the dark at room temperature for 1 h (Wang et al., 2014). A BD C6Plus (BD Biosciences, United States) flow cytometer was used to measure the phagocytosis of haemocytes. The same haemocyte concentration without fluorescent microspheres in each group was analyzed by flow cytometry to obtain the cell concentration, and we set the threshold to be Gate A (cell only). The relative size of haemocytes (FSC value) and FITC fluorescence intensity (FITC value) were selected to limit the data. Finally, the data was extracted for analysis and figure mapping.

The metabolic activity of haemocytes was measured with a Cell Counting Kit-8 (CCK-8, Dojindo, Japan) assay. In every parallel group, 20 μL haemocyte suspension solution was diluted with TBS to 200 μL. Then, 20 μL of CCK-8 reagent was added and incubated at 28°C for 3 h. For the CK, 20 μL serum was obtained from the haemocyte suspension solution, and was diluted with TBS to 200 μL; then 0 μL of CCK-8 was added and incubated at 28°C for 3 h. After the OD value was measured, the relative metabolic activity was calculated using OD value and the total number of haemocytes (obtained from the phagocytosis test).

Calculation and Data Analytical Methods

Survival rate (SR):

$$SR(\%) = 100 \times N_{30}/N_0$$

N_{30} and N_0 are the numbers of surviving individuals in the 30-day and 0-day groups, respectively.

Shell length growth rate (SGR):

$$SGR(\%day^{-1}) = 100 \times (L_{30} - L_0)/T,$$

L_{30} and L_0 are the average lengths of the individuals in the 30-day and 0-day groups, respectively. T is 30 days.

Weight gain rate (WG):

$$WG(\%day^{-1}) = 100 \times (W_{30} - W_0)/T$$

W_{30} and W_0 are the average wet body weights of the individuals in the 30-day and 0-day groups, respectively. T is 30 days.

Oxygen consumption rate:

$$(\text{mgO}_2 \cdot \text{g}^{-1} \cdot \text{L}^{-1} \cdot \text{h}^{-1}) = [A_i(\text{initial oxygen concentrations}) - A_f(\text{final oxygen concentrations})] \times V$$

$$(\text{volume of test water}) / [W(\text{wet body weight}) \times T(\text{test time})]$$

Phagocytosis rate and relative metabolic activity:

$$\text{Phagocytosis rate\%} = (H_t - H_u)/H_t \times 100$$

$$\text{Relative metabolic activity} = [(OD_G - OD_{GB})/H_{tG} \times 1000] / [(OD_{CK} - OD_{CKB})/H_{tCK} \times 1000]$$

H_t and H_u are the total haemocyte and un-phagocytic haemocyte numbers, respectively. OD_G , OD_{GB} , and H_{tG} are the OD of test sample, the blank OD of test sample, and the total haemocyte counts of the test sample, respectively. OD_{CK} , OD_{CKB} , and H_{tCK} are the OD of control sample, the blank OD of control sample and the total haemocyte count of the control sample, respectively.

Statistical Analysis

Sigmaplot 12.3 software was used to identify significant differences analysis and to plot results. Significant differences were analyzed by using single factor analysis of variance (one-way ANOVA analysis) and the LSD method.

RESULTS

Survival and Growth of JSC Under Long-Term Ca^{2+} or Mg^{2+} Stress

During the long-term stress, we found that the concentrations of Mg^{2+} (higher than $73.86 \text{ mmol L}^{-1}$) and Ca^{2+} (higher than $207.55 \text{ mmol L}^{-1}$) cause JSCs to die rapidly (all JSC death groups are listed in Table 2). The concentrations of Ca^{2+} (lower than $0.093 \text{ mmol L}^{-1}$) causes JSCs to die over an extended period of time. Meanwhile, we observed that the bodies of JSCs were lacked calcium carbonate shells in groups stressed with Ca^{2+} concentrations lower than $0.093 \text{ mmol L}^{-1}$. However, the JSCs stressed with low concentrations of Mg^{2+} remained viable (Figure 1). In the range of Mg^{2+} concentration from 0 mmol L^{-1} to $29.54 \text{ mmol L}^{-1}$, there is a trend of JSC survival ratio show as first rise and then fall. Similarly, in the range of Ca^{2+} concentration from 0.19 mmol L^{-1} to $19.46 \text{ mmol L}^{-1}$, there is a trend for JSC survival to first rise and then fall. The Mg^{2+} concentration from 0.18 mmol L^{-1} to 3.69 mmol L^{-1} and Ca^{2+} concentration from 0.93 mmol L^{-1} to 2.32 mmol L^{-1} showed no significant differences in survival compared to the CK.

The shell growth rate was significantly inhibited in the Mg^{2+} concentration from 0 mmol L^{-1} to 0.37 mmol L^{-1} ($P < 0.05$), and from $14.77 \text{ mmol L}^{-1}$ to $29.54 \text{ mmol L}^{-1}$ ($P < 0.001$), and was significantly inhibited in the Ca^{2+} concentration 0.19 – 0.65 mmol L^{-1} ($P < 0.05$) and 6.49 – $19.46 \text{ mmol L}^{-1}$ ($P < 0.05$) (Figure 2). The body weight gain rate was significantly inhibited

in the Mg^{2+} concentration 0 – 0.37 mmol L^{-1} ($P < 0.05$) and 14.77 – $29.54 \text{ mmol L}^{-1}$ ($P < 0.001$), and was significantly inhibited in the Ca^{2+} concentration 0.19 – 0.65 mmol L^{-1} ($P < 0.05$) and 3.24 – $19.46 \text{ mmol L}^{-1}$ ($P < 0.05$) (Figure 3).

Enzyme Activity of JSC Under Long-Term Ca^{2+} or Mg^{2+} Stress

Na^+/K^+ -ATPase activity was significantly decreased in JSCs stressed with low concentrations range (0 – $0.046 \text{ mmol L}^{-1}$) of Mg^{2+} (Figure 4). However, $14.77 \text{ mmol L}^{-1}$ of Mg^{2+} significantly increased NKA activity in JSCs ($P < 0.05$). Low concentrations and high concentrations of Ca^{2+} affected JSC NKA activity. NKA activity was increased significantly in the Ca^{2+} concentration of 0.19 mmol L^{-1} ($P < 0.001$), 0.28 mmol L^{-1} ($P < 0.001$), and $19.46 \text{ mmol L}^{-1}$ ($P < 0.05$). NKA and SOD activity patterns are totally consistent when Ca^{2+} varying. In contrast with NKA, SOD activity is not affected by low Mg^{2+} (Figure 5). AST activity (Figure 6) was significantly reduced in low concentrations (0 – 0.37 mmol L^{-1}) of Mg^{2+} ($P < 0.05$), but was significantly increased in the high Mg^{2+} concentration, $14.77 \text{ mmol L}^{-1}$ ($P < 0.05$) and $29.54 \text{ mmol L}^{-1}$ ($P < 0.001$). However, both 0.19 – 0.65 mmol L^{-1} ($P < 0.05$) and 6.49 – $19.46 \text{ mmol L}^{-1}$ ($P < 0.05$) concentrations of Ca^{2+} significantly reduced AST activity in JSCs. AChE activity increased as the concentration of Mg^{2+} increased (Figure 7). AChE activity was inhibited significantly in the Mg^{2+} concentration of 0 mmol L^{-1} and $0.046 \text{ mmol L}^{-1}$ ($P < 0.05$), but was increased significantly in the of $14.77 \text{ mmol L}^{-1}$ and $29.54 \text{ mmol L}^{-1}$ ($P < 0.001$). The same trend was observed in the effects of Ca^{2+} concentration on AChE activity. AChE activity was inhibited significantly in the Ca^{2+} concentration of 0.19 mmol L^{-1} and 0.28 mmol L^{-1} ($P < 0.001$), but was increased significantly in the 6.49 mmol L^{-1} and $19.46 \text{ mmol L}^{-1}$ ($P < 0.001$). Only the Mg^{2+} – 1.85 mmol L^{-1} , Mg^{2+} – 3.69 mmol L^{-1} , Ca^{2+} – 2.32 mmol L^{-1} , and Ca^{2+} – 3.24 mmol L^{-1} showed no significant inhibition of LZM activity in JSCs (Figure 8).

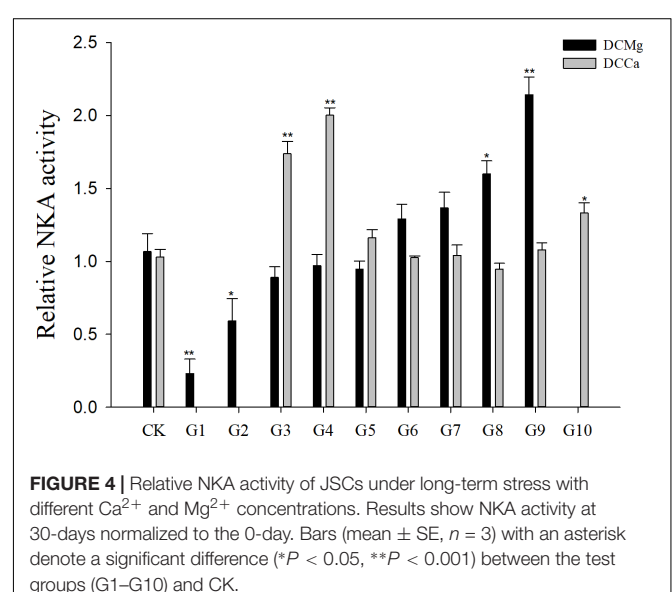
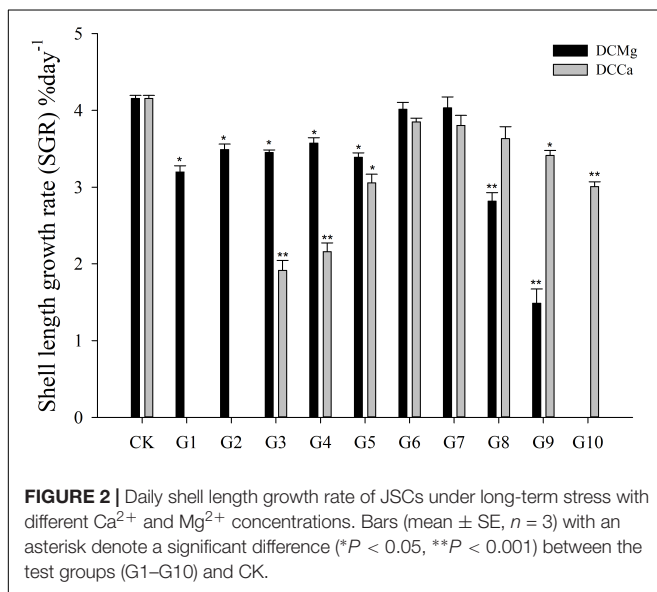
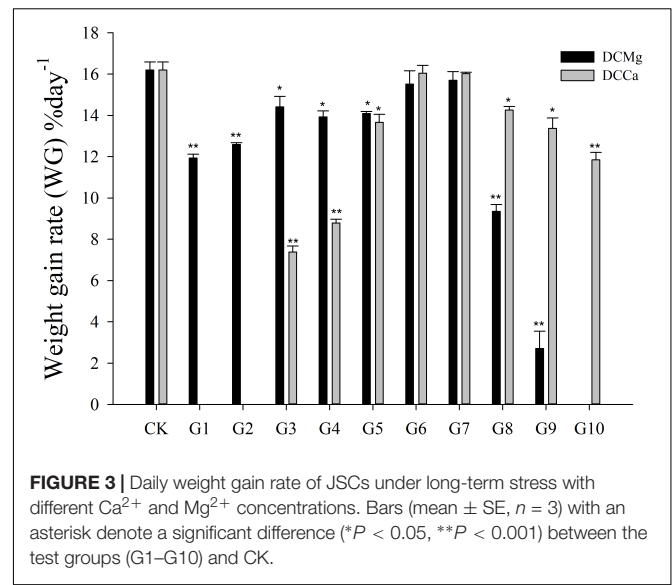
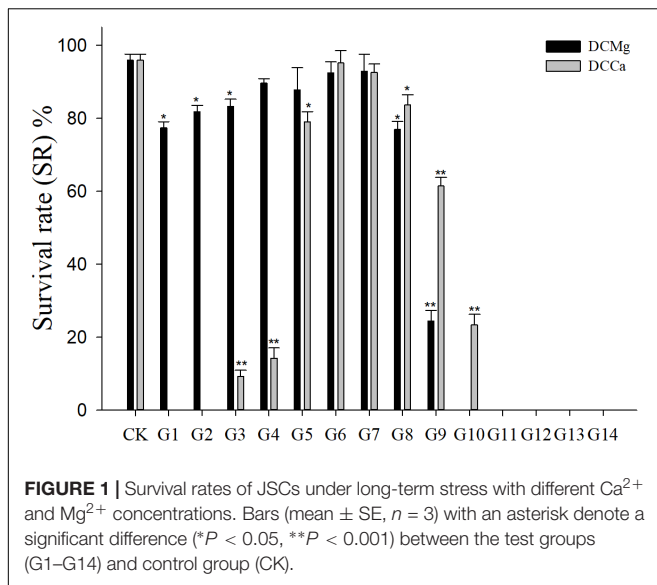
Oxygen Consumption Rate, Phagocytic Rate, and Metabolism Activity of JSC Under Acute Ca^{2+} or Mg^{2+} Stress

The oxygen consumption rate of JSCs changed under acute stress due to different concentrations of Ca^{2+} and Mg^{2+} (Figure 9). Within 48 h, the oxygen consumption rate at 0 mmol L^{-1} of Mg^{2+} did not show any significant difference. However, the oxygen consumption rate in the Mg^{2+} concentration $73.86 \text{ mmol L}^{-1}$ was significantly increased at 24 h, and then was significantly decreased at 48 h. The oxygen consumption rate of the Ca^{2+} concentration 0 mmol L^{-1} was significantly increased at 48 h. The oxygen consumption rate of the Ca^{2+} concentration $207.55 \text{ mmol L}^{-1}$ was significantly increased at 12 and 24 h, and then was significantly decreased at 48 h. The phagocytosis rate of all groups decreased over time (Figure 10). Among them, the Mg^{2+} concentration 0 mmol L^{-1} showed a significant decreased at 48 h ($P < 0.05$), the Mg^{2+} concentration $73.86 \text{ mmol L}^{-1}$ showed a decrease at 24 h ($P < 0.05$) and 48 h ($P < 0.001$),

TABLE 2 | Detailed juvenile *Sinonovacula constricta* mortality information in the test groups which cannot survive under long-term stress with different Ca²⁺ and Mg²⁺ concentrations.

Time	Number of death events										
	DCCa-G1	DCCa-G2	DCCa-G11	DCCa-G12	DCCa-G13	DCCa-G14	DCMg-G10	DCMg-G11	DCMg-G12	DCMg-G13	DCMg-G14
1-day	0.33 ± 0.58	0.00 ± 0.00	0.67 ± 0.58	0.67 ± 0.58	57.33 ± 4.04	90 ± 0.00	18.33 ± 4.04	90 ± 0.00	90 ± 0.00	90 ± 0.00	90 ± 0.00
2-day	0.00 ± 0.00	0.67 ± 0.58	1.00 ± 1.00	8.33 ± 1.16	24.33 ± 2.52	–	55.67 ± 4.62	–	–	–	–
3-day	1.00 ± 1.00	0.33 ± 0.58	3.33 ± 1.53	17.67 ± 2.52	8.33 ± 1.53	–	16.00 ± 1.73	–	–	–	–
4-day	0.33 ± 0.58	0.33 ± 0.58	31.00 ± 2.65	48.67 ± 4.04	–	–	–	–	–	–	–
5-day	2.00 ± 2.00	0.67 ± 1.16	18.67 ± 1.53	14.67 ± 3.06	–	–	–	–	–	–	–
6-day	1.33 ± 0.58	0.67 ± 0.58	8.33 ± 1.53	–	–	–	–	–	–	–	–
7-day	1.67 ± 1.53	2.00 ± 1.00	4.33 ± 1.16	–	–	–	–	–	–	–	–
8-day	0.67 ± 1.16	1.33 ± 1.16	7.00 ± 1.00	–	–	–	–	–	–	–	–
9-day	1.67 ± 1.53	0.67 ± 1.16	4.67 ± 1.53	–	–	–	–	–	–	–	–
10-day	1.67 ± 2.08	1.67 ± 1.16	4.00 ± 1.00	–	–	–	–	–	–	–	–
11-day	0.67 ± 1.16	0.67 ± 0.58	3.00 ± 1.00	–	–	–	–	–	–	–	–
12-day	1.00 ± 1.00	0.67 ± 1.16	2.33 ± 0.58	–	–	–	–	–	–	–	–
13-day	8.00 ± 2.00	0.33 ± 0.58	0.67 ± 0.58	–	–	–	–	–	–	–	–
14-day	8.00 ± 1.00	4.67 ± 2.52	1.00 ± 1.00	–	–	–	–	–	–	–	–
15-day	10.33 ± 6.66	4.33 ± 3.22	–	–	–	–	–	–	–	–	–
16-day	33.33 ± 7.02	6.33 ± 1.53	–	–	–	–	–	–	–	–	–
17-day	4.67 ± 4.16	7.67 ± 1.53	–	–	–	–	–	–	–	–	–
18-day	7.33 ± 2.31	19.33 ± 2.52	–	–	–	–	–	–	–	–	–
19-day	2.33 ± 0.58	8.33 ± 2.08	–	–	–	–	–	–	–	–	–
20-day	3.67 ± 4.04	7.67 ± 1.53	–	–	–	–	–	–	–	–	–
21-day	–	9.33 ± 1.16	–	–	–	–	–	–	–	–	–
22-day	–	11.00 ± 4.58	–	–	–	–	–	–	–	–	–
23-day	–	1.33 ± 1.53	–	–	–	–	–	–	–	–	–

*Data present in this table as mean ± standard error.



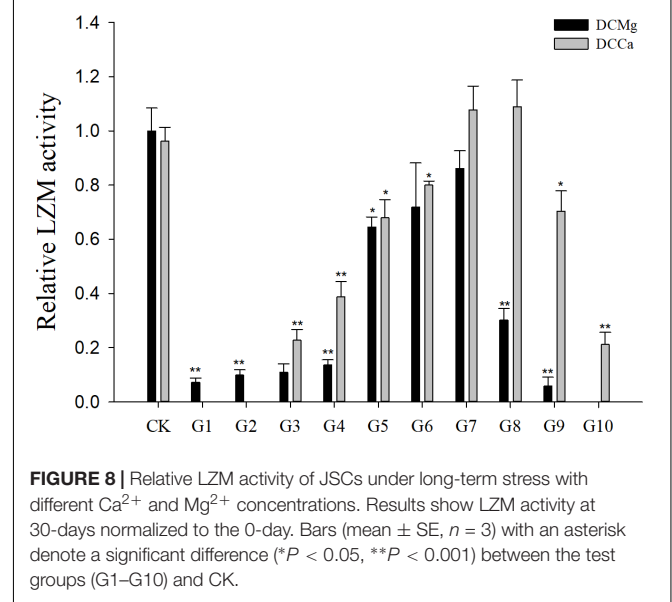
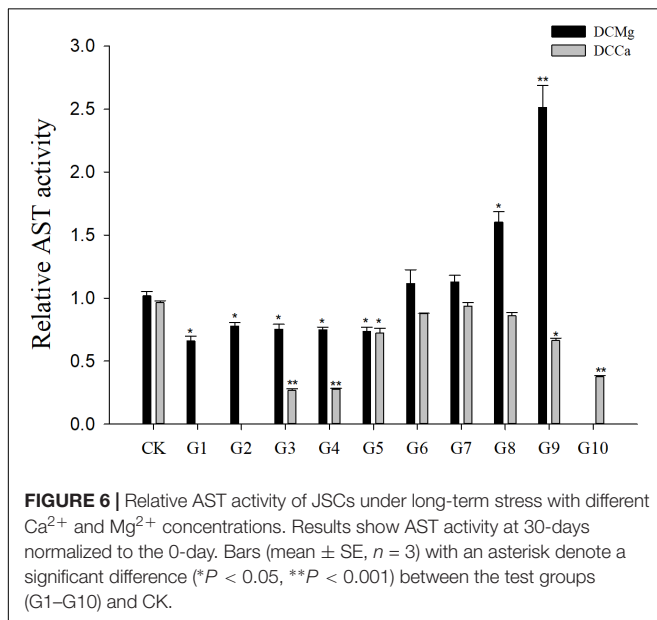
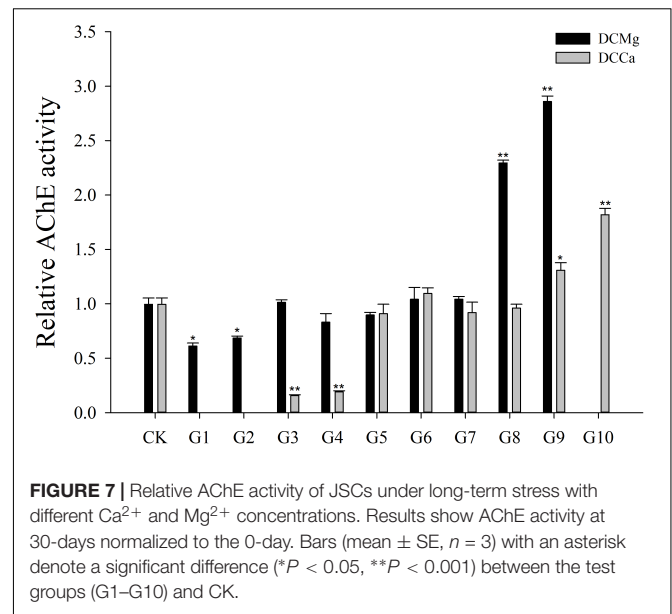
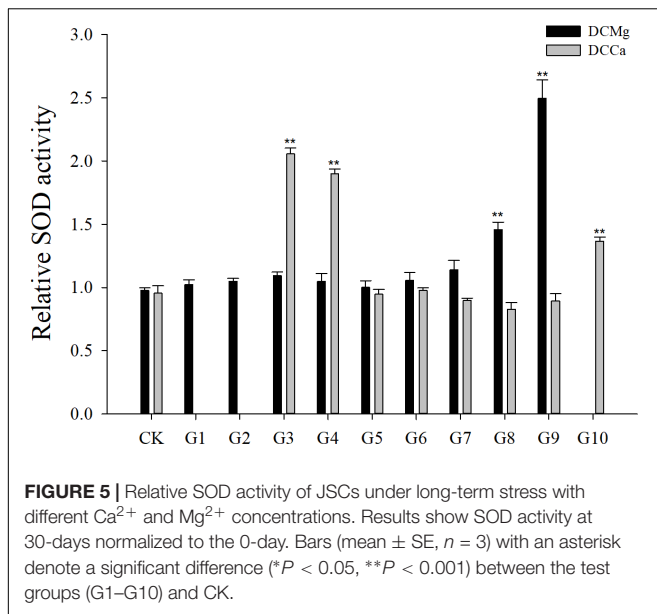
and the Ca^{2+} concentration $0 \text{ mmol}\cdot\text{L}^{-1}$ and $207.55 \text{ mmol}\cdot\text{L}^{-1}$ began to decrease significantly at 12 h. The Mg^{2+} concentration $0 \text{ mmol}\cdot\text{L}^{-1}$ showed no significant change in metabolic activity (Figure 11). Metabolic activity was only significantly increased at 48 h in the Ca^{2+} concentration $0 \text{ mmol}\cdot\text{L}^{-1}$. However, metabolic activity of the Mg^{2+} concentration $73.86 \text{ mmol}\cdot\text{L}^{-1}$ and Ca^{2+} concentration $207.55 \text{ mmol}\cdot\text{L}^{-1}$ began to increase significantly at 12 h.

DISCUSSION

Changes of Ca^{2+} and Mg^{2+} content in water not only affect the survival and growth of bivalves, but also affect their physiological responses. ISW in northwest China, especially the Gansu area (a target area for the transplantation of

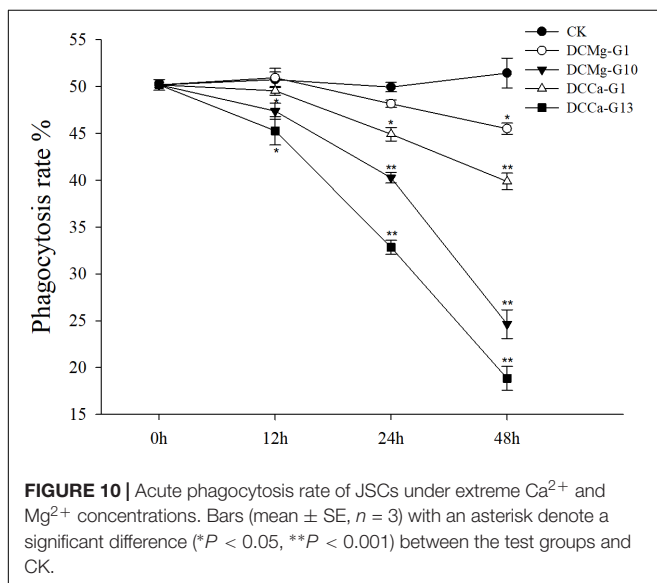
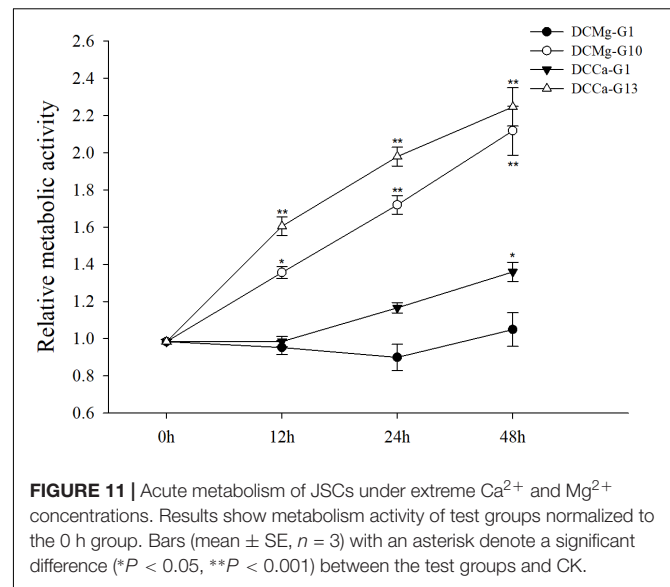
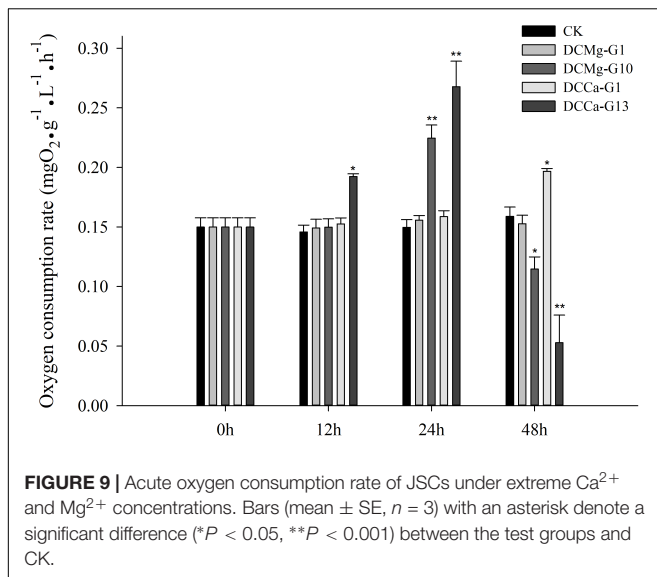
S. constricta), is characterized by broad variation in pH, carbonate alkalinity, and Ca^{2+} ($0.775 \text{ mmol}\cdot\text{L}^{-1}$ – $6.825 \text{ mmol}\cdot\text{L}^{-1}$) and Mg^{2+} ($3.07 \text{ mmol}\cdot\text{L}^{-1}$ – $33.69 \text{ mmol}\cdot\text{L}^{-1}$) content. Studying the ion tolerance range of *S. constricta* is the first and crucial step toward transplanting *S. constricta* cultures to ISW.

There is a significant difference in the tolerance of Ca^{2+} and Mg^{2+} between different invertebrate species. Zhou et al. (2007) reported that the optimal concentrations of Ca^{2+} and Mg^{2+} for the survival of *L. vannamei* larvae were 0.76 – $8.78 \text{ mmol}\cdot\text{L}^{-1}$ and 0.36 – $35.51 \text{ mmol}\cdot\text{L}^{-1}$, respectively. Roy et al. (2007) found that Mg^{2+} concentrations are very important for shrimp. The concentration of Mg^{2+} in inland saline-alkali water is at least 25% higher than that of seawater at the same salinity. Sprung (1987) reported that if environmental Ca^{2+} concentrations are less than $0.3 \text{ mmol}\cdot\text{L}^{-1}$, the veliger larvae of the zebra mussel (*Dreissena*



polymorpha) cannot survive. Furthermore, the New Zealand mud snail (*Potamopyrgus antipodarum*) is reported to have a minimum Ca^{2+} concentration tolerance of $0.125 \text{ mmol}\cdot\text{L}^{-1}$ (Herbst et al., 2008). Lin et al. (2012) studied the effects of acute Ca^{2+} and Mg^{2+} concentrations on the survival of *C. sinensis* to assess the possibility of cultivating *C. sinensis* as a cultured species in ISW. It was found that when Ca^{2+} concentrations were between $0.11 \text{ mmol}\cdot\text{L}^{-1}$ and $115 \text{ mmol}\cdot\text{L}^{-1}$, and Mg^{2+} concentrations were between $0 \text{ mmol}\cdot\text{L}^{-1}$ and $126.67 \text{ mmol}\cdot\text{L}^{-1}$, the *C. sinensis* survival rates were 66 and 91.5%, respectively. It was determined that Mg^{2+} concentration may not be a primary limiting factor for transplanting *C. sinensis* in ISW. Our findings are similar to those of Lin et al. (2012) namely that JSC can

maintain a high survival rate for at least a month when under Mg^{2+} ($0 \text{ mmol}\cdot\text{L}^{-1}$) stress. However, the tolerance of JSC to high concentrations of Mg^{2+} is far less than that of *C. sinensis*. It is worth noting that the maximum tolerated concentration of JSC for Mg^{2+} is also slightly lower than the maximum measured value of the target-ISW. This suggests that perhaps Mg^{2+} be one of the limiting factors to the *S. constricta* transplantation. Thus special attention have to be paid to whether the concentration of Mg^{2+} ions too high when practicing transplantation. Mg^{2+} -free medium have little effect on the survival rates of JSC and *C. sinensis*; this may be caused by the presence of dietary Mg^{2+} ions, or may indicate that the effect of free Mg^{2+} on survival is a long-term process. Because Dietz et al. (1994) have reported that *D. polymorpha* depleted of Mg^{2+} did not survive beyond



51 days. JSC can survival in a wide range of concentrations of Ca^{2+} (0.19–19.46 $\text{mmol} \cdot \text{L}^{-1}$), suggesting that transplantation of JSC to target-ISW may without effect of Ca^{2+} , and could be performed in a broad range of Ca^{2+} concentrations.

The shell of bivalves serves as the first barrier to resist external threats, and the main component of the shell is calcium carbonate. The growth of shells is directly related to the growth of the bivalve. Since Ca^{2+} is the main cation involved in the shell formation of bivalves, it was chosen in previous growth models (Hincks and Mackie, 1997). Most of the environmental calcium (approximately 80%) that is actively taken up from the water is deposited in the shell (Van Der Borgh and Van Puymbroeck, 1966). However, these reactions are reversible, and under condition of low Ca^{2+} concentrations, calcium is removed from the shell (Van Der Borgh and Van Puymbroeck, 1966). Accordingly, in this test we observed that JSCs died from calcium

carbonate shell loss when Ca^{2+} was less than 0.19 $\text{mmol} \cdot \text{L}^{-1}$. Such a situation may stem from the results of Ca^{2+} regulation. On the one hand, it is the release of Ca^{2+} in the shell caused by the efflux of free Ca^{2+} from the hemolymph and shell-mantle fluid. On the other hand, the efflux of Ca^{2+} also causes an increase in free amino acids and acidic metabolites in the body. These acidic end products are buffered by the mobilization of Ca^{2+} from the shell (Akberali et al., 1977). In addition, Wu et al. (2003) studied the effect of Ca^{2+} on the activity of digestive enzymes in *S. constricta*. It was found that an imbalance of Ca^{2+} concentration significantly decreased the activities of amylase and protease, thereby inhibiting growth. The higher Ca^{2+} concentration range of target-ISW 6.49–6.825 $\text{mmol} \cdot \text{L}^{-1}$ and 3.24–6.825 $\text{mmol} \cdot \text{L}^{-1}$ may significantly inhibit the shell length and body weight growth of JSC, respectively. This suggest that transplanting *S. constricta* in the target-ISW may not have growth inhibition caused by Ca^{2+} deficiency, and there may be only a physiological response by excessive Ca^{2+} . Other hand suggest that the effect of Ca^{2+} on the growth of JSC may be more than one aspect, which weight is more likely to be affected than shell.

Mg^{2+} is the most abundant divalent ion within living cells. It is a cofactor in many enzymes, and its free ionic concentration regulates metabolic and shell formation processes (Bentov and Erez, 2006; Roy et al., 2010; Marin et al., 2012; Suzuki and Nagasawa, 2013; Wilbur and Yonge, 2013; González-Vera and Brown, 2017). However, Wu et al. (2003) found that the concentration of Mg^{2+} in the water did not affect the digestive enzyme activity of *S. constricta*, indicating that the effects of Mg^{2+} on JSC growth likely involves the osmotic and ionic regulation caused by Mg^{2+} imbalance and its role as the activity center of metabolic and mineral enzymes. From the Mg^{2+} concentration distribution of the target-ISW, the higher Mg^{2+} concentration range 14.77–33.69 $\text{mmol} \cdot \text{L}^{-1}$ will significantly inhibit the shell length and body weight growth of JSC. This suggest that transplanting *S. constricta* in the target-ISW will not

have growth inhibition caused by Mg^{2+} deficiency, and there may be only a response caused by excessive Mg^{2+} .

Most evidences in bivalves is based on observations that on simulative conditions, oxygen consumption rapidly rises to levels above normal (Moon and Pritchard, 1970; Akberali et al., 1977; Almada-Villela, 1984). Both cell volume regulation and taurine efflux are inhibited in *Noetia ponderosa* erythrocytes and *Glycera dibranchiata* coelomocytes exposed to Ca^{2+} -free hypoosmotic artificial seawater, and both are potentiated in high Ca^{2+} hypoosmotic artificial seawater (Smith and Pierce, 1987). Divalent cations also stabilize the cell membrane. The increased membrane permeability to taurine caused by Ca^{2+} -free isosmotic media may be due to a lack of divalent cations that bind to and stabilize membranes (Swinehart et al., 1980). Not difficult to imagine the volume regulation of JSC haemocytes is extremely active, it may be the main reason sustained increase in the metabolism. Meanwhile, we found that the oxygen consumption increases within 24 h but it decreases sharply in 48 h. This suggest that high Ca^{2+} and Mg^{2+} stresses affects ventilatory activity causing internal hypoxia at 48 h. This may be the main reason for the rapid death of JSCs. On the contrary, Lee et al. (2005) noted that in Mg^{2+} -free artificial seawater, the respiration rate of hard clams is decreased. No significant changes in oxygen consumption were observed in the DCMg-G1 group (Mg^{2+} -free group), and it is likely that this inhibitory effect will take longer in JSCs.

When osmotic concentration and the ionic composition of the extracellular fluid be changed, the cells release cytoplasmic ions and organic osmolytes into the extracellular fluid to eliminate the osmotic gradient across the plasma membrane (Deaton, 2008; Larsen et al., 2014). Changes in environmental Ca^{2+} and Mg^{2+} have important regulation for both inorganic ion and organic osmotic regulation. In research of *G. dibranchiata*, it was found that the lack of Ca^{2+} ions in the environment did not lead to the efflux of Na^+ and Cl^- , but it caused the efflux of K^+ (Costa and Pierce, 1983). Comparing our results, it suggesting that the effect of extracellular fluid Ca^{2+} concentration on NKA activity may be directly related to the flux of K^+ . We believe that the ionic regulation caused by Ca^{2+} may be much more than that. A decrease in the concentration of Mg^{2+} ions in water tends to inactivate NKA in shrimp (Roy et al., 2010). Similar results were also seen in our study, but Watts and Pierce (1978) reported that the ATPase in *Modiolus demissus* ventricles requires a minimum of 5 mM Mg^{2+} in body to maintain constant activity. These suggest that *S. constricta* may existence very high regulation ability. In addition, when cultured in Mg^{2+} -free water, the NKA activity in JSC is not completely lost, as Mg^{2+} may be obtained through dietary Mg^{2+} ions in food (algae). Mollusc farming does not utilize compound feed directly, like shrimp farming, so the impact of dietary Mg^{2+} is difficult to eliminate. Of course, the target-ISW does not have the Mg^{2+} -free type of water, and the target-ISW does not significantly inhibit the NKA activity when the Mg^{2+} content is the lowest. But the higher Mg^{2+} concentration range 14.77–29.54 $\text{mmol}\cdot\text{L}^{-1}$ may significantly increase the NKA activity of

JSC. In addition, the Ca^{2+} concentration range of target-ISW may not significant impact on NKA activity of JSC. This suggest that when transplanting *S. constricta* in the target-ISW, Mg^{2+} may be one of the main factors to induce individuals to produce intense osmotic and ionic regulation.

Mg^{2+} operates synergistically to facilitate glutamate dehydrogenase activity and interactions between this glutamate dehydrogenase and aminotransferase (Fahien et al., 1985). In bivalve, inorganic ions are the first efflux components that respond to ambient medium cation ions (Na^+ , K^+ , Ca^{2+} , and Mg^{2+}) balance changes (Akberali et al., 1977; Shumway, 1977; Smith and Pierce, 1987). The efflux of inorganic ions induces the osmotic regulation from the organic components (free amino acids). The regulation of divalent cations in JSC may affect the concentration of free amino acids and may be directly related to changes in AST activity. Decreases and increases in Mg^{2+} concentration directly affect AST activity, and these results were recapitulated in our study. However, decreases and increases of Ca^{2+} concentration inhibit AST activity in JSC. So from the Ca^{2+} and Mg^{2+} concentration distribution of the target-ISW, we can imagine that both the higher Ca^{2+} (6.49–6.825 $\text{mmol}\cdot\text{L}^{-1}$) and Mg^{2+} (14.77–33.69 $\text{mmol}\cdot\text{L}^{-1}$) concentrations range may significantly affect AST activity. This indicating that changes of Ca^{2+} concentration in water not only affect digestive enzyme activity but also affect enzyme activity related to protein metabolism, thereby inhibiting growth (Wu et al., 2003).

The imbalance between Ca^{2+} and Mg^{2+} in water leads to increased oxygen consumption in aquatic animals, resulting in the production of more oxygen free radicals (Lin and Kao, 2000; Culotta, 2001). The increase in oxygen free radicals induces an increase in SOD activity to scavenge these radicals. In the present study, SOD activity is only augmented at moderately low calcium concentrations, which are coincident with high NKA activity and partial restoring of shell growth. SOD is also increased at the highest concentrations of both ions, which are very stressful conditions. But in target-ISW may be only the higher Mg^{2+} concentration range (14.77–33.69 $\text{mmol}\cdot\text{L}^{-1}$) may significantly increase the SOD activity of JSC. This suggest that Mg^{2+} may cause excessive metabolism and produce a large amount of oxygen free radicals when transplanting *S. constricta* in the target-ISW.

The AChE is a hydrolase for the signal termination at cholinergic synapses by rapid hydrolysis of the neurotransmitter acetylcholine (Quinn, 1987). AChE paly as a important role in neurotransmission process also in fish and invertebrates (Fulton and Key, 2001). The inhibition of AChE activity has been used widely as a biomarker of exposure to organototoxic substance and heavy metal ions in mollusk (Rickwood and Galloway, 2004; Devin et al., 2017). In addition, Pfeifer et al. (2005) reported that the activity of AChE in *Mytilus* sp. is sensitive to environmental salinity. Although the effect of the concentration of Ca^{2+} and Mg^{2+} on AChE activity in mollusc has not been clearly reported, in fish, Tomlinson et al. (1981) reported that AChE activity reached maximum saturation when both Ca^{2+} and Mg^{2+} concentrations were on the order of magnitude of

10 mmol, and the activity level were maintained at more higher Ca^{2+} and Mg^{2+} concentrations. The same situation be found in our study, AChE activity was continuously increased with increasing concentrations of Ca^{2+} and Mg^{2+} in water. Although there are some differences in the physiological functions of fish and bivalve, the physiological roles and functional mechanisms of AChE (the perspective of heavy metal ion binding) are relatively conservative. This indicating that the molecular mechanism of bivalve and fish AChE are similar, and the difference between the them may occur in the magnitude of the cation strength affecting the enzyme activity. In our study, indicating that the neurotransmission process of JSC be significant inhibition when the concentration of Ca^{2+} higher than 6.49 mmol L^{-1} and concentration of Mg^{2+} higher than $14.77 \text{ mmol L}^{-1}$. Then the corresponding physiological processes of neuromodulation are inhibited. Obviously, this effect may be existed in both the higher Ca^{2+} ($6.49\text{--}6.825 \text{ mmol}\cdot\text{L}^{-1}$) and Mg^{2+} concentration range ($14.77\text{--}33.69 \text{ mmol}\cdot\text{L}^{-1}$) of target-ISW.

Lysozyme is a typical immunoenzyme that specifically lyses bacteria (Bachali et al., 2002; Xue et al., 2010). Usually LZM is divided into two categories, based on whether it can be combined with Ca^{2+} (Jielian et al., 2017). In mollusc, there is a class of LZM that binds to Ca^{2+} and interacts with calcium carbonate to participate in the shell formation process (Jimenez-Lopez et al., 2003). However, in mollusc is reported that LZM activity has a low tolerance to Ca^{2+} and Mg^{2+} concentrations (Nilsen et al., 1999; Olsen et al., 2003). In addition, the results of SOD and phagocytosis in this study also reflect the immune response of JSCs. So, we propose that the effects of Ca^{2+} and Mg^{2+} on the immune capacity of JSC are comprehensive. We suggest that (1) Ca^{2+} concentration (lower than 0.28 mmol L^{-1} , higher than $19.46 \text{ mmol L}^{-1}$) and Mg^{2+} concentration (higher than $14.77 \text{ mmol L}^{-1}$) affect metabolic immune enzymes; (2) function of haemocyte phagocytosis will be inhibited when the concentration of Ca^{2+} and Mg^{2+} in hemolymph sharply rise or fall in short period of time. Because adhesion and aggregation function be limited (Chen and Bayne, 1995); (3) Ca^{2+} concentration (lower than 0.93 mmol L^{-1} , higher than 6.49 mmol L^{-1}) and Mg^{2+} concentration (lower than 0.37 mmol L^{-1} , higher than $14.77 \text{ mmol L}^{-1}$) affect antibacterial enzyme activity. Therefore, this means that when the *S. constricta* is transplanted to the target-ISW, both the higher Ca^{2+} ($6.49\text{--}6.825 \text{ mmol}\cdot\text{L}^{-1}$) and Mg^{2+} ($14.77\text{--}33.69 \text{ mmol}\cdot\text{L}^{-1}$) concentration range may significantly enhances the metabolic immunity, but the cellular immunity (haemocyte) and bacterial immunity are weakened.

The $\text{Ca}^{2+}/\text{Mg}^{2+}$ ratio is often reported in shrimp and has important effects on survival, growth and physiology (Pan et al., 2006; González-Vera and Brown, 2017). The $\text{Ca}^{2+}/\text{Mg}^{2+}$ ratio not only has strong species specificity, but also has been widely researched in shrimp species. Tavabe et al. (2013) found that a $\text{Ca}^{2+}/\text{Mg}^{2+}$ ratio of 0.8 was optimal for larval development, while Latif (1992) found that a 1:1 $\text{Ca}^{2+}/\text{Mg}^{2+}$ ratio was desirable for juveniles of *Macrobrachium rosenbergii*. This suggests that the $\text{Ca}^{2+}/\text{Mg}^{2+}$ ratio is an important indicator for shrimp, but further study is needed to elucidate its importance in other aquatic species. The $\text{Ca}^{2+}/\text{Mg}^{2+}$ ratio in mollusc has not been

reported on. We do not have definitive evidence to obtain the significant correlation between $\text{Ca}^{2+}/\text{Mg}^{2+}$ ratio and the survival, growth, and physiology of JSC, but this does not mean that $\text{Ca}^{2+}/\text{Mg}^{2+}$ ratio has no effect, so this requires future validation with independent test.

Over the past 250 years, atmospheric carbon dioxide (CO_2) levels increased by nearly 40% (Solomon et al., 2007). Ocean CO_2 uptake, causes pH reductions and alterations in fundamental chemical balances that together are commonly referred to as ocean acidification (Millero et al., 2009). Orr et al. (2005) predicted that ocean acidification will cause a pH drop of 0.3–0.4 for the 21st century, is equivalent to approximately a 150% increase in H^+ and 50% decrease in CO_3^{2-} concentrations. Marine carbonates are mainly in the form of CaCO_3 and MgCO_3 , which means that the concentration of Ca^{2+} and Mg^{2+} in the 21st century will increase within 50% than it is now. Comparing our findings, we can see that such an increase may not have a negative impact on the survival, growth and physiology of JSC. However, the negative impact of ocean acidification on the survival and growth of corals and molluscs is a proven fact (Hoegh-Guldberg et al., 2007; Fabry et al., 2008). Ocean acidification is a complex environmental change process involving not only changes in the concentration of metal cations, but also pH, $p\text{CO}_2$, CO_3^{2-} , temperature and marine food chains (Fabry et al., 2008). Therefore, we believe that other factors or comprehensive factors other than Ca^{2+} and Mg^{2+} may eventually lead to the negative impact of ocean acidification on the survival and growth of molluscs.

CONCLUSION

Stress caused by changes in the concentration of Ca^{2+} and Mg^{2+} in the environment may cause efflux and reflux of Ca^{2+} and Mg^{2+} in JSC. Metabolism and oxygen consumption are rapidly increased but haemocyte phagocytosis are decreased in short time. At this time, a large number of JSCs died when Ca^{2+} and Mg^{2+} exceed physiological limits. In the long-term stress, the imbalance both Ca^{2+} and Mg^{2+} may cause a comprehensive physiological response including ionic regulation, metabolic levels, neurotransmission process, and immunity. Then ultimately affect growth and even survival. When using such a “standard ruler” to measure the effect of Ca^{2+} and Mg^{2+} concentrations in the target-ISW on *S. constricta*, we found that only high concentrations of Ca^{2+} ($3.24\text{--}6.825 \text{ mmol}\cdot\text{L}^{-1}$) and Mg^{2+} ($14.77\text{--}33.69 \text{ mmol}\cdot\text{L}^{-1}$) had significantly impact on transplantation practice. In physiology, such effects include accelerated ionic regulation, accelerated metabolism, increased oxygen free radical production, increased metabolic immunity, decreased bacterial and cellular immunity, and inhibit neurotransmission process. The concentrations of Ca^{2+} and Mg^{2+} in the target-ISW does not cause all *S. constricta* to die in the short term, but it inhibits growth and causes a part of individual death during long-term aquaculture. Those implies that we have to choose the right concentration of Ca^{2+} and Mg^{2+} or to improve the water quality in target-ISW. Fortunately,

the Ca^{2+} and Mg^{2+} content of most area target-ISWs is suitable for long-term transplantation of *S. constricta*. Our study also suggests that the increase in Ca^{2+} and Mg^{2+} ion concentrations caused by ocean acidification will not affect the survival, growth and physiology of *S. constricta*.

DATA AVAILABILITY

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

ETHICS STATEMENT

In this study, the animals were artificially propagated as juvenile clams.

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AUTHOR CONTRIBUTIONS

MP was responsible for experimental design, test operations, data processing, and manuscript writing. ZL was responsible for data processing and manuscript modification. XL was responsible for experimental design. BY was responsible for test operations. TL was responsible for experimental data collection. JL, DN, and ZD were responsible for experimental program guidance.

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Conflict of Interest Statement: 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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Adenosine Monophosphate-Activated Protein Kinase Signaling Regulates Lipid Metabolism in Response to Salinity Stress in the Red-Eared Slider Turtle *Trachemys scripta elegans*

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Aquatic animals have developed various mechanisms to live in either hyperionomic or hypoionomic environments, and, as such, not many species are capable of surviving in both. The red-eared slider turtle, *Trachemys scripta elegans*, a well-known freshwater species, has recently been found to invade and inhabit brackish water. Herein, we focus on some of the metabolic adaptations that are required to survive and cope with salinity stress. The regulation of the adenosine monophosphate (AMP)-activated protein kinase (AMPK), a main cellular “energy sensor,” and its influence on lipid metabolism were evaluated with a comparison of three groups of turtles: controls in freshwater, and turtles held in water of either 5‰ salinity (S5) or 15‰ salinity (S15) with sampling at 6, 24, and 48 h and 30 days of exposure. When subjected to elevated salinities of 5 or 15‰, AMPK mRNA levels and AMPK enzyme activity increased strongly. In addition, the high expression of the peroxisome proliferator activated receptor- α (PPAR α) transcription factor that, in turn, facilitated upregulation of target genes including carnitine palmitoyltransferase (CPT) and acyl-CoA oxidase (ACO). Furthermore, the expression of transcription factors involved in lipid synthesis such as the carbohydrate-responsive element-binding protein (ChREBP) and sterol regulatory element-binding protein 1c (SREBP-1c) was inhibited, and two of their target genes, acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS), were significantly decreased. Moreover, exposure to saline environments also increased plasma triglyceride (TG) content. Interestingly, the content of low-density lipoprotein cholesterol (LDL-C) and total cholesterol (TC) in plasma was markedly higher than the control in the S15 group after 30 days, which indicated that lipid metabolism was disrupted by chronic exposure to high salinity. These findings demonstrate that activation of AMPK might regulate lipid metabolism in response to salinity stress through the inhibition of lipid synthesis and promotion of lipid oxidation in the liver of *T. s. elegans*. This may be an important component of the observed salinity tolerance of these turtles that allow for invasion of brackish waters.

Keywords: *Trachemys scripta elegans*, salinity adaptation, fatty acid oxidation, lipid synthesis, lipid metabolism-related transcription factors

INTRODUCTION

Salinity is a crucial environmental stress factor for aquatic species and can disrupt electrolyte balance, cell energetics, and various other physiological responses (Verity and Smetacek, 1996). Shifts from optimal salinity cause stress in aquatic organisms and increase the energy demand for osmoregulation (Devreker et al., 2009). Lipid metabolism is highly dependent on the cellular energy (adenosine triphosphate – ATP), and the *de novo* lipogenesis pathway requires cytosolic citrate from mitochondria in positive nutrition status (Osellame et al., 2012). Changes in lipid content and fatty acid composition were associated with different salinities (0, 7, 14, 21, and 28 ppt) in juvenile American shad (*Alosa sapidissima*; Liu et al., 2016). However, upon environmental stressors, the normal function of mitochondria can be disrupted, leading to lipid metabolism dysfunction in aquatic animals. Thus, it is important to understand lipid metabolism in-depth to further understand the mechanisms behind biological processes involving the lipid metabolic pathway.

The adenosine monophosphate (AMP)-activated protein kinase (AMPK) is regarded as the energy sensor of cells and plays a very important role in regulating fuel metabolism for anabolic versus catabolic purposes (Ewart and Kennedy, 2011; Hardie et al., 2012). The regulation of energy metabolism via AMPK-mediated signaling is an active area of research that ranges from studies of the protein kinase itself (structure, cellular distribution, activity) to its roles in multiple organisms including mammals, turtles, frogs, fish, shrimps, and crabs (Mihaylova and Shaw, 2011; Hardie, 2015; Xu et al., 2016). These studies, among others, have emphasized its role in maintaining energy equilibrium, particularly in regulating hepatic lipid metabolism (Towler and Hardie, 2007; Mihaylova and Shaw, 2011). Interestingly, studies with animal models that show natural metabolic rate depression have found that AMPK signaling is involved in the anoxia tolerance of *T. s. elegans* (Rider et al., 2009) and in freezing and dehydration tolerance of wood frogs (*Rana sylvatica*; Horman et al., 2005; Rider et al., 2006). AMPK phosphorylation can inhibit enzymes related to fatty acid, glycogen, protein, and cholesterol synthesis while stimulating enzymes of glycolysis and lipolysis (Hardie and Pan, 2002; Horman et al., 2002; Munday, 2002). In addition to post-translational regulation of various rate-limiting enzymes, AMPK also regulates the gene expression of key glycolytic and lipogenic enzymes via regulating the carbohydrate-responsive element-binding protein (*ChREBP*) and sterol regulatory element-binding protein 1c (*SREBP-1c*) transcription factors, respectively (Kawaguchi et al., 2002; Kohjima et al., 2008), to allow the incorporation of fatty acids into triglycerides that function as a long-term energy reservoir (Dentin et al., 2005).

In recent years, the freshwater turtle, red-eared slider (*Trachemys scripta elegans*), has successfully invaded five continents, and this has been in part due to its strong ability to adapt to diverse environments (Semenov, 2010). Field surveys have indicated that *T. s. elegans* can inhabit and lay eggs in the estuary of the Nanduijiang river (Hainan Province, China) and that their hatchlings can be found at the shore of brackish water (Yang and Shi, 2014). Variations of sea surface salinity

and temperature were negatively correlated during the deglaciation induced by climate warming, which is expected to reduce surface salinity and expand the brackish water regions (Paterne et al., 1993; Groisman et al., 1999). Therefore, the potential invasion danger of *T. s. elegans* would presumably be serious.

Our previous studies have shown that *T. s. elegans* can survive for more than 3 months in saline environments (<15‰; Hong et al., 2014) or promote the antioxidant defense system against the oxidative stress induced by salinity (Ding et al., 2019) and that during this time, they increase their plasma triglyceride and glucose levels, probably supporting increased fuel/energy needs for survival in the saline environment (Shu et al., 2012). Transcriptomic analyses of *T. s. elegans* exposed to saline environments have shown that lipid metabolism pathways are differentially regulated, relative to controls in freshwater, suggesting a strong link between lipid metabolism and salinity-tolerance (Hong et al., 2018). However, the molecular mechanisms behind this targeting of lipid metabolism in *T. s. elegans* under salinity stress remain unknown. The objective of this study was to investigate the role of AMPK in the regulation of lipid metabolism in *T. s. elegans* under salinity stress. Understanding the molecular mechanisms of lipid metabolism in *T. s. elegans* under salinity-responsive AMPK signaling can provide a basis for studying the molecular mechanism of salinity tolerance.

MATERIALS AND METHODS

Experimental Animals and Salinity Exposure

T. s. elegans (3 years old, female, mean mass 392.26 ± 20.57 g) was purchased from the Hongwang turtle farm (Haikou, China). Turtles were acclimated in a cement pool (190 cm × 65 cm × 32 cm) at room temperature with dechlorinated water for 2 weeks prior to salinity exposure. Two treatment groups with salinities of 5‰ (S5) and 15‰ (S15) were generated using sea salt crystals and measured with a digital high precision electronic salt meter (Taihua, Chengdu, China). An equal volume of dechlorinated freshwater was added to another tank for the control group. Ceramic tiles were placed in each pond for shelter and basking. Turtles were fed with a commercial diet twice a week, and any remaining food was removed and the water changed in the pools after 24 h. The salinity of the experimental groups was monitored and adjusted accordingly each day. Water temperature in pools was maintained near 26°C, and 12–15 h of light was received by these turtles daily.

Six turtles from each pool were randomly sampled at indicated time of experimental exposure (6, 24, and 48 h and 30 days) and were anesthetized by freezing for 20 min. Blood was sampled from cardiovascular vessels in heart. Heart, liver, skeletal muscle, kidney, lung, and intestine were quickly sampled, frozen in liquid nitrogen, and stored at –80°C until further use. All experimental procedures had the prior approval of the Hainan Provincial Ecological Environment Education Center Experimental Animal Ethics Committee and were conducted under standard protocols for the care and use of laboratory animals at Hainan Normal University (No. HNECEE-2014-004).

RNA Extraction and Quantitative Real-Time Polymerase Chain Reaction

Total RNA was extracted from heart, liver, skeletal muscle, kidney, lung, and intestine using Trizol (Invitrogen, Carlsbad, USA), according to the manufacturer's protocol. The quality and concentration of total RNA were assessed using a NanoDrop™ One/OneC spectrophotometer (Thermo Fisher, MA, USA), and the integrity of the RNA was determined by electrophoresis on a 1.2% agarose gel that showed the presence of two sharp 18S and 28S rRNA bands. Approximately 1 µg of total RNA was reverse transcribed to cDNA in 20 µl reactions using Prime Script Reverse Transcriptase (TaKaRa, Tokyo, Japan) according to the manufacturer's instructions.

Primers for *T. s. elegans* mRNA were designed using Primer-BLAST (Table 1). Relative mRNA expression levels were measured using quantitative real-time polymerase chain reaction (qRT-PCR) analysis using the TB Green QuantiTect RT-PCR Kit (TaKaRa, Tokyo, Japan) and a LightCycler®480 Real-Time PCR System (Roche Diagnostics, Basel, Switzerland). The melting curve, amplification curve, and standard curve of each gene were gained by Roche Light Cycler® 480II Real-Time PCR software. The correlation coefficient of standard curves was >0.99, and all genes amplification efficiency were 98–99%. The following transcripts were examined: acetyl-CoA carboxylase (ACC), fatty acid synthase (FAS), stearoyl CoA desaturase (SCD), carnitine palmitoyltransferase 1 (CPT-1), carnitine palmitoyltransferase 2 (CPT-2), acyl-CoA oxidase (ACO), and acyl-CoA synthetase long-chain 1 (ACSL-1). These were analyzed by the $2^{-\Delta\Delta C_t}$ method of processing data after normalization to β -actin as the reference gene (β -actin was expressed in the three groups and unaffected under salinity stress). The values of control group at 6 h were standardized to 1.0.

Biochemical Analysis

Liver tissue (200 mg) was homogenized in 2 ml 0.7% saline and centrifuged at $3,000 \times g$ for 15 min at 4°C. The protein-containing supernatant was used to measure enzyme activities (AMPK, ACC, FAS, CPT-1, and ACO) using customized ELISA kits (Zike Biological Technology Co., Ltd., Shenzhen, China) according to the manufacturer's instructions.

The concentration of plasma triglycerides (TG), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), and total cholesterol (TC) was measured within 24 h of blood collection in a refrigerator by an Olympus AU640 automatic biochemical analyzer (Olympus, Tokyo, Japan).

Data Analysis

All experimental data are expressed as mean $\pm$ SE and were analyzed by Excel 2016 and SPSS 16.0 statistical software. The interaction of two factors (salinity and time) was verified by two-way analysis of variance. After analyzed homoscedasticity of variances, one-way analysis of variance (ANOVA) was run to examine the influence of environmental salinity. Significance differences between different groups were assessed using LSD multi-comparison, and a p lower than 0.05 was considered statistically significant.

RESULTS

Tissue-Specific mRNA Expression of AMPK Subunits

AMPK is a heterotrimeric complex with one catalytic (α) and two regulatory (β and γ) subunits. The tissue-specific expression of AMPK subunit mRNA in control turtles is presented in Figure 1. The expression of AMPK α 1 mRNA was highest in liver and heart and the lowest in lung (Figure 1A). The levels of AMPK α 2 mRNA were highest in kidney, with skeletal muscle and heart being the second most abundant, and the other three tissues showing the lowest levels of this subunit (Figure 1B). The mRNA expression levels of AMPK β 1 were from highest to lowest: skeletal muscle, kidney, liver, heart, intestine, and lung (Figure 1C). The mRNA expression levels of AMPK β 2 were highest in skeletal muscle and lowest in intestine and lung (Figure 1D). The mRNA expression levels of AMPK γ 1 and AMPK γ 3 were highest in skeletal muscle (Figures 1E,G), and AMPK γ 2 was highest in heart (Figure 1F). However, there was no expression in liver for AMPK γ 3 (Figure 1G).

AMPK α Gene Expression and Adenosine Monophosphate-Activated Protein Kinase Activity Changes After Salinity Stress

The mRNA expression level of AMPK α 1 in liver of *T. s. elegans* increased markedly only in S15 group at 48 h when compared with the control ($p = 0.028$), whereas S5 group showed no statistical differences at each sampling time. With exposure time extended, there was a peak at 48 h, and significant differences were found in S5 group between 48 and 24 h ($p = 0.027$) and in S15 group between 48 h and 30 days ($p = 0.009$; Figure 2A). Compared with the control, the expression level of AMPK α 2 decreased markedly in S5 and S15 groups at 24 h ($p = 0.031$, $p = 0.017$), began rising again at 48 h ($p = 0.082$, $p = 0.172$), and returned to baseline expression levels at 30 days ($p = 0.051$, $p = 0.270$; Figure 2B). As for AMPK activity, it increased with salinity increased. With exposure time extended, there was a peak at 48 h (Figure 2C). Moreover, there was extremely remarkable interaction effect between exposure time and salinity as for AMPK activity ($F = 10.636$, $p = 0.000$).

Relative Expression of AMPK-Responsive Lipid Metabolism Transcription Factors in Liver

As shown in Figure 3, ChREBP expression decreased in the liver of S5 group after 24 h ($p = 0.025$) but then rose to maximum expression at 48 h ($p = 0.012$) before decreasing by 30 days ($p = 0.014$), relative to the control. In S15 group, ChREBP expression showed a marked decrease except at 24 h ($p = 0.051$; Figure 3A). As for SREBP-1c expression in liver, there were significant differences only between S5 and the control at 6 h ($p = 0.004$) and between S15 and the control at 6 h ($p = 0.003$; Figure 3B). By contrast, the mRNA expression level of PPAR α exhibited a significant increasing over time except S5 group at 24 h, with 48 h exposures displaying the highest abundance (Figure 3C). The mRNA expression levels

TABLE 1 | The specific primer for *T. s. elegans*.

Genes	Accession number in GenBank	Primer sequence (5'–3')	Product length (bp)
<i>β-actin</i>	FJ514826.1	F: GCACCCTGTGCTGCTTACA R: CACAGTGTGGGTGACACCAT	190
<i>AMPKα1</i>	XM_005282144.3	F: TGGAGCAGTGGGGTTATTCTC R: CCCGAATGTCTCTGATTGTAGC	199
<i>AMPKα2</i>	XM_005284772.3	F: GCTGATTTTGGGTTGTCGAA R: TTGAGTGGGTCAACCTGCA	299
<i>AMPK-β1</i>	XM_005288707.2	F: GCTTACTGGGAGGAGAGCGA R: AGGCACTCATCCAGCTCCTG	171
<i>AMPK-β2</i>	XM_005311511.3	F: GGCTCGTCCCACTGTCATAC R: CTCGGGCAGGTCCAGAATAG	136
<i>AMPK-γ1</i>	XM_008173877.2	F: GCTGACCATCACCGACTTCA R: CCAGCGGTTTGAAGGAGTCT	134
<i>AMPK-γ2</i>	XM_024114117.1	F: TCTCCAGCACCGTTCTCAGT R: TCAGAGAGGGAGATGATACCCA	156
<i>AMPK-γ3</i>	XM_008167850.2	F: TGGCCCTGGAGATCTTCGT R: TAGGTCTTCTGGGCAGCTAGG	121
<i>ChREBP</i>	XM_005294339.2	F: GTCTGTCCCCATCACACTGG R: GGAGTGATGCAGCGAGATCC	166
<i>SREBP-1c</i>	XM_008164848.2	F: CCAGTGCAGAGAAAGCCAG R: CTCAGCTGTTGGCTGACAT	162
<i>PPARα</i>	XM_024100408.1	F: TGCCAAATCTATCCCTGGCTTC R: AGGCTACCAGCATCCCATCT	131
<i>ACC</i>	XM_005298822.3	F: GCCAGCTGAAGGACAACACT R: GCATGGTGGAGTGAACGAGT	185
<i>FAS</i>	XM_005283095.3	F: CGTTGGATCAGCACCTCCAT R: GCAATCTCCACCACAACAGC	155
<i>SCD-1</i>	XM_005285549.3	F: CTGTTCCCAACCCTAGTGCC R: GGCTCCAAATGTGACGAAGC	195
<i>CPT-1</i>	XM_005312860.2	F: TTATCCATGCCACCCTGCTC R: CTCCTGGGATACGGGAGGTA	135
<i>CPT-2</i>	XM_005284832.3	F: GCTGTCACCCCACAATCTCA R: TCGAGAGTCAAGGTTTCCACAG	146
<i>ACO</i>	XM_005297505.3	F: GTTGCACTGGCCTTCTGAT R: TGTTGCCCTGAGAGGTCGTT	178
<i>ACSL-1</i>	XM_005281979.2	F: TCTGACAAGGCCAAGCTGC R: CTTCTGGATTGGAGGCACAGG	199
<i>L-FABP</i>	XM_008169713.1	F: TGATCCAAAAGGCCAAGGACA R: CTCTCCAGTTGGGCTCTCCA	149

of *ChREBP* and *PPARα* were highly significant in the interaction effects of exposure time and salinity ($F = 4.178$, $p = 0.003$; $F = 10.955$, $p = 0.000$).

Relative Expression of Lipid Metabolism Genes in Liver

In S15 group, the mRNA expression level of *ACC* in liver decreased 3–4-fold after 48 h ($p = 0.0006$) and 30 days ($p = 0.000$) compared with the control, and *FAS* decreased at 24 h ($p = 0.020$; **Figure 4**). In S5 group, there was a significant decrease in *ACC* mRNA expression at 30 days ($p = 0.002$), but no significant differences were found in *FAS* mRNA expression, compared with the control (**Figures 4A,B**). Relative mRNA abundance of stearoyl CoA desaturase 1 (*SCD1*) decreased in S5 group only after 30 days ($p = 0.021$), and there was no

significant difference between the other groups (**Figure 4C**). The mRNA expression levels of *CPT-1*, *CPT-2*, *ACO*, and *ACSL-1* showed a significant trend of enhanced abundance with increased salinity exposures, relative to the control (**Figures 4D–G**). However, the expression level of *L-FABP* decreased by almost 5-fold in S5 group as the exposure time increased to 30 days ($p = 0.019$; **Figure 4H**). Only *CPT-1* and *ACO* among them were controlled by the interaction effects of salinity and exposure time dramatically ($F = 3.323$, $p = 0.010$; $F = 4.346$, $p = 0.002$).

The Enzymatic Activities of Lipid Metabolism-Related Enzymes in Liver

The activities of *ACC* and *FAS* in liver decreased strongly as ambient salinity increased (**Figures 5A,B**). Activity of *CPT-1*

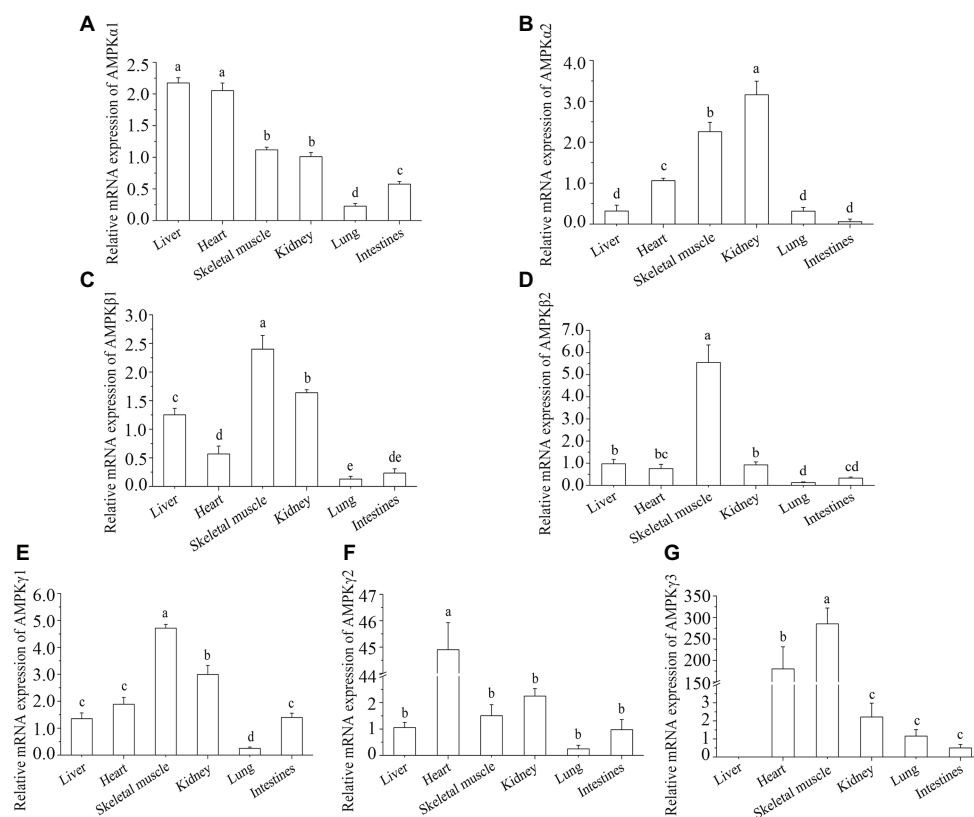


FIGURE 1 | Tissue distribution of AMPK subunits in control turtles, *T. s. elegans*. (A) *AMPKα1*; (B) *AMPKα2*; (C) *AMPKβ1*; (D) *AMPKβ2*; (E) *AMPKγ1*; (F) *AMPKγ2*; (G) *AMPKγ3*. Note: The ordinate shows the fold change in the relative mRNA expression of AMPK subunits in tissues relative to liver. The superscripts a, b, and c indicate significant differences among tissues.

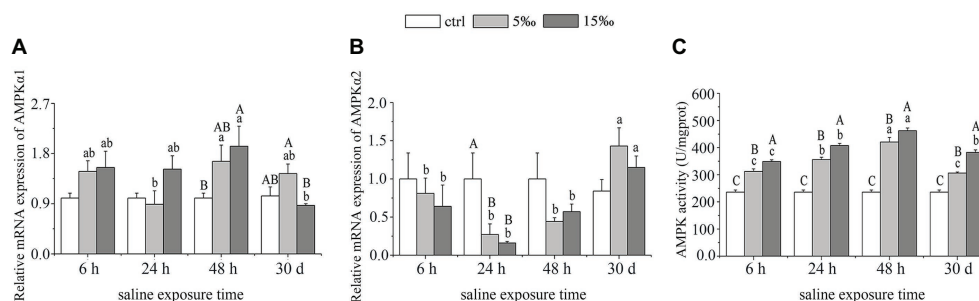


FIGURE 2 | AMPK response to salinity stress in liver. (A) mRNA expression of *AMPKα1*; (B) mRNA expression of *AMPKα2*; and (C) activity of AMPK. Different uppercase letters (A, B, and C) represent significant differences between different salinity groups at the same time point, and different lowercase letters (a, b, and c) represent significant differences between different stress times at the same salinity, $p < 0.05$.

related to lipid oxidation increased significantly in S5 group at 48 h ($p = 0.000$) and 30 days ($p = 0.000$) and also increased in S15 group at 6 h ($p = 0.045$), 24 h ($p = 0.002$), and 30 days ($p = 0.028$), relative to control levels (Figure 5C). However, ACO activity in S5 was higher than that in S15 and the control, and there was no significant difference between S15 and the control, except after 24 h salinity treatment (Figure 5D). Analysis of variance results shows that there was a significant interaction effect within salinity and exposure time on the activities of ACC, FAS, CPT-1, and ACO ($F = 12.388$, $p = 0.000$;

$F = 10.666$, $p = 0.000$; $F = 10.350$, $p = 0.000$; $F = 4.640$, $p = 0.001$).

Lipid Markers in Plasma: Triglyceride, Low-Density Lipoprotein Cholesterol, High-Density Lipoprotein Cholesterol, and Total Cholesterol

The content of TG in plasma increased markedly as salinity increased, and the levels in both salinity treatment groups

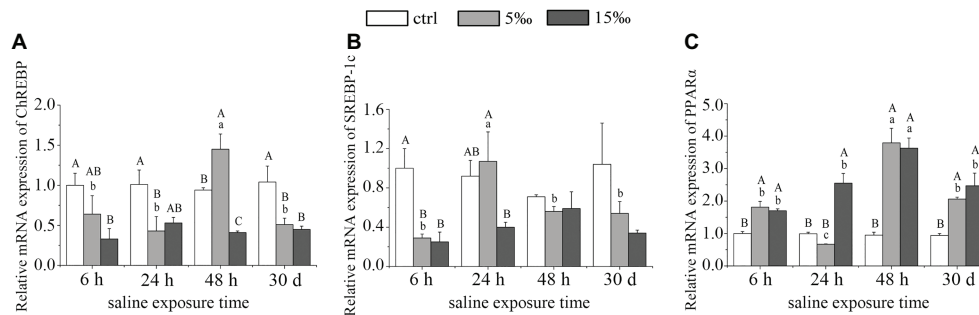


FIGURE 3 | AMPK activates the expression of major lipid metabolism transcription factors in liver of salinity-stressed turtles. **(A)** mRNA expression of *ChREBP*; **(B)** mRNA expression of *SREBP-1c*; and **(C)** mRNA expression of *PPARα*. Different uppercase letters (A, B, and C) represent significant differences between different salinity groups at the same time points, and different lowercase letters (a, b, and c) represent significant differences between different stress times at the same salinity, $p < 0.05$.

were highest at 24 h ($p = 0.000$, $p = 0.000$) and 48 h ($p = 0.001$, $p = 0.000$), relative to the control (Figure 6A). The contents of plasma LDL-C and TC in S15 group were notably higher than the other two groups after 30 days (Figures 6B,D). There was no significant difference in the content of HDL-C among these three groups over the course of the different salinity exposures (Figure 6C). As for the interaction effects between salinity and exposure time, there were significant effects on the contents of plasma TG, LDL, and TC ($F = 7.719$, $p = 0.000$; $F = 3.412$, $p = 0.007$; $F = 2.981$, $p = 0.015$).

DISCUSSION

The mRNA Expression and Activity of Adenosine Monophosphate-Activated Protein Kinase During Salinity Stress

An analysis of tissue-specific gene expression is useful to achieve a better understanding of physiological role of a gene and its protein product. AMPK is a heterotrimeric complex composed of the catalytic α , the scaffolding β , and the nucleotide binding γ subunits that are widely distributed in all tissues (Steinberg and Kemp, 2009). The enzyme plays an important role in both regulating intracellular energy balance and the cellular response to stress (Park et al., 2002). There are two or three isoforms of each subunit in mammalian tissues ($\alpha 1$ and $\alpha 2$; $\beta 1$ and $\beta 2$; $\gamma 1$, $\gamma 2$, and $\gamma 3$), each encoded by a different gene. In our study, mRNA transcripts of all seven subunits were detected in *T. s. elegans* tissues. The expression of *AMPKα1* was highest in liver, and this corresponds with analogous findings in fish and mammals, where *AMPKα1* was highly expressed in metabolically active tissues, such as liver, kidney, and brain (Stapleton et al., 2016). In this study, *AMPKα2* expression was higher in kidney, skeletal muscle, and heart. Previous research reported that *AMPKα2* plays an important role in glucose metabolism in mammalian muscles (Jørgensen et al., 2007); however, elucidating the regulatory role of *AMPKα2* on glucose metabolism in turtles still requires further study. The $\beta 1$ and $\beta 2$ subunits were also higher in skeletal muscle of *T. s. elegans* than other tissues. They provide the scaffold on which the α and γ subunits assemble (Hardie et al., 1998). Of the γ isoforms,

$\gamma 1$ and $\gamma 2$ were expressed quite uniformly across all tissues examined, with the exception of low level of $\gamma 1$ in lung and high level of $\gamma 2$ in heart. As for the mRNA expression level of $\gamma 3$, it was mainly detected in skeletal muscle and heart of the tissues examined; however, there was no expression in liver. The result is in accord with the study in mammalian in which significant expression of $\gamma 3$ mRNA was found only in skeletal muscle (Cheung et al., 2000). Previous work suggested that there were no selective associations among the $\alpha 1$ and $\alpha 2$ subunits; the $\beta 1$ and $\beta 2$ subunits; and the $\gamma 1$, $\gamma 2$, or $\gamma 3$ subunits (Thornton et al., 1998). Therefore, the catalytic subunits, *AMPKα1* and *AMPKα2*, in liver were selected for further analysis to attempt to determine if they had separate functions in the regulation of lipid metabolism in *T. s. elegans* under salinity stress.

Salinity exposures were found to significantly alter the gene expression levels of *AMPKα1* and $\alpha 2$ subunits in a salinity- and time-dependent manner, suggesting that *AMPKα1* and $\alpha 2$ are involved in mediating metabolic responses to salinity stress (Zeng et al., 2016). Indeed, studies on other salt-stressed organisms, such as the Pacific white shrimp *Litopenaeus vannamei*, found that *AMPKα* mRNA levels were upregulated in response to elevated nitrite levels in water (Xu et al., 2016). Interestingly, in the present study, the activity of AMPK was significantly increased by salinity stress and was found to closely resemble the mRNA expression level of *AMPKα1*. Future studies should explore the role of phosphorylation in AMPK activation during salinity stress of turtle by examining the reversible phosphorylation at Thr172 within the activation loop of the α -subunit, as this posttranslational modification is the most potent activator of AMPK (Oakhill et al., 2012).

Lipid Metabolism Activated by AMPK Signaling During Salinity Stress

The activation of AMPK can directly phosphorylate *SREBP-1c* and *ChREBP*, both of which are important lipid-related transcription factors that enter the nucleus and activate the transcription of lipogenic genes in liver (Yamashita et al., 2001). In recent years, *ChREBP* has emerged as an important regulator of glycolytic and lipogenic genes by binding to glucose-responsive DNA elements, termed carbohydrate response elements (*ChRE*), in the promoter regions of selected genes (Rufo et al., 2001). In our study, the gene expression of *SREBP-1c* and *ChREBP*

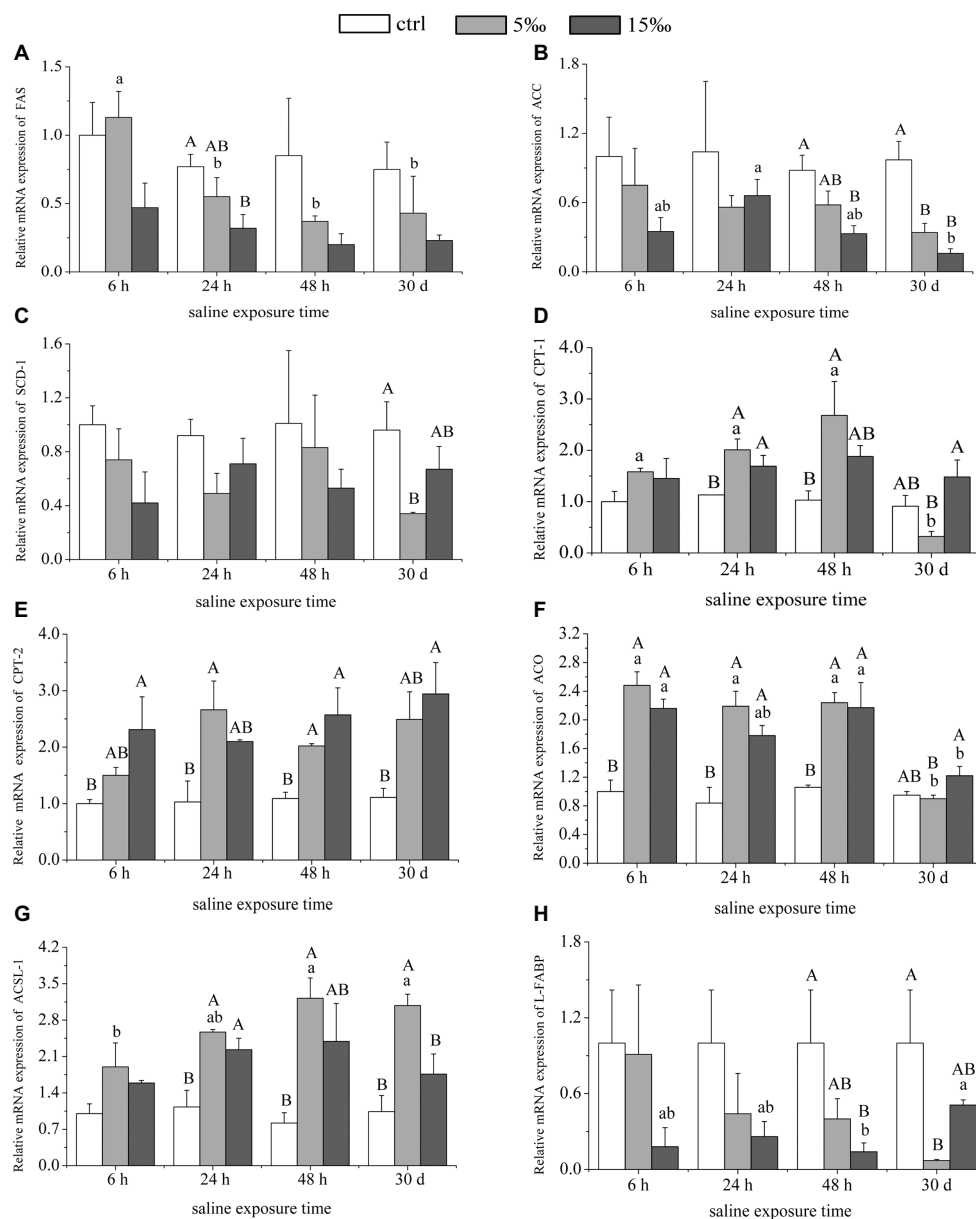


FIGURE 4 | The expressions of lipid metabolism genes in liver. (A–C) mRNA expression levels of genes involved in lipid synthesis: *FAS*, *ACC*, and *SCD-1*.

(D–H) mRNA expression levels of genes involved in lipid oxidation: *CPT-1*, *CPT-2*, *ACO*, *ACSL-1*, and *L-FABP*. Different uppercase letters (A, B, and C) represent significant differences between different salinity groups at the same time point, and different lowercase letters (a, b, and c) represent significant differences between different stress times at the same salinity, $p < 0.05$.

shows a general decreasing trend as salinity increased, indicating the potential suppression of the downstream lipogenic genes under its control. Interestingly, the reduced level of *ChREBP* during salinity stress was different from the response turtles during anoxia. Indeed, when exposed to 5 h of acute anoxia, both transcript and protein levels of *ChREBP* increased in liver and kidney of *T. s. elegans* (Krivoruchko and Storey, 2014).

SREBP-1c is also able to induce lipogenic genes through its ability to bind to sterol regulatory elements (SREs) present in gene promoters (Koo et al., 2001). Transgenic mice that over-express *SREBP-1c* in liver exhibited liver steatosis and increased

mRNA of most lipogenic genes (Shimano et al., 1997). With this in mind, the decrease in *SREBP-1c* transcript level with increased salinity suggests that the lipogenic enzyme that it controls would also be reduced by salinity stress. *SREBP-1c*, together with *ChREBP*, preferentially regulates the lipogenic process by activating genes involved in fatty acid and triglyceride synthesis such as acetyl-CoA carboxylase (*ACC*), fatty acid synthase (*FAS*), and stearoyl CoA desaturase (*SCD*; Dentin et al., 2005; Xu et al., 2013). In our study, the transcript levels of *ACC*, *FAS*, and *SCD-1* were significantly decreased, along with their enzyme activities, suggesting that salinity stress is playing a role to reduce lipogenesis

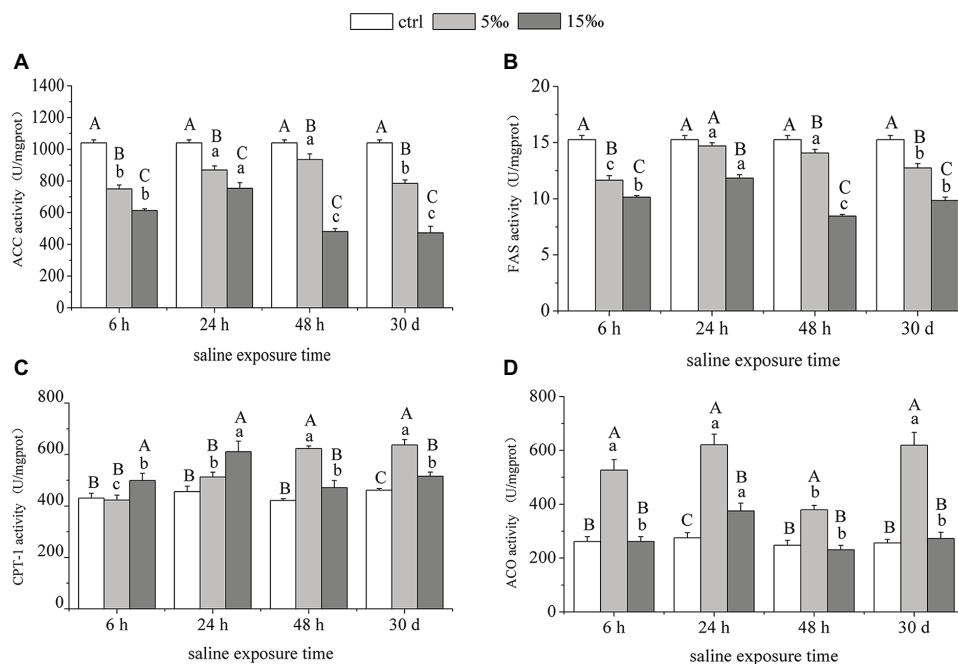


FIGURE 5 | Enzymatic activities of lipid metabolism-related enzymes in liver under salinity stress. **(A,B)** ACC and FAS. **(C,D)** CPT-1 and ACO. Different uppercase letters (A, B, and C) represent significant differences between different salinity groups at the same time point, and different lowercase letters (a, b, and c) represent significant differences between different stress times at the same salinity, $p < 0.05$.

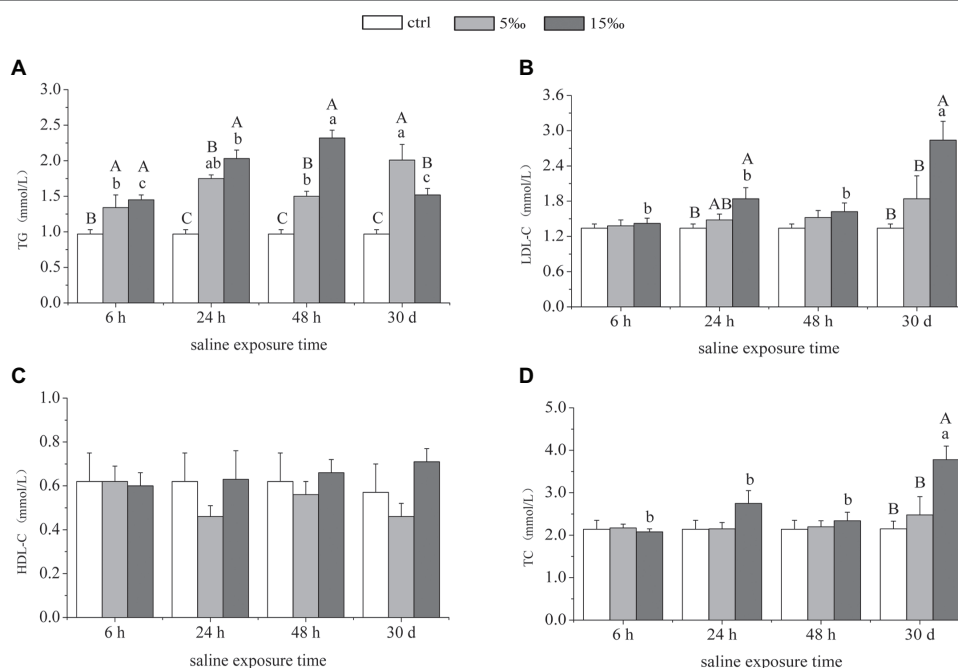
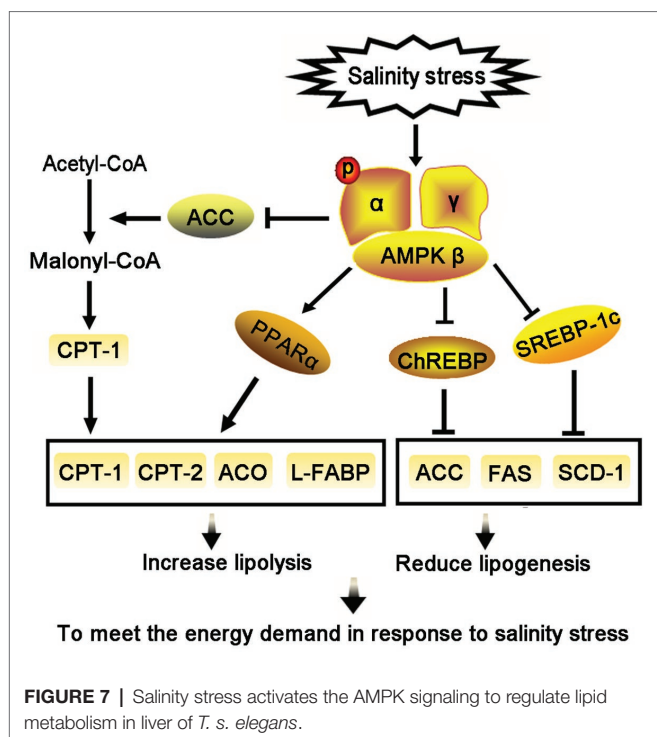


FIGURE 6 | Lipid contents in plasma in response to salinity exposure in *T. s. elegans*. Levels of TG **(A)**, LDL-C **(B)**, HDL-C **(C)**, and TC **(D)**. Different uppercase letters (A, B, and C) represent significant difference between different salinity groups at the same time, and different lowercase letters (a, b, and c) represent significant difference between different stress time at the same salinity, $p < 0.05$.

in the liver of *T. s. elegans*. ACC is a well-defined substrate of AMPK, converts acetyl-CoA to malonyl-CoA, and is highly expressed in lipogenic tissues such as liver and adipose where

it regulates fatty acid synthesis (O'Neill et al., 2013). Interestingly, the activity of ACC (more than 1,000 U/mg prot.) in liver of turtle was higher than that of FAS (about 10 U/mg prot.)



in our study, suggesting that ACC is more stress-responsive in hepatic lipogenesis and subject to inhibition (or degradation) in *T. s. elegans* in response to salinity stress. Cytosolic ACC1 is the rate-limiting enzyme in fatty acid synthesis, whereas ACC2 co-localizes with mitochondria and its product, and malonyl-CoA serves to inhibit carnitine palmitoyl transferase (CPT1), an outer mitochondrial membrane protein that catalyzes fatty acid transport into mitochondria for oxidation (Hardie and Pan, 2002). Therefore, the increased expression of CPT1 during increased salinity was in accordance with the reduced level of ACC.

PPAR α is a major regulator of genes involved in fatty acid oxidation in liver mitochondria and peroxisomes (Kohjima et al., 2007; Monsalve et al., 2013). It regulates lipid oxidation enzymes in liver, such as CPT-1, CPT-2, ACO, ACSL-1, and liver fatty acid-binding protein (L-FABP; Kersten et al., 2000). The activated AMPK likely facilitated the observed increase of PPAR α transcript level with increased salinity stress (Joly et al., 2010). Furthermore, the downstream lipid oxidation genes under PPAR α regulation, CPT-1, CPT-2, ACO, and ACSL-1, were also significantly upregulated in liver of salinity-stressed turtles. Accordingly, the CPT activity in liver increased as salinity increased, whereas ACO activity peaked at S5 group, suggesting that the lower salinity stress was able to stimulate lipid oxidation, and ACO activity is not as involved in providing energy at higher salinity.

With exposed time extended, the expression of ChREBP was highest in S5 group at 48 h, and SREBP-1c was highest in S5 group at 24 h, while there was no significance between other groups. As for PPAR α , the expression level was highest in S5 group at 48 h, and the same trend existed in S15 group. Also, there was no significance in the gene expressions of SCD-1 and CPT-2 between different exposed times, which indicated that *T. s. elegans* could adapt itself into salinity stress to some extent

after longer exposure. However, some gene expressions and enzyme activities such as CPT-1 decreased with exposure time extended.

Content of Triglyceride, Low-Density Lipoprotein Cholesterol, High-Density Lipoprotein Cholesterol, and Total Cholesterol in Plasma During Salinity Stress

The activation of compensatory acclimation mechanisms *via* a substantial energetic reorganization over a relatively short period of time has been reported in other organisms such as euryhaline fish that experience osmotic stress (Kültz, 2015). Herein, several blood parameters were evaluated to analyze the potential effects of hyperosmotic stress on lipid-related products in blood. The levels of TG in plasma increased as salinity increased, which is consistent with increased levels plasma TG found in sea bream (*Sparus auratus*) after long-term seawater acclimation (AasHansen et al., 2005). Increase in TG level suggests an enhanced capacity for oxidizing lipids in the species. However, it should be noted that studies have found that plasma TG levels in sea bream and Arctic char (*S. alpinus*) did not change during 96-h acclimation to seawater, suggesting that lipid metabolism was unchanged (Nordgarden et al., 2002; Susana et al., 2005; Bystriansky et al., 2007). These inconsistencies between species may be partially due to acclimation salinities, exposure durations, and other experimental conditions.

By contrast, the plasma levels of LDL-C, HDL-C, and TC in S5 group did not change significantly, relative to control, which suggested that *T. s. elegans* could adapt to salinity under 5‰ while maintaining crucial blood parameters at homeostatic levels. However, the content of LDL-C and TC in S15 group increased significantly after 30 days of chronic, long-term salinity stress, potentially indicating that the energy balance of the organism was being affected. Furthermore, excessive LDL-C and TC in plasma has been shown to lead to endoplasmic reticulum stress (Jiang et al., 2015), and so when the environmental salinity increased up to 15‰, the salinity adaptation of *T. s. elegans* was challenged.

CONCLUSION

In our study, AMPK activity increased dramatically when *T. s. elegans* was subjected to increased ambient salinity, along with AMPK α mRNA levels. Salinity-activated AMPK signaling was shown to potentially inhibit the expression of SREBP-1c and ChREBP transcription factors, which led to a reduction in mRNA transcripts and enzymatic activities of their downstream targets, ACC and FAS. However, AMPK activation was found to promote the expression of PPAR α that could, in turn, mediate upregulation of the expression of its target genes CPT and ACO. The AMPK signaling networks examined herein indicated an inhibition of lipogenesis and a promotion of lipid oxidation to meet the energy demand in response to salinity stress (Figure 7). The AMPK-mediated regulation of lipid metabolism in response to salinity sets the stage for future work on other molecular mechanisms involved in salinity tolerance and survival in *T. s. elegans*.

DATA AVAILABILITY

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

ETHICS STATEMENT

All experimental procedures had the prior approval of the Hainan Provincial Ecological Environment Education Center Experimental Animal Ethics Committee and were conducted under standard protocols for the care and use of laboratory animals at Hainan Normal University (No. HNECEE-2014-004).

AUTHOR CONTRIBUTIONS

All authors contributed to the preparation of this manuscript. MH conceived and designed the experiments, wrote the paper,

and reviewed drafts of the paper. NL, JL, WL, and LL were integral in the collection and initial processing of turtle samples used for this study. NL, QL, and RW all contributed to the data collection and analysis in this study. KS and HS reviewed drafts of the paper and approved the final draft. LD contributed reagents, materials and analysis tools, prepared figures and tables, reviewed drafts of the paper, and approved the final draft. All authors have approved this statement and the final article.

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Physiological Responses of Pacific White Shrimp *Litopenaeus vannamei* to Temperature Fluctuation in Low-Salinity Water

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Temperature is a significant environmental factor in aquaculture. To investigate the physiological responses during temperature fluctuation (28~13°C), experimental shrimps (*Litopenaeus vannamei*) were treated with gradual cooling from acclimation temperature (AT, 28°C) to 13°C with a cooling rate of 7.5°C/day and rose back to 28°C at the same rate after 13°C for 24 h. Hepatopancreas histological changes, plasma metabolites concentrations, relative mRNA expression of unfolded protein response (UPR) pathway and apoptosis in hepatopancreas and hemocyte were investigated. The results showed that with the decline of temperature, the number and volume of the secretory cells in hepatopancreas increased significantly, the tubule lumen appeared dilatated, and the epithelial cell layer became thinner. The contents of glucose (Glu) significantly decreased to the minimum value of 13°C for 24 h. The contents of triglyceride (TG), total cholesterol (TC), and total protein (TP) increased and reached the peak of 13°C for 24 h. Alkaline phosphatase (ALP) and alanine aminotransferase (ALT) activities in plasma reached the lowest and highest value in 13°C, respectively. The expressions of all genes related to UPR and apoptosis in the hepatopancreas and hemocytes were significantly changed during the cooling process and reached the highest level of 13 and 13°C for 24 h, respectively. During re-warming stage, the histopathological symptoms got remission and each of the plasma metabolite concentrations and gene expressions returned to AT levels. These results revealed that pacific white shrimp can adapt to a certain level of temperature fluctuation by self-regulation.

Keywords: *Litopenaeus vannamei*, temperature, endoplasmic reticulum stress, apoptosis, self-regulation

INTRODUCTION

The pacific white shrimp *Litopenaeus vannamei*, with a wide range of salt-tolerance, rapid growth, and other characteristics suitable for intensive aquaculture, has become one of the most important aquaculture shrimps in the world. However, a variety of environmental stimuli affect the growth of shrimp, such as changes in pH (Han et al., 2018a), salinity (Li et al., 2008;

Wang et al., 2016), dissolved oxygen (DO) (Han et al., 2018b), temperature (Madeira et al., 2015), and pollutants like nitrite, ammonia, and sulfide (Duan et al., 2018b).

Since the start of global climate change, various extreme climates have happened frequently. Previous studies have indicated that many extreme weather events which were associated with drastic temperature fluctuation can directly affect the growth, physiological performance, and survival of animals (He et al., 2018; Zhang et al., 2019). It has been studied that temperature changes may lead to growth arrest, stoppage of feeding, and swimming or even death in 13°C (Fan et al., 2013; Huang et al., 2017; Xu et al., 2018). Our previous study has indicated that glucose-regulated protein 78 kDa (GRP78) was significantly up-regulated in the hepatopancreas of *L. vannamei* under 13°C for 24 h cold-stress (Fan et al., 2016). GRP78, also known as immunoglobulin-binding protein (BIP), is a central regulator of endoplasmic reticulum stress (ERS) and regulated the process of unfolded protein response (UPR) and apoptosis (Dejean et al., 2006; Nakka et al., 2010). At present, studies on ERS are mainly focused on mammals and the UPR pathway (Cao and Kaufman, 2012).

UPR is a self-protective mechanism which can promote cell survival in response to ERS. It includes three classical signaling pathways: the activating transcription factor 6 (ATF6) pathway, the inositol-requiring enzyme-1-X-box binding protein 1 (IRE1-XBP1) pathway, and the protein kinase RNA (PKR)-like ER kinase-eIF2 α (PERK-eIF2 α) pathway (Mori, 2009; Costa et al., 2011). In addition, apoptosis signals will be generated if stress is prolonged for protecting the organism by eliminating the damaged cells. Apoptosis signal-regulated kinase 1 (ASK1) is essential for the continuous activation of c-Jun NH2-terminal kinases (JNK) and induces cell apoptosis (Tobiume et al., 2001). Cysteine-containing aspartate-specific proteases (caspases) are a family of proteases that perform apoptosis in animals. Apoptosis mediated by ERS triggers a specific cascade of caspase 12, 9, and 3, and the activation of caspase 3 (CASP3) indicates that apoptosis has entered an irreversible stage (Morishima et al., 2002). In invertebrates, UPR is widely recognized as the key to ER stress response (Chen and He, 2019). However, studies of the UPR signaling pathway and apoptosis in *L. vannamei* mainly focused on the immune function, especially in response to WSSV infection (Chen et al., 2012; Wang et al., 2013; Xu et al., 2014; Yuan et al., 2016, 2017, 2018). UPR in response to temperature fluctuation has not been reported.

Additionally, it has been identified that hepatopancreas histology could be used to monitor the impact of a stressed environment, showing ultrastructural alterations at the early stage of stress (Collins, 2010). Environmental changes like pH stress can cause change or damaged of hepatopancreas cells (Tao et al., 2016). However, it is still not clear about the change of hepatopancreas histology during temperature fluctuation process.

In the present study, based on the statistics of weather conditions from the winter (November, December and January) in Guangdong from 2017 to 2018 (China Meteorological Administration, www.cma.gov.cn), we found that the average daily temperature difference of winter in Guangdong was 7.52°C.

The annual cold wave causes huge economic losses to the *L. vannamei* breeding industry in China. However, little was known about the responses of the shrimp during the process of temperature gradual cooling and warming. Thus, we investigated (1) histological section of the hepatopancreas, (2) metabolite concentrations of plasma, and (3) UPR gene and cell apoptosis gene expressions of hepatopancreas and hemocyte in *L. vannamei* during temperature fluctuations. These results could provide valuable reference to analyze the adaptation mechanism of the shrimp in response to temperature fluctuation.

MATERIALS AND METHODS

Experimental Shrimp and Culture Conditions

The experimental shrimps, with an average weight of 5.4 ± 0.7 g, were obtained from a commercial farm in Panyu (Guangdong, China). The shrimps were immediately transported to the lab and acclimated in 500 L filtered, aerated (oxygen pump, HAP-120, HAILEA, Guangdong, China) seawater tanks (Guanzhong, K500 L, Jiangsu, China) at least 4 days before experiments. During the acclimation stage, the water salinity and temperature in tanks were consistent with that of the culture ponds (salinity 5‰, pH 8.3 ± 0.1 and temperature $28 \pm 1^\circ\text{C}$). Commercial shrimp feeds (Haida Group Feed, Jieyang, China) were given two times per day (5% of shrimp body weight per time).

Treatment

Sixty-three healthy shrimps were randomly divided into three replicate tanks. They were placed in an artificial climate incubator (Laifu, Ningbo, China), and the water temperature was decreased from acclimation temperature (AT, 28°C) to 13°C with a cooling rate of 7.5°C/day (2.5°C/8 h). After 13°C for 24 h, the water temperature rose back to 28°C at the same rate.

Sample Collection

Tissue Slice

At each temperature point [28, 23, 18, 13, and 13°C for 24 h during cooling process, 18 and 28°C during return process (r18 and r28°C)], the whole hepatopancreas of one shrimp from each tank were dissected from the cephalothoraxes and fixed with 4% paraformaldehyde (Biosharp, China) for tissue fixation and then stored in 4°C for paraffin sections by Servicebio (Wuhan, China).

Plasma and Gene Expression Analysis

Hemolymph was extracted from the ventral sinus of shrimp at each temperature point as same as tissue slice, using a 1 ml sterile syringe containing an equal volume of ice-cold anticoagulant solution (27 mM trisodium citrate, 385 mM sodium chloride, 115 mM glucose, pH 7.5). Hemolymph of two shrimps from each tank was mixed as one sample, three repeats. After being centrifuged at 3000 rpm (844g) for 10 min

in 4°C, the supernatant fluid was immediately stored in −80°C for analysis of plasma metabolite concentrations. The pelleted hemocytes were collected, instantly frozen in liquid nitrogen and then stored at −80°C for analysis of gene expression (Xu et al., 2018). After hemolymph sampling, hepatopancreases were dissected, frozen in liquid nitrogen, and stored in −80°C for gene expression analysis.

RNA Extraction and cDNA Synthesis

Total RNA was extracted from hemocytes and hepatopancreases using RNAiso Plus reagent (TaKaRa, Japan) following the manufacturer's protocol. RNA quality was assessed by electrophoresis on 1.0% agarose gel, and concentration was tested by mySPEC (VWR, USA). Total RNA was purified, and first-strand cDNA was synthesized using ReverTra Ace® qPCR RT Master Mix with gDNA Remover (TOYOBO, Shanghai) according to the manufacturer's instructions.

Real-Time Quantitative PCR

The SYBR Green real-time Polymerase Chain Reaction (PCR) assays were carried out on a CFX Connect™ Real-Time System (Bio-Rad) using THUNDERBIRD® SYBR® qPCR Mix (TOYOBO). Previous studies showed that the expressions of β -actin were constant after environmental stimuli such as ammonia (Duan et al., 2018a), dissolved oxygen (Han et al., 2018b), and pH stress (Han et al., 2018a). Therefore, we used β -actin as the housekeeping gene, and specific primer sequences were designed based on the coding sequence of the target genes using Primer Premier 6.0 software (Table 1). The real-time PCR program was 95°C for 1 min, followed by 40 cycles of 95°C for 15 s, 60°C for 15 s, and 72°C for 45 s, followed by 1 step of 95°C for 10 s. Melting curves were obtained by increasing the temperature from 65 to 95°C (0.5°C/s) to denature the double-stranded DNA. The relative mRNA expressions were calculated by the comparative Ct method ($2^{-\Delta\Delta C_t}$).

Statistical Analyses

All the data were presented as mean $\pm$ SD of triplicates. Data were statistically analyzed by SPSS 19.0 with one-way ANOVA and Tukey test. $p < 0.05$ was significant difference.

RESULTS

Hepatopancreas Histological Analysis

According to the results of hepatopancreas with HE staining, the hepatocytes in 28°C exhibited the well-organized tubules. With the decrease of temperature, stellate tubule lumen appeared dilatation, and some vacuoles appeared and ruptured to make the epithelial cell layer thinner. The secretory cells ("blasenzellen", B-cells), which are the main site for synthesis of digestive enzymes, typically contain a single large secretory vesicle. The number and volume of B-cells significantly increased during the cooling process. All these symptoms got remission during the temperature return process (Figure 1).

Plasma Analysis

Analysis of plasma metabolite concentrations is shown in Figure 2. Compared to the AT group, the contents of glucose (Glu) decreased to the minimum value (50.78 mmol/L) at 13°C for 24 h. After temperature rose back to 28°C, it recovered to the level nearly of AT ($r_{28^\circ\text{C}} = 62.82$ mmol/L, AT = 64.81 mmol/L) (Figure 2A). The contents of triglyceride (TG) and total cholesterol (TC) decreased after cooling and reached the lowest (TG = 0.25 mmol/L, TC = 0.39 mmol/L) at 23°C, and then increased gradually and reached the peak of 0.49 mmol/L and 1.12 mmol/L, respectively, in 13°C for 24 h (Figure 2B), while the change of TG contents was not statistically different with the AT group. Changes of total protein (TP) content during the cooling process were similar to the TC and TG. During the return process, the content

TABLE 1 | The real-time PCR primers used in this study.

Primer names	Nucleotide sequences (5'–3')	Protein ID
LvGRP78-F	TCATTGCCAACGACCGGGT	AFQ62791.1
LvGRP78-R	TCCGATGAGACGCTTGGCAT	
LvPERK-F	TCCTGACATCATATTATCATCTCC	XP_027239142.1
LvPERK-R	TGAAGCTCATGCTCTCTGCCAATCC	
Lvelf2 α -F	GGAACCTGTCTGTTGTCATCAGAGTAG	AGI97278.1
Lvelf2 α -R	AGAAGCTCTCCAACATGCCGAATG	
LvATF4-F	GCCACGATTCAAGATGCTGC	AGI97279.1
LvATF4-R	TCCTCCTCGTCCATGCCATA	
LvATF6-F	CTGTTGGGACAGGACCATAAGC	AYM00394.1
LvATF6-R	GAATTGTAGGTGTGGCAGCTGTTA	
LvIRE1-F	TGGTGAGAAGCAGCTTGTGTTGG	AFQ62792.1
LvIRE1-R	ACTGTTGATGAAGAGCCACTTGTAGC	
LvXBP1-F	GTGGATCAGCAGTATCCCAACC	AFQ62793.1
LvXBP1-R	TGCCAAGGCAGCTGTATTGA	
LvCasp3-F	ACATTCTGGGCGGAACACC	AGL61582.1
LvCasp3-R	GTGACACCCGTGCTTGTACA	
LvASK1-F	GCTGTGTTGAAGTCCGAGGAGAAG	AKI88007.1
LvASK1-R	AGCCAAGCAACCAACTCCATATCG	
LvActin-F	GACTACCTGATGAAGATCC	AAG16253.1
LvActin-R	TCGTTGCCGATGGTGATCA	

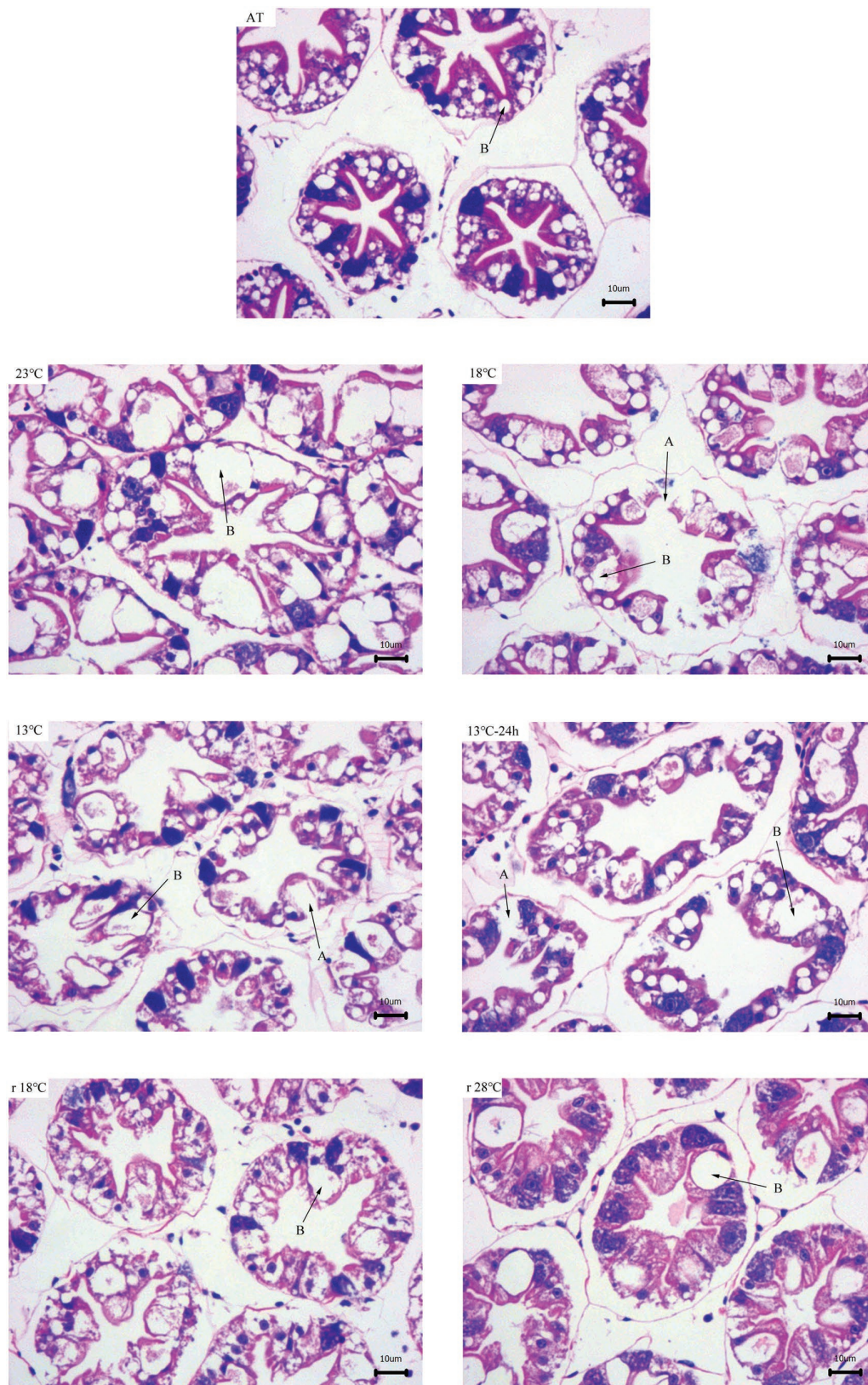


FIGURE 1 | Hepatopancreas tissue structure ($\times 400$) of *L. vannamei* with HE dying during temperature fluctuation. AT represents acclimation temperature (28°C), and r18 and r28°C represent 18 and 28°C in temperature return process, respectively. The letters in the figure indicate: A, ruptured cells; B, B-cell.

Plasma

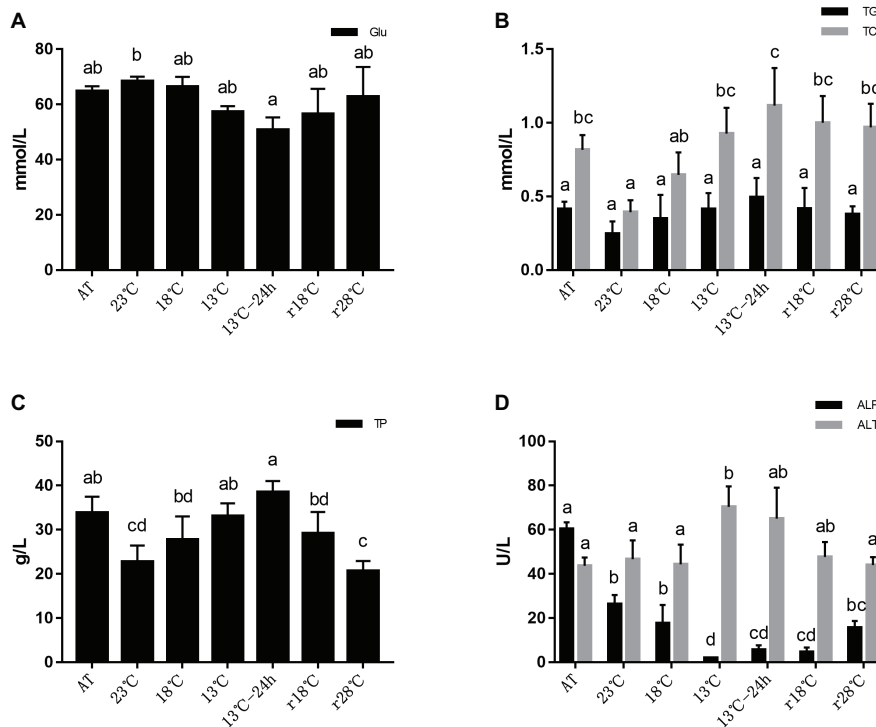


FIGURE 2 | Contents of glucose (Glu, **A**), triglyceride (TG) and total cholesterol (TC) (**B**), total protein (TP, **C**) and alkaline phosphatase (ALP) and alanine aminotransferase (ALT) (**D**) activities in plasma of *L. vannamei* during temperature fluctuation. AT represents acclimation temperature (28°C), r18 and r28°C represent 18 and 28°C in temperature return process, respectively. The bars represent the mean $\pm$ S.D. ($n = 3$). Statistical significance was calculated by one-way ANOVA. Bars with different letters indicate statistical differences ($p < 0.05$).

of TP reduced to 20.67 g/L in r28°C (Figure 2C). Alkaline phosphatase (ALP) activities in plasma decreased significantly after cooling and reached the lowest (2 U/L) at 13°C. After temperature rose back to 28°C, ALP activities increased to 15.67 U/L, which is near the activities at 18°C (17.67 U/L) in the cooling process. Alanine aminotransferase (ALT) activities remained stable from AT to 18°C and then significantly increased and reached the highest level (70.33 U/L) at 13°C. During the return process, ALT activities decreased to the level near AT (43.67 U/L) (Figure 2D).

Unfolded Protein Response and Related Apoptosis Gene Expression to Temperature Fluctuation in the Hepatopancreas

In the hepatopancreas, the relative expressions of GRP78 increased significantly at 18°C and reached the peak at 13°C, which is about fourfold that at AT. During the next 24 h maintaining in 13°C and the return process, GRP78 expressions significantly decreased and they were near to the level of AT in r28°C (Figure 3A). Expressions of apoptosis related genes including CASP3 and ASK1 showed the same trend with the PERK sub-pathway and their highest expression levels appeared in 13°C (Figure 3B). In UPR, expressions of ATF6 showed the same trend found in GRP78 (Figure 3C). In the IRE1

sub-pathway, expressions of IRE1 and XBP1 reached the peak at 18°C (Figure 3D). In the PERK sub-pathway, expressions of PERK, eIF2 α , and ATF4 increased gradually during the cooling process, and the highest expressions (16.67, 9.74, and 6.21 folds compared with that at AT, respectively) appeared at 13°C and then decreased significantly. There was no obvious difference among the expressions of eIF2 α and ATF4 between r28°C and AT (Figure 3E).

Unfolded Protein Response and Related Apoptosis Gene Expression to Temperature Fluctuation in Hemocyte

In the hemocyte, the expression level of GRP78 remained stable from AT to 13°C and then significantly increased in 13°C for 24 h, which is more than twofold the level in AT. After temperature rose back, expressions of GRP78 were approximate to the level of AT in r28°C (Figure 4A). For genes related to apoptosis, expressions of CASP3 and ASK1 showed the same trend with the PERK sub-pathway (Figure 4B). In UPR, the expressions of ATF6 decreased significantly after cooling in 23°C and then increased gradually and reached the highest level in 13°C for 24 h compared with the expression in AT. After temperature rose back, it returned to nearly the level of AT (Figure 4C). In the IRE1 sub-pathway, IRE1 and XBP1 showed a similar trend as ATF6 (Figure 4D).

Hepatopancreas

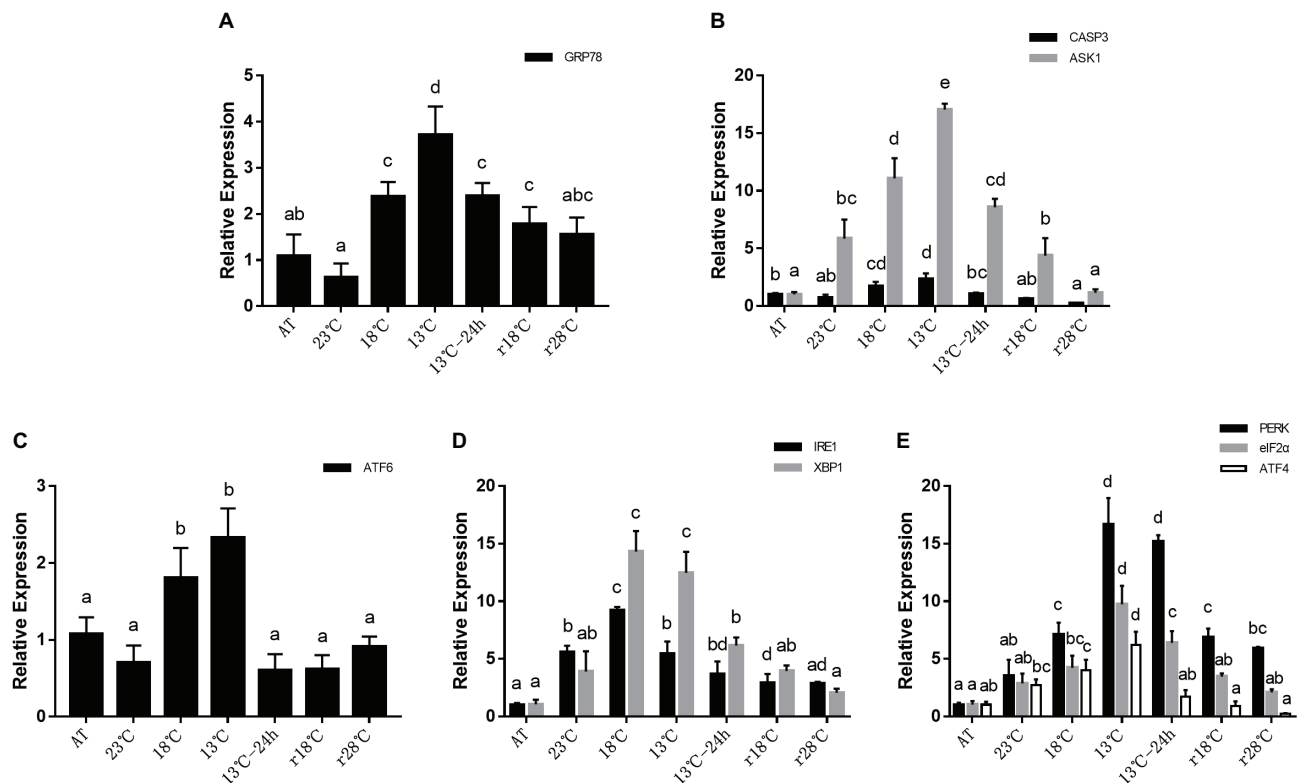


FIGURE 3 | Relative expression of UPR and apoptosis related genes in hepatopancreas during temperature fluctuation. Water temperature was changed from 28 to 13°C with a cooling rate of 7.5°C/day. After 13°C for 24 h, the water temperature rose back to 28°C at the same rate. AT represents acclimation temperature (28°C), r18 and r28°C represent 18 and 28°C in temperature return process, respectively. The relative mRNA expression levels of GRP78 (A), apoptosis related genes (CASP3, ASK1) (B), ATF6 pathway (ATF6) (C), IRE1 pathway (IRE1, XBP1) (D), and PERK pathway (PERK, eIF2α, ATF4) (E) were compared with those at AT. The bars represent the mean ± S.D. ($n = 3$). Statistical significance was calculated by one-way ANOVA. Bars with different letters indicate statistical differences ($p < 0.05$).

In the PERK sub-pathway, expressions of PERK, eIF2α, and ATF4 increased gradually after cooling, reaching at 13°C for 24 h approximately seven-, four-, and twofold, respectively, of the levels found in AT, but after temperature rose back the expressions decreased (Figure 4E).

DISCUSSION

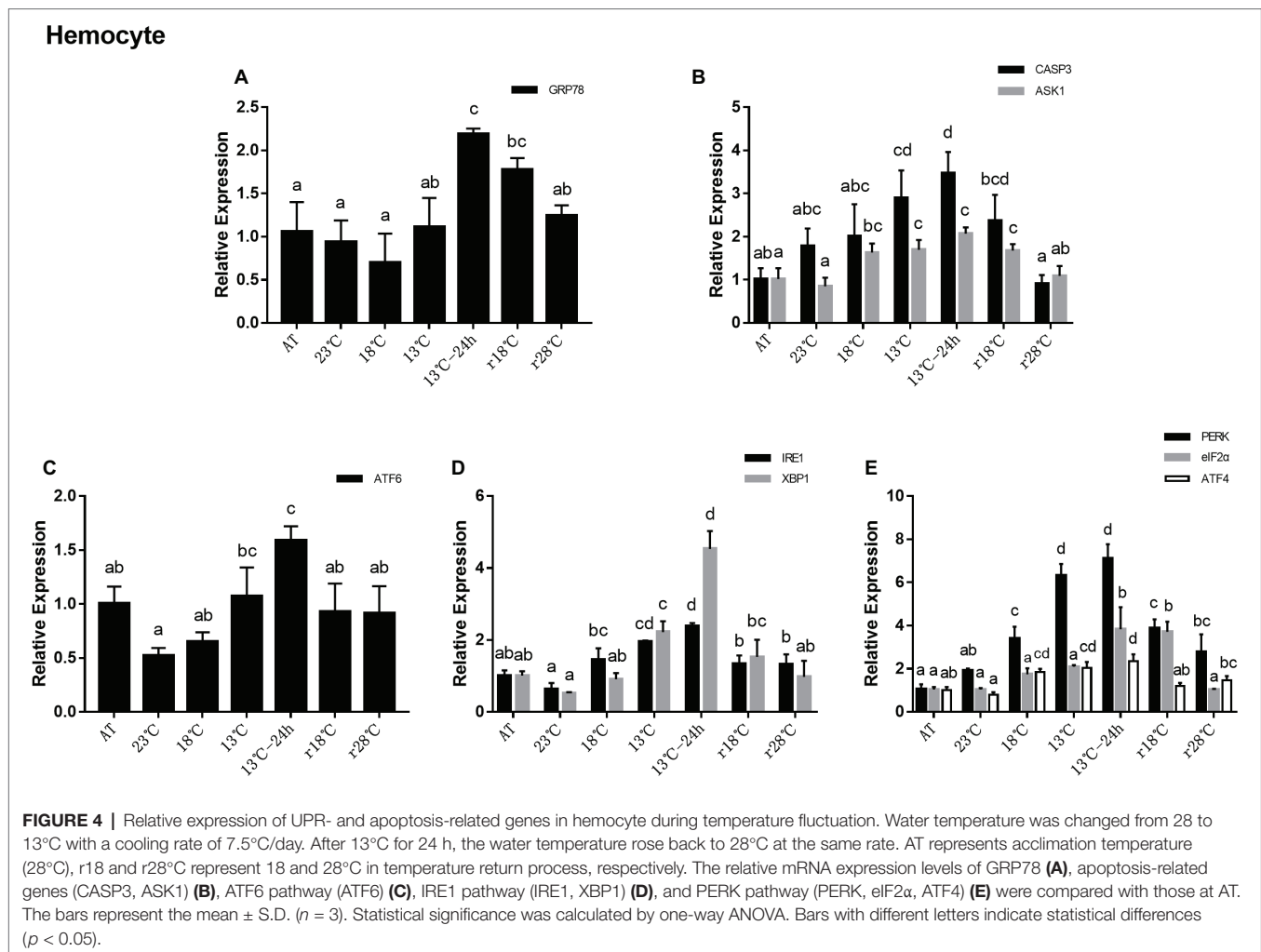
Global climate change is impacting marine and estuarine aquaculture. It is generally known that coastal marine systems are some of the most important ecologically and socio-economically on the planet. Temperature, as one of the interacting climatic variables, will drive future ecological changes in marine systems. Understanding how temperature change will affect the aquatic organisms is a key issue worldwide. *L. vannamei*, mainly distributed in the sea of Ecuador and introduced to China in 1988, is one of the most important aquaculture shrimps in the world. As its origins are tropical, temperature fluctuation is a serious challenge to its survival, growth, and distribution (Peng et al., 2015; Cottin et al., 2016).

In this study, the hepatopancreas histological changes, plasma metabolites concentrations, and relative mRNA expression in the UPR signaling pathway and apoptosis genes induced by ERS in *L. vannamei* during cold stress were studied.

Hepatopancreas Histological Change During Temperature Fluctuation

The hepatopancreas as a vital organ of crustaceans is involved in excretion, molting, diverse metabolic activities, and storage of energy reserves (Yepiz-Plascencia et al., 2000; Verri et al., 2001). In this study, the number and volume of B-cells in hepatopancreas tubules was significantly increased after suffering cold stress. This may be related to the fact that B-cells are the main site of absorption and digestion of nutrients (Almohanna and Nott, 1989; Wang et al., 2016). We suspected that the high rate of synthesis and release of digestive enzymes in B-cells accelerated the mobilization of nutrients in hepatopancreas tubules, by which shrimp can adapt to environmental stress.

The hepatopancreas of shrimp has high self-repairing ability. *L. vannamei* can repair hepatopancreas injury after long-term



exposure to low zinc (Jui-Pin et al., 2008) and low pH (Han et al., 2018a). The hepatopancreas weight of *L. vannamei* significantly declined after fasting, but then increased immediately after re-feeding (Pascual et al., 2006; Sanchez-Paz et al., 2007). In the present study, histological damage of the hepatopancreas got remission after temperature return, confirming the shrimp's self-repair ability.

Plasma Metabolite Concentrations Change During Temperature Fluctuation

It has been widely accepted that protein acts as the main energy source for shrimp (New, 1976; Zhang et al., 2006; Cuzon et al., 2010). Research has shown that lipids are the main energy source of tilapia (*Oreochromis niloticus*) during long-time hypoxia stress (Li et al., 2018). In this study, the results showed that lipids (TC, the major components of lipids, supply and store energy) and protein (TP provides energy and transports various metabolites) in plasma responded more rapidly to temperature fluctuation, while Glu remained stable before 13°C and recovered to AT levels after temperature rose back to 28°C. It has been reported that the hepatopancreas is typically high in lipids and appears to be the main site for gluconeogenesis

in decapod crustaceans (Hervant et al., 1999; Vinagre and Silva, 2011; Reyes-Ramos et al., 2018; Berry et al., 2019). Thus, combined with hepatopancreas histology and plasma results, we deduced that the increase of B-cells facilitates the gluconeogenesis to synthesize glucose from protein and lipid, by which shrimps maintain glucose demand under cold stress. However, after temperature dropped to 13°C, the rupture of hepatopancreas tubules causes lipids and proteins to enter hemolymph, resulting in an increase of lipid and protein content in plasma. The glucose content decreased at the same time due to the damage of the hepatopancreas.

It is known to all that nonspecific immunity plays an important role in the immune defense of aquatic animals. *L. vannamei* depends entirely on cellular and humoral immunity to prevent external injury (Iwanaga and Lee, 2005). ALP is directly involved in the transfer and metabolism of phosphoric acid groups in organisms and plays a significant role in the immune system against pathogens. The present study showed that ALP played a major role during the cold stress response in *Sparus aurata* and *L. vannamei*, and this is probably because ALP can help protect the hepatopancreas and hemolymph from cold-stress damage (Mateus et al., 2017; Peng et al., 2018).

The activity of ALT in plasma can reflect the damage of the hepatopancreas (Jiang et al., 2014; Yan et al., 2016). It has been shown that various forms of stress can cause an increase in plasma ALT activity in fish (Cho et al., 1994), and it is responsive to temperature change in fish (Costas et al., 2012). In the present study, the ALP activity decreased to the lowest level, and the ALT activity increased to the highest level at 13°C, indicating that the shrimp was damaged at this temperature. However, there was no obvious change in the next 24 h when the temperature was kept at 13°C, but it increased significantly after the temperature rose back to 28°C. Thus, we deduced that shrimp has the ability to adapt low-temperature stress to a certain extent, and these results were consistent with those found in the hepatopancreas histological analysis.

Differential Gene Expression About Unfolded Protein Response Pathway and Apoptosis During Temperature Fluctuation

UPR is a feedback regulatory system, capable of controlling the elimination of misfolded proteins in the ER, thereby maintaining the homeostasis of the endoplasmic reticulum. Appropriate ERS can activate UPR to improve the ER function and protect cells. But if the imbalance exceeds its regulating ability, it will lead to apoptosis. In invertebrates, apoptosis is also an effector factor of immune response. Environmental stresses such as temperature stimulation, pH changes, and toxic substances can induce apoptosis. In this study, the relative mRNA expressions of all UPR pathway- and apoptosis-related genes in the hepatopancreas and hemocytes were significantly changed during the cooling and re-warming process, which indicated that the UPR pathway and apoptosis participated in this process.

Previous studies indicated that shrimp can adapt to the environmental changes by self-regulation to a certain degree. In these studies, it was observed that the glutamate-oxalacetate transaminase and glutamate-pyruvate transaminase activities increased after shrimp were exposed to Zn for 7 days but declined after exposure for 14 and 28 days (Jui-Pin et al., 2008). Additionally, the lipid peroxidation levels in shrimp had no significant changes between 10 and 15 days after Cd exposure (Chiodi Boudet et al., 2015). In our study, the expressions of genes (GRP78, ATF6, IRE1, XBP1, PERK, eIF2 α , and ATF4) in the hepatopancreas reached their highest level at 13°C instead of 13°C for 24 h. The plasma metabolites concentration analysis also showed that ALT activity got its highest point at 13°C, and the activity of ALT in plasma is inversely proportional to the health of hepatopancreas. This finding is consistent with previous studies and confirms the self-repair ability of shrimp. In addition, all these related gene

expressions reached their highest level in 13°C in hepatopancreas, while in hemocytes their peak appeared in 13°C for 24 h. Thus, we deduced that the shrimp response to temperature fluctuation in the hepatopancreas may be relatively rapid compared to that in hemolymph.

CONCLUSIONS

In this study, protein and lipid were observed to be the main energy source of *L. vannamei* during temperature fluctuation. All the three UPR pathways were involved in temperature fluctuation process, and their responses in the hepatopancreas were relatively rapid compared to that in hemolymph. All the results suggest that *L. vannamei* can adapt to a certain level of temperature fluctuation by self-regulation. However, the detailed adaptation mechanism in *L. vannamei* still needs further study.

DATA AVAILABILITY

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

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the guidelines for animal care and use for scientific research in China. The protocol was approved by the Ministry of Agriculture and Rural Affairs of the China.

AUTHOR CONTRIBUTIONS

ZW performed the study, analyzed the results, and drafted the manuscript. YQ and JL coordinated the study. MY, JZ and LF set the experimental design. All authors reviewed and approved the final manuscript.

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Characterization of Gut Microbiome in the Mussel *Mytilus galloprovincialis* in Response to Thermal Stress

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The gut microbiota is essential for utilization of energy and nutrition and may have a role in host immunity in response to environmental shifts. The present study evaluated the temperature stress (increasing from 21 to 27°C) on gut microbiome and dynamics of the mussel *Mytilus galloprovincialis* by 16S rRNA gene sequencing with the aim of discovering the gut microbiome resilience to warming. Exposure to high temperature of 27°C significantly reduced the survival of *M. galloprovincialis* associated with increased microbial diversity of gut. The microbial communities were shifted with elevated temperature (from 21 to 27°C) and different exposure time (from day 0 to day 7) by principal coordinate analysis (PCoA). Linear discriminant analysis effect size (LEfSe) revealed that the relative abundance of *Vibrio* and *Arcobacter* presented in live animals as the top genus-level biomarkers during the initial exposure to 27°C and followed by microbiomes fluctuation with increasing exposure time at day 4 and day 7. The proliferation of opportunistic pathogens such as genus *Vibrio* and *Arcobacter* might increase host susceptibility to disease and contributed greatly to mortality. The results obtained in this study provide the knowledge on ecological adaptation for south domestication of *M. galloprovincialis* and host–bacteria interaction during temperature stress (27°C).

Keywords: gut microbiome, thermal stress, physiological response, Illumina HiSeq sequencing, *Mytilus galloprovincialis*

INTRODUCTION

Global climate change has profound effects on marine ecosystem by altering the physiological functioning and behavior of marine organisms (Brierley and Kingsford, 2009). Variation in temperature is affecting the metabolic and respiratory rates of ectothermic animals. Presumably low stress-tolerance organisms are susceptible to disease outbreaks, which are associated with decline of population (Harvell et al., 2002; Doney et al., 2012). Although a shift of biogeographic distribution has occurred in mobile marine organisms (Beaugrand et al., 2002), the sedentary organisms such as mussel and oyster are more prone to mass mortality in summer (Myrand et al., 2000; Malham et al., 2009).

The mussel *Mytilus galloprovincialis* is an important commercial aquaculture species and mainly cultured in China and Europe (Lazo and Pita, 2012). *M. galloprovincialis* is widely distributed along the northern shores of Liaodong and Shandong peninsulas in north of China and the seawater temperature reaches to 25°C in these habitats during summer season (Xiao et al., 2005; Lin et al., 2011). Recently, *M. galloprovincialis* has been cultured in the coast of Zhoushan (Zhejiang Province, China), where is a natural habitat of the mussel *M. coruscus* (Wang et al., 2012; Yang et al., 2014). The average of surface seawater temperature was 27°C during the summer season in Zhoushan (Li et al., 2018) and it might result in the reduction of fitness and increment of mortality.

The gut of marine animals shelter species-rich microbial communities, which provides biological function for the host such as competition with pathogens and disease resistance (Gatesoupe, 1999). Previous evidences showed that exposure to high water temperature of 31°C altered the *M. coruscus* gut microbiome by facilitating the proliferation of opportunistic bacteria in mussel adults (Li et al., 2018). Therefore, the disturbance of gut microbiota may reflect the healthy status of the host. To determine physiological responses of *M. galloprovincialis* acclimating to seawater temperature increasing from 21 to 27°C, the effects of temperature stress (27°C) on *M. galloprovincialis* gut microbiota using Illumina HiSeq sequencing of 16S rRNA gene was analyzed. The change of microbial community was determined in response to temperature stress (27°C). The knowledge obtained in this study will provide the information for south domestication and mariculture of *M. galloprovincialis* in changing environment.

MATERIALS AND METHODS

Ethics Statement

The experimental protocol for mussel acclimation and experimentation was approved by the Animal Ethics committee of Shanghai Ocean University, Shanghai, China.

Biological Material

Wild population of juvenile *M. galloprovincialis* (1.8 ± 0.3 cm in shell length and 1.4 ± 0.3 cm in shell width) were collected at Lvshunkou district (38°45' N, 121°13' E), Dalian, Liaoning Province, China, in October 2018. The mussels were reared in 10 L polycarbonate tanks (30 mussels/tank) containing seawater (salinity: 30) with aeration at 21°C, which was the average seawater temperature in mussel collection site. The seawater was renewed daily. The algae *Platymonas helgolandica* var. *tsingtaoensis* and *Isochrysis zhanjiangensis* were supplied to the mussel as food source as previous described (Li et al., 2018). Mussels were acclimated for 1 week before the start of experiment at 21°C.

Experimental Design and Gut Sampling

Mussels were exposed for 7 days to determine the effects of temperature stress on the gut microbiota of the mussel *M. galloprovincialis*. The mussels ($n = 300$) were kept in triplicate

15-L polycarbonate tanks (0.12 m^2) at $21 \pm 1^\circ\text{C}$ (control) or $27 \pm 1^\circ\text{C}$ (treatment) and fed daily with *I. zhanjiangensis* or *P. helgolandica* (algal cell density of 8×10^4 cells/mL). For treatment group at 27°C, an increase of temperature from 21 to 27°C was performed by gradually increased 1°C/day to minimize heat shock (Webster et al., 2011). The mussels were sampled at 0, 4, and 7 days in control and treatment groups (20 individuals per replicate tank).

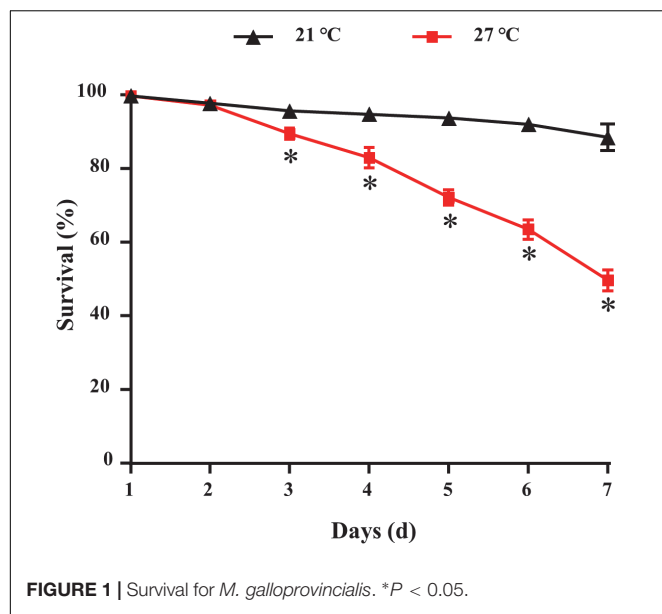
The seawater was renewed every day in each treatment groups. The tanks were frequently checked for removing the dead mussels when identified and the cumulative number of deaths were calculated on a 24 h basis. The gut samples from live mussels were obtained by dissecting mussels with tweezer and scissor. Each gut sample was collected from 10 individual juvenile mussels and stored at -80°C prior to bacterial DNA extraction.

Bacterial DNA Extraction and PCR Amplification

Total DNA was isolated from the stored gut samples ($n = 3$) using a MOBIO PowerSoil® DNA Isolation Kit (MOBIO Laboratories, Carlsbad, CA, United States) and following the protocol provided by manufacturer. The DNA concentration and purity were checked using the NanoDrop One (Thermo Fisher Scientific, Waltham, MA, United States) and followed by the amplification of the 16S ribosomal RNA gene (V3–V4 region) using universal bacterial primers, 338F (5'-ACTCCTACGGGAGGCAGCA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') tagged with 12 bp barcode. PCR reactions, containing 25 µL 2× Premix Taq (Takara Biotechnology, Dalian, Co., Ltd., China), 1 µL each primer (10 mM) and 3 µL DNA (20 ng/µL) template in a volume of 50 µL, were amplified by thermocycling: 5 min at 94°C for initialization; 30 cycles of 30 s denaturation at 94°C, 30 s annealing at 52°C, and 30 s extension at 72°C; followed by 10 min final elongation at 72°C.

Illumina HiSeq Sequencing

The PCR products were examined on 1% agarose gel electrophoresis. PCR products were mixed in equidensity ratios using the GeneTools analysis software (Version4.03.05.0, SynGene) prior to the purification of the mixture PCR products using E.Z.N.A. gel extraction kit (Omega, United States). Sequencing libraries of each group were generated using NEBNext® Ultra™ DNA Library Prep Kit for Illumina® (New England Biolabs, Ipswich, MA, United States) following manufacturer's instructions. The quality of sequencing libraries was checked on the Qubit® 2.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA, United States) and Agilent Bioanalyzer 2100 system (Agilent Technologies, Waldbronn, Germany). Sequencing was performed on an Illumina HiSeq 2500 platform and 250 bp paired-end reads were generated (Guangdong Magigene Biotechnology, Co., Ltd., Guangzhou, China). Raw sequences have been submitted to the NCBI sequence read archive database under the accession number: SRP197453.



Statistical and Bioinformatics Analysis

The statistical analyses of microbial diversity indices (Chao1, Shannon, and Simpson) were performed by

Wilcoxon/Kruskal–Wallis test after tested for normality (Shapiro–Wilk test) and homogeneity (O’Brien test) using JMP™ software (SAS Institute, Shanghai, China) and P -value < 0.05 was considered significantly different.

Trimmomatic (V0.33¹) was used to remove the low quality sequences, reads with N and sequence < 100 bp long of the raw reads. FLASH (V1.2.11²) was used to assemble the filtered sequences followed by removing barcodes and primers by Mothur software (V1.35.1³) for obtaining the clean Tags. The OTUs were determined using a similarity threshold of 97% by the Usearch software (version 10.0.⁴). Taxonomic annotation of 16S rRNA gene sequence was determined with the RDP classifier⁵ against the database of Silva (Release132⁶) with a confidence threshold of 0.5. R software was performed to construct the histogram, heatmap, principal coordinate analysis (PCoA), the diversity indices of Chao1 Simpson and Shannon for analyzing the bacterial community composition. Linear discriminant analysis effect size (LEfSe) was performed to

¹<http://www.usadellab.org/cms/?page=trimmomatic>

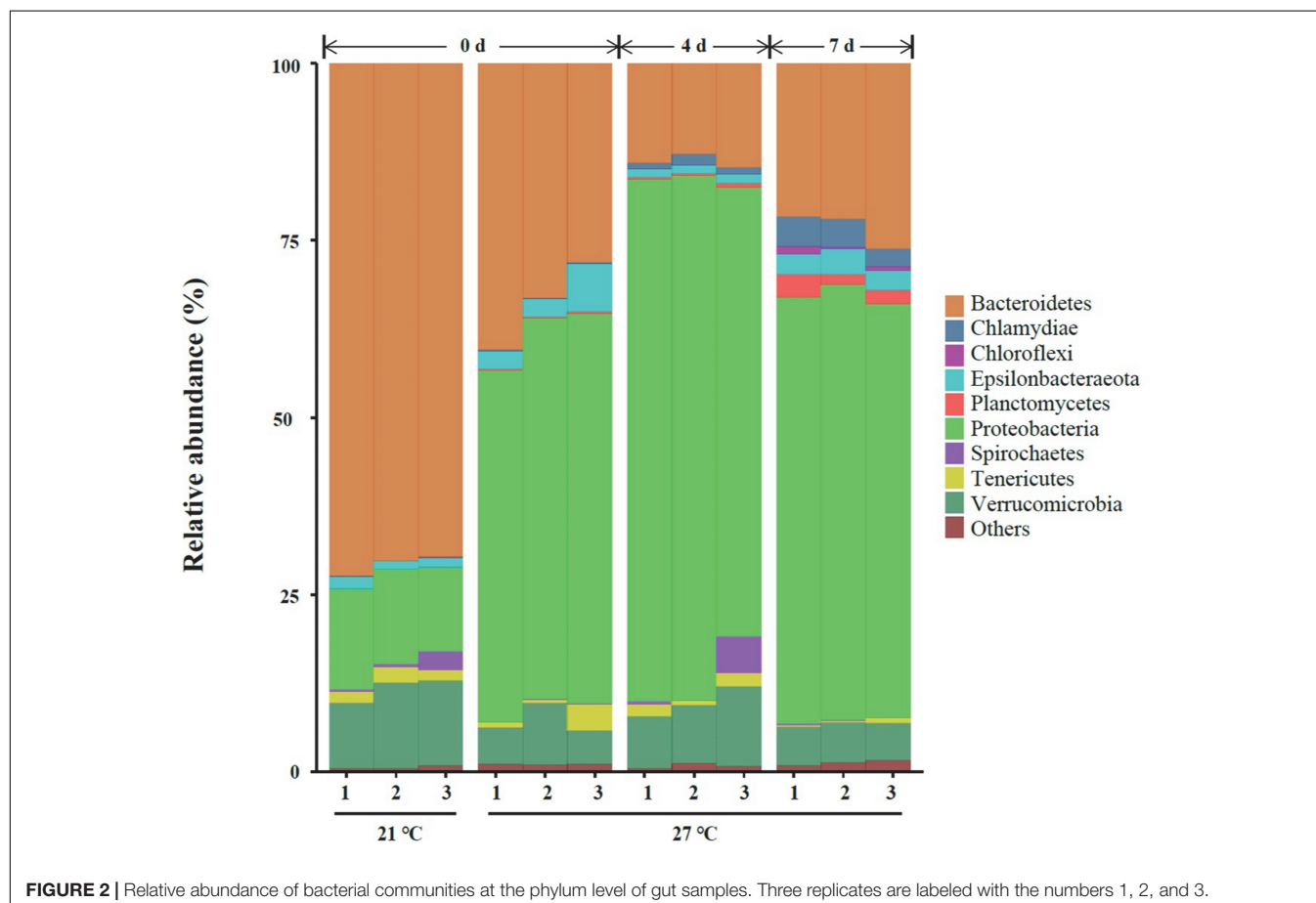
²<https://ccb.jhu.edu/software/FLASH/>

³<http://www.mothur.org>

⁴<http://www.drive5.com/usearch/>

⁵<http://rdp.cme.msu.edu/>

⁶<http://www.arb-silva.de>



determine differences of the relative abundance of bacteria at all taxonomic levels between the treatment and control. The Kruskal–Wallis test was conducted to identify bacterial taxa that are significantly different in relative abundance among different samples. The linear discriminant analysis (LDA) identifies the effect size with which these taxa differentiate the samples with thresholds of a log-transformed LDA score of 2.0.

RESULTS

M. galloprovincialis Survival

The survival of mussels cultured at 21 and 27°C for 7 days were shown in **Figure 1**. Significant difference was observed between control and warm acclimated mussels after 3 days exposure ($P < 0.05$). After 7 days, the lowest survival rate ($50 \pm 2\%$) was observed in the mussels exposed to higher temperature at 27°C.

Gut Microbiome Analysis

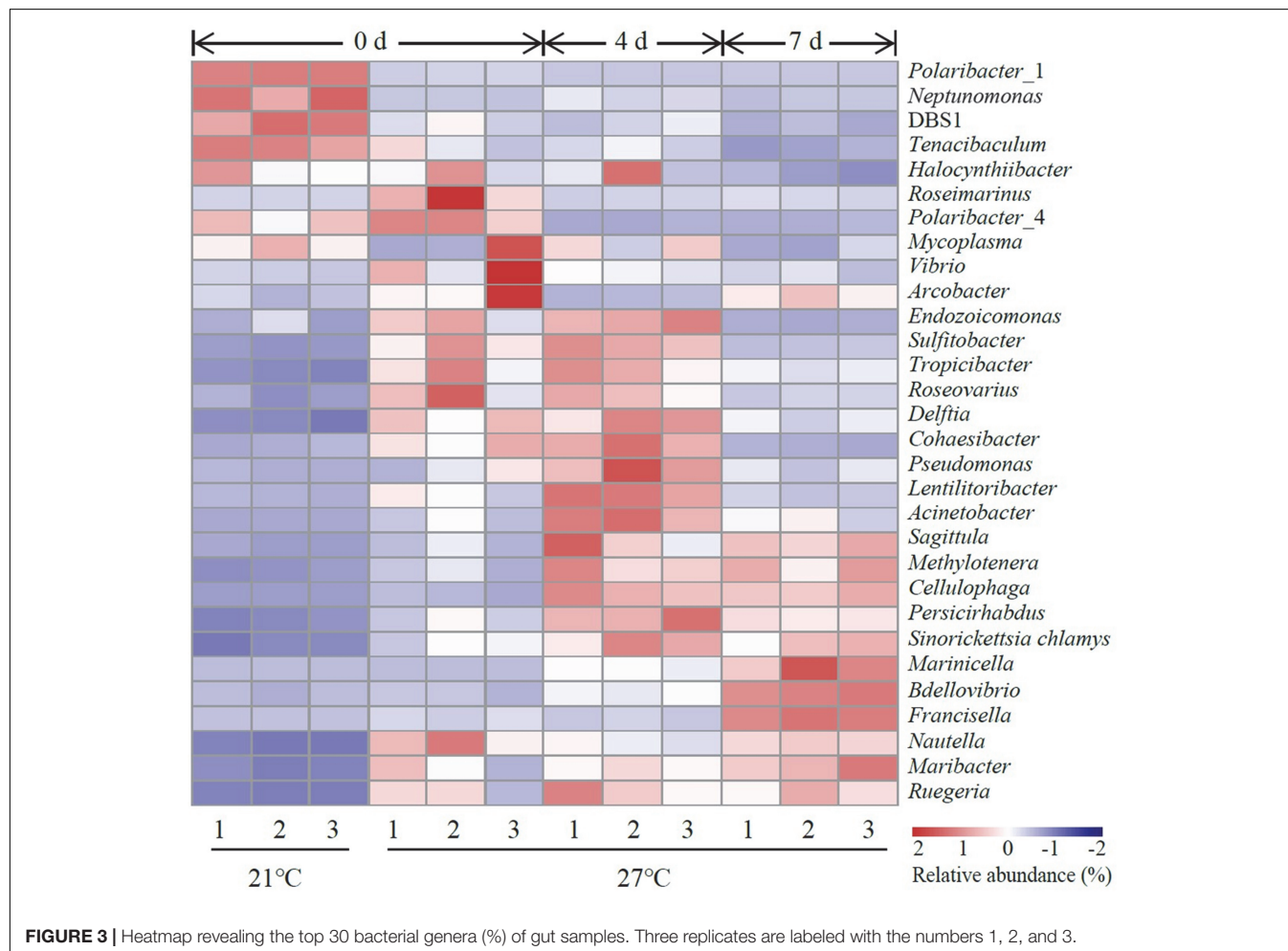
Globally 1152 operational taxonomic units (OTUs) were identified from the gut samples. At a 3% dissimilarity level, Good's coverage showed that 99.7 to 99.9% OTUs were identified for all of the groups. Based on OTUs at 3% dissimilarity, the

rarefaction curve of each group tended to approach the saturation plateau (**Supplementary Figure S1**).

Composition of Gut Microbiota at Phylum Level

A total of nine different phyla in all samples with an abundance of $> 1\%$ were characterized, and the abundance of nine phyla $< 1\%$ were all classified as “others” (**Figure 2**). Three dominant phyla Bacteroidetes, Proteobacteria and Verrucomicrobia were present in gut samples, which accounted for 87.2–95.9% of total reads (**Figure 2** and **Supplementary Table S1**).

High temperature (27°C) significantly increased the relative abundance of Chlamydiae, Epsilonbacteraeota, Planctomycetes, and Proteobacteria on day 0. A significant reduction of the relative abundance of Bacteroidetes, Spirochaetes, and Verrucomicrobia were observed in warm acclimated mussels compared to control mussels on day 0 ($P < 0.05$, **Supplementary Table S1**). At 27°C, the relative abundance of Chlamydiae, Planctomycetes, and Proteobacteria were significantly increased in mussels collected on day 4 compared to day 0 ($P < 0.05$, **Supplementary Table S1**). Warm acclimated



mussels significantly reduced the relative abundance of Bacteroidetes and Epsilonbacteraeota in mussels collected on day 4 compared to day 0 ($P < 0.05$, **Supplementary Table S1**). In a comparison between day 4 and day 7, the relative abundance of Bacteroidetes, Chlamydiae, Chloroflexi, Epsilonbacteraeota, and Planctomycetes were significantly increased, and this was accompanied by a significant reduction in the relative abundance of Proteobacteria and Verrucomicrobia ($P < 0.05$, **Supplementary Table S1**).

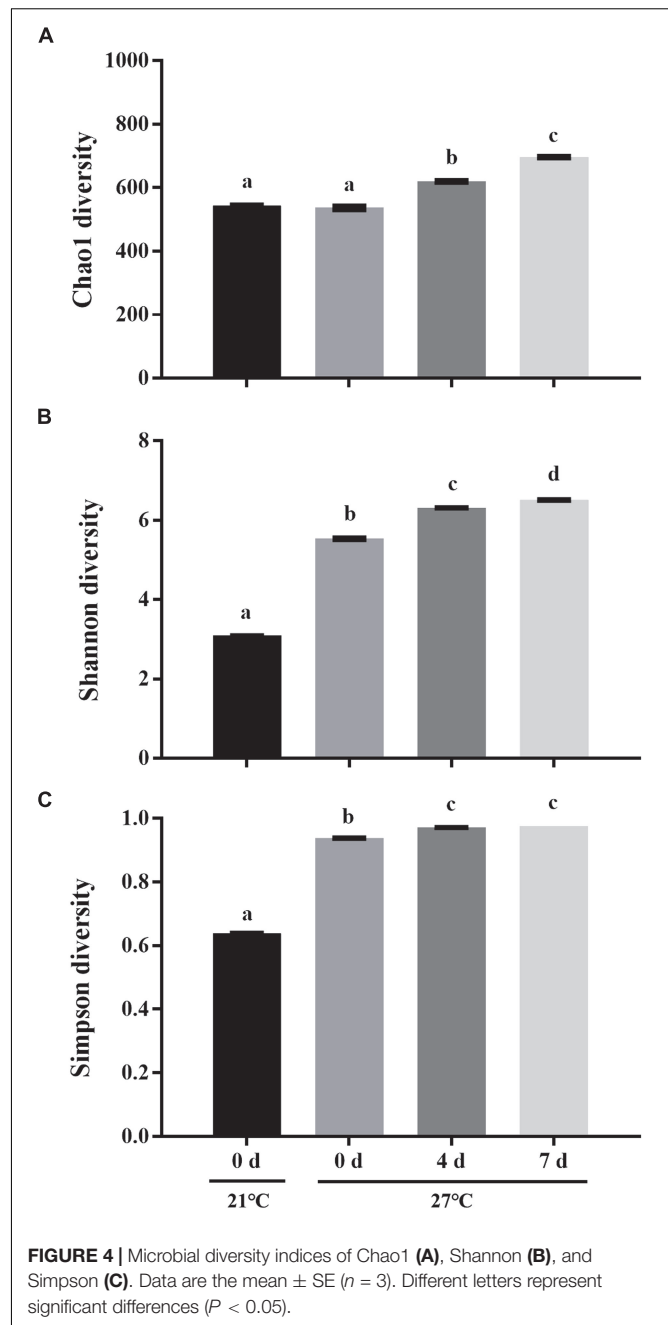
Composition of Gut Microbiota at Genus Level

The top 30 abundant genera were constructed for comparative analysis (**Figure 3**). *Polaribacter_1* was a dominant genus in control groups which accounted for 57.8–59.6% of total reads (**Supplementary Table S2**). *Vibrio* was a dominant genus in treatment groups at 27°C on day 0 and the relative abundance of *Vibrio* was significantly higher in treatment groups (27°C) than control groups (21°C) on day 0 ($P < 0.05$, **Supplementary Table S2**). The relative abundance of *Acinetobacter*, *Bdellovibrio*, *Cellulophaga*, *Lentilitoribacter*, *Marinicella*, *Methylothermobacter*, *Neptunomonas*, *Persicirhabdus*, *Pseudomonas*, and *Sinorickettsia_chlamys* were significantly increased in treatment groups on day 4 compared to day 0 and associated with a significant reduction in the relative abundance of *Arcobacter*, *Nautella*, *Polaribacter_1*, and *Roseimarinus* ($P < 0.05$, **Supplementary Table S2**). In the comparison groups between day 4 and day 7, continuous exposure to warm temperature favored proliferation of a dominant genus *Marinicella* as well as other genera such as *Arcobacter*, *Bdellovibrio*, *Francisella*, *Maribacter*, *Nautella*, and *Roseimarinus* ($P < 0.05$, **Supplementary Table S2**).

To characterize the microbial diversity of the gut samples, alpha diversity metrics such as total species abundance index (Chao1, **Figure 4A**) and species diversity indices (Shannon, **Figure 4B**; Simpson, **Figure 4C**) were analyzed. No significant difference of Chao1 index was observed in the treatment group at 27°C relative to control groups ($P > 0.05$). Warm temperature significantly increased Chao1 index with increasing exposure time ($P < 0.05$). Exposure to high temperature significantly increased the Shannon index on day 0 relative to control groups ($P < 0.05$). A rise of the Shannon index value was observed in groups at 27°C with increasing exposure time. Similarly, the Simpson index revealed significantly higher diversity in the treatment groups at 27°C relative to control groups ($P < 0.05$). PCoA analysis revealed that temperature shaped the microbial communities by separating the control and treatment groups on day 0 with the groups on day 4 and 7 at 27°C by PC1, which made up > 22.90% of the variance (**Figure 5**). Clear dissimilarities were observed in the treatment groups within various exposure times.

Unique Biomarkers Detected in the Mussel Gut

LEfSe analysis revealed that *Polaribacter_1* and *Tenacibaculum* were the top genus-level biomarkers distinguished the control group at 21°C from all other groups (**Figure 6** and



Supplementary Table S3). The gut samples collected in treatment groups at 27°C on day 0 were distinguished from all other groups by the relative abundance of *Vibrio* and *Arcobacter*. *Endozoicomonas*, *Persicirhabdus*, *Sulfitobacter*, and *Tropicibacter* were the top genus-level biomarkers in treatment groups on day 4 compared to all other groups.

DISCUSSION

The present study provides the knowledge of mussel physiological responses to temperature stress. The results showed

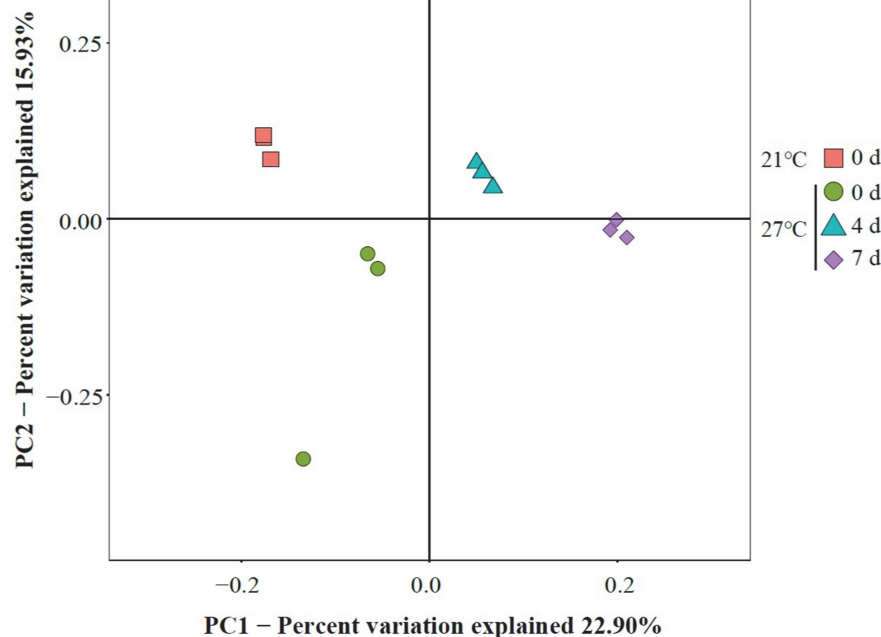


FIGURE 5 | Principal coordinate analysis (PCoA) of gut microbiome.

that increased mortality of the mussel *M. galloprovincialis* with raising seawater temperature from 21 to 27°C. Temperature affected microbial communities and dynamics in mussel gut as determined by 16S rRNA gene sequencing in live mussels and higher microbial diversity was found in live gut samples within short-term exposure to warm temperature. PCoA analysis revealed that the bacterial communities were affected by continuous heat stress. Unique species biomarkers determined by LEfSe analysis were presented good indicators of healthy status.

The geographic distributions of individual variation are determined by both adaptation and phenotypic plasticity in different latitudinal gradient, which is related to thermal tolerance range in species level (Sorte et al., 2011). Temperature stress was reported as a key factor in summer mortality syndrome (SMS) of marine bivalves (Myrand et al., 2000; Xiao et al., 2005; Malham et al., 2009). Generally, *M. edulis* was unable to tolerate temperatures above 28.5°C (Chapple et al., 1998). Laboratory trials demonstrated that temperature stress (31°C) had been directly linked to adult mussel *M. coruscus* mortality (29 and 49%) when fed two different diets, whereas exposure to 27°C for 3 days caused nearly 10% mortality rate (Li et al., 2018). In the present study, *M. galloprovincialis* exposed to 27°C for 7 days had contributed to 50% mortality, which indicates that the homeostasis of mussels held at 27°C may be disrupted. The divergent geographic distribution of *M. galloprovincialis* and *M. coruscus* might indicate that thermal tolerance could be an important physiological limit.

Warm temperature significantly increased microbial diversity in gut microbiota of mussels as indicated by Chao1, Shannon, and Simpson indexes. However, a significant reduction of microbial diversity was observed in gut microbiome of live mussel *M. coruscus* under heat stress (Li et al., 2018). This discrepancy may be explained by the alteration of community structure together with species-rich microbiome contributed to the high resilience threshold of animals under adverse conditions (Lozupone et al., 2012). Species diversity is strongly positively correlated with ecosystem stability which enables the community less susceptible to perturbation (Kühnel and Blüthgen, 2015; Sommer et al., 2017). It was reported that warm temperature impaired health condition in oysters associated with decreased microbial diversity (Lokmer and Wegner, 2015). It remains to be established whether the variation of microbial community diversity is caused by the mussel physiological responses to heat stress or a direct effect by temperature *per se*.

In the present study, PCoA analysis revealed that warm temperature altered major bacterial species. This is supported by previous study that a separation was observed between the gut microbiota of live mussel *M. coruscus* challenged by high temperature (Li et al., 2018). Furthermore, the results of present study extended our previous study by showing continuously exposure at 27°C reduced the similarity of community composition, and the shift of core microbial community over time could possibly be an indicator of high mortality. Similarly, the microbial dynamics and community

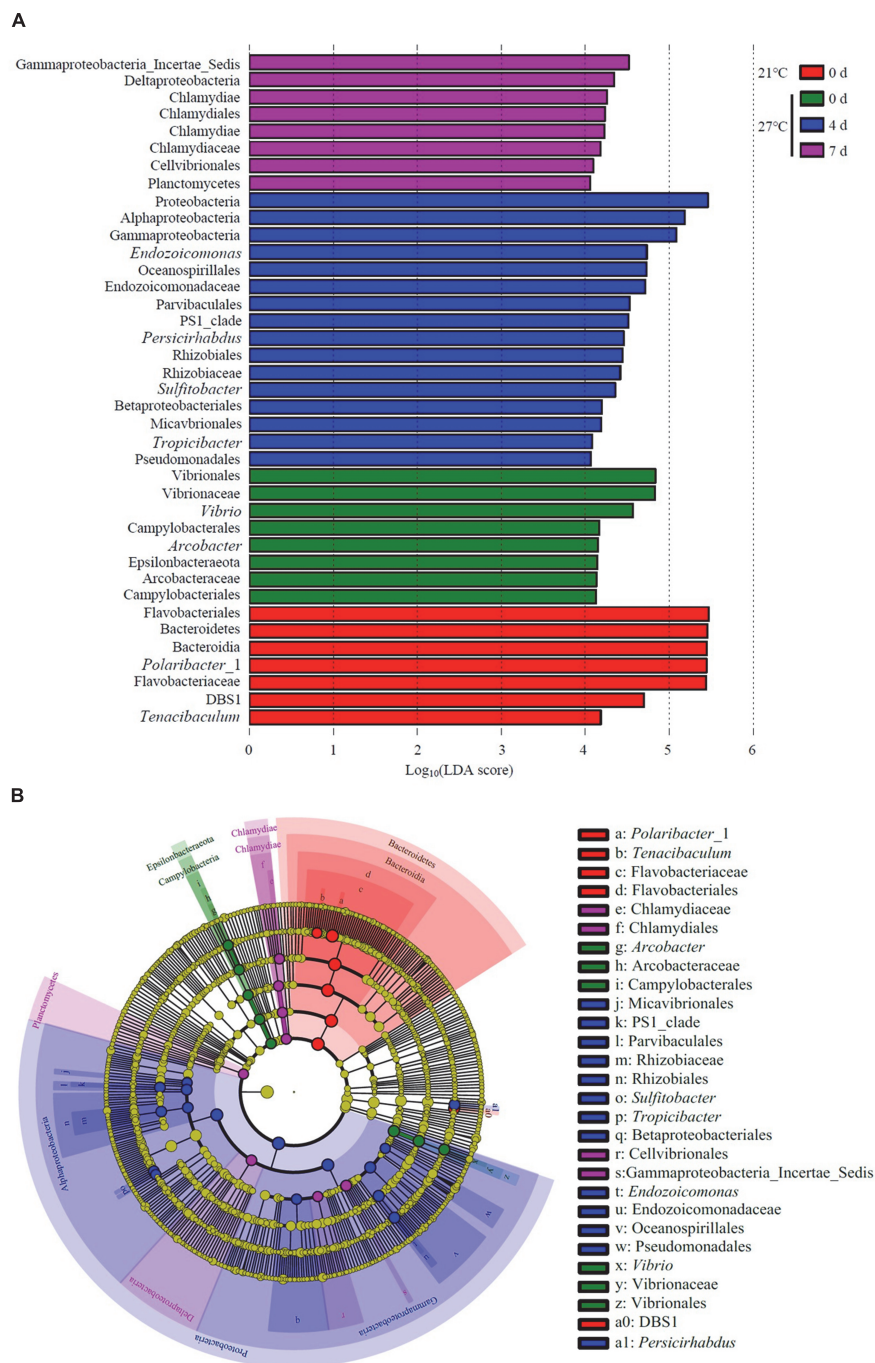


FIGURE 6 | Unique community composition of biomarkers in mussel gut. **(A)** Bar chart showing the log-transformed LDA scores of bacterial taxa identified by LEfSe analysis. A log-transformed LDA score of 2 was used as a threshold for identification of significant taxa, 39 taxa were identified by LEfSe analysis and are shown (Supplementary Table S3). **(B)** Cladogram showing the phylogenetic relationships of 47 bacterial taxa revealed by LEfSe.

composition in the oyster haemolymph was influenced by heat stress and suggested that instability of microbiome might contribute to high mortality (Lokmer and Wegner, 2015). Although the exact mechanisms of host–bacteria interaction are elusive, shifts of bacterial communities in gut may closely relate to host's physiology (Chen et al., 2018).

The present study revealed that Bacteroidetes, Proteobacteria, and Verrucomicrobia were three dominant bacterial phyla in *M. galloprovincialis* which differ of the previous study where Bacteroidetes, Proteobacteria and Firmicutes were prevalent in *M. coruscus* gut (Li et al., 2018). Bacteroidetes and Proteobacteria were abundantly found in guts of small abalone *Haliotis*

diversicolor (Zhao et al., 2018), crab *Callinectes sapidus* (Givens et al., 2013) and sea urchin *Lytechinus variegatus* (Hakim et al., 2015). Verrucomicrobia is commonly found in soil and marine environment (Freitas et al., 2012) as well as gut samples from mammalian (Dubourg et al., 2013), amphibian (Kohl et al., 2013), and bivalve (King et al., 2012). In the case of the mammalian, the relative abundance of Verrucomicrobia is suggested to be associated with the adaptive immunity (Zhang et al., 2015). It is noteworthy that *M. galloprovincialis* harboring a high proportion of microbes belonging to the phyla Verrucomicrobia even under heat stress.

An increase in water temperature (from 21 to 27°C) reduced the abundances of genus *Polaribacter* belong to the phylum Bacteroidetes. *Polaribacter* species have been detected in marine environment such as seawater (Yoon et al., 2006; Fukui et al., 2013). This species have been isolated from diatom phytoplankton blooms, which may be involved in the decomposition of sulfated polysaccharides (Xing et al., 2015). In the gut of *M. galloprovincialis* exposed to 27°C on day 0, there was a high abundance of the genus *Vibrio* and *Arcobacter* as revealed by LEfSe analysis. *Vibrio* species is ubiquitous microbes in marine environment. Warm temperature favored the proliferation of *Vibrio* and contributed to the mass mortality in bivalve shellfish aquaculture (Elston et al., 2008; Le Roux et al., 2016). Previous study on the oyster indicated that moribund oysters were dominated by the genus *Arcobacter*, which suggested as opportunistic pathogens (Lokmer and Wegner, 2015). The genome of *Endozoicomonas* bacteria showed their enrichment genes related to metabolic function such as utilization of carbohydrates and the supply of proteins to their host (Neave et al., 2017). In addition, *Endozoicomonas* species seems to have symbiotic relationships with the host by producing antimicrobial substance to deter potential invading microbes (Bourne et al., 2008). The genus *Endozoicomonas* dominated in gut of *M. galloprovincialis* exposed to 27°C on day 4, indicating those microorganisms were suspected to play a crucial role in maintaining health.

The present study showed that temperature was a key factor determining gut microbial community in *M. galloprovincialis*. The responses to heat stress were through the shifts of bacterial communities. Proliferation of opportunistic pathogens such as genus *Vibrio* and *Arcobacter* may increase host susceptibility to disease in response to heat stress and contributed to the mass mortality in mussel *M. galloprovincialis* aquaculture. The shifts of those opportunistic pathogens are suggested to be good indicators of mussel's physiology. The results obtained in this study provide the knowledge on mariculture of *M. galloprovincialis* associate with ecological adaptation for south domestication in changing environment and host–bacteria interaction during temperature stress.

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DATA AVAILABILITY

Publicly available datasets were analyzed in this study. This data can be found here: Raw sequences have been submitted to the NCBI sequence read archive database under the accession number: SRP197453.

ETHICS STATEMENT

The experimental protocol for mussel acclimation and experimentation was approved by the Animal Ethics committee of Shanghai Ocean University, Shanghai, China.

AUTHOR CONTRIBUTIONS

J-LY, XL, and Y-FL conceived and designed the experiments. J-KX, Y-WC, W-YD, and A-QS performed the experiments. Y-FL, J-KX, XL, and J-LY analyzed the data. Y-FL, Y-TZ, J-LY, and XL wrote the manuscript. All authors reviewed the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fphys.2019.01086/full#supplementary-material>

FIGURE S1 | Operational taxonomic units (OTUs) represented by rarefaction curve of all gut samples.

TABLE S1 | Relative abundance (in%) of the sequences in gut assigned to different phyla.

TABLE S2 | Relative abundance (in%) of the sequences in gut assigned to different genera.

TABLE S3 | All bacterial taxa (log-transformed LDA > 2) identified by LEfSe analysis of *M. galloprovincialis*.

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Conflict of Interest Statement: 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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Growth and Lipidomic Responses of Juvenile Pacific White Shrimp *Litopenaeus vannamei* to Low Salinity

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The Pacific white shrimp (*Litopenaeus vannamei*), a euryhaline penaeid species, can tolerate a wide range of salinities, but little is known on its strategies to cope with low salinity fluctuations from the aspect of lipidomics. Thus, in this study, *L. vannamei* were grown in two different salinities [3 and 30‰ (control)] for 8 weeks, and then an liquid chromatography (LC)–mass spectrometry (MS)-based lipidomics analysis was performed to reveal the lipid profile differences in gill and muscle. *L. vannamei* under low salinity had lower weight gain and condition factor than the control shrimp at 30‰, but no differences were found in survival and hepatopancreas index. A higher number of differential lipid metabolites were identified in gill than in muscle in *L. vannamei* at salinity 3‰ relative to the control shrimp at salinity of 30‰ (159 versus 37), which belonged to 11 and 6 lipids classes, respectively. Of these lipids, phosphatidylcholine (PC), phosphatidylinositol (PI), phosphatidic acid (PA), phosphatidylethanolamine (PE), and triglyceride (TG) were the main lipids in both shrimp gill and muscle, regardless of salinities. Compared with the control shrimp at salinity 30‰, the percentage of PC significantly reduced, but TG and PA significantly increased in gill of shrimp at salinity 3‰. Moreover, the relative fatty acid abundances showed significant changes in *L. vannamei* between the two salinity groups, but the patterns of the changes were complex and were fatty acid dependent. Neither lipid nor fatty acid composition in muscle was affected by salinity. Further pathway analysis showed that these metabolites were closely related to lipid and fatty acid metabolic pathways. All the findings in this study reveal that the lipid variations are closely related to bio-membrane structure, mitochondrial function, energy supply, or organic osmolyte contents in hemolymph for improving osmoregulatory capacity of *L. vannamei* under low salinity.

Keywords: salinity, *Litopenaeus vannamei*, lipidomics, growth, gill, muscle

INTRODUCTION

The Pacific white shrimp (*Litopenaeus vannamei*) is found in tropical waters, from Mexico to Peru, and in the last decade, there was an increase of using this species in inland aquaculture (Valencia-Castañeda et al., 2018). Regarded as a highly efficient osmoregulator, *L. vannamei* can tolerate a wide range of salinities from 0.5 to 50‰ (Saoud et al., 2003; Xu et al., 2018). Salinity is an

important environmental factor, however, has been shown to influence the distribution, abundance, and physiological response of aquatic animals extensively (Lv et al., 2013; Penney et al., 2016). Poor survival and growth, as well as low stress tolerance and disease resistance, have become restrictive factors for inland low salinity (<5‰) *L. vannamei* farming (Lin and Chen, 2003; Li et al., 2007, 2008). To solve these problems osmoregulatory adjustments are necessary to control hemolymph osmotic pressure, as well as the development of compensatory mechanisms allowing re-establishment of homeostasis before cellular structure and function are severely disturbed (Brito et al., 2000; Lucu et al., 2008; Silva et al., 2010).

Aquatic animals require more energy from growth for osmoregulation at low salinity (Spaargaren, 1975; Li et al., 2008; Huang et al., 2010). A great amount of extra energy in *L. vannamei* (20–50% of total metabolic energy) are needed for osmoregulatory processes including increasing metabolic rate, modification of cellular membrane components, alterations in ion transport enzyme activity, highly unsaturated fatty acids (HUFAs) concentration, free amino acids (FAAs) concentration, water permeability of gills, and synthesis of certain non-essential amino acids under hyposaline stress (Evans et al., 2005; Hurtado et al., 2007; Lv et al., 2013; Chen et al., 2014; Penney et al., 2016; Faleiros et al., 2018). Thus, it is reasonable to explore the effect of energy provision on osmoregulatory efficiency of *L. vannamei* grown in a low-salinity environment.

Of the three energy-yielding nutrients, lipids may be closely related to osmoregulation, because lipids have the greatest energy density, and many fatty acids derived from lipid metabolism are essential for normal growth and various metabolic functions in shrimp (Li et al., 2017). Previous studies have shown that large amounts of neutral lipids could be stored in cells for membrane synthesis and energy supply in times of starvation (Li et al., 2007; Tseng and Hwang, 2008). Moreover, phospholipids and glycolipids are indispensable components of the cell membrane, effecting osmoregulatory capacity of crustaceans by changing their cell membrane structures (Chapelle and Zwingelstein, 1984; Li et al., 2006; Chen et al., 2014). Furthermore, increasing dietary lipid level from 6 to 9% can alleviate the osmoregulatory pressure of *L. vannamei* under hyposaline stress (Xu et al., 2018). However, information is limited on the physiological functions of specific lipids and fatty acid metabolites in improving osmoregulatory efficiency of shrimp under low salinity.

Lipidomics is a mass spectrometry-based science for exploring the structure, composition, and even quantity of lipids in biological systems such as cells, organs, and body fluids (Smith et al., 2014). Gills, as the organs primarily responsible for osmoregulation of hemolymph, specialize in exchange with the exterior medium, and muscle is the material repository or nutrient pool for many of the aquatic organisms (Tseng and Hwang, 2008). However, little is known on the lipidomic characteristics in either gill or muscle in *L. vannamei* under low salinity. Therefore, this study investigated the significant differences in lipid metabolites between shrimp grown in salinity of 3 and 30‰ via ultra performance liquid chromatography–mass spectrometry (UPLC–MS) analysis. The current study is first investigation examining the effect of hyposaline stress

on *L. vannamei* using lipidomics analysis. The results in this study would help to elucidate the physiological strategies of *L. vannamei* for adapting to low salinity, and provide new insights into the significance of lipids for osmoregulation of *L. vannamei*.

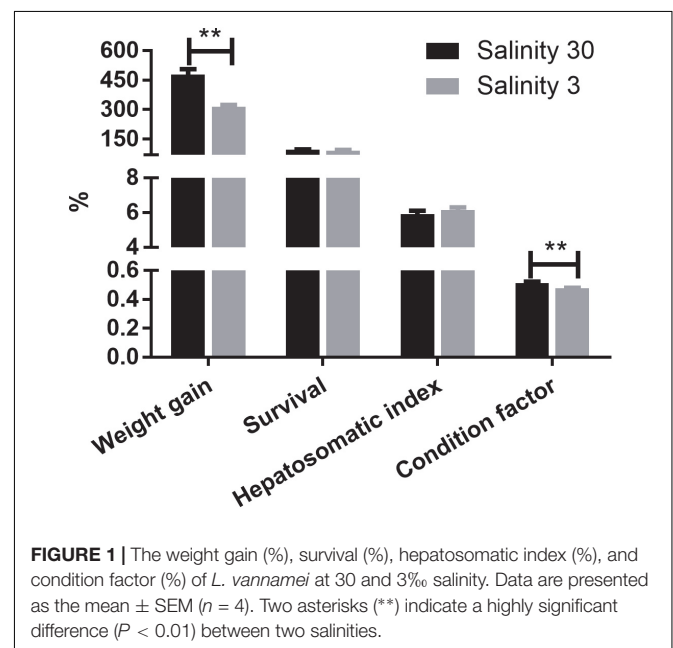
MATERIALS AND METHODS

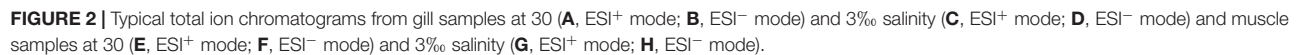
Experimental Animals and Design

Healthy juvenile *L. vannamei* were obtained from a shrimp larvae breeding base in Danzhou, Hainan, China. Shrimp were acclimatized for 1 week at 30‰ salinity in three tanks. During acclimation, salinity of two tanks was adjusted to 3‰ by decrease water salinity 3–5‰ per day by aerated and de-chlorinated tap water. Then, a total of 160 juvenile *L. vannamei* (0.75 ± 0.03 g) were randomly divided into separated tanks with four replicates for each salinity group with 20 shrimps per tank. During the acclimation and experimental periods, shrimp were fed three times daily at 08:00, 16:00, and 22:00 with commercial feed for *L. vannamei*. Based on the amount of residual food, daily rations were adjusted to a feeding level slightly more than satiation. The uneaten food and excrement were removed with a siphon tube once daily with water renewal (50%) until the end of the tests. The photoperiod was 12 h light and 12 h dark. Water pH (7.5–7.9), temperature (26–28°C), dissolved oxygen (4.8–6.4 mg/L), and total ammonia nitrogen concentration (< 0.02 mg/L) were monitored twice a week and maintained throughout the experiment.

Growth Evaluation and Sampling

After 8 weeks, all shrimp were fasted for 24 h prior to sampling. Shrimp in each tank were bulk weighed and counted. Then, each shrimp was dissected rapidly to obtain the hepatopancreas, gills, and muscle tissues on ice. Hepatopancreases were weighed to





determine the hepatosomatic index. Gills and muscles from four individuals in each tank were pooled as one sample and frozen in liquid nitrogen immediately, and then were kept at -80°C for lipidomics analysis. Growth performance-related parameters were calculated as follows:

$$\begin{aligned}\text{Survival (\%)} &= (\text{final number of prawns}/\text{initial number of prawns}) \times 100; \\ \text{Weight gain (\%)} &= ((\text{final weight}-\text{initial weight})/\text{initial weight}) \times 100; \\ \text{Condition factor (\%)} &= (\text{final weight (g)}/(\text{body length (cm)})^3) \times 100; \\ \text{Hepatosomatic index (\%)} &= (\text{wet hepatopancreas weight (g)}/\text{wet body weight (g)}) \times 100.\end{aligned}$$

Lipid Extraction

The methods for lipid extraction were according to Bi et al. (2013) with modifications. Gill and muscle samples selected from each tank were excised (50 mg) and dissolved in 1.5 mL trichloromethane/methanol (2/1, v/v), then mixed with 0.5 mL of ultrapure water (Merck, Germany). After being vortexed for 1 min, the solutions were separated by centrifugation at 3000 rpm for 10 min. The total organic phase of each was separated and placed in a clean test tube and dried under N_2 , then re-dissolved in 400 μL isopropanol/methanol (1/1, v/v). After centrifugation at 3000 rpm for 10 min at 4°C , the upper phase was transferred for following analysis.

LC-MS Analysis

Untargeted UPLC analysis was performed using the Ultimate 3000 LC system (Dionex, United States) with a Kinetex C18 analytical column (100×2.1 mm, $1.9 \mu\text{m}$). The injection volume was 4 μL . The mobile phase was composed of solvents A (60% acetonitrile/40% H_2O , v/v) and B (10% acetonitrile/90% isopropanol, v/v); the former contained 10 mmol/L ammonium formate and the latter contained 10 mmol/L ammonium formate and 0.1% formic acid. In addition, the concentration of solvent B underwent an optimized gradient program. It was kept at 30% for the first 2 min (0–2 min), then reached 100% in 18 min (20 min) and held for 20 min (40 min), linearly changed to 30% in 0.01 min (40.01 min), and finally maintained for 4.99 min (45 min). The flow rate was 0.40 mL/min and the column temperature was set at 45°C .

Mass spectrometry was performed using a Thermo Scientific Orbitrap Elite mass spectrometer with a heated electrospray ion source (HESI-II) (Thermo Scientific, United States), and the eluents were monitored in both positive and negative modes. Briefly, for the positive mode, the parameters were as follows: heater temperature 300°C ; sheath gas flow rate 45 arb; Aux gas flow rate 15 arb; sweep gas flow rate 1 arb; spray voltage 3.0 kV; capillary temperature 350°C ; S-lens RF level 30%; and scan ranges 200–1500. For the negative mode, the parameters were as follows: heater temperature 300°C ; sheath gas flow rate 45 arb; Aux gas flow rate 15 arb; sweep gas flow rate 1 arb; spray voltage 2.5 kV; capillary temperature 350°C ; S-lens RF level 60%; and scan ranges 200–1500.

LC-MS Data Processing and Lipid Identification

The raw data obtained from LC-MS analysis of all samples were processed initially by using Lipid Search v4.0.20 software (Thermo Scientific, United States). The data from each sample were then normalized to total area, and all data about variates [including retention time (rt) and charge-to-mass ratio (m/z)], sample number, and normalized peak intensities were imported into SIMCA-P⁺ 12.0 software (Umetrics, Umea, Sweden), where multivariate analyses including principal component analysis (PCA) and orthogonal partial least squares discrimination analysis (OPLS-DA) were performed to classify lipid samples. In addition, the OPLS-DA models were validated using a permutation test with 200 as the permutation number (Su et al., 2013).

The lipid metabolite information, including lipid types, number of saturated bonds, and differences in chain length of lipid molecules, were obtained by qualitative analysis using Lipid Search software (Thermo Scientific, United States). To select potential lipid biomarkers, the variable importance in the projection (VIP, $\text{VIP} > 1$) values of lipid metabolites in the OPLS-DA model and P -values ($P < 0.05$) acquired from the t -test analysis were regarded as the screening condition. In addition, fold change (FC) analysis was also conducted.

Statistical Analysis

The growth performance, relative abundance (%) of lipids, and position distribution of fatty acids [including 16:0 (palmitic acid), 18:1 n –9 (oleic acid), 18:2 n –6 (linoleic acid), 18:3 n –3 (linolenic acid), 20:4 n –6 (arachidonic acid), 20:5 n –3 (eicosapentaenoic acid), and 22:6 n –3 (docosahexaenoic acid)] were assessed by using Student's t -tests in SPSS 17.0 for Windows (SPSS Inc., New York, NY, United States) (Liu et al., 2019). The data were expressed as the mean $\pm$ SEM, and the level of significant difference was set at $P < 0.05$.

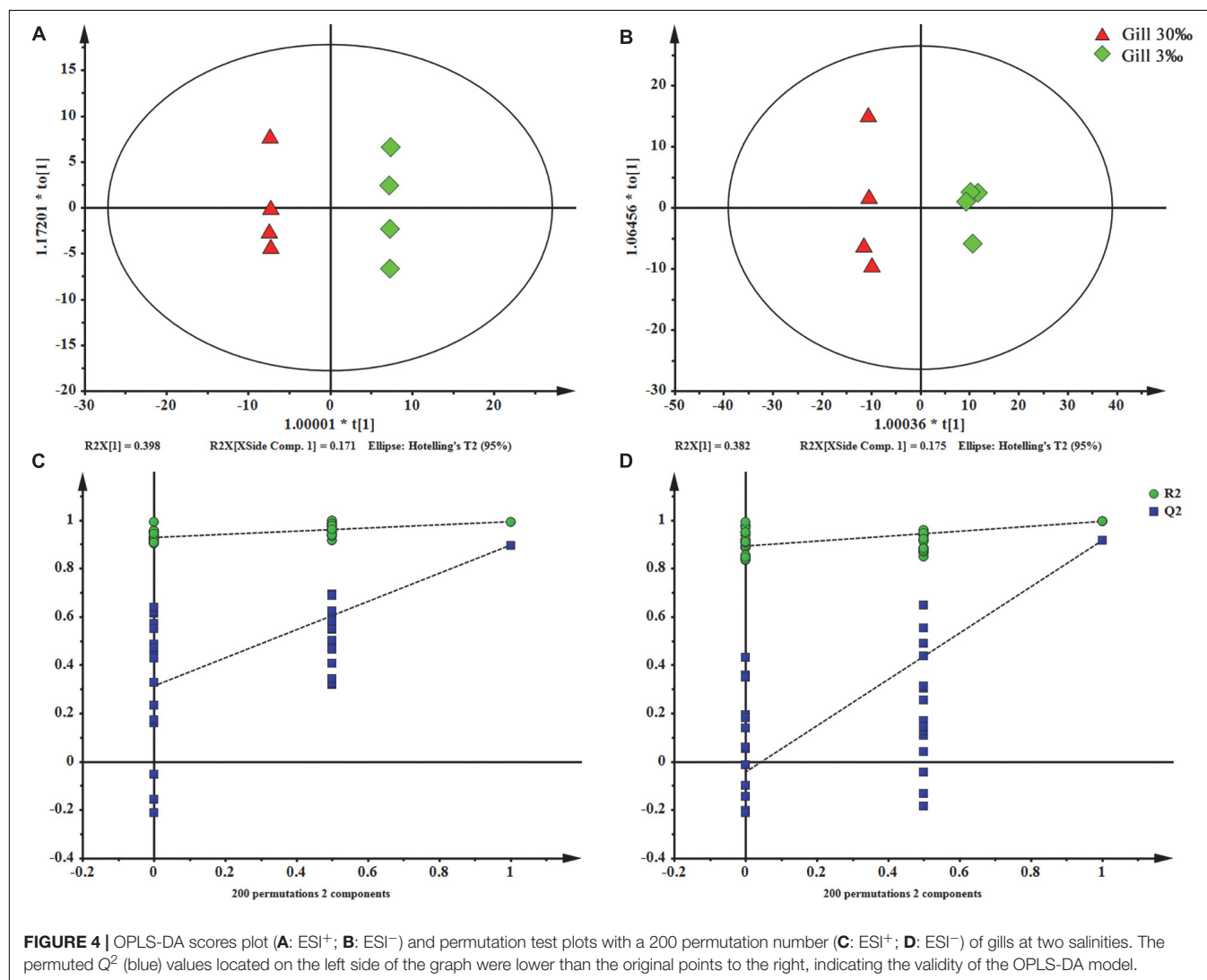
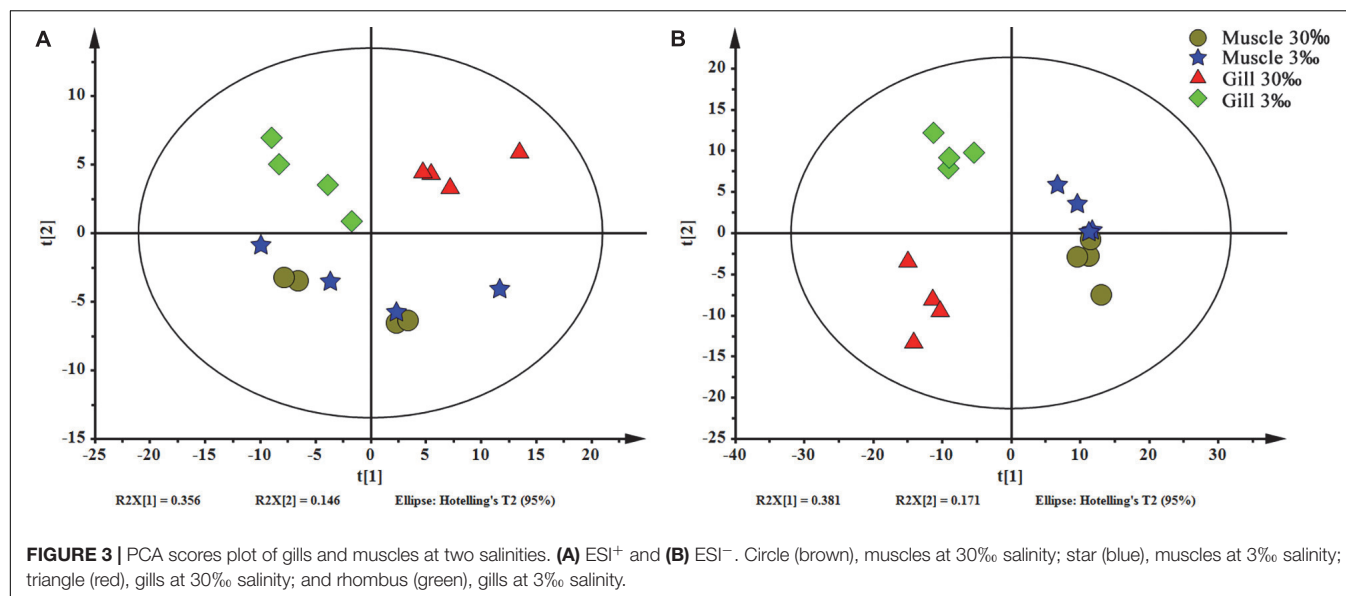
RESULTS

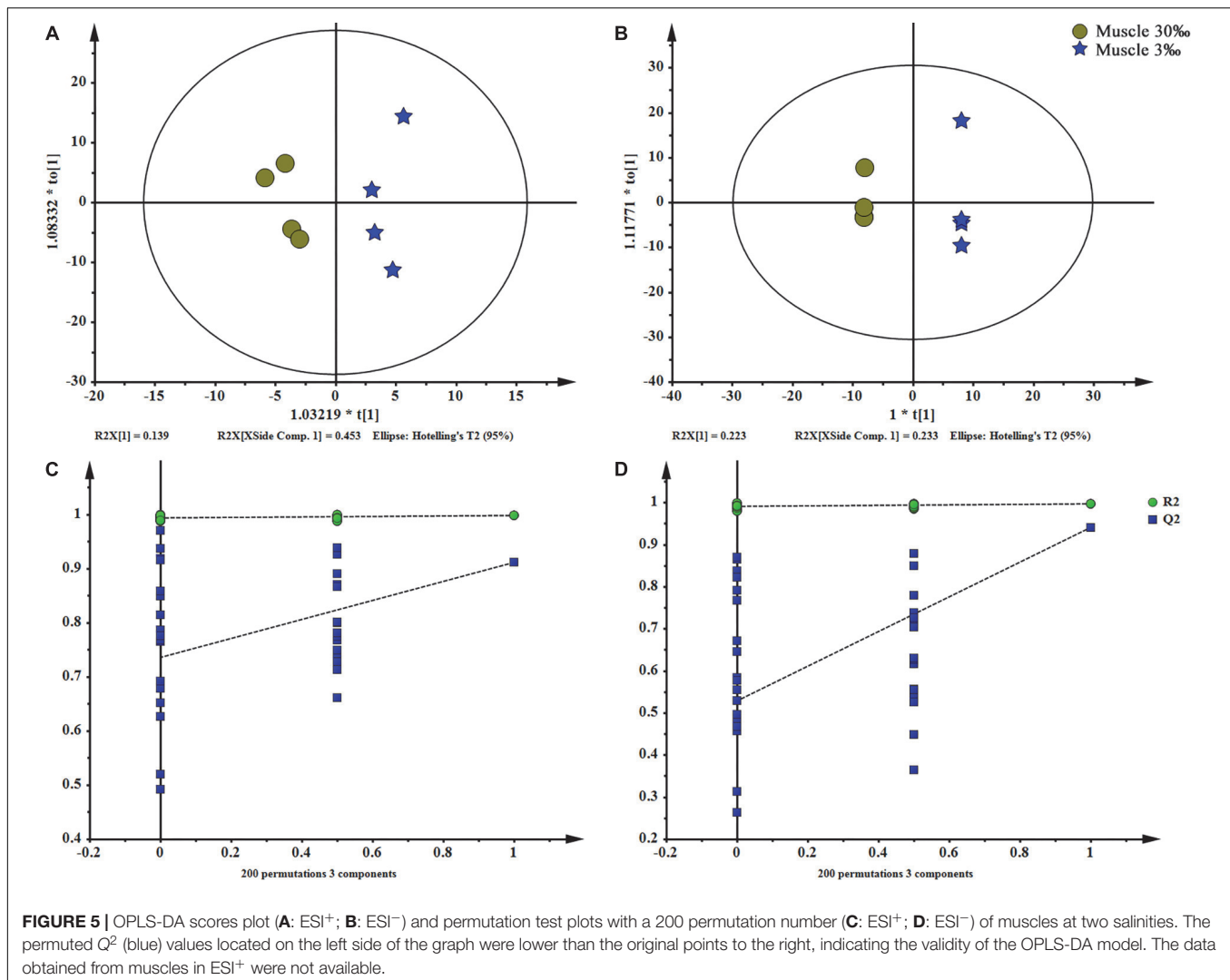
Weight gain and condition factor ($P < 0.01$) of the shrimp grown at 3‰ were significantly lower than those grown at 30‰. However, there were no significant differences in shrimp survival rate and hepatosomatic index between the two salinity groups (Figure 1).

TABLE 1 | Summary of the parameters in PCA model.

Title	POS model			NEG model		
	C	R^2X (cum)	Q^2 (cum)	C	R^2X (cum)	Q^2 (cum)
All groups	2	0.502	0.266	3	0.673	0.400
G30‰ vs. G3‰	2	0.612	0.155	2	0.588	0.111
M30‰ vs. M3‰	2	0.641	0.279	2	0.550	–0.071

The letters “G” and “M” are the abbreviations for gills and muscles, respectively. C is the number of components. R^2X (cum) is the cumulative modeled variation in X-matrix. Q^2 (cum) is the cumulative predicted variation in Y-matrix.





In total, 404 metabolite peaks, including 93 peaks in positive mode and 311 peaks in negative mode, were selected for multivariate analysis (Figure 2). According to the values of the parameters for the PCA model (Table 1), all R^2X (cum) were higher than 0.4, indicating the model was reliable. The PCA score plots obtained from four comparison groups showed four clusters in positive and negative ion scan modes by the first two components (Figure 3), respectively. Moreover, the two clusters corresponding to the gill samples in the score plots were separated more clearly in both ion scan modes by the first two components than those corresponding to muscle samples at the two salinities. The OPLS-DA score plots of gill groups (Figures 4A,B) and muscle groups (Figures 5A,B) showed clear separation. However, based on the R^2Y values (0.937–1) and Q^2 values (0.433–0.893) (Table 2), and the permutation test plots of positive and negative modes (gill group, Figures 4C,D; muscle group, Figures 5C,D), the data obtained from muscle groups in ESI⁺ were not available.

A total of 37 significantly different lipid metabolites belonged to 6 lipid classes were then identified in muscle comparison groups, and 159 in gill comparison groups were

classified into 11 lipid classes (Table 3). Among all lipid classes, phosphatidylcholine (PC), phosphatidylinositol (PI), phosphatidic acid (PA), phosphatidylethanolamine (PE), and triglyceride (TG) were the main lipid classes in both gills and muscles, regardless of salinities (Figures 6A, 7A). In detailed, the percentage of PC significantly reduced, but TG and PA significantly increased in gills of shrimp grown at 3‰. No lipid classes in muscle were affected by low salinity. Although PI was not significantly different between two salinity groups, regardless of muscle or gill, it showed opposite trends in both tissues; that is, it increased in the gill but decreased in the muscle. The percentage of PC tended to be higher in muscle tissue of shrimp grown at 3‰ compared to those grown at 30‰ salinity.

The fatty acid compositions of each position of specific lipids were similar regardless of salinities or tissues. No differences were found in fatty acid compositions in all lipid classes in muscle of *L. vannamei* between the two salinity groups (Figures 7B–F). However, compared with shrimp at salinity 30‰, shrimp at 3‰ had significantly lower percentages 16:0 at the sn-1 position of PC, but higher percentage of 18:0, 18:2n-6, 18:3n-3, 20:5n-3,

TABLE 2 | Summary of the parameters in OPLS-DA model.

Title	POS model				NEG model			
	C	R ² X (cum)	R ² Y (cum)	Q ² (cum)	C	R ² X (cum)	R ² Y (cum)	Q ² (cum)
G30‰ vs. G3‰	1 + 2	0.659	1	0.863	1 + 1	0.557	0.995	0.893
M30‰ vs. M3‰	1 + 1	0.592	0.937	0.433	1 + 4	0.835	1	0.886

The letters “G” and “M” are the abbreviations for gills and muscles, respectively. C is the number of components, the numbers before and after the plus sign are the principal component and orthogonal components, respectively. R²X (cum) is the cumulative modeled variation in the X-matrix. R²Y (cum) is the cumulative modeled variation in the Y-matrix. Q² (cum) is the cumulative predicted variation in the Y-matrix.

TABLE 3 | Differential lipid metabolites in muscles and gills of *L. vannamei*.

Lipid class	Muscle			Gill			Total
	Positive ion mode	Negative ion mode	Total	Positive ion mode	Negative ion mode	Total	
Phosphatidylcholine (PC)	–	17	17	11	48	59	76
Phosphatidylethanolamine (PE)	–	9	9	9	36	45	54
Phosphatidylglycerol (PG)	–	–	–	8	–	8	8
Phosphatidic acid (PA)	–	2	2	–	6	6	8
Sphingomyelin (SM)	–	6	6	2	6	8	14
Lyso-phosphatidylcholine (LPC)	–	2	2	–	5	5	7
Lyso-phosphatidylethanolamine (LPE)	–	–	–	–	1	1	1
Phosphatidylinositol (PI)	–	–	–	2	–	2	2
Triglyceride (TG)	–	–	–	10	–	10	10
Diacylglycerol (DG)	–	–	–	2	–	2	2
Phosphatidylserine (PS)	–	1	1	–	13	13	14
Total	–	37	37	44	115	159	196

“–” means fatty acid was not detected.

and 38:6 (Figure 6C). The percentage of 16:0, 20:5n–3, and 22:6n–3 except for 18:1n–9 was significantly higher in shrimp grown at 3‰ in the sn-2 position of PC (Figure 6D). At the sn-1 position of PE molecules, 18:1n–9, 18:2n–6, 18:3n–3, 20:5n–3, 22:6n–3, and 41:5 reduced significantly. Meanwhile, 27:0, 37:1, and 39:5 significantly increased (Figure 6E). At the sn-2 position, the percentages of 18:2n–6, 20:4n–6, and 22:6n–3 decreased, but percentages of 21:0 and 23:5 increased (Figure 6F). In the TG class, only the percentage of 9:0 at the sn-1 position significantly decreased in gills of shrimp at 3‰ than at 30‰ (Figure 6B).

The potential target metabolic pathway analysis based on the differential lipid metabolites obtained from gill and muscle samples (Supplementary Tables S1, S2, respectively) revealed that those metabolites were responsible for the metabolism of glycerophospholipid, linoleic acid, alpha-linolenic acid, arachidonic acid, glycerolipid, and for the biosynthesis of glycosylphosphatidylinositol (GPI)-anchor (impact-value ≥ 0.01, Figure 8).

DISCUSSION

In this study, *L. vannamei* cultured at 30‰ salinity exhibited better growth than those at 3‰ salinity. Similarly, growth of *L. vannamei* at 2 and 4‰ were significantly lower than that of shrimp at 30‰ (Laramore et al., 2001; Gao et al., 2016). As a

euryhaline species, *L. vannamei* was reported to have an optimal salinity range of 20–25‰ for growth (Huang et al., 2004; Li et al., 2017). It is worth noting that 30‰ salinity is the actual salinity of local marine aquaculture. Our study suggested that shrimp had better growth performance at 30‰ salinity for being close to the hemolymph isotonic point, also further confirmed that ambient water at 3‰ was definitely stressful for *L. vannamei*.

In this study, the lipid components in gill tissues obtained from *L. vannamei* were very similar to those of muscle, regardless of ambient salinities, and phospholipids were the principal lipids. The phospholipid composition was found to be principally formed of relatively large amounts of PC, followed by PI and PA in the present study. As reported, phospholipids also proved to be the principal lipids in *Eriocheir sinensis*, but it was interesting to note that PC was the main phospholipid, and PE was the second most abundant phospholipid in both gill and muscle tissue (Chapelle, 1977), illustrating the differences in lipid content between the two crustacean species. Classes and composition of phospholipids present in different animals have been characterized, and each animal tissue has its own pattern of phospholipid classes (Ju et al., 2011). PC and PE were the two most important phospholipids in muscle tissue of Nile tilapia (Liu et al., 2019). Interestingly, PE was the dominant phospholipid in *Mya truncate* and in the muscle tissue of terrestrial animals (Bevers et al., 1999; Gillis and Ballantyne, 1999), indicating that composition of lipids is species-dependent.

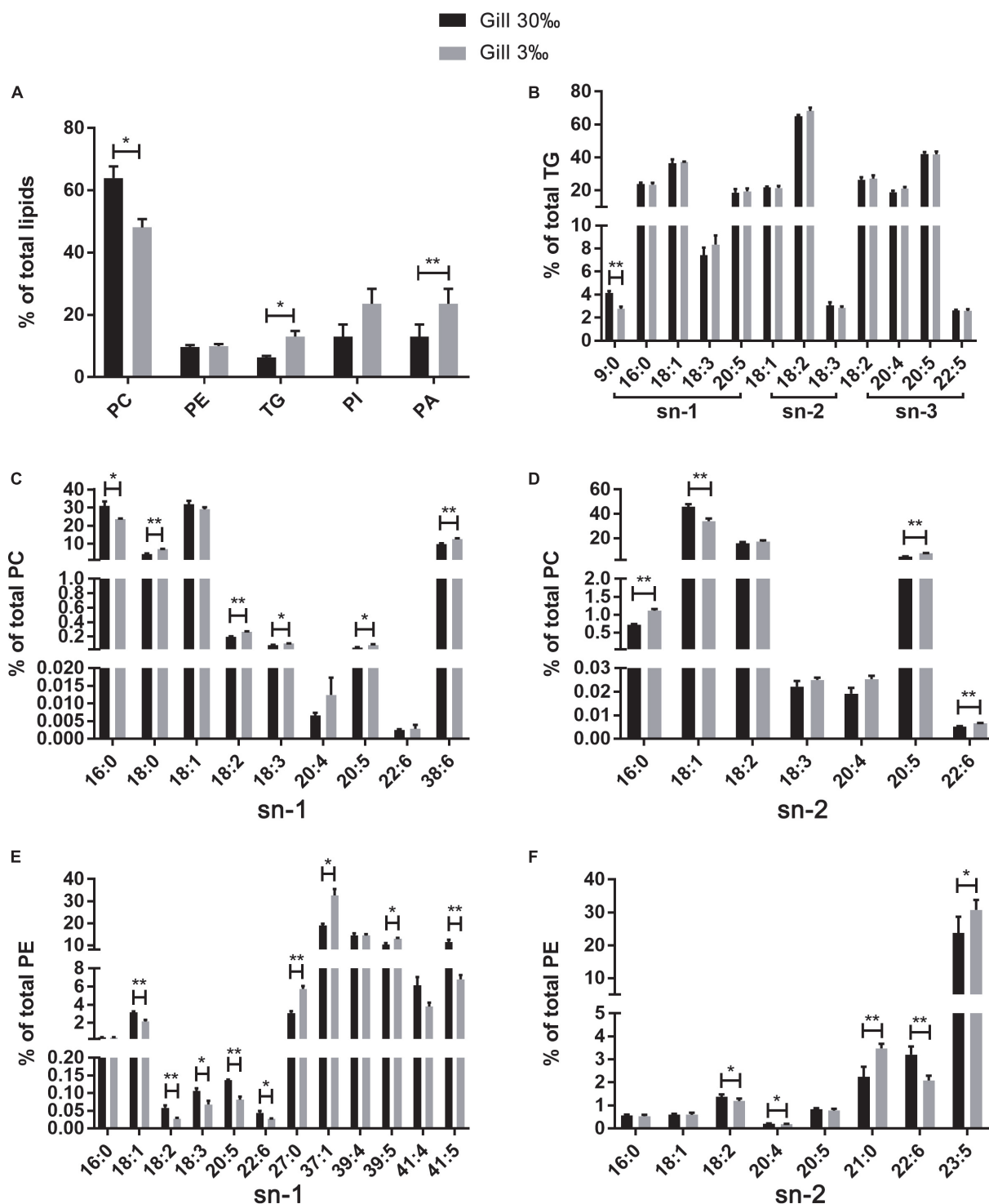


FIGURE 6 | The relative abundance of specific lipids in total lipids (A) and the positional distribution of individual fatty acids in total TG (B), total PC (C: sn-1 position; D: sn-2 position), and total PE (E: sn-1 position; F: sn-2 position) in gills of *L. vannamei*. Values are means $\pm$ SEM ($n = 4$). One asterisk (*) and two asterisks (**) indicate significant differences ($P < 0.05$) and highly significant differences ($P < 0.01$) between two salinities, respectively.

According to the analysis of percentages, the relative abundances of PC significantly reduced, while TG and PA increased, in gills of shrimp grown at 3‰ salinity. PC acted as a choline

“storage” molecule, and the choline could be metabolized to organic osmolytes such as betaine via glycine, serine, and threonine metabolic pathways (Athamena et al., 2011; Jiang et al., 2019).

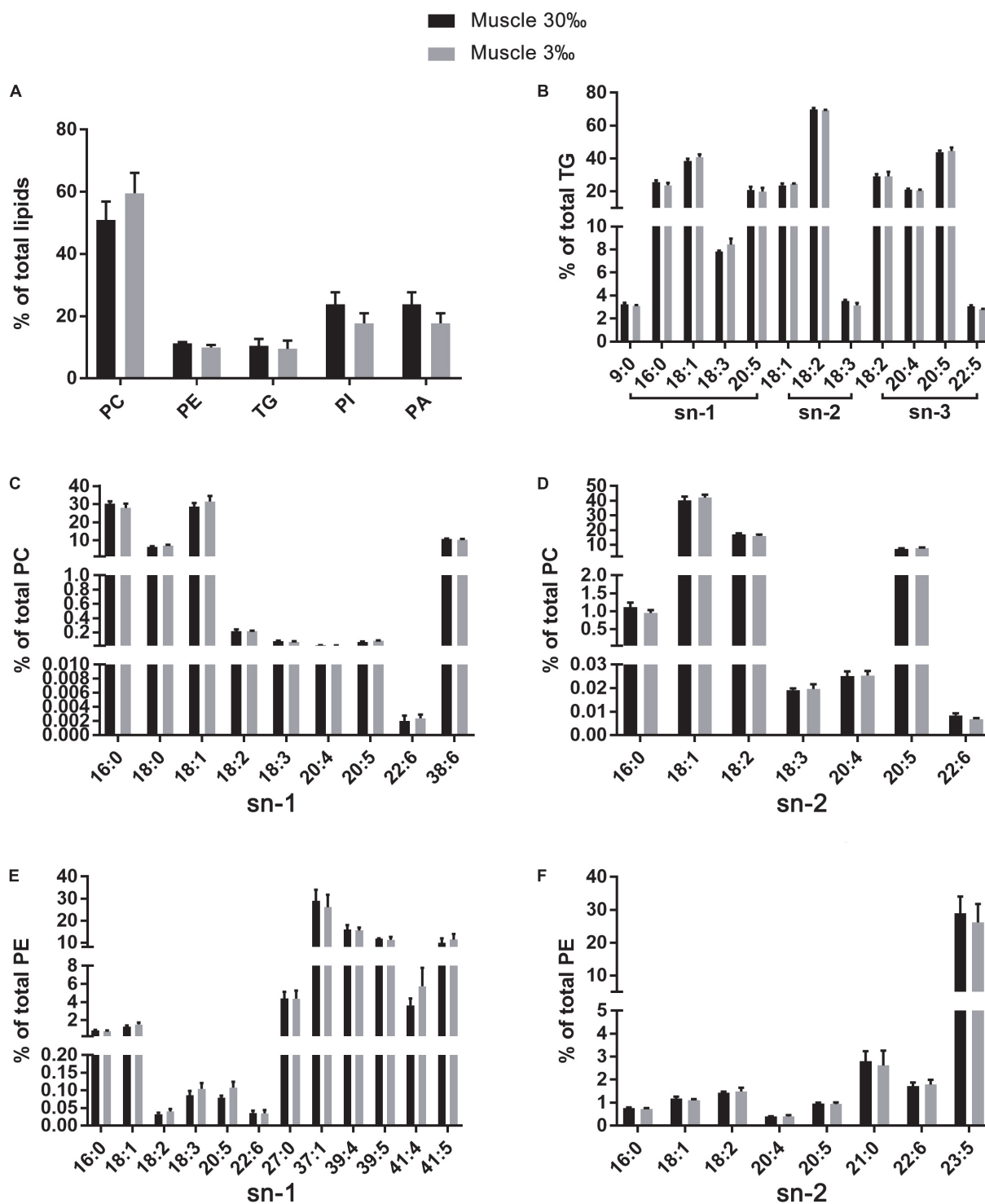


FIGURE 7 | The relative abundance of specific lipids in total lipids (A) and the positional distribution of individual fatty acids in total TG (B), total PC (C: sn-1 position; D: sn-2 position), and total PE (E: sn-1 position; F: sn-2 position) in muscles of *L. vannamei*. Values are means $\pm$ SEM ($n = 4$). One asterisk (*) and two asterisks (**) indicate significant differences ($P < 0.05$) and highly significant differences ($P < 0.01$) between two salinities, respectively.

As reported, choline was reduced under hyperosmotic stress while elevated under hypo-osmotic stress in the gills of tongue sole (*Cynoglossus semilaevis*) (Jiang et al., 2019). The osmolality of

hemolymph of crustaceans decreased after acclimation to dilute seawater (Lucu et al., 2008). Kettunen et al. (2001) demonstrated that betaine in the duodenal epithelium of broiler chicks played

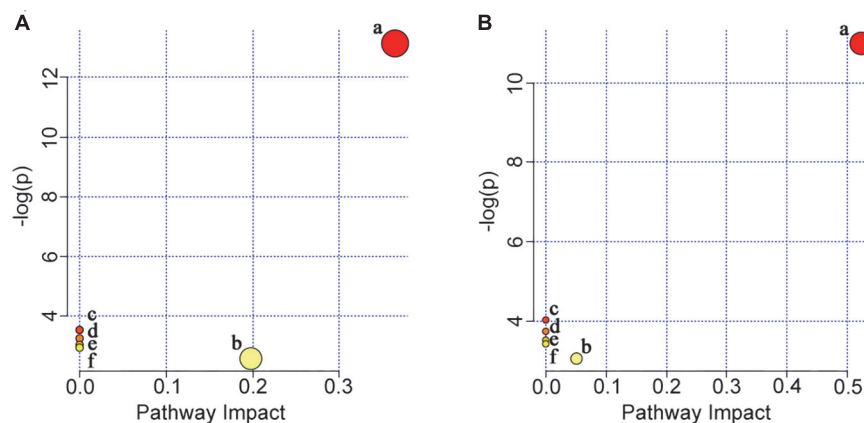


FIGURE 8 | Summary of pathway analysis in gill (A) and muscle (B) with MetaboAnalyst 4.0. (a) Glycerophospholipid metabolism; (b) glycerolipid metabolism; (c) linoleic acid metabolism; (d) alpha-linolenic acid metabolism; (e) arachidonic acid metabolism; (f) glycosylphosphatidylinositol (GPI)-anchor biosynthesis.

a crucial role to maintain water balance, thereby improving the osmoregulatory ability. Therefore, the reduction of PC in gill tissue of shrimp grown at 3‰ may be related to choline elevation under hypo-osmotic stress, regulation of the water permeability of gills, and maintenance of the normal morphology and function of cells.

It is worth to note that gill PI showed an increased trend in shrimp grown at 3‰, which was regarded as the phospholipid the least implicated in acclimation mechanisms in crabs (Chapelle, 1986). Furthermore, saturated fatty acids (SFAs) constituted the prevalent class of fatty acids in phospholipid PI detected in gill samples, including 17:0 and 34:0. Similar results were found in *Serripes groenlandicus* and *M. truncata* due to the high levels of 16:0 in PI of these two Arctic marine bivalve mollusks (Gillis and Ballantyne, 1999). At present, it is difficult to explain the exact functions of these specific fatty acids, 17:0 and 34:0. Perhaps both SFA and simpler fatty acids composition could help to improve the stability of PI function, suggesting PI plays highly specific roles in bio-membrane function. In cells, PI was used as a substrate in the plasma membrane, and PI cycle was the major metabolic pathway for PI biosynthesis, linked to actin polymerization and able to change membrane shape (Bozelli and Eband, 2019). Hence, we hypothesized that PI might be one of the important structural lipid components in membranes and had the capability to regulate bio-membrane structure or change cell shape, or could quickly fill the “vacancy” due to PC reduction.

The proportion of PA extremely significantly increased in gill of shrimp grown at 3‰ salinity. In the PI-cycle reaction, PI is transferred from the endoplasmic reticulum to the plasma membrane in exchange for PA moving in the opposite direction (Bozelli and Eband, 2019). Part of PA is synthesized in the endoplasmic reticulum and transported to the mitochondrial outer membrane. Moreover, PA has been reported to inhibit mitochondrial division and to stimulate mitochondrial outer membrane fusion, preventing the creation of too many mitochondria by excess division that might potentially compromise oxidative phosphorylation (Kameoka et al., 2018). Hence, the increase in PA may be closely related to mitochondrial

fission and fusion, which play critical roles in maintaining physiological functions of mitochondria when gill cells are under environmental stresses, enhancing the synthesis of ATP and providing sufficient energy for osmoregulation. Also as reported, PA was related to the enhancement of diphosphatidylglycerol (DPG) synthesis when *E. sinensis* was transferred from sea water to fresh water, and the rapid renewal of DPG was thought to directly enhance mitochondrial activity (Chapelle and Zwingelstein, 1984). The changes in number or activity of mitochondria organelles might occur in response to the increased energy requirement required by the active transport mechanism because mitochondria have the principal function to form ATP (Chapelle and Zwingelstein, 1984; Lucu et al., 2008). In contrast, the lack of mitochondrial division led to the enlargement of mitochondria, decreased the efficiency of mitochondrial transport into subcellular regions, and consumed more additional ATP compound (Youle and van der Bliek, 2012). Furthermore, it was reported that PA played a fundamental role in the biosynthesis and metabolism of TG (Stanley et al., 2013). To maintain the balance of PA content in the cells, a portion of the PA might be used to synthesize TG, further providing sufficient energy for the gills.

Osmoregulation is an energy-dependent process, and aquatic animals are forced to spend more additional energy for modulating and stimulating ion transport mechanisms when challenged with salinity stress (Tseng and Hwang, 2008; Li et al., 2017). As good indicators for assessing the energy utilization, oxygen consumption and respiratory quotients of shrimp at 3‰ were significantly higher than those of shrimp at 17 and 32‰ salinity (Li et al., 2007). Similarly, higher oxygen consumption was needed in juvenile pompanos reared at 3‰ salinity than for those cultured in the other experimental salinities (6, 12, and 32‰) (Abou Anni et al., 2016). TG is a major class of neutral lipid used for energy storage (Liu et al., 2019), and the increase in TG synthesis was expected. The present research also indicates that shrimp grown at salinity of 3‰ require more energy than at 30‰.

Both the total content and positional distribution of fatty acids in lipid molecules affected the nutritional values of the lipids

and their utilization in metabolism (Karupaiah and Sundram, 2007; Liu et al., 2019). In this study, the composition and relative abundance of fatty acids at the same position of the specific lipid class was similar in *L. vannamei* regardless of ambient salinities or tissues, indicating that distribution of fatty acids in lipids was far from random, and the utilization of lipid classes is highly specific in *L. vannamei*. Fatty acids, such as 20:5n-3 and 22:6n-3, showed significantly positive genetic correlations, suggesting that the high values have potential for genetic improvement (Nolasco-Alzaga et al., 2018). However, other fatty acids like 20:4n-6 showed an exceptionally low level of heritability, indicating a larger role for environmental regulation than genetic. As reported, the lipid molecules and fatty acid contents could also be modified by different dietary oils and different salinities (Chapelle et al., 1982; Chen et al., 2014; Chen et al., 2015; Liu et al., 2019). Thus, work on this topic should be further conducted.

In this study, a variety of unsaturated fatty acids were found in PE, which was regarded as the most highly unsaturated phospholipid in a marine animal, playing an important role in the permeability of biological membranes via regulation of fluidity (Chapelle et al., 1982). Seven specific fatty acids including 20:4n-6 and 20:5n-3 had important nutritional value in organisms (Liu et al., 2019). As reported, 20:4n-6 and 20:5n-3 were abundant in the posterior gills of crabs (Chapelle and Zwingelstein, 1984), which were found mainly in TG in this study. Importantly, dietary supplementation with 20:5n-3 and 22:6n-3 could change gill water permeability of *L. vannamei* exposed to low salinities (Hurtado et al., 2007). Thus, we hypothesized that the role of 20:4n-6 and 20:5n-3 is related to energy supplying and changing water permeability of gills, and *L. vannamei* could rapidly respond to changes in environmental salinity, but more information should be obtained in the future to further confirm this.

CONCLUSION

In conclusion, growth performance of *L. vannamei* would be inhibited when grown at salinity 3‰. Shrimp gill, as the organ directly connects with ambient water, is more sensitive than muscle because higher number of significantly changed lipids was found in shrimp gill. The significantly changed lipids were related to bio-membrane structure, mitochondrial function, fatty acid utilization, energy supply, or organic osmolyte content, for improving shrimp osmoregulatory capacity. The dramatic change in lipid profile is a significant physiological strategy that *L. vannamei* employs to cope with low salinity stress.

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DATA AVAILABILITY

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

ETHICS STATEMENT

All experimental procedures were conducted in conformity with institutional guidelines for the care and use of laboratory animals in Hainan University, Haikou, China.

AUTHOR CONTRIBUTIONS

EL and QZ conceived and designed the research. MH, JX, and CX conducted the research. YD and MH finished the cultured experiment. YD, MH, YZ, and QC performed the statistical analysis. MH and EL wrote the manuscript. All authors contributed to the manuscript revision and approved the submitted version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fphys.2019.01087/full#supplementary-material>

TABLE S1 | The raw data of differential lipid metabolites using for pathway analysis in gill. VIP > 1 and *P*-values < 0.05 were regarded as the screening condition. *m/z* is the mass-to-charge ratio of metabolites. FA is the abbreviation of fatty acid and VIP is the abbreviation of variable importance in the projection.

TABLE S2 | The raw data of differential lipid metabolites using for pathway analysis in muscle. VIP > 1 and *P*-values < 0.05 were regarded as the screening condition. *m/z* is the mass-to-charge ratio of metabolites. FA is the abbreviation of fatty acid and VIP is the abbreviation of variable importance in the projection.

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Differential Coping Strategies in Response to Salinity Challenge in Olive Flounder

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To examine how different fish coping strategies respond to salinity challenge, olive flounder (*Paralichthys olivaceus*) with active coping style (AC) and passive coping style (PC) were transferred from seawater (SW) to freshwater (FW) and their behavior and physiology were analyzed. Different behavioral coping strategies, in terms of escape and feeding tendencies, were confirmed in AC and PC fish without FW exposure. Differences in swimming distance between AC and PC flounder were then assessed after 1 and 2 days of FW transfer. Plasma parameters and branchial gene expression were also determined 2, 5, 8, and 14 days after transfer, with comparisons between AC and PC fish and against a SW-acclimated control group. The results showed that: (1) PC flounder exhibited a significant reduction in swimming activity, while AC flounder significantly increased locomotion 2 days after transfer. (2) The plasma osmolality and plasma ionic (Na^+ and Cl^-) concentration of FW-acclimated PC flounder declined in a continuous fashion over time but this contrasted against the plasma parameters of AC flounder which fluctuated below the baseline level of a SW-acclimated control group. (3) The expression of NKA- $\alpha 1$ and NHE-3-like mRNA in PC flounder gill increased significantly from 5 days, but the expression of these two genes in AC flounder only increased after 8 days of transfer. (4) There were no remarkable differences observed in Rhcg expressions between AC and PC flounder. This study indicates for the first time that PC flounder adopt a “freeze-passive tolerance” strategy while AC flounder adopt a “flight-active resistance” defense strategy in response to salinity challenge.

Keywords: flounder, salinity challenge, coping strategy, NKA- $\alpha 1$, NHE-3-like

INTRODUCTION

In animals, including fish, coping style is defined as a consistent individual response (CIR) to environmental challenge that is maintained over time and/or across contexts (Castanheira et al., 2017). Moreover, the CIRs of fish in response to environmental challenges are associated with behavioral and physiological differences (Overli et al., 2007), and have increasingly been recognized as adaptive variation (Castanheira et al., 2017). Differences in osmoregulatory ability and behavioral patterns (e.g., feeding attempts, captured and uncaptured) also exist in teleosts

with different coping styles (Sloman et al., 2003, 2004, 2008; Louison et al., 2017). In common with the rainbow trout *Oncorhynchus mykiss* (Frost et al., 2007), active coping (AC) individuals and passive coping (PC) individuals are observed in olive flounder *Paralichthys olivaceus* (Rupia et al., 2016). Compared to PC flounder, AC flounder show differences in response to simulated capture, feed propensity and metabolic rate (Rupia et al., 2016). For example, during acute stress, passive coping individuals adopt a passive “freeze-hide” strategy by reducing their oxygen consumption rates and remaining immobile whereas active coping individuals adopt an active “fight-flight” defense strategy by increasing their rates of respiration and activity levels (Rupia et al., 2016).

In fish, marine teleosts are hypoosmotic to their environment, which requires them to ingest seawater (SW) to offset the osmotic loss of water and excrete NaCl to reduce the excessive diffusive gain of salt. In contrast, freshwater teleosts are hyperosmotic to their environment which requires that they extract NaCl from their surrounding freshwater (Hwang and Lee, 2007; Evans, 2008). Euryhaline fish such as olive flounder, encounter complicated salinity changes during their spawning migration (Bolasina et al., 2007) and need to maintain body fluid homeostasis through sophisticated mechanisms of ionic and osmotic regulation (Kang et al., 2008). Although Na⁺ and Cl⁻ are the dominant ions in plasma, euryhaline marine teleosts in freshwater have lower salt content compared to euryhaline fish in seawater (Evans, 2008). The fish gill is an essential organ that plays a dominant role in osmotic and ionic regulation, mainly due to the presence of chloride cells that perform ion transport (Evans et al., 2005; Hwang, 2009). In freshwater (FW), ionocytes actively transport Na⁺ and Cl⁻ from the external aquatic environment to compensate the passive loss of ions in the urine and diffusion from the permeable surfaces of the body and gill (Hsu et al., 2014). Na⁺-K⁺-ATPase (NKA) as a primary Na⁺ transporter has been localized to the basolateral region of ionocytes, and provides an electrochemical gradient for active ion movements (e.g., Na⁺ and Cl⁻) across the branchial epithelia to maintain intracellular homeostasis (Hwang et al., 2011). It had also been demonstrated that salinity challenge increases the abundance of NKA- α -subunit mRNA (Scott et al., 2004; Hwang and Lee, 2007) and most euryhaline teleosts exhibit adaptive changes in NKA activity in response to salinity challenge (Feng et al., 2002; Weng et al., 2002; Hwang and Lee, 2007). Moreover, another Na⁺/H⁺ exchanger (NHE3) and a Rh glycoprotein (Rhcg) are both suggested to be involved in the model for ammonium-dependent Na⁺ uptake: NHE3 down the extracellular apical H⁺ gradient created by ammonia diffusion of Rhcg, simultaneously extract sodium from environment to compensate the Na⁺ uptake (Esaki et al., 2007; Yan et al., 2007; Hwang et al., 2011). Therefore, based on the accumulating evidence, a model of NaCl movement across the gill of euryhaline teleosts has been established (Hwang and Lee, 2007). Salinity challenge is common for flounder as they inhabit shallow coastal embayments and estuaries that fluctuate in salinity level. An increase in the intensity and frequency of extreme events is predicted to occur as a result of climate change.

In the estuary, hurricanes, flooding and heavy rainfall can cause dramatic changes in water conditions, especially salinity. Flounder is therefore forced to face with unusual salinity challenge (Bailey and Secor, 2016; Williams et al., 2017). Flounder also encounter complicated salinity changes during their spawning migration. The ability of individuals to “cope” with such a challenge will therefore influence their ecology and, hence, their vulnerability to capture. An understanding of individual coping strategy is therefore warranted.

A link between coping style and osmoregulation in teleost fishes has been reported previously (Sloman et al., 2003). But there is still a shortage of information that links the behavior and physiology of euryhaline fish with different coping styles to salinity stress. For example, do these fish adopt different behavioral and physiological coping strategies under salinity stress? In order to answer this question and advance knowledge in the field, our study aims to provide more detail regarding the behavioral and physiological mechanisms by which olive flounder, with different active and passive coping strategies, contend with salinity challenge. To achieve this aim, the following parameters were measured in euryhaline flounder with different coping styles transferred from SW to FW: (1) swimming activity, behavioral feeding and escape response, (2) dynamic change in plasma osmolality and ionic concentration (Na⁺ and Cl⁻), and (3) the branchial gene expression levels of NKA- α 1, NHE-3-like, and Rhcg.

MATERIALS AND METHODS

Experimental Fish

To reduce the potential influence of sexual dimorphism in fish (Yuan et al., 2017), gynogenetic olive flounder (body weights: 500 $\pm$ 50 g) were produced and reared in SW (30‰) recirculating aquaculture systems at the Central Experimental Station of Chinese Academy of Fisheries Sciences in November 2018 (Beidaihe, Hebei, China). About 80 active coping (AC) individuals and 80 passive coping (PC) individuals were selected from PC and AC broodstock screened according to behavioral tests developed by Rupia et al. (2016). Gynogenetic olive flounder of each coping style were then distributed evenly into each of the 20 holding tanks (500 L) and maintained for 2 weeks with filtered flow-through SW at a salinity level of 30‰. Water temperature was maintained at 18 $\pm$ 1°C, with a constant 12 h light/12 h dark cycle (8:00 am–20:00 pm). The mean light intensity, measured centrally at the bottom of each tank, was approximately 40 lux. Fish were starved throughout the experiment to ensure that differential rates of feeding did not influence the results of the study (Yuan et al., 2017).

Behavioral Test to Confirm the Presence of Passive Coping Style and Active Coping Style Strategies

To confirm the behavioral type of each AC and PC family, gynogenetic olive flounder were selected at random from the main holding tanks and isolated individually in smaller holding tanks (size 300 L). About 48 experimental fish (n = 24 for

AC and $n = 24$ for PC) then had their behavior screened for two different aspects of coping. Propensity to feed and air exposure are commonly used in fish studies to distinguish behavioral types (Rupia et al., 2016), and these two behavioral tests were also used in this experiment for confirmatory purposes. In the first feeding behavior test, a piece of raw fish was attached to a rod with a thin piece of line and held approximately 10 cm above the head of an individual fish for 2 min. The time taken to initiate the first feeding strike in 2 min was recorded as “feeding tendency.” To ensure that the fish did not actually feed during the trial, the food item was pulled out of the water if the fish approached it within about half a body length. In the second test, an individual was removed from the holding tank in a net and held in air for 2 min. The time taken to initiate the first escape attempt was recorded as “escape tendency” and the total number of escape attempts was recorded as “escape attempt,” defined as the number of body movements that resulted in an elevation of the body from the net (Rupia et al., 2016).

The Behavioral Response of Active and Passive Coping Styles to Acute Salinity Challenge

Olive flounder with confirmed active coping style (AC, $n = 8$) and confirmed passive coping style (PC, $n = 8$) were selected at random and placed individually into 16 opaque 100 L seawater tanks ($n = 1$ per tank; salinity = 30‰; water depth = 20 cm). Individuals could not see each other in these boxes. Cameras were mounted centrally above the tanks and trials began at 8:00 am. After recording the behavior of fish for 2 days, the flounder were transferred from 30‰ seawater (control group) to different set of 16 freshwater tanks at 3‰, with behavior monitored for a further 2 days. The swimming distance of flounder from video recordings was then computed using specialized behavioral software (Big Brother version 3, ACTIMETRICS, Wilmette, Illinois, USA).

The Ionic and Gene Expression Response of Active and Passive Coping Styles to Salinity Challenge

Olive flounder with confirmed active coping style (AC, $n = 48$) and confirmed passive coping style (PC, $n = 48$) were netted and randomly divided into mixed coping groups with direct transference to four seawater (SW, 30‰, control group) tanks or four freshwater (FW, 3‰) tanks ($n = 6$ for each coping style in each tank). SW-acclimated and FW-transferred fish were then sampled during daylight hours 2, 5, 8, and 14 days after initial transfer. All fish in each tank were rapidly netted at each time point tank and blood samples (3–5 ml) were collected within 90 s using a heparinized needle and syringe (200 U/ml heparin, Sigma) by caudal venipuncture. Blood was then aliquoted into ammonium-heparinized tubes (200 U/ml, Sigma) and plasma separated by centrifugation for 5 min at 13,000 rpm and stored at -80°C for the subsequent measurement of plasma osmolality and plasma ionic concentrations (Na^+ and Cl^-). Fish were then

killed humanely by severing the spinal cord and destruction of the brain. The gill was removed and stored at -80°C for the later analysis of branchial gene expression.

Plasma osmolality was measured using a vapor pressure osmometer (Wescor 5200, Logan, UT, USA). Plasma Na^+ and Cl^- concentration was measured using DIONEX ICS-1500 for cation and DIONEX ICS-900 for anion (Thermo, scientific, USA).

Total RNA was extracted from the gills using RNAiso Plus (TaKaRa, Japan). About 500 ng of total RNA was reverse-transcribed into cDNA using PrimeScript™ RT reagent (Takara, Dalian, China) following the standard protocol. NKA- $\alpha 1$, NHE-3-like and Rhcg gene expressions were collected on ABI 7500 (Applied Biosystems, Carlsbad, CA) with SYBR Premix Ex Taq™ (Takara, Dalian, China). β -actin as the housekeeping gene, was using to normalized branchial gene expression levels. The following PCR conditions were used: 10 min at 95°C , followed by 36 cycles of 95°C for 10 s, 30 s at 60°C . Gene sequences are obtained from NCBI and all the primers designed by NCBI primer blast. The forward and reverse primers span an exon-exon junction. All the primers are tested and showed to be viable and specific. The primer sets for the quantitative RT-PCR are shown in Table 1.

Statistical Analysis

The $2^{-\Delta\Delta C_t}$ method was used to analyze the quantitative RT-PCR data (Yuan et al., 2017), and the expression level in all plots of each gene are presented as the relative change in values from SW-acclimated AC at 14 days, which was set at 1. All statistical analysis was carried out using SPSS Statistics 20 or XLSTAT@2014. The normality and homogeneity of variance of data were tested using Shapiro-Wilk's test and Levene's test respectively. A principal component analysis (PCA) according to behavioral test, examining feeding and escape behavior was performed in XLSTAT@2014. Two-way analysis of variance (ANOVA) with Tukey *post hoc* comparisons were used to determine the effect of coping style and salinity (SW versus FW) on swimming activity at day 1 and day 2. The effects of coping style, salinity and time on plasma osmolality concentration were assessed using a three-way mixed ANOVA. Once the interactions among the factors were determined, plasma osmolality and ionic concentration were compared within each coping style using one-way ANOVA with time set as within-subjects factor and salinity set as a between-subjects factor. Plasma osmolality and ionic concentration and branchial gene expression

TABLE 1 | Primer sequences used for real-time PCR amplifications.

Gene	GenBank	Primer(5'-3')
β -actin	HQ386788.1	F: GGAAATCGTGCGTGACATTAAG R: CCTCTGGACAACGGAACCTCT
NKA- $\alpha 1$	XM_020104090.1	F: CATCAGCATCGCTTACGG R: GGGAAGGCACAGAACCAC
NHE-3-like	XM_020108892.1	F: CAGAGCAGGAGCTGGAAT R: GACAGGAGTGTCGGCAAG
Rhcg	XM_020078180.1	F: TTTGTGACAGACTGGAGGTG R: TCTAACGGCAACAAGGGA

were also compared within each coping style using one-way ANOVA only with salinity set as a between-subjects factor. All one-way ANOVA tests were followed by Tukey's HSD test. All data are expressed as mean $\pm$ SEM, and the significance level was $p < 0.05$.

RESULTS

The Principal Component Analysis of the Behavioral Experiment

In the series of behavioral tests, AC and PC flounder showed a divergence in escape and feeding behavior. The AC flounder were more motivated to feed and showed a higher tendency to escape during the confinement than PC flounder. The two principal components, PC1 and PC2, explained 85.62 and 10.53% of total variance, respectively, showing a clear way to discriminate between the two coping styles. According to PCA, escape attempts, feeding tendency and escape tendency all loaded positively onto PC1 (Figure 1). The eigenvectors and correlation between variables of PC1 and PC2 are shown in Table 2.

Swimming Activity of Flounder After Transfer From Seawater to Freshwater

The swimming activities of PC and AC flounder were both modified 2 days after being transferred from SW to FW. The swimming distance of PC flounder appeared higher than that of AC flounder in SW at 1 day and 2 days but no significant

difference was seen between these groups (Figure 2A). However, after FW transfer, AC flounder moved significantly further than PC flounder after 1 day (ANOVA, $F = 29.69$, $p = 0.015$) and 2 days (ANOVA, $F = 29.71$, $p = 0.014$) of salinity challenge (Figure 2B). A marked significant difference in the distance moved by two coping styles was therefore seen with PC flounder significantly reducing its swimming distance and AC flounder significantly increasing its swimming distance after both 1 day (ANOVA, $F = 0.907$, $p = 0.01$) and 2 days (ANOVA, $F = 0.266$, $p = 0.004$) of FW transfer (Figure 2C). The trend for increasing distance in AC fish but decreasing distance for PC fish was revealed as a significant interactive effect between salinity and coping style at both day 1 (ANOVA, $F = 8.4$, $p < 0.01$) and day 2 (ANOVA, $F = 10.8$, $p < 0.01$).

Plasma Osmolality

The plasma osmolality of PC (ANOVA, $F = 5.748$, $p < 0.001$) and AC flounder (ANOVA, $F = 0.473$, $p = 0.003$) transferred to FW were lower than those of fish in SW (Figure 3). There were no significant changes observed in plasma osmolality of SW-acclimated AC and PC flounder. The plasma osmolality of PC flounder gradually declined from 2 to 14 days after transference to FW, and showed a significant difference between SW and FW at 5 days (ANOVA, $F = 1.525$, $p = 0.002$), 8 days (ANOVA, $F = 7.593$, $p = 0.001$), and 14 days (ANOVA, $F = 5.313$, $p = 0.001$), respectively (Figure 3A). The plasma osmolality of PC flounder decreased to below 223 mOsm at 14 days after transference to FW, and was significantly different to the osmolality of the PC freshwater fish at 2 days (ANOVA, $F = 3.85$, $p = 0.024$; Figure 3A). In contrast, the plasma osmolality of FW-transferred AC flounder did not show a consistent decline over time but instead fluctuated around an average level of 260 mOsm from 5 days with a significant difference between SW and FW only at 5 days (ANOVA, $F = 0.978$, $p = 0.005$) and 14 days (ANOVA, $F = 0.005$, $p = 0.003$; Figure 3B). The impact of coping style, time, and salinity on plasma osmolality was explained by interactions between salinity and time (ANOVA, $F = 5.086$, $p = 0.003$), and coping style and salinity (ANOVA, $F = 5.429$, $p = 0.023$). Four AC flounders began to die after FW-transfer since 8 days after transfer to FW.

Plasma Ionic Concentration

Plasma Na^+ and Cl^- in both PC (Na^+ , ANOVA, $F = 2.688$, $p < 0.001$; Cl^- , ANOVA, $F = 0.605$, $p < 0.001$) and AC flounders (Na^+ , ANOVA, $F = 1.015$, $p = 0.007$; Cl^- , ANOVA, $F = 0.256$, $p < 0.001$) were higher in SW-acclimated fish than FW-acclimated

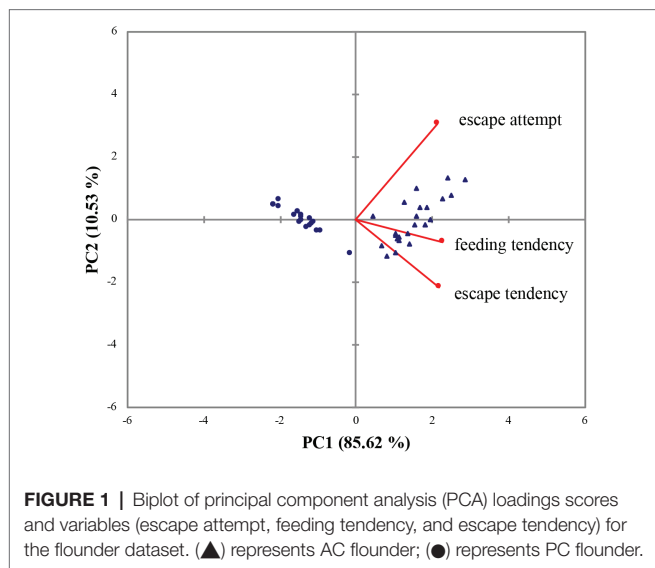


TABLE 2 | Principal component analysis; and eigenvectors and correlation between variables of two principal components PC1 and PC2.

Principal component	% Variability		Feeding tendency	Escape attempt	Escape tendency
PC1	85.62%	Eigenvectors	0.598	0.578	0.555
		Correlation	0.958	0.927	0.890
PC2	10.53%	Eigenvectors	-0.193	-0.568	0.8000
		Correlation	-0.109	-0.319	0.450

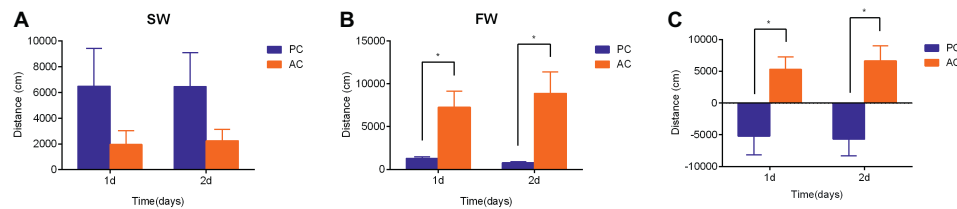


FIGURE 2 | The swimming activity (i.e., distance moved in cm) of passive coping style (PC) and active coping style (AC) olive flounder after transference from SW to SW (A) and FW (B). (C) Summary of the direction and magnitude of difference in distance moved for PC and AC fish after transfer from SW to FW. The data in (C) were calculated using the values of total swimming distance of each fish in FW minus the total swimming distance of fish in SW at each time point (1 day and 2 days post transfer). Data are mean $\pm$ SEM ($n = 8$). An asterisk denotes a significant difference between the data within the same time group ($p < 0.05$).

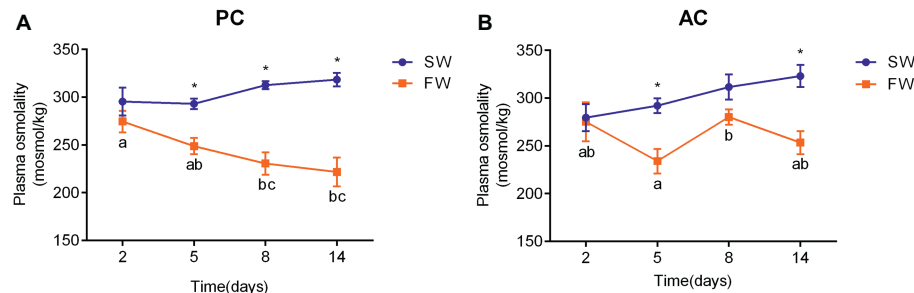


FIGURE 3 | Temporal changes in the plasma osmolality of olive flounder (A) with passive coping style (PC) and (B) with active coping style (AC) with fish either remaining in seawater (the SW control, ●) or after transference to freshwater (FW, ■). Data are mean $\pm$ SEM ($n = 6$). Different letters denote a significant difference across time within each SW or FW group ($p < 0.05$). An asterisk signifies a significant difference between the SW and FW groups at each time point ($p < 0.05$).

fish (Figure 4). Plasma Na^+ and Cl^- of PC gradually decline from 2 to 14 days after transferred from SW to FW and a significant difference was observed between SW and FW at 5 days (Na^+ : ANOVA, $F = 0.01$, $p = 0.048$ and Cl^- : ANOVA, $F = 2.078$, $p = 0.026$), 8 days (Na^+ : ANOVA, $F = 0.228$, $p = 0.031$ and Cl^- : ANOVA, $F = 0.569$, $p = 0.006$), and 14 days (Na^+ : ANOVA, $F = 1.136$, $p = 0.001$ and Cl^- : ANOVA, $F = 0.064$, $p = 0.008$), respectively (Figures 4A,C). The plasma Cl^- of FW-acclimated PC at 14 days was significantly different to the values of the same group at 2 days (ANOVA, $F = 4.294$, $p = 0.003$) and 5 days (ANOVA, $F = 4.294$, $p = 0.044$), respectively (Figure 4C), while plasma Na^+ of FW-acclimated PC at 14 days was only significantly different to the values of the same group at 2 days (ANOVA, $F = 3.228$, $p = 0.008$; Figure 4A). In contrast, plasma Na^+ of FW-acclimated AC flounder declined from 2 to 5 days, and elevated sharply to above 165 mmol/L at 8 days, and declined again at 14 days. There was a significant difference in AC flounder between SW and FW at 5 days (ANOVA, $F = 0.257$, $p = 0.03$; Figure 4B). The pattern of plasma Cl^- of AC flounder was similar to plasma Na^+ as it declined from 2 to 5 days, and elevated at 8 days, but remained stable from 8 to 14 days after transference to FW, and a significant difference was seen at 5 days between SW and FW (ANOVA, $F = 0.287$, $p = 0.026$; Figure 4D). In contrast to the steady decline in plasma Na^+ and Cl^- of PC flounder during FW-acclimation, the plasma Na^+ and Cl^- of AC flounder fluctuated below the baseline level of SW-acclimated control group (Figure 4).

Branchial Gene Expression of NKA- $\alpha 1$, NHE-3-like, and Rhcg

In comparison with the time matched control group, the expression of NKA- $\alpha 1$ mRNA in PC flounder significantly increased from 5 to 14 days (5 days: ANOVA, $F = 12.209$, $p = 0.029$; 8 days: ANOVA, $F = 1.23$, $p < 0.001$, and 14 days: ANOVA, $F = 7.403$, $p = 0.022$; Figure 5A), while the NKA- $\alpha 1$ expression of AC flounder only significantly increased at 8 days (ANOVA, $F = 9.535$, $p = 0.02$; Figure 5B). Again compared to the time matched control, the expression of NHE-3-like mRNA in PC flounder was significantly decreased at 2 days (ANOVA, $F = 1.939$, $p = 0.033$), but remarkably increased at 5 days (ANOVA, $F = 0.49$, $p = 0.049$) and 8 days (ANOVA, $F = 0.117$, $p = 0.001$) (Figure 5C). In AC flounder, the NHE-3-like expression of FW-acclimated flounder was significantly increased at 8 days (ANOVA, $F = 8.548$, $p = 0.042$) and 14 days (ANOVA, $F = 1.618$, $p = 0.046$) compared with the SW-acclimated controls (Figure 5D). The expression of Rhcg mRNA in both of PC and AC flounders observed no significant differences between SW and FW (Figures 5E,F). Interestingly, in similarity with the expression profile of NHE-3-like gene, Rhcg expression in FW-acclimated PC flounder tended to be lower than that of SW group at 2 days and slightly higher from 5 to 14 days, but without significant difference (Figure 5E), while Rhcg expression of FW-acclimated AC flounder tended to be higher than that of SW group at 8 and 14 days, but there was no significant difference (Figure 5F).

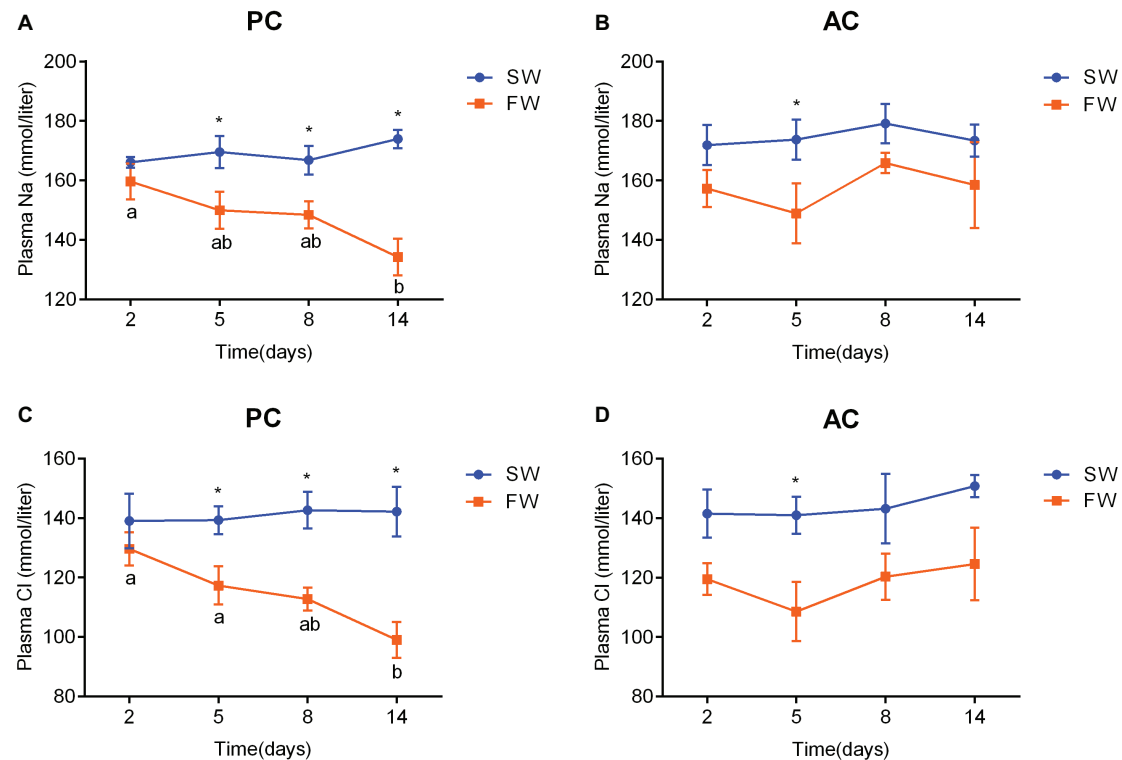


FIGURE 4 | Changes in plasma Na⁺ (A,B) and Cl⁻ concentration (C,D) of olive flounder with passive coping style (PC) and active coping style (AC) either in seawater (the SW control, ●) or after transference to freshwater (FW, ■). Data are mean ± SEM (n = 6). Different letters denote a significant difference across time within each SW or FW group (p < 0.05). An asterisk signifies a significant difference between the SW and FW groups at each time point (p < 0.05).

Schematic Model for Coping Strategies in Passive Coping and Active Coping Flounder Under Salinity Challenge

As shown in **Figure 6**, PC flounder reduced swimming activity after transfer from SW to FW within 2 days, while AC flounder showed an increase in locomotion. For osmolality and ionic content of flounder plasma, FW-acclimated PC flounder show a constant decline in their physiological profile but FW-acclimated AC flounder show a fluctuating level compared to the physiological baseline of control fish in SW. According to gene expression related to Na⁺ uptake, the gene expression of PC flounder increases sharply at 2 days after transfer to FW while the gene expression of AC flounder reveal a more delayed response, only showing a modest increase from 2 to 8 days after transfer.

DISCUSSION

Our study demonstrates marked differences in the behavioral and physiological coping strategies of PC and AC olive flounder in response to salinity challenge. The AC flounder showed higher feeding tendency and more willingness to escape under net confinement than PC flounder. AC individuals also adopt an “active resistance” defense strategy to retain plasma composition during hypoosmotic challenge, which is underpinned by initially increasing swimming activity level to escape and gradually increasing

the expression of branchial osmoregulatory genes for hypoosmotic adaptation. Conversely, PC individuals appear to employ a “passive tolerance” strategy with a decrease in the plasma osmolality and ionic content, underpinned by a decrease in swimming activity level as well as a sharp increase in the expression of branchial osmoregulatory genes including NKA-α1, NHE-3-like, and Rhcg. This is therefore the first study showing that coping strategies influence the way fish deal with salinity challenge.

Previous studies suggest that passive and active individuals respond differently to novel situations or a change in the environment and a number of different parameters can be recorded to quantify this difference in coping style. For example, the time taken to resume feeding in a novel environment, or after stress, has become the most useful and informative ways of assessing different coping styles in fish (Overli et al., 2006, 2007; Kittilsen et al., 2009; Silva et al., 2010; Martins et al., 2011c; Basic et al., 2012). Indeed, fish that resume feeding faster after transfer to a novel environment are typically ascribed to being active individuals (Martins et al., 2011a; Rupia et al., 2016). But other parameters are valuable too because the time taken to react to the presence of food or time taken to avoid stressors have also been used to assess coping style in animals (Brelvi et al., 2005; Silva et al., 2010; Laursen et al., 2011; Martins et al., 2011d). Many different parameters can therefore be used so propensity to feed and air exposure has been used in this study to distinguish behavioral coping styles. According

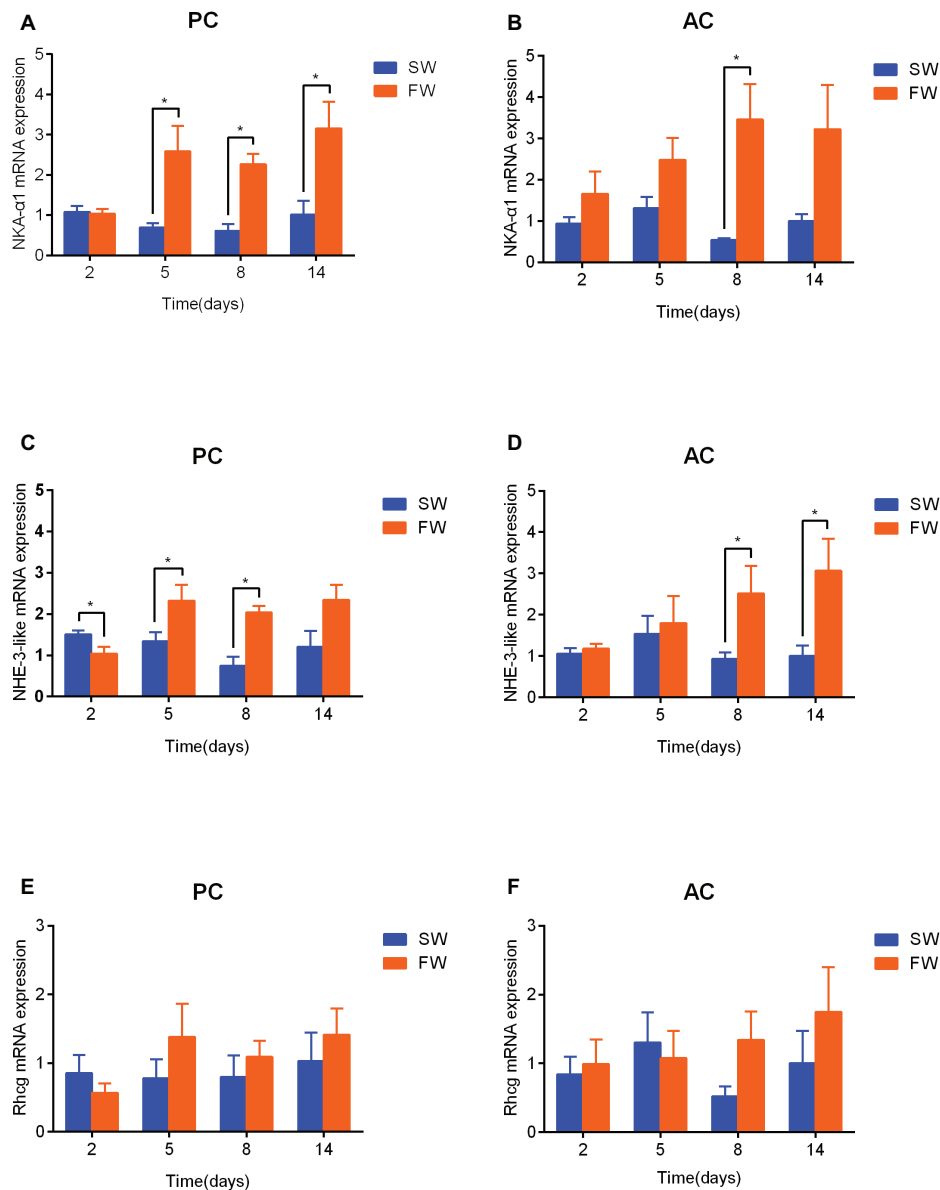
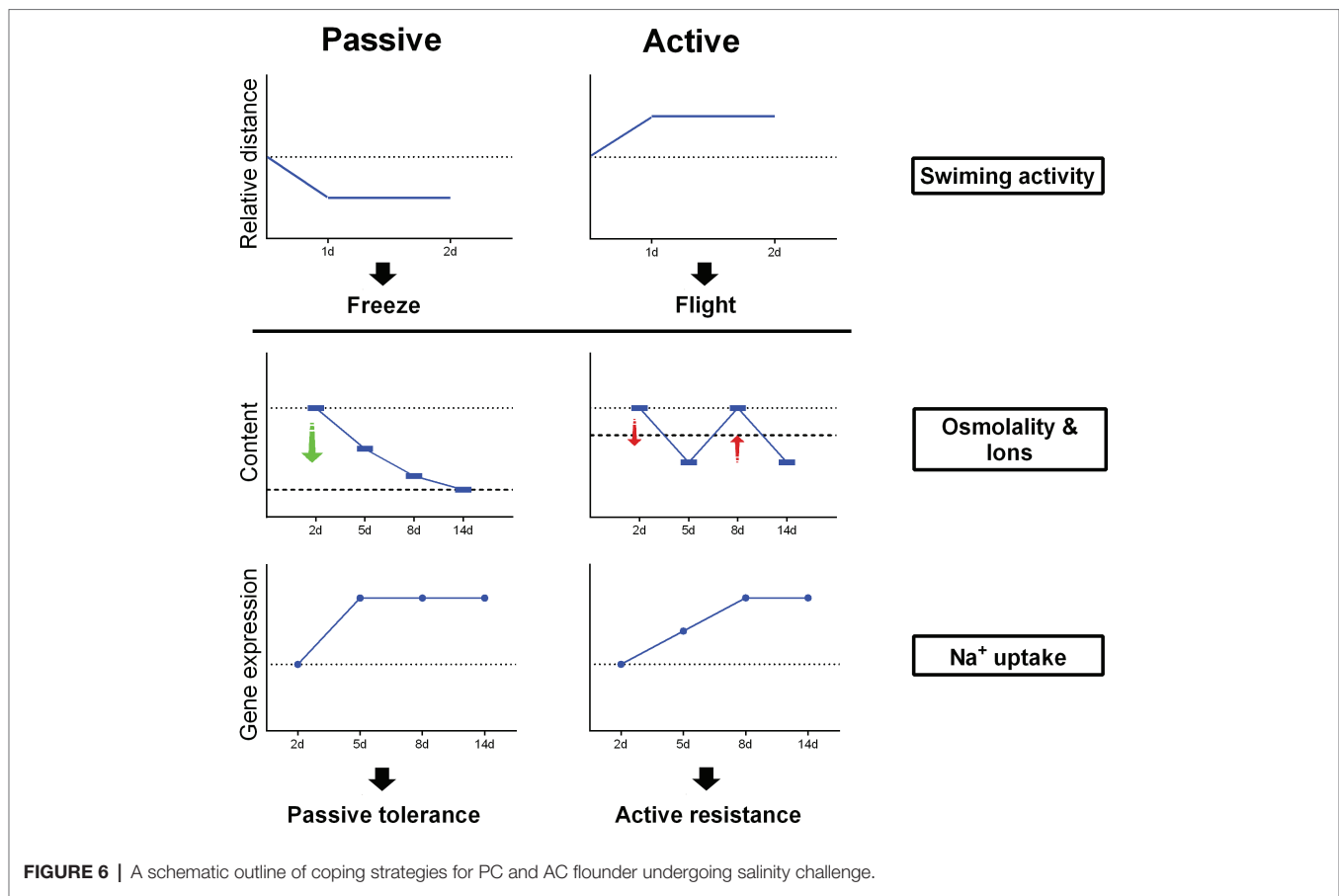


FIGURE 5 | The branchial gene expression of NKA- α 1 (A,B), NHE-3-like (C,D) and Rhcg (E,F) in passive coping style (PC) and active coping style (AC) of olive flounder after transfer from seawater (SW) to freshwater (FW). Data are mean $\pm$ SEM ($n = 6$). An asterisk signifies a significant difference between the SW and FW groups at each time point ($p < 0.05$).

to behavioral tests, the results of the PCA indicated that AC and PC flounders were divergent in the way they responded to food and net confinement. AC flounder were more active and exhibited a higher tendency to feed after isolation than PC flounder. Similar results have also been seen in wild-caught olive flounder *P. olivaceus* (Rupia et al., 2016). Likewise, active dominant Nile tilapia *Oreochromis niloticus* and Atlantic salmon *Salmo salar* have a strong appetite and are more successful in competitive feeding interactions compared to passive subordinate individuals of the same species (Fernandes and Volpato, 1993; Metcalfe, 1994). A number of studies therefore suggest that active fish had an actively escape to stressor and

have high feeding motivation and these are supportive of the results from our own study (Brelvi et al., 2005; Silva et al., 2010; Laursen et al., 2011; Martins et al., 2011b).

Bimodality in the distribution of behavioral or physiological traits or both is not uncommon in animal populations and has been documented in insects (Riechert and Jones, 2008), fish (Brown and Bibost, 2014), birds (Favati et al., 2014), and mammals (Oortmerissen and Busser, 1989). Locomotor activity is among the commonly measured behaviors in animal personality research (Le Galliard et al., 2013). In our study, both AC and PC flounder exhibited behavioral changes after transference from SW to FW within 2 days but nature of change differed between



the two coping style types. PC flounder exhibited a significant reduction in swimming activity while AC flounder significantly increased locomotion after transference during 2 days of recording. Previous studies demonstrate that active individuals employ a proactive defense response, such as flight or fight, while passive individuals employ a reactive defense response, such as freeze or hide when startled (Rupia et al., 2016). In general, the ability of animal to alter their behavior provides an adaptive advantage when facing a challenge (Dall et al., 2004) because escaping from stressors is facilitated. Moreover, active animals are willing to investigate novel environments, while passive animals are more reclusive when faced with unfamiliar situations (Overli et al., 2007; Oswald et al., 2012). Accordingly, we suggest that PC individuals faced with a hypoosmotic environment reduce their activity levels and therefore adopt a passive less energetically expensive form of “freeze” behavior, to conserve energy for FW acclimation (Brown et al., 1993). On the other hand, AC individuals adopt a proactive “flight” strategy where they increase swimming activity levels that presumably allow them to investigate a novel environment and escape. However, some AC flounders maintained higher swimming activity and began to die 8 days after transfer to FW (data not shown). These phenomena were similar to the rainbow trout *Oncorhynchus mykiss* which were exposed to severe hypoxia; non-surviving fish displayed strenuous avoidance behavior involving burst type activity while surviving fish did not panic and remained quiet (van Raaij et al., 1996).

Some researchers considered that individual variation exists in the threshold for when a stimulus becomes inhibitory or stimulatory whereby coping style is linked with the subjective experience of that stimulus in a particular situation (Martins et al., 2011a). Accordingly, AC flounder are willing to escape when faced a hypoosmotic environment, when this stimulus could not become inhibitory signals in cultured condition, finally caused energy exhaustion and become distress. The success of both coping styles might therefore depend upon the variability or stability of the environment (Koolhaas et al., 1999). But only a few studies have addressed the survival value of distinct active and passive coping styles. Notably, unpublished studies in feral populations of birds indicate that the fitness of different coping styles depends on the stability of the environment in terms of social structure and food availability (Koolhaas et al., 1999). Thus, behavioral coping strategies under environmental stress appear to be highly related to the chance of survival.

When faced with a hypoosmotic environment, the plasma osmolality and ionic levels of marine fish remain stable or decline slightly (Kelly et al., 1999; Motohashi et al., 2009), while euryhaline fish significantly change plasma conditions for freshwater adaption (Evans, 2008; Kang et al., 2008). As euryhaline fish, flounder also reduce their plasma osmolality and plasma Na⁺ and Cl⁻ (which are the dominant ions in plasma) content during SW to FW-adaption (Evans, 2008; Yuan et al., 2017). In this study, differential and dynamic changes

in plasma osmolality and ionic content of olive flounder with active or passive coping styles (AC and PC) were observed during FW acclimation. The plasma osmolality of FW-acclimated PC flounder gradually declined to below 223 mOsm at 14 days, while it fluctuated around 260 mOsm in FW-acclimated AC flounder. The pattern of plasma osmolality and ionic content of FW-acclimated PC flounder showed a significant and lasting decrease during FW-acclimation and is similar to that seen in other euryhaline teleosts such as brackish medaka *Oryzias dancena* (Kang et al., 2008) and European sea bass *Dicentrarchus labrax* (Jensen et al., 1998). In contrast, the pattern of plasma parameters in FW-acclimated AC flounder showed a fluctuation under the baseline level of the control group and is similar to the response seen in marine fish. Such results indicate that markedly different iono- and osmoregulatory processes exist between AC and PC flounders. Previous studies suggest that subordinate rainbow trout *Oncorhynchus mykiss* display higher Na^+ uptake and excretion than dominant fish under social stress (Sloman et al., 2002, 2004), with the assumption that chronic stress impacts the ionoregulatory ability of subordinate trout in a negative way. Indeed, that subordination stress results in an increased branchial efflux of sodium suggests that the ionoregulatory ability of subordinate trout is compromised (Sloman et al., 2004). Therefore, on one hand, it could be considered that PC individuals have a compromised ionoregulation based on the higher ion efflux rate compared to AC fish. Indeed, the salinity stress associated with PC flounder appeared to result in an increased branchial efflux of Na^+ , driving the need for higher uptake of Na^+ across the gills and then increasing the expression of branchial Na^+ -related gene. In contrast, it would appear that AC flounder adopt an “active resistance” defense strategy by maintaining original plasma conditions. However, on the other hand, the metabolic performance of *P. olivaceus* with active coping style or passive coping style appears to be constant between SW and FW (Rupia et al., 2016), indicating that the energy consumption of olive flounder secreting NaCl in seawater or extracting NaCl in freshwater is approximately the same. Iono- and osmoregulatory processes are achieved by various enzymes and transporters, and the synthesis and operation of these transport related proteins is energetically expensive (Tseng and Hwang, 2008). Boeuf and Payan theorized that osmoregulation costs 20–68% of the total energy expenditure in different species (Boeuf and Payan, 2001). Active individuals, however, have a prominently higher standard metabolic rate (SMR), maximum metabolic rate (MMR), and absolute aerobic scope (AAS) than passive individuals (Rupia et al., 2016) meaning that AC individuals can potentially accommodate metabolic costs more easily. Therefore, when faced with a hypoosmotic environment, AC flounder are possibly capable of spending more energy on escape and iono- and osmoregulation and thus performed higher levels of swimming activity while simultaneously maintaining original plasma osmolality and ionic concentrations. In contrast, as a result of a lower metabolic space, PC flounder were possibly forced to reduce their swimming activity and conserve energy in FW adaption with a less energetically demanding decline in plasma osmolality and ionic content.

“FW-type” ionocytes of killifish *Fundulus heteroclitus* located with NKA and NHE3-like, are responsible for NaCl uptake and appear in the fish gill after transfer from SW to FW (Hwang and Lee, 2007; Takei et al., 2014). NKA, NHE3, and Rhcg are also both involved with “NHE3 cells,” which is a major type of ionocytes (over 90%) in FW-acclimated medaka *Oryzias latipes* (Lin et al., 2012) and are responsible for Na^+ uptake, acid secretion and NH_4^+ excretion (Hsu et al., 2014). NKA creates an electrochemical gradient to drive ions (like Na^+ and Cl^-) actively across the branchial epithelia to maintain intracellular homeostasis in teleosts (Hwang et al., 2011). It had also been demonstrated that salinity challenge would change NKA activity as well as the abundance of NKA- α -subunit mRNA in fishes (Scott et al., 2004; Hwang and Lee, 2007; Moorman et al., 2014). Previous studies found that sea bass *Dicentrarchus labrax* (Varsamos et al., 2002), milkfish *Chanos chanos* (Lin et al., 2003; Hwang and Lee, 2007) and brackish medaka *Oryzias dancena* (Kang et al., 2008) both increased the NKA activity after FW acclimation. This study confirmed that branchial expression of NKA α 1 was up-regulated in both AC and PC flounders during FW-acclimation. On the other hand, NHE as a transporter to compensate Na^+ uptake has been well established (Shih et al., 2012; Chang et al., 2013; Guh et al., 2015). More recent studies demonstrated that branchial NHE expression levels and Na^+ uptake were both upregulated by low- Na^+ FW acclimation in tilapia *Oreochromis mossambicus* (Inokuchi et al., 2009), medaka *Oryzias latipes* (Wu et al., 2010) and zebrafish *Danio rerio* (Lin et al., 2008). Similarly, branchial Rhcg1 expression was also induced by low- Na^+ FW acclimation in medaka *Oryzias latipes* (Wu et al., 2010). In flounder, our study indicated that the branchial NHE-3-like and Rhcg mRNA expression of AC and PC were both up-regulated in response to FW adaption. Further evidence suggests that NHE3 and Rhcg1 were involved in fish ammonium-dependent Na^+ uptake model (Hwang et al., 2011). Thus, NKA- α 1, NHE3-like, and Rhcg are all suggested to play critical roles in sodium uptake in FW-acclimated olive flounder. However, notably, AC flounders delayed these sodium uptake related gene expression than PC flounders. Indeed, the gene expression of PC flounders increased sharply at 2 days after transference from SW to FW, while the gene expressions of AC flounders only increased modestly from 2 to 8 days after transference. Accordingly, such differences indicated that: AC flounders gradually increased the expression of Na^+ uptake related genes (i.e., NKA α 1, NHE3, and Rhcg), and was accompanied by an up-regulation of acid secretion and ammonia excretion and also enhanced the function of “NHE3 cell like” or “FW-type like” ionocytes for adaption to a hypoosmotic environment. In contrast, PC flounder show a sharply increase in these gene expressions after transfer to FW. However, a previous study has reported that SW-acclimated active flounder show a higher expression level of NKCC1 compared to passive flounder (Zou et al., 2019). NKCC, a major secretory ion transporter, is located on the basolateral side of ionocytes and is responsible for NaCl secretion in most SW-acclimated euryhaline teleost (Kang et al., 2010; Hwang et al., 2011).

Moreover, NKCC is reported to play a role in Na^+/Cl^- uptake of FW-acclimated tilapia (Hiroi et al., 2005; Hwang and Lee, 2007) and other studies propose that NKCC may also be involved in ammonia dependent Na^+ uptake by transporting the basolateral NH_4^+ (Hiroi and McCormick, 2012; Wright and Wood, 2012). Thus, due to the higher expression and reserve level of NKCC seen in AC flounder in seawater, it is suggested that AC flounder gradually increase Na^+ uptake related gene expression when faced with FW while PC flounder sharply increase these gene expression and quickly increase sodium uptake from ambient water environment. Consequently, when considering gene expression and plasma parameters collectively, PC flounder appear to adopt a “passive tolerance” strategy in their adaptation to hypoosmotic environments because plasma osmolality and plasma ionic content decreases while the expression of Na^+ uptake related osmoregulatory genes (i.e., NKA- α 1, NHE-3-like and Rhcg) increases quickly. In contrast, AC flounder retain original plasma conditions, they gradually increase the expression of NKA- α 1, NHE-3-like and Rhcg, adopt a more “active resistance” defense strategy in response to salinity challenge.

CONCLUSION

Our results demonstrate clear differences in the way that AC and PC flounders respond to acute stress. The AC flounder showed higher feeding tendency and more willing to escape under net confinement than PC flounder. AC individuals adopt “active resistance” defense strategy to retain the plasma composition during hypoosmotic challenge, which was underpinned by an initially increasing swimming activity level to escape and a gradually increasing level of osmoregulatory gene expression in the gills for hypoosmotic adaptation. Conversely, PC individuals employed a “passive tolerance” strategy with decreasing the plasma osmolality and ionic content, which was underpinned by decreasing swimming activity level and a sharp increase in the expression of branchial osmoregulatory gene, including NKA- α 1, NHE-3-like and Rhcg, to conserve energy with the osmoregulatory process. This study clearly

indicates that olive flounder with active or passive coping styles adopt different coping strategies in respond to salinity challenge which are “flight-active resistance” and “freeze-passive tolerance,” respectively. In the context of heavy rainfall and flooding, fish encounter salinity challenge for extended periods of time, and adopting different coping strategies may affect their vulnerability and mortality. This detailed insight into the coping strategies of fish improves our understanding of individual variability and welfare may therefore contribute to improved sustainability of the aquaculture industry. In addition, as the global average surface temperature increase, causing ocean warming, future studies should consider the long-term effects of wide temperature range associated with coping style for this species.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the figshare: https://figshare.com/articles/Original_Data_xlsx/9987797.

ETHICS STATEMENT

The animal study was reviewed and approved by The Animal Ethics Committee of Shanghai Ocean University (Shanghai, China). Animal procedures abide by the Guidelines on Ethical Treatment of Experimental Animals established by the Ministry of Science and Technology, China.

AUTHOR CONTRIBUTIONS

JZ, NH, and WL designed experiments and wrote the manuscript. JZ carried out experiments and analyzed experimental results.

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Dynamic Responses of the Caudal Neurosecretory System (CNSS) Under Thermal Stress in Olive Flounder (*Paralichthys olivaceus*)

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Temperature is a critical environmental factor that affect most biological and physiological processes in fish. The caudal neurosecretory system (CNSS) is unique to fish and is proved to maintain homeostasis during seasonal alterations. However, the dynamic expression and secretion pattern of its major hormones, corticotrophin-releasing hormone (CRH), urotensin I (UI), and urotensin II (UII), and their response to thermal stress has not been studied. CRH, UII and cortisol in plasma, gene expression levels of CRH, UI, and UII in the CNSS of olive flounder (*Paralichthys olivaceus*) were therefore characterized. UI- and UII-positive Dahlgren cells, as well as cell proliferation in the CNSS, were also quantified. The results showed that plasma cortisol and CRH were increased in both low temperature (LT) and high temperature (HT) groups. However, there was no difference in plasma UI and UII during thermal stress. In CNSS, CRH, UI, and UII mRNA levels were all significantly elevated in response to acute hypothermal stress and recovered back to the control (normal) level after 8 days of adaptation. During hyperthermal challenge, gene expression of CRH and UI only significantly increased after 8-days of transfer but no change in UII was observed. We also demonstrated an increasing percent of UI-positive Dahlgren cells in the CNSS of 8-days hyperthermal stressed fish. However, no BrdU-labeled Dahlgren cells were found among the three treatment groups. Collectively, our results demonstrate that the CNSS is subjected to dynamic responses under thermal stress and expands upon the role of the CNSS in thermoregulation. The dynamic responses of hormone levels and the gene expression of CRH, UI and UII in CNSS are all involved in the process of hyper- or hypo-thermal stress and adaptation.

Keywords: caudal neurosecretory system, corticotrophin-releasing hormone, urotensin I, urotensin II, thermal stress, fish

INTRODUCTION

Temperature is one of the critical abiotic factors that would affect many aspects of animals. Active thermoregulation inherent in homeothermic animals is important to preserve molecular and cellular functions critical for life (Nakamura and Morrison, 2010). In addition, almost all fish, with the exception of a few large pelagic fish (e.g., tuna, mako sharks), lack the ability to retain endogenous heat because the gills and body surface consistently and rapidly exchanges heat with the

surrounding environment (Stevens and Sutterlin, 1976). Therefore, fish cannot maintain a constant body temperature that is different from the external environment meaning that temperature is a critical abiotic factor that affects most biological and physiological processes, including growth, reproduction, digestive ability, and immune function (Hazel and Prosser, 1974; Marley et al., 2008). When fish encounter an acute or long-term change in optimal temperature, homeostatic responses and energetic reorganization are needed for survival in allostatic conditions (Juriaán et al., 2003; McEwen and Wingfield, 2003). In fish, neuroendocrine signaling affects and becomes regulated by the onset of immune responses, due to the peculiar organization of the head kidney, a hematopoietic tissue made from a mixture of endocrine, hematopoietic and immune cell populations. Besides that, responses could also be mediated by the activation of two hormonal axes in fish, the Sympatho-Chromaffin (SC) axis and the Hypothalamic-Pituitary-Interrenal (HPI) axis (Carles Balasch and Tort, 2019).

Cortisol, a common global marker of the stress, acts as a regulatory component of neuro-immuno-endocrine circuitry by binding to glucocorticoid (GR) or mineralocorticoid (MR) receptors, eliciting stress-induced immunosuppression and allostatic imbalances (Benítez-Dorta et al., 2017). Plasma cortisol is traditionally regulated by a signal pathway of corticotrophin-releasing hormone (CRH), adrenocorticotropin hormone (ACTH), within the HPI axis (Nesan and Vijayan, 2016). However, there is an additional set of neuroendocrine cells in the caudal neurosecretory system (CNSS), which can release CRH into the circulation of fish. The CNSS is located in the terminal segments of the spinal cord, including magnocellular peptide-synthesizing neuroendocrine neurons (Dahlgren cells) and a neurohemal organ (the urophysis), in which synthesized peptides can be stored and released into circulation from the capillaries of the caudal vein (Winter et al., 2000; Lu et al., 2004, 2006).

The CNSS is the major circulating source of CRH, Urotensin I (UI), and Urotensin II (UII) in fish (Lu et al., 2004, 2006). UI is a 41-amino acid peptide (Lederis et al., 1982) belonging to the superfamily of CRH, which also includes the mammalian UI ortholog, urocortin (Lovejoy and Balment, 1999; Lu et al., 2004). UII is a cyclic peptide formed by a disulfide bond at the C terminus, in which the “-Cys-Phe-Trp-Lys-Tyr-Cys-” sequence is fully conserved across vertebrate species (Lu et al., 2006). CRH and UI are known to regulate and integrate the neuroendocrine, autonomic, immune, and behavioral response of fish to stressors by affecting stress-related cortisol production (Kelsall and Balment, 1998; Craig et al., 2005). Additionally, UII is found to maintain body fluid homeostasis by regulating water and ion transport (Lu et al., 2006). Therefore, the CNSS is proposed to play a role in many aspects of adaptive physiology, including osmoregulation, reproduction, nutrition and stress-related responses (Winter et al., 2000; Lu et al., 2004, 2006). Previous studies have found that the CNSS might be functionally reprogrammed to cope with changes in physiological challenge during seasonal alternation (Lu et al., 2007; Chen and Mu, 2008). As temperature is arguably the most critical seasonal factor, the role that the

CNSS plays in thermal stress and adaptation should be resolved (McCrohan and Bernier, 2007).

The olive flounder (*Paralichthys olivaceus*) is a marine demersal species found along the coasts of Japan, Korea, and China (Sabate et al., 2008; Tomiyama et al., 2008). This flounder migrates from deep (cold) seas to shallow (warm) areas for reproduction, so thermoregulation is an important way to keep their internal environment steady. In order to better understand the physiological roles of CNSS in thermoregulation, we investigated the dynamic changes in CRH, UI and UII peptides, gene expression levels, as well as the cellular level in CNSS during thermal stress. We hypothesized that the adaptability of the olive flounder to thermal stress is associated with the dynamic response of CRH, UI, and UII in the CNSS.

MATERIALS AND METHODS

Fish and Ethics Approval

The gynogenetic olive flounder is a very popular fish for aquaculture in China. Sexual dimorphisms are potentially seen when fish are subject to stress so gynogenetic fish were used in this study. Gynogenetic olive flounder were produced as previously described (Liu et al., 2012) and reared in recirculating aquaculture systems at the Central Experimental Station of Chinese Academy of Fisheries Sciences (Beidaihe, China). The experiment was conducted in September, 2016. A total of 108 gynogenetic olive flounders (body weights: 500 ± 50 g) were randomly allocated to 24 tanks with flow-through, filtered seawater (30‰) systems at $18 \pm 1^\circ\text{C}$ for more than 2 weeks. Black plastic light-proof curtains surrounded each set of tanks and artificial illumination was provided with white fluorescent lamps. Mean light intensity was approximately 40 lux measured centrally at the bottom of each seawater tank. Fish were not fed during the experiment in order to reduce the influence of feeding. The experimental protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of Shanghai Ocean University (SHOU), Shanghai, China, and abides by the Guidelines on Ethical Treatment of Experimental Animals established by the Ministry of Science and Technology, China.

Thermal Stress Experiments

Fish were divided into six treatment groups: (1) low temperature group (LT, 12°C ; $n = 24$), (2) normal temperature group (NT, 18°C ; $n = 24$), (3) high temperature group (HT, 24°C ; $n = 24$), (4) low temperature group injected with 5'-bromo-2'-deoxyuridine, BrdU (LT + BrdU, 12°C ; $n = 12$), (5) normal temperature group injected with BrdU (NT + BrdU, 18°C ; $n = 12$), (6) high temperature group injected with BrdU (HT + BrdU, 24°C ; $n = 12$). In the BrdU treatment groups, fish were injected intraperitoneally with a single dose of saline-BrdU (Sigma, Germany; 0.2 mg/g body weight) solution within 30 s before the fish were transferred into the corresponding tank. All fish were acclimated in each experimental condition for 8 days and sampled during daytime from 10 am to 3 pm at 2 h, 1, 2, and 8 days after transfer. Fish were removed from each time point tank and, without using anesthetic, blood samples (3–5 ml)

were collected within 90 s into ammonium-heparinized syringes by caudal venepuncture. Blood was aliquoted into ammonium-heparinized tubes and plasma separated by centrifugation for 5 min at $13,000 \times g$ and stored at -80°C until the measurement of plasma hormones by ELISA. Fish were then humanely killed using spinal cord severance and brain excision. The CNSS of the fish from the LT, NT and HT groups were removed and instantly frozen in liquid nitrogen for subsequent analysis of gene expression. The CNSS from BrdU treatment groups were removed and stored in 4% paraformaldehyde (PFA) at 4°C . All samples were taken during daylight hours.

Plasma Measurements

Plasma levels of CRH, cortisol (COR), and UII were quantified by ELISA commercially (Qiyi Biotechnology Co., Ltd., Shanghai, China). According to the manufacturer's instructions, the circulating level range of COR was detected between 100 and 1800 ng/L, CRH was detected between 15 and 900 ng/L and UII was detected between 3 and 120 ng/L. In detail, -80°C stored plasma supernatant fractions were naturally warmed in an ice box. Samples were then diluted to the appropriate concentration. Commercially available ELISA kits (Qiyi Biotechnology Co., Ltd., Shanghai, China) were subsequently used to measure serum COR, CRH, and UII levels in duplicate as per manufacturer instructions (Mechesso et al., 2019).

Relative Quantitative RT-PCR

The CNSS mRNA expression levels were analyzed by quantitative real-time PCR on ABI 7500 Real-Time PCR System (Applied Biosystems, Singapore). Relative quantification of the target gene transcripts was analyzed using β -actin gene expression as the reference gene (Yuan et al., 2017). Sequences of CRH, UI, UII were obtained from GeneBank. The primers were designed using Primer Premier 5 software (PREMIER Biosoft International, Palo Alto, CA, United States), and synthesized commercially (Sangon Biotech, Shanghai, China) (Table 1). The optimization and validation of primers and probes were performed using standard ABI protocols.

Total RNA was extracted from the tissues of each individual by RNAiso Plus (TaKaRa, Japan). One microgram of total RNA was treated by PrimeScriptTM RT reagent kit with gDNA Eraser. Briefly, quantitative real-time PCR assays were run using FastStart Universal SYBR Green Master kit (Roche,

United States), in 20 μl reaction volume, under a standard amplification procedure (2 min at 50°C , 10 min at 95°C and then 40 cycles of the following process: 15 s at 95°C and 30 s at 60°C).

UI, UII, and BrdU Immunofluorescence Chemistry

Paraformaldehyde-fixed CNSS were dehydrated in ethanol, cleared in xylene and embedded in paraplast. Every section (5- μm -thick) was cut on a microtome and mounted on glass slides with a positive charge. Briefly, tissue sections were dewaxed in xylene and rehydrated in gradient alcohol. Endogenous peroxidase activity was blocked with 3% H_2O_2 in methanol before slides were placed in 0.01M citrate buffer and heated in a water bath for 20 min at 95°C . After cooling, sections were rinsed in PBS. For UI and UII immunofluorescence chemistry, sections were treated with fetal bovine serum (FBS) blocking solution (1% blocking, dissolved in MABT, and 5% FBS in PBST, PBS with 0.1% Triton X-100) for 1 h at room temperature (RT) to reduce non-specific staining and incubated with 1:500/1:1000 rabbit anti-UI/UII antibodies [produced by Lu et al. (2004, 2006)] diluted with PBS in a moist chamber at 4°C overnight. The moist chamber was transferred into an air oven at 37°C for 45 min, then washed six times at RT in PBST for 15 min each time and incubated with 1:100 DAPI and 1:500 goat anti-rabbit IgG (H + L) highly cross-adsorbed secondary antibody, Alexa Fluor Plus 488 (Thermo Fisher Scientific, United States), diluted with PBS for 1 h in dark. The slides were washed six times in 5% FBS (in PBST) for 90 min. For BrdU immunofluorescence, sections were incubated in 2 N HCl for 30 min at RT, followed by thorough washing in PBS and blocked with 5% FBS in PBST for 1 h at RT. Sections were incubated with 1:100 mouse anti-BrdU antibody (Sigma-Aldrich, Germany) diluted with PBS in a moist chamber at 4°C overnight. After thorough buffer rinses, the sections were incubated with 1:100 DAPI (Sangon Biotech, Shanghai, China) and 1:500 goat anti-mouse IgG (H + L) highly cross-absorbed secondary antibody, Alexa Fluor Plus 568 (Thermo Fisher Scientific, United States), diluted with PBS for 1 h in dark. Then the sections were washed three times in PBST. Control experiments were carried out by omission of the primary antibody and preabsorption of the antibody with an excess of antigenic peptide.

Quantification of Labeled Cells

Single-labeling UI/UII and BrdU experiments of short and long term thermal stress were used for quantification of protein secretion and cell proliferation. Cell counts of UI/UII and BrdU labeling were obtained within each CNSS in every 20 sections from all sections sampled (5 μm -spaced serial sections parallel to the entire central canal axis of the CNSS) at $400\times$ magnification with a fluorescence microscope (Nikon ECLIPSE 55i, Nikon Corporation, Japan). The UI-/UII- and BrdU-positive cells, as well as all Dahlgren cells, were counted. The numbers of UI-/UII-positive Dahlgren cells were expressed as the proportion of total Dahlgren cells, while the numbers of BrdU-positive cells were counted as the fraction of BrdU-positive cells per unit area of the CNSS. Size and luminosity of the figures were modified

TABLE 1 | Gene specific primers for β -actin, CRH, UI, and UII of olive flounder *P. olivaceus*.

Gene	GeneBank	Primers (5'-3')
β -actin	HQ386788.1	F: GGAAATCGTGCGTGACATTAAG R: CCTCTGGACAACGGAACCTCT
CRH	XM_020087578.1	F: AAAGGAGGTGAAGGAGGA R: AAGAAGGCAACAAGCAGA
UI	XM_020105024.1	F: GACCTGCTGAGCGACAA R: TCATCCTCGGCTATCTGG
UII	XM_020096040.1	F: ATCTGCTGAGATGCCCTATC R: CTGTTGTTCTCCACCGTCTC

with NIS-Elements Version 4.0 (Nikon, Japan). The graphs and drawings were prepared using Adobe PhotoshopCS 6 (Adobe System, United States).

Statistics

The $2^{-\Delta\Delta C_t}$ method was used to analyze the real-time PCR data (Livak and Schmittgen, 2001), and amplified transcripts were expressed as the fold change relative to the mean value of the standard sample. Data were tested for normality by the Shapiro-Wilk's test and homogeneity of variance by Levene's test, and expressed as means $\pm$ SEM. Significant analyses were conducted by two-way ANOVA with treatment and time as independent variables, followed by the Tukey's multiple comparison test when changes in data were assessed for each treatment or time. Significant effects of temperature treatment in BrdU positive cells were conducted by one-way ANOVA with Dunnett's test. Results were considered significantly different when $P < 0.05$. All analyses were conducted using a computer program, GraphPad Prism 5.0 (San Diego, CA, United States).

RESULTS

Plasma Hormones

Significant interactions between temperature and time were only detected for cortisol concentrations in plasma [two-way ANOVA,

$F(6,30) = 6.677$, $P = 0.0027$], indicating that stress hormone responses were different among the temperature treatments at different time points. After 2 h thermal stress, plasma cortisol was significantly increased to the highest level in both LT ($P = 0.0075$) and HT ($P = 0.0036$) groups. Subsequently, cortisol levels declined significantly with time in both HT (Tukey's test, 2 h vs. 1 day: $P = 0.0036$; 2 h vs. 2 days: $P < 0.0001$; 2 h vs. 8 days: $P < 0.0001$; 1 day vs. 2 days: $P = 0.0042$) and LT (Tukey's test, 2 h vs. 1 day: $P = 0.0075$; 2 h vs. 2 days: $P = 0.0009$; 2 h vs. 8 days: $P < 0.0001$; 1 day vs. 8 days: $P = 0.0472$) treatments (**Figure 1A**). Plasma CRH level increased but not significantly more than the NT group in both LT and HT groups during thermal stress treatments. A statistically significant increase of CRH level was only found in LT group at 1 day ($P = 0.0058$, **Figure 1B**). There was no remarkable difference with time or temperature treatments for plasma UII during the experiment (**Figure 1C**).

CRH, UI, and UII Gene Expression in CNSS

Significant interactions between temperature and time were observed for CRH [two-way ANOVA, $F(6,30) = 3.51$, $P = 0.0123$], UI [two-way ANOVA, $F(6,30) = 6.045$, $P = 0.0006$] and UII [two-way ANOVA, $F(6,30) = 13.08$, $P < 0.0001$] gene expression in CNSS, indicating that gene expression of CRH, UI and UII in CNSS was different among the temperature treatments at

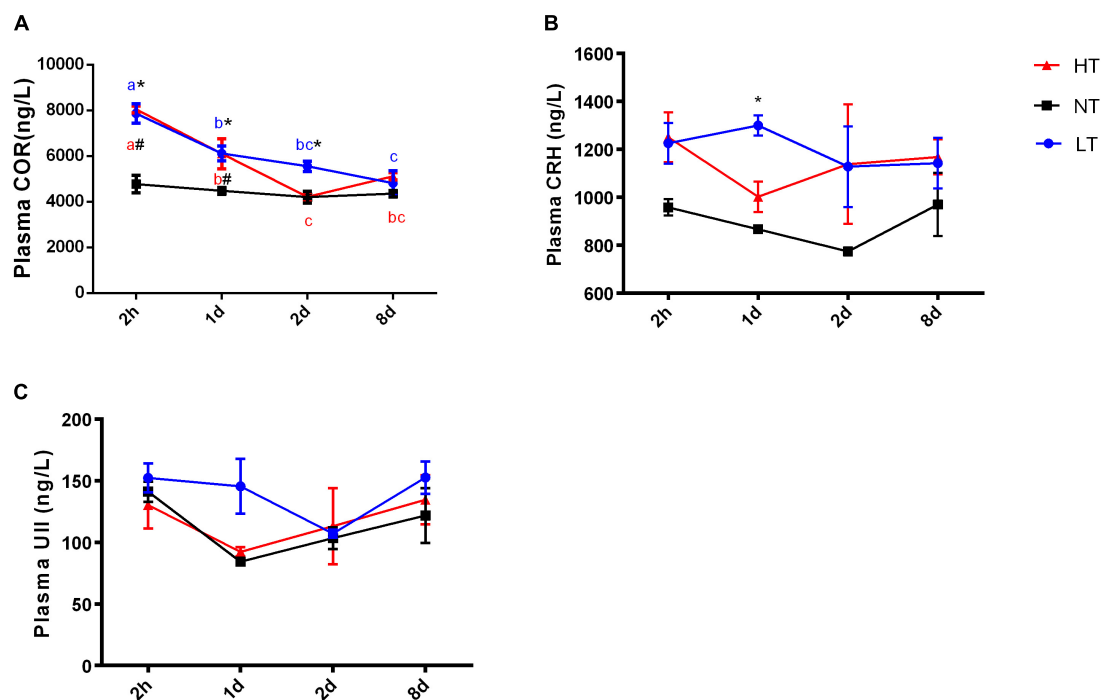


FIGURE 1 | Dynamic changes in plasma cortisol (A), CRH (B), and UII (C) after transfer of olive flounder from NT to LT (●) or HT (▲). Mean $\pm$ SEM ($n = 6$). Control fish were maintained in NT and sampled across the same time course (■). Significant analyses were conducted by two-way ANOVA with treatment and time as independent variables, followed by the Tukey's test. Results were considered significantly different when $P < 0.05$. Symbol "*" or "#" was used to represent the significant difference between NT and LT or HT within a time point. Different small letters were used to represent a significant difference across time points within a treatment group.

different time points. The expression of these three genes was therefore compared between the different thermal stress and control treatments at each sampling time, and changes in gene expression were assessed for each thermal stress.

Two hours after hypothermia, the gene expression of CRH and UII in CNSS was significantly higher than the control group, but UI did not show a statistically significant increase (Tukey's test, CRH: $P = 0.0054$, UII: $P < 0.0001$). Subsequently, gene expression of CRH (Tukey's test, 8 days vs. 2 h: $P = 0.0058$; 8 days vs. 1 day: $P = 0.0127$) and UII (Tukey's test, 2 h vs. 1 day: $P < 0.0001$; 2 h vs. 2 days: $P < 0.0001$; 2 h vs. 8 days: $P < 0.0001$; 2 days vs. 8 days: $P = 0.0272$) declined significantly with time (Figures 2A–C). In contrast, the gene expression of UI showed a remarkable increase in CNSS 8 days after hyperthermia (Tukey's test, $P = 0.0001$) with a significant increase with time (Tukey's test, 8 days vs. 2 h: $P = 0.0004$, 8 days vs. 1 day: $P = 0.0007$, 8 days vs. 2 days: $P = 0.003$; Figure 2B). There was no significant difference with time in CRH and UII mRNA expression during the experiment (Figures 2A,C).

Count of UI and UII Positive Dahlgren Cells in CNSS

Since UI and UII were produced by Dahlgren cells in CNSS, a count of UI and UII positive Dahlgren cells could represent the storage and secretion level of these two hormones in the CNSS. Dahlgren cells were distributed in the spinal cord dorsal to the 1st–6th preterminal vertebrae and appeared laterally and ventrolaterally to the central canal in olive flounder (Figures 3C–H). Significant interactions between temperature and time were measured for the percent of UI-positive Dahlgren cells [two-way ANOVA, $F(6, 12) = 5.412$, $P = 0.0064$]. The percent of UI-positive Dahlgren cells showed a remarkable increase in CNSS after 8 days of hyperthermia (Tukey's test, $P = 0.0017$) with a significant increase over time (Tukey's test, 8 days vs. 2 h: $P = 0.0007$; 8 days vs. 1 day: $P = 0.0032$; 8 days vs. 2 days: $P = 0.0173$; Figure 3A). For the LT group, the percent of UI-positive Dahlgren cells did not show any difference with time. Meanwhile, the percent of UII-positive Dahlgren cells increased. But, it had no significant effect of temperature or time (Figure 3B).

Count of BrdU Positive Cells in CNSS

According to the changes of UI and UII positive Dahlgren cells in CNSS, BrdU positive cells in CNSS were counted after 8 days of thermal stress. However, no BrdU-positive Dahlgren cells were observed in either LT or HT treatment groups. BrdU-labeled cells were found near the central canal (Figures 4B–D). Compared to the NT group a significant decrease in BrdU-positive cells was found in both LT (Dunnett's test, $P = 0.0491$) and HT (Dunnett's test, $P = 0.0244$) groups (Figure 4A).

DISCUSSION

When fish encounter an acute thermal stress, as with many other stressors, stress-related physiological responses are immediately activated. For instance, the stimulation of the HPI axis elicits

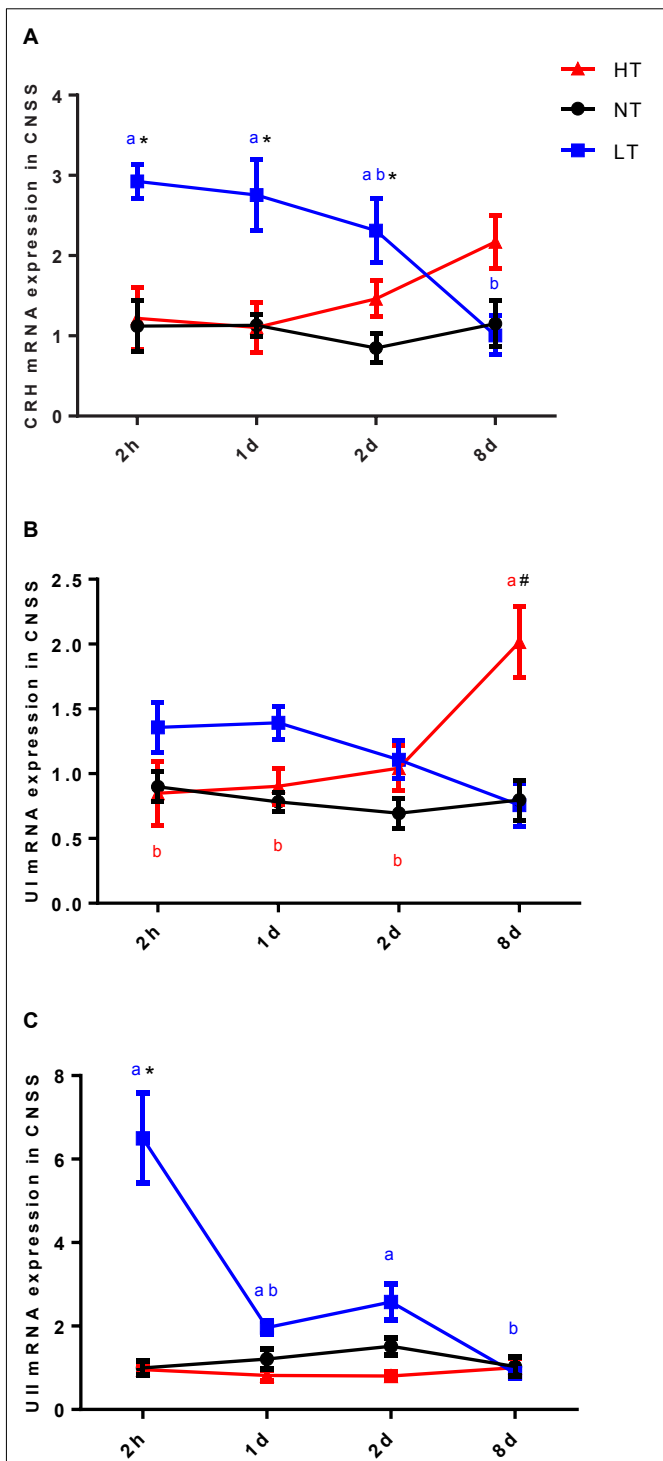
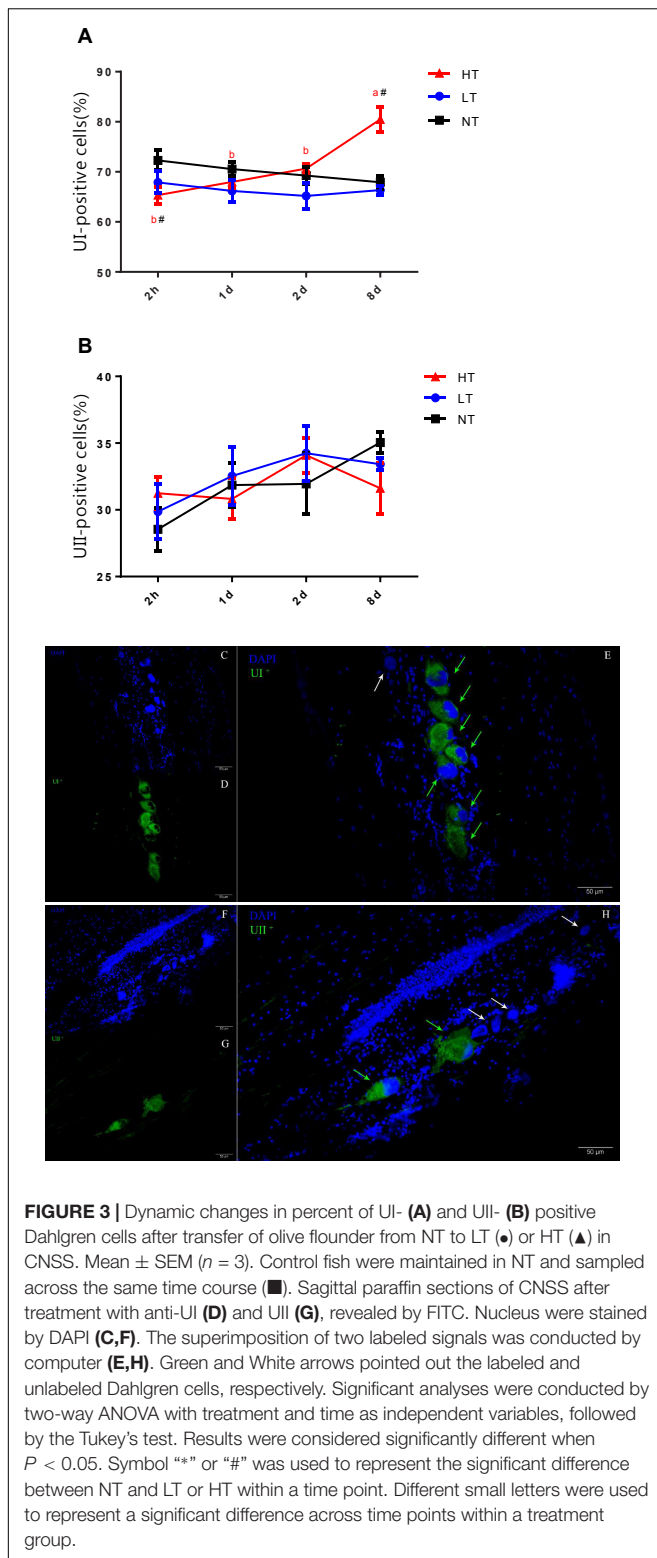
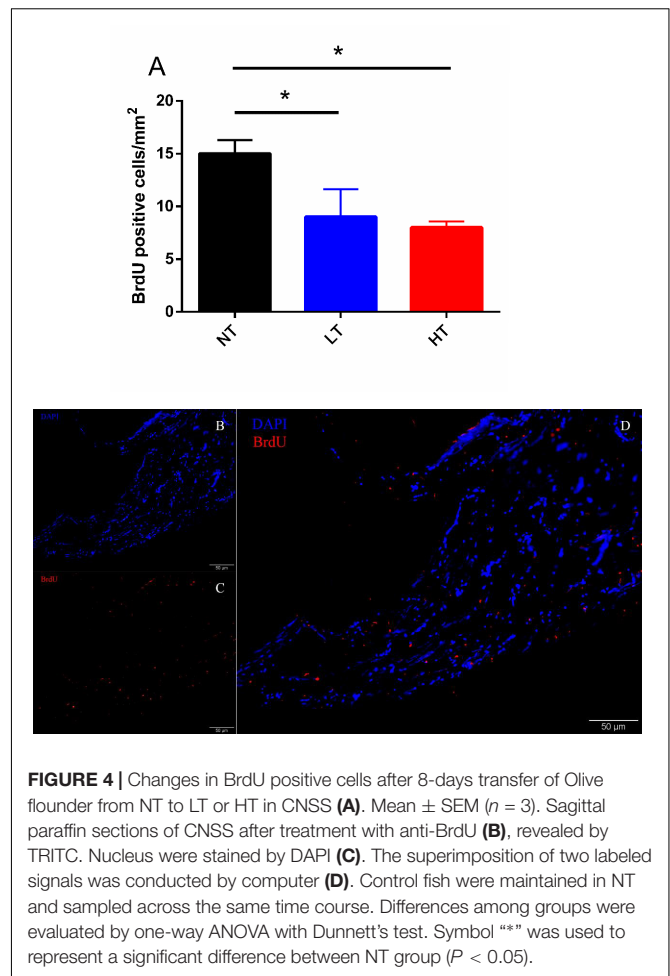


FIGURE 2 | Dynamic changes in gene expression of CRH (A), UI (B), and UII (C) after transfer of Olive flounder from NT to LT (●) or HT (▲) in CNSS. Mean $\pm$ SEM ($n = 6$). Control fish were maintained in NT and sampled across the same time course (■). Significant analyses were conducted by two-way ANOVA with treatment and time as independent variables, followed by the Tukey's test. Results were considered significantly different when $P < 0.05$. Symbol “*” or “#” was used to represent the significant difference between NT and LT or HT within a time point. Different small letters were used to represent a significant difference across time points within a treatment group.



an increase in plasma cortisol. Therefore, previous studies had generally put more attention on stress-related regulation of CRH and UI in the brain (Pepels et al., 2004; Pinkel et al., 2007). However, there is an additional area in central nervous system



(CNS) which is an important source of CRH, UI and UII in fish, the CNSS, but its function still remains unclear. In this study, we elaborated the dynamic changes of cortisol, CRH and UII in plasma, as well as their expression, changes in UI:UII Dahlgren cell ratio and the rate of cell proliferation in the CNSS during thermal stress. Overall, our results confirmed that there was a dynamic response of hormone levels and gene expression of CRH, UI and UII in CNSS associated with hyper- and hypo-thermal stress and thermal adaptation.

Using the ELISA kits to analyze the plasma cortisol in olive flounder, previous study showed that the plasma cortisol level in control group was around 5 ng/mL which was similar with our results (around 4 ng/mL) (Mechesso et al., 2019). Comparing with the results of RIA, the control level of cortisol was around 8 ng/mL (Zou et al., 2018). It had also been found that acute stress elevated plasma cortisol levels within minutes to hours (Pavlidis et al., 2015) but plasma cortisol declined back to pre-stress levels after long-term acclimation (Hosoya et al., 2007). Our results showed that plasma cortisol in both LT and HT group were dramatically elevated about 65–69% after 2 h thermal stress and declined with time to the control level at 8 d. Previous evidence showed that CRH played a key role in regulating the secretion of cortisol (Flik et al., 2006). However, UI and UII were

also shown to stimulate the secretion of cortisol from isolated interrenal/head kidney preparations of seawater (SW)-adapted flounder (Kelsall and Balment, 1998), and UI also appeared to potentiate the steroidogenic actions of ACTH *in vitro* (Arnold-Reed and Balment, 1994). Therefore, plasma CRH and UII concentration were measured in the current study and it was found that the profile of plasma CRH matched closely with the trend in cortisol secretion but plasma UII did not respond to thermal stress. Studies pointed out that changes in CRH-related peptide secretion from the preoptic area (POA) and CNSS could impact upon the activity of the HPI axis (Huising et al., 2004; Bernier et al., 2008). As the CNSS is the major source of circulating CRH, UI and UII, we therefore, investigated the gene expression and secretion of these hormones from the CNSS.

Previous studies had found that thermal stress significantly changed CRH, UI, and UII expression at early developmental stage in zebrafish (Luo et al., 2014). In adult rainbow trout, salinity challenge produced a remarkable elevation in CRH and UI mRNA expression in CNSS (Craig et al., 2005). However, in this study, we found that CRH, UI, and UII mRNA levels exhibited different expression profiles not only as a result of the thermal conditions but also as a result of exposure time. Previous studies showed that additional UI and UII directly stimulate interrenal cortisol secretion in fish (Kelsall and Balment, 1998; Craig et al., 2005). The mRNA expression of CRH, UI and UII in CNSS was elevated after a 2 h period of hypothermal acclimation and returned back to the control level at 8 days. In contrast, gene expression of CRH, UI, and UII did not increase at the beginning under hyperthermal condition in CNSS. An increasing expression of CRH and UI was observed at 8 days hyperthermal treatment and a similar trend was seen in rainbow trout subject to a hyperammonemia condition (Bernier et al., 2008). The current mRNA expression results supported the view that CRH-, UI- and UII-expressing neurons in the CNSS could be recruited differentially and only in response to specific stimuli. From the expression profiles of these three genes, it is plausible that CRH, UI and UII in CNSS respond to acute hypothermal stress. By contrast, CRH and UI in CNSS did not respond to acute hyperthermal stress, but appeared to play an important role in hyperthermal adaptation, although the functional mechanism is less clear.

The number of Dahlgren cell were altered across seasons (Chen and Mu, 2008). However, until now, it was not known whether alterations in temperature would affect the number of Dahlgren cells and their associated secretions. Morphology and immunohistochemistry studies of the CNSS suggested that CRH, UI and UII were produced and secreted from the Dahlgren cells in the CNSS (Cioni and Bordieri, 2003; Lu et al., 2004). Therefore, the number of CRH-, UI- and UII-positive Dahlgren cells could represent the required secretory level of these genes in CNSS. In this study, we calculated the changes in UI:UII Dahlgren cell ratio and our results demonstrated that the percent of UI-positive Dahlgren cells increased after 8 d of hyperthermal treatment, and significantly higher than the NT group, with an additional match to the mRNA expression profile of UI in CNSS. No changes on percent of UI- or UII- positive Dahlgren cells were observed in the other treatment groups. To find out where

the increasing percent of UI-positive Dahlgren cells came from, we used BrdU to label the new cells after thermal treatment and calculated the BrdU-labeled cells in CNSS. However, the number of BrdU-labeled cells in both LT and HT group were significantly lower than the NT group after 8 days thermal treatment. Additionally, no BrdU-labeled Dahlgren cells were observed in the CNSS (Figures 4B–D). Previous studies found that the neuroblasts lateral to the central canal were the precursor cells of Dahlgren cells (Fridberg, 1962). Therefore, the BrdU-labeled cells might partially be the regenerative neuroblasts, and the increased UI-positive Dahlgren cells might not be differentiated from newly regenerative neuroblast cells. Previous morphology and immunohistochemistry studies of the CNSS have found that CRH and UI were co-expressed (Cioni and Bordieri, 2003; Lu et al., 2004). Meanwhile, UI and UII could also be co-expressed in a fraction of Dahlgren cells (Parmentier et al., 2006). Therefore, the increased UI-positive cells might be recruited from the Dahlgren cells that expressed UII peptides. In brief, our results found that the percent of UI-positive Dahlgren cells increased after 8 days of hyperthermal treatment and the increasing percent of UI-positive Dahlgren cells might be due to the increased number of Dahlgren cells that co-expressed both UI and UII.

CONCLUSION

In summary, this study demonstrates that the dynamic responses of hormone levels and the gene expression of CRH, UI and UII in the CNSS are associated with the whole process of hyper- or hypo-thermal stress response and adaptation. However, CRH-, UI- and UII-expressing neurons of the CNSS are recruited differentially and only in response to specific stimuli. Specifically, CRH, UI and UII in CNSS respond to acute hypothermal stress. By contrast, CRH and UI do not respond to acute hyperthermal stress, but appear to play a currently unknown role in hyperthermal adaptation. Our results indicate that the increase of UI-positive cells might originate from the increased Dahlgren cells that co-expressed both UI and UII during long-term hyperthermal adaptation in CNSS. Overall, our results supplements evidence that CNSS plays an important physiological role in thermal stress. Further research should focus on the specific thermal neural inputs that trigger the recruitment of CRH-, UI-, and UII-expressing cells in the CNSS.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The experimental protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of Shanghai Ocean University (SHOU), Shanghai, China, and abides by

the Guidelines on Ethical Treatment of Experimental Animals established by the Ministry of Science and Technology, China.

AUTHOR CONTRIBUTIONS

WL and MY designed the study. MY and YC collated the data. MY, XL, and TL carried out the experiment, data analyses, and produced the initial draft of the manuscript. WL and MY contributed to drafting the manuscript. All authors participated in the manuscript revision, and have read and approved the final version of the manuscript.

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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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Broad Thermal Tolerance in the Cold-Water Coral *Lophelia pertusa* From Arctic and Boreal Reefs

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Along the Norwegian coasts and margins, extensive reefs of the stony coral *Lophelia pertusa* act as hotspots for local biodiversity. Climate models project that the temperature of Atlantic deep waters could rise by 1–3°C by 2100. In this context, understanding the effects of temperature on the physiology of cold-water species will help in evaluating their resilience to future oceanic changes. We investigated the response of *L. pertusa* to stepwise short-term increases in temperature. We sampled corals from four reefs, two located north of the Arctic circle and two at the mid-Norwegian shelf (boreal). In on-board experiments (one per reef), the sampled fragments were exposed to increasing temperatures from 5 to 15°C over 58 h. Respiration increased linearly by threefold for a 10°C increase. The short-term temperature increase did not induce mortality, cellular (neutral red assay for lysosome membrane stability; but one exception) or oxidative stress (lipid peroxidation assay) – to a few exceptions. However, the variability of the respiration responses depended on the experiment (i.e., reef location), possibly linked to the genetic structure of the individuals that we sampled (e.g., clones or siblings). The corals from the Arctic and boreal regions appear to have a high tolerance to the rapid temperature fluctuations they experience in the field. Over extended periods of time however, an increased metabolism could deplete the energy stored by the corals, if not met by an increased food availability and/or uptake. Empirical data on organisms' thermal performance curves, such as the one presented in this study for *L. pertusa*, will be useful to implement predictive models on the responses of species and populations to climate change.

Keywords: cold-water corals, global warming, stress physiology, temperature, respiration

INTRODUCTION

Reefs of *Lophelia pertusa* (syn. *Desmophyllum pertusum*), a widely spread cold-water coral species, are found in cold (4–15°C) waters around the globe, at depths of 40–3300 m (Roberts et al., 2009). The main proportion of large and healthy-looking *L. pertusa* reefs occur in temperatures between 6 and 9°C. Most reefs occur in the NE Atlantic, but reefs have also been observed on the NW and SE Atlantic and the Mediterranean Sea, and with some records in the Pacific and Indian oceans (see review by Roberts et al., 2006, 2009; Freiwald et al., 2017). *L. pertusa* reefs act as local biodiversity hotspots in these regions, offering a three-dimensional structure to support a diverse community

(e.g., Henry and Roberts, 2017). At least 1300 associated species have been identified in *L. pertusa* reefs from the NE Atlantic (Roberts et al., 2006).

Due to climate change, bathyal waters of the Atlantic are warming nearly as fast as the surface waters (Chen and Tung, 2014) and models project abyssal/bathyal waters to further increase by 1–3°C before 2100 (see review by Sweetman et al., 2017). In this context, it is critical to estimate the vulnerability of deep-sea ectotherms to temperature changes, in particular cold-water species such as *L. pertusa*. In ectotherms, respiration is expected to increase exponentially to almost linearly with acute temperature elevation, until reaching a short plateau and then collapsing at high, lethal temperatures (see review by Schulte, 2015). The breadth of the respiratory thermal window (narrow vs. broad) has been used as an indicator of the thermal tolerance/phenotypic plasticity of corals (e.g., Coles and Jokiel, 1978; Lesser, 1997; Nyström et al., 2001; Castillo and Helmuth, 2005; Castillo et al., 2014). Thermal tolerance of tropical and a few temperate corals has been correlated to their thermal history, with more tolerant corals in variable environments or pre-exposure to high temperatures (e.g., Brown et al., 2002; Oliver and Palumbi, 2011; Guest et al., 2012) although this relationship is in part linked to the presence of zooxanthellae (absent in *L. pertusa*).

Despite living in deep waters, *L. pertusa* reefs probably experience a relatively high variability in temperature. The reefs are often situated in areas with complex hydrodynamics where cold water masses can be rapidly displaced by warmer water masses either horizontally or vertically, e.g., by the meandering of the Gulf Current in the NW Atlantic or by the action of internal waves in the NE Atlantic (e.g., Mienis et al., 2007; Brooke et al., 2013; Findlay et al., 2015). Moreover, seasonal changes can be broad, for instance, Godø et al. (2012) recorded temperatures from 5.5 (April 2010) to 9.0°C (November 2010) at the Hola Reef (northern-Norway). The Hola Reef also experiences small-scale variations with a strong short-term and cyclic pattern over a period of 12 h (Van Engeland et al., 2019). In other *L. pertusa* reefs, temperature changed by 1°C over periods of two (Mingulay Reef in west-Scotland, Dodds et al., 2007) to three weeks (Sula Reef in mid-Norway, Roberts et al., 2005). Even broader changes were recorded at the Tisler Reef (southern-Norway), where temperature changed by 4°C in <24 h as the consequence of rapid downwelling of warm surface water (Guihen et al., 2012).

While predictive modeling established that temperature is a major factor controlling the distribution of cold-water corals (Davies et al., 2008; Davies and Guinotte, 2011 but see Tittensor et al., 2009), the knowledge on the relationship between a broad range of temperature and cold-water coral physiology is fragmentary. Brooke et al. (2013) observed full survival in *L. pertusa* from the West Atlantic exposed to 5–15°C for 24 h, but 100% mortality when corals were exposed to 25°C (see also Lunden et al., 2014: full mortality for seven days at 16°C). *L. pertusa*'s respiration increased by 35% when temperature was increased from 9 to 11°C (Dodds et al., 2007). These last authors also observed that respiration decreased by 45% for a rapid temperature change from 9 to 7.5°C. Yet, Naumann et al. (2014) found no difference in respiration rates when decreasing

temperature gradually over three months from 12 to 6°C, a possible sign of long-term acclimatization.

As temperature is rising in the ocean, and particularly in the deep waters of the Atlantic (+0.2–0.3°C since 2000; Chen and Tung, 2014), understanding the effects of temperature on the physiology of cold-water species will help in evaluating their resilience to future oceanic changes. In this study, we investigated the response of *L. pertusa* to a short-term temperature rise (+10°C in 58 h) in metabolism (respiration and NH_4^+ excretion) and in cellular stress (lysosome stability and lipid peroxidation). This temperature range encompasses the known distribution of *L. pertusa* (from 4°C in Greenland to 14°C in the West Atlantic). The design of this study allows to identify potential physiological “tipping-points” within this range (e.g., linear or bell-shaped relationship between respiration and temperature) and to explore mechanisms underlying physiological processes. Corals were sampled from four reefs along the Norwegian coasts and margins (Figure 1A). We present insight on the potential resilience of this cold-water coral to increasing temperature.

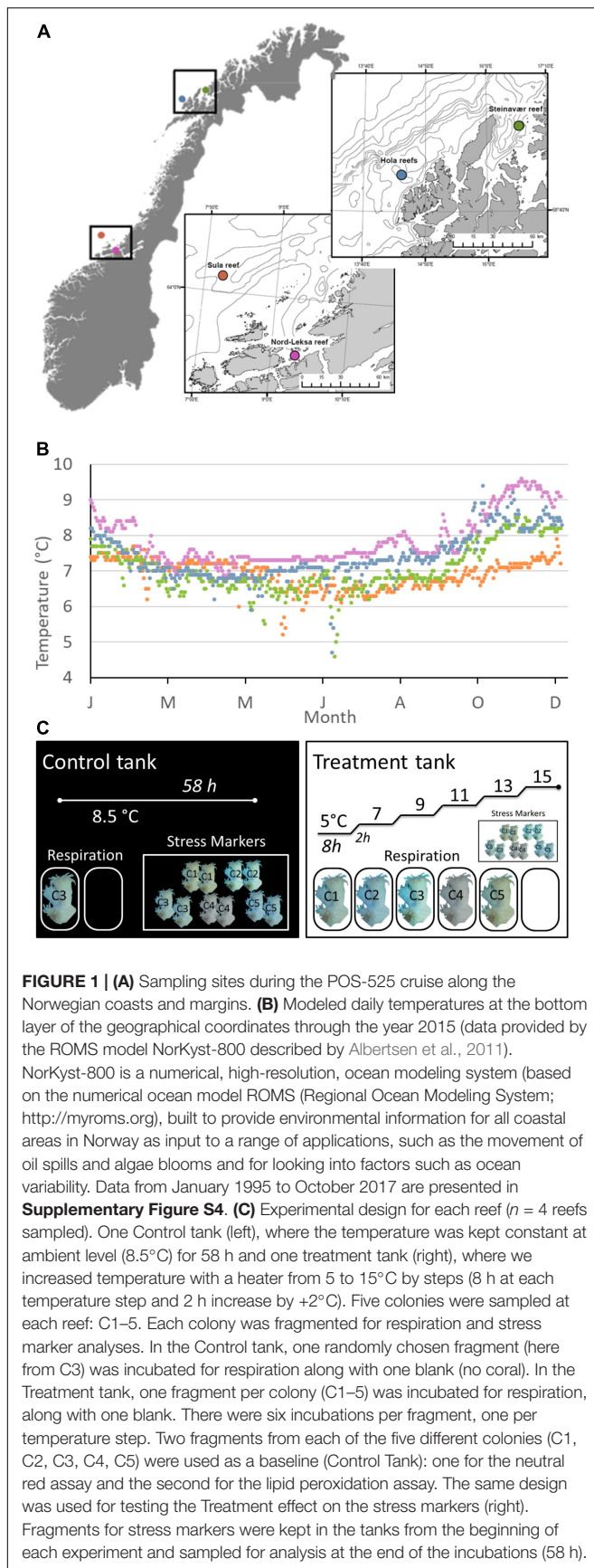
MATERIALS AND METHODS

Sampling Sites: Hydrography and Reef Characteristics

Two water masses dominate the deeper water layers of the Norwegian shelf. Firstly, the Atlantic Water mass (salinity > 35.0) enters the Norwegian Sea in the Faeroe Shetland Channel flowing northwards, roughly following the 500 m isobath on the shelf break. Secondly, the Norwegian Coastal Water mass (salinity < 35.0) flows northwards from the eastern Skagerrak. This water mass is a mixture of Atlantic Water, fresh water from the Baltic Sea and land run-off (Sætre, 2007). The Norwegian Coastal Water mass extends between 50 and 150 m depth (depending on latitude), while the Atlantic Water mass predominates below it.

Reefs of the Norwegian continental shelf and fjords are affected by variations in temperature and salinity primarily driven by meteorological conditions, fresh water input, and variations in the inflow of Atlantic Water into the Norwegian Sea (Sætre, 2007). Wind events and tidal motions can cause up-welling and down-welling and rapid short-term changes in temperature and salinity at depth (e.g., Thiem et al., 2006; Davies et al., 2009). The reefs – and more so reefs closer to the continental shelf break – can also be sporadically affected by deeper masses: e.g., seasonal fluctuations in bottom water temperature (of 1–2°C) in the Hola Reef is correlated to the seasonal fluctuation of the cline separating Atlantic Water and the Norwegian Sea Arctic Intermediate Water (Mork and Skagseth, 2010; Godø et al., 2012).

For the purpose of this study, we sampled corals from four reefs of the Norwegian waters: two reefs were situated in the boreal realm (mid-Norway) and two in the Arctic realm (northern Norway); within each realm, one coastal and one continental shelf reef were sampled (Figures 1A,B). In the boreal realm, the Sula Reef Complex (64°06.57' N, 08°07.12' E, 300 m depth) is located on the mid-Norwegian continental shelf. It



is the second largest reef complex documented in Norway and is composed of densely packed *L. pertusa* dome-shaped reefs growing along lineations and iceberg scour marks on three main ridges (Mortensen et al., 2001). The Nord-Leksa Reef (63°36.46 N, 09°22.89 E, 200 m depth) is located 90 km east of the Sula Reef, in the mouth of Trondheimsfjorden. Nord-Leksa Reef is composed of very densely packed *L. pertusa* domes forming one large (500 × 200 m) and two smaller patch reefs on a north-eastward trending sub-marine ridge (Mortensen et al., 2001). In the Arctic realm, the Steinavaer Reef (69°14.68 N, 16°38.47 E, 200 m depth) is a large coastal hill reef (700 × 1500 m) growing in steep terrain on top of a northwesterly/south-southeasterly directed topographical elevation between Selfjorden and Andfjorden (Fosså et al., 2015). The last sampling was done within the Hola Reef aggregation (68°54.96 N, 14°24.10 E, 300 m depth). Located in the Hola Trough, this cold-water coral reef aggregation is situated ~25 km from the shelf break, and includes 414 elongated coral reefs (100–200 m long) that have been detected using underwater video and multi-beam bathymetry (Bøe et al., 2009).

Animal Collection

All required permits were obtained from the Norwegian Fisheries Directorate for collection (reference number: 17/19618). Colonies of *L. pertusa* were collected in July 2018 at the four different reefs described above with RV POSEIDON (GEOMAR Helmholtz-Zentrum für Ozeanforschung, 2015). At each of the four reefs, five coral colonies were collected from different locations within the reef using the manned submersible JAGO (GEOMAR Helmholtz-Zentrum für Ozeanforschung, 2017), to increase the likelihood of sampling genetically distinct individuals. Within each reef, corals were collected on a single dive to the exception of Hola, where samples were taken on two different dives. The largest distance between samples was ~70 m at Sula (280 m depth) and Nord-Leksa (165 m depth) and ~270 m at Steinavaer (200–220 m depth) and ~400 m at Hola (260 m depth).

Corals were immediately transferred to a 3000-l PVC tank filled with water collected from 100 m depth and maintained at near-ambient temperature of 8.5°C (4000 W cooling unit, Aqua Medic Titan 4000, Aqua Medic, GmbH, Bissendorf, Germany). Corals were acclimated to tank conditions for 5–12 h before the start of the experiment. It was taken care that all experimental fragments contained younger generations from the upper 10–15 cm of the coral colonies, and that associated organisms (e.g., sponges, polychaetes) were removed from the fragments. Experiments were performed in the wet lab onboard the ship.

Experimental Design

The experimental design is presented in **Figure 1C**. At each of the four reefs, the five coral colonies were fragmented into five pieces each to obtain one fragment of 10–15 cm (~20–50 polyps, 29 ± 10 gWM) for respiration, and four smaller fragments (~5 polyps each) for stress marker analyses. From the latter group, two fragments were used for the neutral red assay and two for the lipid peroxidation assay

(described in detail in the section “Stress Markers”). The large fragments for coral respiration analysis and two of the small fragments were transferred to a tray submerged inside a square tank of 1000 L of recirculating seawater (Experimental Tank). Temperature was regulated by a cooler (395 W, Aqua Medic Titan 500, Germany) and an aquarium heater (300 W thermocontrol, EHEIM GmbH, Germany) activated by a regulator when needed (InkBird ITC-308, InkBird, Shenzhen, China). The temperature in the Experimental Tank increased from 5 to 15°C by steps of 2°C. Corals were exposed to the desired temperature for 8 h and then temperature was increased by 2°C within 2 h. To control for the effect of experimental manipulation on stress response, the last two small fragments from each of the five colonies were transferred to a tray submerged in a Control Tank (1000 L recirculating seawater), where temperature was kept constant at ambient conditions (8.5°C, 790 W, Aqua Medic Titan 1500, Germany) for the entire duration of each experiment. One additional 10–15 cm fragment from one of the five colonies from each reef site was maintained in the Control Tank to measure respiration at ambient condition throughout the four experiments. Each experiment (one per reef) lasted 58 h: the corals in the Experimental Tank experienced six stable temperature steps of 8 h and five temperature increases of 2 h per experiment.

New unfiltered seawater was pumped from ~100 m depth into each of the two tanks one or two days before the start of each experiment. Temperature was monitored continuously in the Experimental Tank using a surface temperature sensor ($\pm 1^\circ\text{C}$ accuracy, LabQuest2, Vernier, Beaverton, United States; see **Supplementary Figure S1**). Salinity was checked in both tanks right after they were filled with newly pumped seawater (Sula: $S = 34.8$, Nord-Leksa: $S = 34.5$, Steinavaer and Høla: $S = 34.0$), using a conductivity meter (Cond 3210, WTW GmbH, Weilheim, Germany).

Coral Metabolism Respiration

The 10–15 cm coral fragments were placed in cylindrical respiration chambers (800–850 ml) equipped with mini USB-pumps ($\sim 30\text{--}40\text{ L h}^{-1}$; JT-160A 5V, SAILFLO, Xiamen, China) for water circulation. Oxygen concentration inside the chambers was measured every 15 s using optodes (sensor spots oxy-SP-Pt3_NAU, PreSens GmbH, Regensburg, Germany) connected to a 10-channel oxygen meter with polymer optical fibers (Oxy-10 mini, PreSens GmbH, Regensburg, Germany). The probes were calibrated prior the experiment, as advised by the manufacturer, for 0% (oxygen-free seawater: 1 g of Na_2SO_3 per 100 mL of seawater) and 100% (air-saturated seawater) and re-adjusted to 100% prior each run.

Six respiration chambers were placed in the Experimental Tank: one for each coral fragment and one seawater control (accounting for bacterial/microbial respiration). The Control Tank contained two respiration chambers: one with a coral fragment and one bacterial control. The eight chambers were closed simultaneously at the beginning of each temperature step.

Oxygen concentration always remained $>70\%$ air-saturation. Respiration runs lasted 30 min to 8 h depending on the speed of oxygen consumption of the individual fragments and/or the temperature. At the end of each respiration run, the USB pump connections to the chambers were opened to flush the chambers with fresh seawater and the oxygen concentrations increased to 100% again within minutes.

At the end of each 58 h-experiment, the volume of the water in each chamber was measured and a water sample was taken (20–30 mL) and immediately frozen for determination of the ammonium concentration. All coral fragments were dabbed dry with a cloth and immediately frozen in liquid nitrogen and stored at -80°C . The mass of each coral fragment was measured in the laboratory back on shore (WM: wet mass, DM: dry mass, ADM: ash dry mass; balance: Mettler Toledo XPE205, United States, precision: 0.01 mg). Dry mass (seven days at 60°C in the drying oven; Series 9000, Termaks, Norway) and ADM (5 h at 550°C in a LH30/12 furnace with C30 controller, Nabertherm, Germany) of the respiration pieces were estimated from sub-samples (7–28 g_{WM}) of each fragment after homogenization by crushing it with a mortar. The ash-free dry mass of the tissue (AFDM) was calculated for each fragment as $\text{AFDM} = \text{DM} - \text{ADM}$.

For each coral fragment and each temperature step, the respiration rate (oxygen consumption rate: MO_2) was calculated as the regression coefficient of the linear decrease in oxygen concentration ($\mu\text{mol O}_2\text{ h}^{-1}\text{ g}_{\text{AFDM}}^{-1}$) in a given volume of water, which was sampled from each chamber after incubation for volume analysis, accounting for the water displacement of the particular coral fragments. Before the regression analysis, oxygen concentrations (%) were converted in $\mu\text{mol L}^{-1}$ using the package *Respirometry* (Birk, 2018) in the software *R* (R Core Team, 2017). The oxygen depletion calculated in the bacterial control chambers was subtracted from each coral's respiration rate. The oxygen data from the Sula Reef at 5°C and respiration of four out of five corals of Steinavaer at 11°C was lost when saving the files and are therefore not included in the analyses.

Ammonium Excretion and O:N Ratios

Ammonium concentrations (NH_4^+) were measured in seawater samples taken prior to and after the respiration incubations from each chamber with the fluorometric method described in Kérouel and Aminot (1997) and Holmes et al. (2011) using a Flow Solution VI autoanalyzer (OI Analytical, Xylem, United States). Ammonium excretion rates were calculated as the increase in NH_4^+ concentrations and expressed as $\mu\text{mol NH}_4^+\text{ h}^{-1}\text{ g}_{\text{AFDM}}^{-1}$. Ammonia (NH_3) was considered as negligible in our conditions (1–2% of total ammonium/ammonia at pH 8.0, for a pK_a of 9.68–9.99, Bell et al., 2007). The samples were not filtered before the analysis as we considered that the water from this experiment was virtually particle-free (100 m depth, away from shore and reefs). Although we are aware that introduced compounds could have altered the measurements, the variation in the repeated measurements in the Control Tank were small (see range presented in the black dots in **Figure 2B**, $n = 6$ incubation of the same coral piece), compared to the variation due to the temperature treatment.

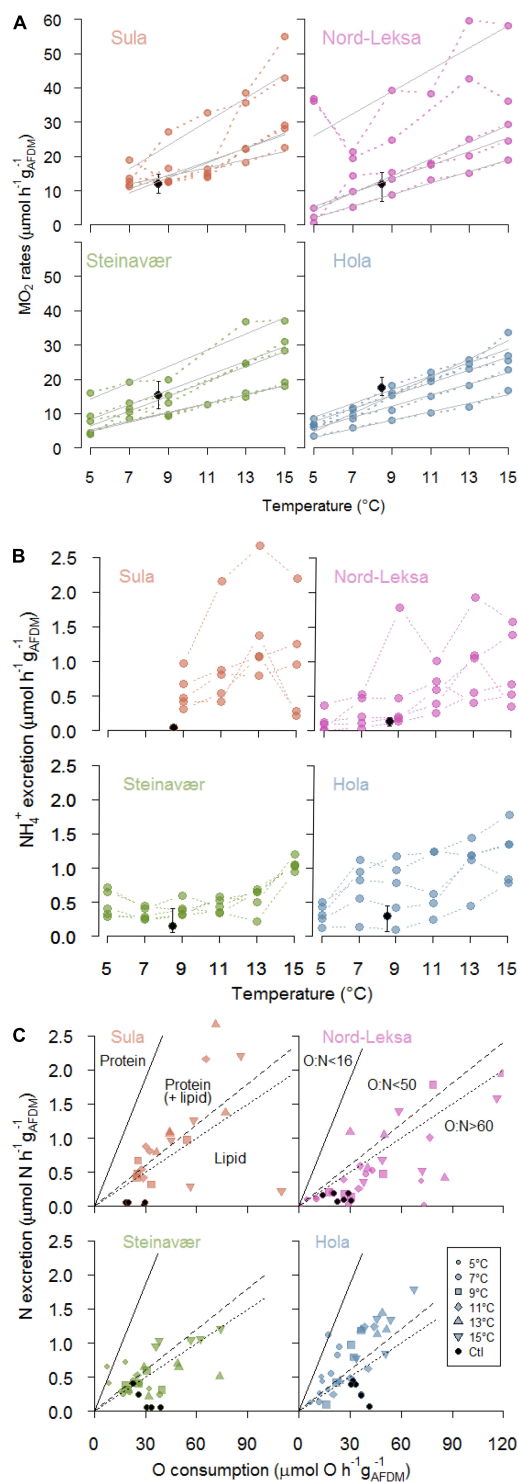


FIGURE 2 | (A) Respiration rates (MO_2 : $\mu\text{mol O}_2 \text{ h}^{-1} \text{ gAFDM}^{-1}$) of each of coral fragments ($n = 5$) at each temperature step (color dots), and each reef (panel). Full gray lines represent significant linear regressions of individual MO_2 rates with temperature ($^{\circ}\text{C}$). Black dots represent the mean MO_2 of the coral fragment incubated in the Control Tank (8.5°C , bars for minimum and maximum rates: $n = 6$ incubations). **(B)** Ammonium excretion rates ($\mu\text{mol NH}_4^+ \text{ h}^{-1} \text{ gAFDM}^{-1}$) of each of the coral fragments ($n = 5$) at each

(Continued)

FIGURE 2 | Continued

temperature step (color dots), and each reef (panel). Black dots represent the mean excretion of the coral fragment incubated in the Control Tank (8.5°C , bars for minimum and maximum rates: $n = 6$ incubations). **(C)** Nitrogen excretion ($\mu\text{mol N h}^{-1} \text{ gAFDM}^{-1}$) as a function of oxygen consumption ($\mu\text{mol O h}^{-1} \text{ gAFDM}^{-1}$). Each point represents one coral fragment at one temperature step (symbols: see legend; one panel per reef). Black dots represent the coral fragment incubated in the Control Tank (8.5°C). The region above the full line corresponds to exclusive protein catabolism ($16 < O:N$), while the regions below are protein-dominated catabolism ($16 < O:N < 50$), catabolism of equal amounts of protein and lipids ($50 < O:N < 60$) and lipid-dominated catabolism ($O:N > 60$). Also see **Supplementary Figure S3** for an alternative representation.

The ratio between the atoms of oxygen consumed per atom of nitrogen excreted ($O:N$ ratio) was calculated for each coral fragment at each experimental temperature using the following equation:

$$O : N = (2 \times MO_2) \times (NH_4^+ \text{ excretion})^{-1} \quad (1)$$

This ratio allows to detect a possible change in the metabolic substrates used: low $O:N$ ratios indicate the metabolic reliance on proteins/amino acids vs. lipids/carbohydrate utilization (Mayzaud and Conover, 1988).

Stress Markers

Cellular stress is a response to an environmental stressor that can be measured with several methods, direct (e.g., visual assessment of cellular damage in the neutral red retention assay) or indirect (production of stress by-products in the lipid peroxide assay).

Lysosomal Destabilization

The neutral red retention assay, a measure of lysosome stability, described in Lowe et al. (1995) and Ringwood et al. (2003) was adapted for corals. At the end of each experiment (58 h), one small coral fragment from each of the five coral colonies was sampled from the Experimental Tank and from the Control Tank for the neutral red assay ($n = 5$ fragments $\times$ 2 tanks) and the assay was performed immediately onboard. The sampled coral fragments were first crushed with a granite pestle in a mortar with 1.5 ml of Ca-Mg-free artificial seawater (20 mM HEPES, 450 mM NaCl, 12.5 mM KCl, and 5 mM tetrasodium EDTA; pH 8.0, 4°C) and transferred to Eppendorf tubes. The tubes were kept on ice and shaken every 5 min for the next 20 min. Samples were filtered through a $80\text{-}\mu\text{m}$ plankton mesh and centrifuged twice for 5 min at 6000 rpm. Cell pellets were resuspended in freshly mixed neutral red solution (0.08 mg ml^{-1}) and incubated in the dark at room temperature for 60 min. For each fragment, 50 cells were scored as either stable (red dye contained inside the cell's lysosomes) or unstable (dye leaked inside the cell's cytoplasm via microscope observation ($40\times$, Nikon Eclipse E-200, Japan)).

Lipid Peroxidation Assay

The oxidative degradation of the cell membrane's lipids leads to the production of, e.g., malondialdehyde (MDA), which can be quantified with the lipid peroxidation assay described in Ringwood et al. (2003). For this analysis, at the end of each

experiment, one small coral fragment from each of the five coral colonies was sampled from the Experimental Tank and from the Control Tank and frozen in liquid nitrogen and stored at -80°C . We crushed 2.05–2.50 g of the thawed samples with a pestle in a mortar with 1.0 mL of K_2PO_4 buffer solution (50 mM, 4°C , pH 7.0). Samples were centrifuged at $13000 \times g$ for 5 min at 4°C to separate tissue and skeleton. The supernatant from each sample (100 μL) was transferred to a micro-centrifuge tube kept on ice until 1414 μL of the assay solution (thiobarbituric acid solution, 0.375% mixed with butylated hydroxytoluene, 2%) was added. For the calibration curve, seven MDA standards were prepared using a serial dilution ($/2$) from a 10 mM stock solution of MDA with the K_2PO_4 buffer. The sample solutions, the standards, and a blank were homogenized and put in a water bath at 100°C for 15 min. They were then centrifuged at $13000 \times g$ for 5 min (20°C). Three replicates of 200 μL of the supernatant of each sample, standard, and blank were distributed in a well of a 96-well plate. Absorbance was read at 532 nm in a spectrophotometer (SpectraMax M5, Molecular Devices, United States). Results are expressed as MDA concentration ($\mu\text{mol L}^{-1} \text{ gAFDM}^{-1}$).

Statistical Analyses

All the analyses were performed with the software *R* (R Core Team, 2017). The level for significance used was 5%. Results are presented as mean $\pm$ SD. Because the data on respiration, NH_4^+ excretion, and O:N ratio were measured on the same individuals (i.e., coral replicates), we used generalized linear mixed effect models (GLMMs) to test for the effect of reef (location/reef origin; i.e., experiment), assigning coral replicates as the random factor and temperature and reef origin as fixed factors. GLMMs were performed using the *R* package *nlme* (Pinheiro et al., 2018). The effect of the temperature treatment on lysosome stability (Control Tank proportion subtracted to Experimental Tank proportion) was analyzed with a *t*-test (significant difference from zero) and a one-way ANOVA (i.e., ANOVA-I) with reef as a factor. The MDA concentration was compared between Control Tank and Experimental Tank using a two-way ANOVA (i.e., ANOVA-II) with reef origin and tank treatment as factors. Normality of the data was tested with Shapiro–Wilk tests and qq-plots, while variance homogeneity was tested with Bartlett, Levene, or Fligner–Killeen tests, depending on the structure of the data. *Post hoc* tests were performed with Tukey's Honest Significant Difference method.

RESULTS

Coral Metabolism

Respiration Rates

We observed no mortality in the corals after the 58 h incubations. The measured decrease in oxygen in the chambers was statistically correlated with time in all the incubation runs (details provided in **Supplementary Figure S2**).

The best GLMM model to represent the respiration was a gamma-GLMM with temperature as a fixed effect with significant influence ($t_{(103)} = 15.1$, $p < 0.001$, AIC = 693, **Supplementary Table S1A**), and the individual fragments from each reef as a random effect. The influence of the

reef origin was not significant ($p = 0.35$). Respiration (MO_2) was linearly correlated to temperature in 18 of the 20 coral fragments investigated (**Figure 2A**), with individual rates ranging from 1.2 to 3.5 $\mu\text{mol O}_2 \text{ h}^{-1} \text{ gAFDM}^{-1} {}^{\circ}\text{C}^{-1}$ (details provided in **Supplementary Table S1B**). On average, raising temperature by 10°C increased the respiration rates by threefold, from $10 \pm 11 \mu\text{mol O}_2 \text{ h}^{-1} \text{ gAFDM}^{-1}$ at 5°C to $30 \pm 11 \mu\text{mol O}_2 \text{ h}^{-1} \text{ gAFDM}^{-1}$ at 15°C (all reefs combined; **Figure 2A**; see details in **Table 1**). This GLMM was significantly better ($\chi^2_{(1)} = 77$, $p < 0.001$) than the gamma-generalized linear model (GLM) without the random effect (AIC = 768), indicating that the random effect (individuals) had significant contribution to the observed variation in respiration rates.

Additionally, the inter-individual deviation from the mean (i.e., variance) of the respiration rates between coral fragments was homogenous at all temperature steps (Levene test: $F_{5,104} = 2.07$, $p = 0.075$; pooled reefs), but depended on the reefs ($F_{3,106} = 4.07$, $p = 0.008$; pooled temperature steps). We observed a significantly higher variance in fragments taken from Nord-Leksa (**Figure 2A**; SD = $15.2 \mu\text{mol O}_2 \text{ h}^{-1} \text{ gAFDM}^{-1}$) than from Steinavaer (SD = $9.1 \mu\text{mol O}_2 \text{ h}^{-1} \text{ gAFDM}^{-1}$; *post hoc* test: $p = 0.03$) and Hola (SD = $7.7 \mu\text{mol O}_2 \text{ h}^{-1} \text{ gAFDM}^{-1}$; *post hoc* test: $p = 0.009$). All the other pairwise comparisons were non-significant, with intermediate variance observed in Sula (SD = $11.4 \mu\text{mol O}_2 \text{ h}^{-1} \text{ gAFDM}^{-1}$).

Ammonium Excretion

Coral ammonium excretion rates ranged from <0.01 up to $2.66 \mu\text{mol NH}_4^+ \text{ h}^{-1} \text{ gAFDM}^{-1}$ (**Figure 2B**).

The best GLMM model to represent ammonium excretion (**Figure 2B**) was a gamma-GLMM with temperature as a fixed effect with significant influence ($t = 9.2$, $p < 0.001$, AIC = 55; **Supplementary Table S2**), and the individual fragments from each reef as a random effect. The influence of the reef origin was not significant ($p > 0.06$). On average, raising temperature by 10°C increased the ammonium excretion rates by threefold, from $0.3 \pm 0.2 \mu\text{mol NH}_4^+ \text{ h}^{-1} \text{ gAFDM}^{-1}$ at 5°C to $1.0 \pm 0.5 \mu\text{mol O}_2 \text{ h}^{-1} \text{ gAFDM}^{-1}$ at 15°C (all reefs combined; **Figure 2B**; see details in **Table 1**) This GLMM was significantly better ($\chi^2_{(1)} = 37$, $p < 0.001$) than the gamma-GLM without random effect (AIC = 90), indicating that the random effect (individuals) had significant contribution to the observed variation in the ammonium excretion rates.

The inter-individual variance of the ammonium excretion rates between coral fragments was homogenous at all temperature steps (Levene test: $F_{5,104} = 1.67$, $p = 0.15$; pooled reefs), but depended on the reefs ($F_{3,106} = 3.90$, $p = 0.01$; pooled temperature steps). From pairwise comparisons, a significant difference was observed only between the fragments taken from Sula (SD = $0.68 \mu\text{mol O}_2 \text{ h}^{-1} \text{ gAFDM}^{-1}$) and Steinavaer (SD = $0.27 \mu\text{mol O}_2 \text{ h}^{-1} \text{ gAFDM}^{-1}$; *post hoc* test: $p = 0.009$; **Figure 2B**).

O:N Ratio

Corals exposed to increasing temperature had an average O:N ratio of 77 ± 90 , meaning that they excreted one molecule of N for every 39 molecules of O_2 consumed.

TABLE 1 | Summary of the results: Averages (+/– SD) for respiration and ammonium excretion rates and O:N ratios are given by reef. Results of the regressions of respiration and ammonium excretion with temperature are presented as regression coefficient (r^2 , df).

Parameter	GLMM	Sula	Nord-Leksa	Steinavaer	Hola
Respiration rates ($\mu\text{mol O}_2 \text{ h}^{-1} \text{ g AFDM}^{-1}$)	FE: - Temp*** - Reef ^{ns} RE: - Indiv***	22 ± 11	23 ± 15	17 ± 9	15 ± 8
Respiration rates increase with temperature ($\mu\text{mol O}_2 \text{ h}^{-1} \text{ g AFDM}^{-1} \text{ } ^\circ\text{C}^{-1}$)		2.76*** (45%, df = 23)	2.06** (20%, 27)	1.84*** (55%, 24)	1.88*** (71%, 28)
Ammonium excretion rates ($\mu\text{mol h}^{-1} \text{ g AFDM}^{-1}$)	FE: - Temp*** - Reef ^{ns} RE: - Indiv***	0.98 ± 0.68	0.58 ± 0.53	0.54 ± 0.27	0.80 ± 0.45
Ammonium excretion rates increase with temperature ($\mu\text{mol h}^{-1} \text{ g AFDM}^{-1} \text{ } ^\circ\text{C}^{-1}$)		0.084 ^{ns} ($p = 0.2$)	0.088*** (31%, 28)	0.051*** (40%, 28)	0.081*** (35%, 28)
O:N ratio (no units)	FE: - Temp ^{ns} - Reef ^{ns} RE: - Indiv***	78 ± 103	118 ± 135	68 ± 38	47 ± 26

Summary of the mixed-effect models used in this study are given under the column GLMM. Summary results of the GLMM models used in this study (details of the GLMM models can be found in **Supplementary Tables S1A, S2, S3**). FE, fixed effect; RE, random effect. *** $p < 0.001$, ** $p < 0.01$ and ^{ns}non-significant.

The best GLMM model to represent the O:N ratio response was a gamma-GLMM with temperature and reef origin as fixed effects (AIC = 1040; details in **Supplementary Table S3**), and the individual fragments from each reef as a random effect. However, the influence of temperature ($t = 0.27$, $p = 0.79$) and reef origin ($t = 1.63$, -0.06 and -1.2 , $p > 0.10$) were not significant, even if O:N ratio seemed higher in Nord-Leksa (118 ± 135) – with most data point in the area of the O:N > 60 (**Figure 2C**) – than in Hola (47 ± 26) – where most data points are situated above this same area (see details in **Table 1**). This GLMM was significantly better ($\chi^2_{(1)} = 32$, $p < 0.001$) than the gamma-GLM without random effect (AIC = 1070), indicating that the random effect (individuals) had significant contribution to the observed variation of the O:N ratio.

The O:N ratio of the fragments experiencing increasing temperatures was on average 3.5 times lower than the fragments from the same colony kept at ambient temperature (275 ± 208 ; **Figure 2C**; black dots: $n = 1$ fragment per reef, seven incubations).

Stress Markers

Lysosome Stability

In three of the four reefs, the occurrence of burst lysosomes did not significantly increase in the corals that experienced elevated temperature (no difference from zero in Nord-Leksa: $t_{(3)} = -0.03$, $p = 0.97$, Steinavaer: $t_{(4)} = 2.26$, $p = 0.08$, Hola: $t_{(4)} = 0.16$, $p = 0.88$; **Figure 3A**). Coral fragments from Sula exposed to elevated temperature significantly increased the proportion of destabilized cells by $10 \pm 7\%$, compared to the ones kept at 8.5°C ($t_{(4)} = 3.30$, $p = 0.03$).

Lipid Peroxidation

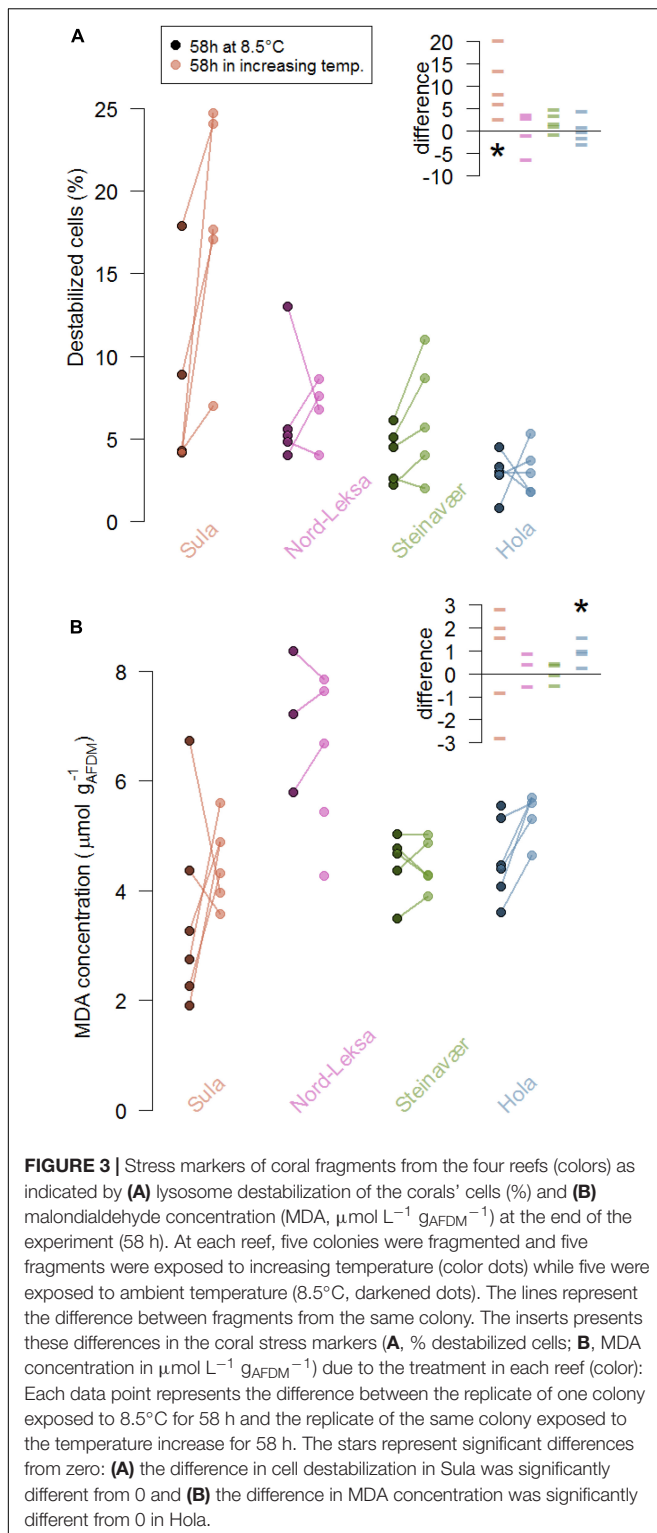
To the exception of corals from Hola, lipid peroxidation products did not significantly increase in corals that experienced elevated temperatures (no difference from zero in Sula: $t_{(4)} = 0.57$, $p = 0.60$, Nord-Leksa: $t_{(2)} = -0.64$, $p = 0.58$, Steinavaer: $t_{(3)} = 0.41$, $p = 0.70$; **Figure 3B**). Coral fragments from Hola

exposed to elevated temperature significantly increased the MDA concentration by an average of $0.96 \pm 0.55 \mu\text{mol L}^{-1} \text{ g AFDM}^{-1}$, compared to the ones kept at 8.5°C ($t_{(3)} = 3.47$, $p = 0.04$). Concentrations of MDA measured in corals from Nord-Leksa were 1.5 times higher than the other three reefs (6.66 ± 1.39 vs. $4.44 \pm 1.03 \mu\text{mol l}^{-1} \text{ g AFDM}^{-1}$).

DISCUSSION

Effect of Temperature on Coral Metabolism

In the present study, the metabolic rates of freshly collected corals increased linearly by threefold for a 10°C increase (i.e., Q10 of 3), suggesting that this increase follows linearly the biochemical reactions' kinetics. It is indeed reasonable to assume that respiration rates would follow temperature-dependent chemical reaction rates and, using the Arrhenius equation (temperature dependence of reaction rates), a 10°C increase in temperature would result in two- to threefold increase in respiration (see discussion on the use of Q10 and Arrhenius models in Kruse et al., 2011). Although this rule is empirical, our results support the hypothesis that the respiration rates we measured are only dependent on the increase in temperature and that no additional process(es) needing respiration were activated (in the range of temperatures we investigated). In contrast, in an experiment from Dodds et al. (2007) on *L. pertusa*, an increase of temperature from 9 to 11°C increased respiration rates by eight times for 10°C , which suggests that additional mechanisms were at play in these corals. Hennige et al. (2014) discusses the effect of fresh vs. laboratory-kept *L. pertusa* metabolism: in particular, the effect of the low-quality food *L. pertusa* is provided with in experimental conditions could affect the metabolism (e.g., Larsson et al., 2013). Although the experimental handling/fragmentation could have affected the respiration rates we measured, we also worked on freshly collected corals and the



discrepancy between our results and Dodds et al. (2007) results may be explained by the food regime the corals from Dodds et al. (2007) were acclimatized to (fed twice a week with dead krill and starved 24 h before incubation). Direct comparisons with *L. pertusa* respiration rates are made difficult by different

size- or weight-standardizations of different studies and authors have also reported biogeographic variability (Georgian et al., 2016). Nevertheless, our results ($8\text{--}30 \mu\text{mol O}_2$ per gram of tissue dry-mass per hour at 9°C) correspond with the respiration rates of *L. pertusa* either freshly collected ($23 \mu\text{mol O}_2$ per gram of tissue dry-mass per hour at 9.5°C ; Hennige et al., 2014) or pre-acclimated to laboratory conditions for a month ($5\text{--}7 \mu\text{mol O}_2$ per gram of tissue dry-mass per hour at 8°C ; Georgian et al., 2016).

We observed that 2°C temperature steps from 5 to 15°C increased the metabolic rates of the corals linearly (Figure 2A). The respiration does not show any sign of marked change in slope in the range of $5\text{--}15^{\circ}\text{C}$, which we would expect to see for either sub-optimal temperature or when approaching the thermal critical maximum. The shape and breadth of Thermal Performance Curves (see review by Schulte, 2015) can inform about the potential tolerance of organisms. According to our results, *L. pertusa* from the Norwegian reefs can be considered as eurytherms, with tolerance to a broad (10°C) range of temperatures. The upper critical temperature has been investigated in *L. pertusa* from the Gulf of Mexico (Brooke et al., 2013): *L. pertusa* survived (100%) to temperatures up to 15°C for 24 h but started dying at higher temperatures (70% survival at 20°C and 0% at 25°C) and longer exposure time (80% survival after seven days at 15°C). In cold waters, it could be advantageous – or even necessary – to be adapted to more variable environments: cold-eurythermy as observed for *L. pertusa* could allow for survival in short-term and seasonal variations (see the section “Sampling Reefs”) as well as in the long-term, for instance during ice ages (Pörtner and Gutt, 2016).

The ammonium excretion followed the increase by two- to threefold described for respiration at all the temperature steps investigated. O:N ratios (1) lower than 16 correspond to exclusive protein catabolism while higher ratios correspond to (2) protein-dominated catabolism ($16 < \text{O:N} < 50$), (3) catabolism of equal amounts of protein and lipids ($50 < \text{O:N} < 60$), and (4) lipid-dominated catabolism ($\text{O:N} > 60$; Mayzaud and Conover, 1988). In our study, only two measurements had O:N ratios lower than 16 (Figure 2C), while most of the other measurements have higher O:N ratios, pointing toward more or equal reliance to lipids. The O:N ratio of ~ 80 (i.e., one molecule of N produced for ~ 40 molecules of O_2 consumed) of the present study suggests that the *L. pertusa* specimens we sampled rely on a mixed substrate of proteins and lipids for catabolism, with a dominance for lipids (Mayzaud and Conover, 1988). Other authors reported O:N ratios of 35, measured *in situ* on *L. pertusa* (Khripounoff et al., 2014). These measurements are lower than the ratios in our study and reflect preferred protein catabolism, which could be a sign of limited lipid reserves, possibly linked to seasonal (reproductive) or geographical differences. The highest O:N ratios are found in individuals kept at ambient temperature, with similar respiration rates (Figure 2A) but lower NH_4^+ excretion rates (Figure 2B) compared to the individuals experiencing increasing temperature. Although the interpretation of this result should be careful (one individual measured per experiment), this may be a sign that tissue

repair has started in the corals kept at ambient temperature (N utilization).

Food supply to *L. pertusa* is thought to be both limited and highly variable, mainly associated with the settling of the spring and autumn plankton blooms (e.g., Duineveld et al., 2007). The reliance on lipids in the metabolism suggests that *L. pertusa* has durable lipid reserves, a strategy that would be helpful in the deep sea, where food availability can fluctuate (Maier et al., 2019). Larsson et al. (2013) proposed that stored fatty acids of *L. pertusa* could be a better proxy for food availability than growth or respiration. Comparing the lipid content of *L. pertusa* at different reefs and understanding seasonal variability therefore seems primordial to assess their health.

While only a few measurements were in the O:N < 50 area in the first three reefs, at Hola nearly half of the ratios were lower than 50 (Figure 2C), suggesting that the corals from this reef were more reliant on proteins than the corals from the other sampled reefs. Hola is situated in a northern, offshore region and food availability at this site, in particular the supply from blooms, could be limited or of poor quality in comparison with the other three reefs. Thus, the corals at Hola could rely more strongly on protein than on stored lipids. During our cruise, visual assessment of the reefs led us to believe that the Hola reef was less healthy than the other three reefs we sampled (Figure 4; Flögel et al., 2014).

From similar deep-sea ecosystems, the O:N ratio of a gorgonian coral, *Primnoa resedaeformis*, decreased by 30% after 33 days of exposure to +6°C, which could reflect a shift in substrate for catabolism since lipids were not as available anymore (Scanes et al., 2018; but see Nordemar et al., 2003 for no evidence of increased catabolism in the tropical coral *Porites cylindrica*). We did not observe any effects of temperature on the O:N ratios, most likely because the exposure time was too short to deplete the lipid reserves and induce a switch in the substrates used. In another – but closely related – cold-water coral species, Gori et al. (2016) observed no change in O:N ratio when *Desmophyllum dianthus* was exposed to +3°C (12 vs. 15°C) for eight months.

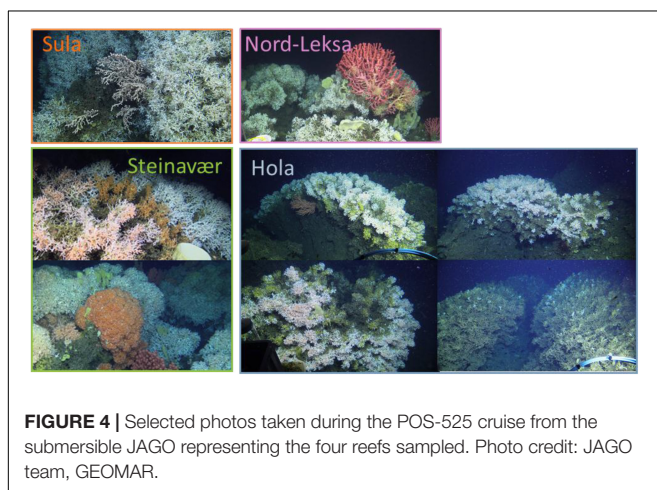


FIGURE 4 | Selected photos taken during the POS-525 cruise from the submersible JAGO representing the four reefs sampled. Photo credit: JAGO team, GEOMAR.

Effect of Temperature on Stress Markers

Quantifying the alteration to the lysosome membrane integrity can reveal cellular stress linked to toxic effects of marine stressors (Moore et al., 2006) such as heavy metals (e.g., Regoli, 1992) or hyperthermia (e.g., Moore et al., 2007). Blue mussels exposed to +18°C for 48 h decreased lysosomal stability by 60% but fully recovered after four days of recovery (Moore et al., 2007). In a cold-water coral, Scanes et al. (2018) observed a decrease in lysosomal stability by 7.5% for corals that were exposed to +6°C for 33 days – but this effect was not significant (and inverse) for 26 or 40 days exposure. Here, we found no effect of the temperature treatment on the lysosomal membrane integrity, to the exception of the corals from Sula. Although this is not confirmed by the results of the lipid peroxidation assay, corals from Sula could be the most sensitive to temperature increases as they are the ones experiencing the least variable, and coldest temperatures along the year (Figure 1B).

In our study, the thermal conditions the corals were exposed to did not increase the production of MDA, a product of lipid peroxidation – to the exception of Hola. However, concentrations of MDA at Nord-Leksa were approximately twice as much than those in corals from the other three reefs (Figure 3B). The yearly average temperatures modeled at Nord-Leksa (7.9°C; Figure 1B) are up to a degree higher than at the other three reefs (7.0°C for Sula, 6.9°C for Steinavær and 7.4°C for Hola; averages from modeled daily temperatures for years 2015 to 2017 at the bottom layer of the geographical coordinates). The baseline concentrations of MDA could therefore be linked to the corals' temperature history and further investigation would be needed to test this hypothesis.

In other invertebrates, thermal stress (+10 and +17°C for 1 h) had no effect on the MDA concentration in the mussel *P. viridis* but MDA increased after a 24 h recovery period (+17°C only, Wang et al., 2018). While we sampled directly after the temperature treatment, a recovery period could have allowed the time necessary for the corals from all reefs to increase their MDA levels.

Ecological Implications

Lophelia pertusa appears to be robust to a wide range of temperatures. A cold-eurythermy that could allow this species to live in an environment with variable temperatures (e.g., seasonal changes by 3–5°C and small-scale changes of a few degrees over a few hours: Godø et al., 2012; Brooke et al., 2013; Osterloff et al., 2019). However, generalizations to accurately project how *L. pertusa* will fare in the future scenario of global change are limited by several factors.

The reef of origin (i.e., experiment) of the corals influenced the variance between replicates of the respiration rates, with higher variance in the corals from Nord-Leksa and lowest in corals from Hola. A similar observation was made in a recent *in situ* study by Büscher et al. (2019) with far higher variances in the growth rates in replicates of Nord-Leksa compared to the offshore site Sula. This result may be linked to the difficult task of sampling genetically diverse colonial animals in the deep sea. The sampling process is costly, resulting in small sample sizes and the same

L. pertusa clone have been shown to be sometimes spread across 300 m² (Tisler Reef in southern Norway by Dahl et al., 2012). Thus, although our sampling sites appeared distant, we could have sampled widely spread clones or siblings at Hola Reef for example, resulting in lower inter-individual variability of the responses. More knowledge is needed on the genetic distribution and structure of this species at each of these reefs to interpret the observed differences. Additionally, inter-individual variability is an important factor for predicting the adaptation of *L. pertusa* to future changes. For instance, Kurman et al. (2017) observed that some genotypes of *L. pertusa* from the Gulf of Mexico were more resilient to ocean acidification than others (see also Georgiann et al., 2016). Differences in the genetic structure of the different reefs or phenotypic responses of the genotypes of each reef would have important ecological implications for conservation. A large genetic pool and the presence of at least a few resilient genotypes would influence the potential for adaptation in this slow-growing species. Even without adaptation, *L. pertusa* could persist by successful acclimatization to the future conditions (see review by Brown and Cossins, 2011). For instance, *L. pertusa* can be acclimated (same respiration/calcification rates) in the laboratory to lower temperatures (−3 and −6°C) in slow steps of 1°C per month (Naumann et al., 2014), therefore hinting at a potential for thermal acclimation in this species.

CONCLUSION

The present study determined the vulnerability of the deep-sea cold-water coral species *L. pertusa* to short-term temperature changes. We exposed freshly collected fragments of *L. pertusa* to an acute, stepwise increase in temperature (5–15°C in 58 h). Respiration and ammonium excretion increased linearly with increasing temperature, while temperature did not induce cellular or oxidative stress – to few exceptions. Our results suggest that *L. pertusa* from the Norwegian continental shelf and fjords is tolerant to a broad thermal range of temperatures (5–15°C), possibly owing to the high thermal variability in their natural environment.

An experimental study with *L. pertusa* from the Gulf of Mexico also showed that this species was able to survive prolonged exposures to increased temperatures up to +4°C, depending on the genotype (Lunden et al., 2014), making it a possibly robust species to ocean warming. While a major challenge for its future could be finding enough food to meet its metabolic demands (+30–100% catches for an increase by 1–3°C by 2100), this species has been described as an opportunistic feeder (Mueller et al., 2014; Murray et al., 2019) and thus changes in food web could have only minimal consequences on *L. pertusa*.

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DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

ND, ØG, TK, and JB designed the work, contributed to the data acquisition and writing, and revising the manuscript. ND carried out the experiment. ND and ØG analyzed and interpreted the data and wrote the first draft of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fphys.2019.01636/full#supplementary-material>

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Effects of Water Acidification on Senegalese Sole *Solea senegalensis* Health Status and Metabolic Rate: Implications for Immune Responses and Energy Use

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Increasing water CO₂, aquatic hypercapnia, leads to higher physiological pCO₂ levels in fish, resulting in an acidosis and compensatory acid-base regulatory response. Senegalese sole is currently farmed in super-intensive recirculating water systems where significant accumulation of CO₂ in the water may occur. Moreover, anthropogenic releases of CO₂ into the atmosphere are linked to ocean acidification. The present study was designed to assess the effects of acute (4 and 24 h) and prolonged exposure (4 weeks) to CO₂ driven acidification (i.e., pH 7.9, 7.6, and 7.3) from normocapnic seawater (pH 8.1) on the innate immune status, gill acid-base ion transporter expression and metabolic rate of juvenile Senegalese sole. The acute exposure to severe hypercapnia clearly affected gill physiology as observed by an increase of NHE3b positive ionocytes and a decrease of cell shape factor. Nonetheless only small physiological adjustments were observed at the systemic level with (1) a modulation of both plasma and skin humoral parameters and (2) an increased expression of HIF-1 expression pointing to an adjustment to the acidic environment even after a short period (i.e., hours). On the other hand, upon prolonged exposure, the expression of several pro-inflammatory and stress related genes was amplified and gill cell shape factor was aggravated with the continued increase of NHE3b positive ionocytes, ultimately impacting fish growth. While these findings indicate limited effects on energy use, deteriorating immune system conditions suggest that Senegalese sole is vulnerable to changes in CO₂ and may be affected in aquaculture where a pH drop is more prominent. Further studies are required to investigate how larval and adult Senegalese sole are affected by changes in CO₂.

Keywords: water acidification, flatfish, gills immunofluorescence, respirometry, immune system

INTRODUCTION

Senegalese sole (*Solea senegalensis*, Kaup) is currently reared in super-intensive recirculation aquaculture systems (RAS) (Morais et al., 2016). This production structure allows high rearing densities together with a minimal water change (Martins et al., 2009). Such efficiency depends on the constant control and maintenance of water parameters, guaranteeing optimal fish performance (Bowden, 2008). Still, because of CO₂ accumulation in the water, there is a possible decrease of pH (Summerfelt et al., 2000) throughout the system, ultimately creating areas where acute or prolonged hypercapnia may be found. In fact, in salmonids production the concentration of CO₂ can reach 10–20 mg L⁻¹ (Mota et al., 2019), while the guidelines for intensive RAS production has proposed a safe limit of 40 mg L⁻¹ CO₂ (Blancheton, 2000; Ellis et al., 2017). Moreover, as a known consequence of anthropogenic activity, the release of CO₂ into the atmosphere is absorbed by the oceans leading to the decrease of oceanic water pH (Bresolin de Souza et al., 2014). In both scenarios, and despite the great despair in pH values found in aquaculture and in oceans (Ellis et al., 2017), the increase of water CO₂ leads to higher physiological pCO₂ in the fish. This could impact fish acid-base balance, which results in the activation of regulatory mechanisms to compensate for the acidosis (Melzner et al., 2009) and initiates reflexes such as bradycardia and hyperventilation (Perry and Abdallah, 2012). Perry and Abdallah (2012) reviewed that changes in water CO₂ can be sensed by chemoreceptors mainly located in fish gills that are involved in elevating plasma HCO₃⁻ concentrations to compensate for the respiratory acidosis (Heuer and Grosell, 2014; Allmon and Esbaugh, 2017; Esbaugh, 2018). The marked effects of modest elevations in CO₂ in fish are due in part to the low physiological pCO₂ levels found in fish (Qin et al., 2010; Clements and Chopin, 2017). The maleficent effects of aquatic hypercapnia rest on CO₂ concentration and duration of the exposure (Santos et al., 2013), and besides being a stress factor, hypercapnia can also be responsible for energy deviation from growth and other metabolic processes (Cech and Crocker, 2002; Stapp et al., 2015). Elevated water CO₂ concentration is associated with a drop of extra- and intra-cellular pH and a concomitant depression of cytosolic and membrane enzymes activity, ion transport and protein and DNA biosynthesis (Busa and Nuccitelli, 1984). In fact, juvenile spotted wolffish (*Anarhichas minor* Olafsen) showed reduced growth and plasma chloride levels after 10 weeks exposure to pH 6.45 (Foss et al., 2003) while Atlantic salmon post-smolt (*Salmo salar*) growth decreased linearly with the increase of water CO₂ (Mota et al., 2019). Moreover, Bresolin de Souza et al. (2014) observed an up-regulated expression of plasma immune system- and gill metabolism- related proteins in response to high CO₂. In addition, Wang et al. (2010) showed that elevated CO₂ inhibits LPS-induced expression of IL6 and TNF and impairs bacterial clearance through inhibition of both phagocytosis and autophagy in macrophages. Atlantic halibut (*Hippoglossus hippoglossus*) exposed to high CO₂ showed up-regulation of plasma immune-related proteins as the complement component C3 and gills metabolism- and cellular turnover-related proteins, possibly due to an increase of energy demand

(Bresolin de Souza et al., 2014). Hence, the fish immune system could also be affected acutely by hypercapnia since immune cells internal milieu is directly affected and reflects the external environment (Boleza et al., 2001).

Short-term hypercapnia exposure is generally alleviated through small adjustments in fish metabolism (Cech and Crocker, 2002), but in response to a chronic exposure or to increased ambient CO₂ level, the metabolic demands may increase and reach the limit of the regulatory mechanisms. In fact, it is suggested that as soon as the compensatory mechanisms are exceeded, fish adopt a passive strategy toward the stress resolution by shifting the available energy to survival related machinery (Walsh et al., 1988; Pörtner et al., 1998; Baker and Brauner, 2012; Stapp et al., 2015). Few studies have investigated the effect of hypercapnia on fish immune mechanisms, which is particularly true for flatfishes. Since fish immunity is highly dependent on both host energy budget and environmental variables, this study assessed the effects of acute and prolonged exposures to CO₂ induced low pH levels on the innate immune status, gill acid-base ion transport and metabolic rates of Senegalese sole. This was accomplished using juvenile fish exposed to pH 7.9, 7.6, and 7.3 and sampled after 4, 24 h and 4 weeks of exposure.

MATERIALS AND METHODS

Experimental Design

The trial was performed at the Centro Interdisciplinar de Investigação Marinha e Ambiental (CIIMAR) facilities in Porto, Portugal. Senegalese sole juveniles (82.74 ± 0.75 g) were obtained from a commercial fish farm, located in north-west Portugal (Aquacria, Aveiro, Portugal). Fish were acclimated for one month in a recirculating sea water system according to standard protocols, with continuous control of water parameters and were daily fed a commercial diet (2% body weight). Water was kept at 19°C, salinity at 24 ppt, dissolved oxygen at 100% air saturation (O_{2sat}), and a photoperiod of 12 h light/12 h dark was adopted. After being weighed, 24 fish were distributed into four independent recirculating seawater systems (*n* = 6 per tank) comprised by a header tank and 6 flat-bottomed tanks (0.05 m²; Temp.: 19 ± 1°C; Salinity: 24 ppt; O_{2sat}: 100%; pH = 8.1, 0.756 mg l⁻¹ CO₂). Each of the systems comprised a biological and mechanical biofiltration system. The water pH of each system was controlled by a pH-stat system (Aqua Medic®, AT Control-SW, version 9.0) (Wilcox-Freeburg et al., 2013). A solenoid valve was used to regulate the delivery of compressed CO₂ gas into the water of the respective header tanks to establish and maintain experimental pH levels at 7.9, 7.6, or 7.3 with an expected final concentration of 1.253, 2.601, and 5.297 mg l⁻¹ of CO₂, respectively, and calculated according to Mojica Prieto and Millero (2002). The solenoid valves were activated when pH values were 0.1 pH units above their pre-set levels. The pH values of all header tanks were recorded with a data-logger. Control conditions were secured in a fourth system with normal seawater pH (8.1). One fish per tank (*n* = 6) was sampled 4 and 24 h after the beginning of the trial to assess the acute effects of a decrease in pH. The remaining fish were fed with a commercial

diet (2.5% biomass per day) and were weighed and sampled after a 4-week period for the assessment of the prolonged water acidification. At all sampling times, fish were fasted for 24 h prior to sampling, anesthetized by immersion in 2-phenoxyethanol (1500 ppm; Sigma-Aldrich, St. Louis MO, United States) and sampled for skin mucus, blood, gills and head-kidney tissue (HK). A second trial was performed, with the identical water conditions, fish size and pH treatments. A final number of 12 fish per treatment was used for analysis of hematology, plasma and mucus humoral parameters.

For respirometry assays, six fish were maintained in an ambient tank at the desired pH (i.e., pH levels at 8.1, 7.9, 7.6, or 7.3) for 1 week acclimatization (Temp.: $19 \pm 1^\circ\text{C}$; Salinity: 24 ppt; O_2 saturation: 100%) and fasted for 24 h before being chased to exhaustion and placed in the respirometer chamber for 24 h. Following previous studies (Svendsen et al., 2012; Baktoft et al., 2016; Peixoto et al., 2016), chasing procedures were used to induce the maximum metabolic rate (MMR), whereas the standard metabolic rate (SMR) was estimated using respirometry data collected over the subsequent 24 h. Aerobic metabolic scope (AMS) was estimated by subtracting SMR from the MMR (Svendsen et al., 2014).

The experiments were approved by the Animal Welfare Committee of the Interdisciplinary Centre of Marine and Environmental Research and carried out in a registered installation (N16091.UDER). Trained scientists performed experiments in full compliance with national rules and following the European Directive 2010/63/EU of the European Parliament and the European Union Council on the protection of animals used for scientific purposes.

Fish Growth Performance

All fish were weighted at the beginning of the trial and 12 fish per treatment ($n = 1$ per tank) were weighted at 4 weeks of exposure to the different pH. Weight gain (WG) and specific growth rate (SGR) were calculated as follows:

$$\text{WG} = \frac{\text{Final weight} \times 100}{\text{Initial weight}} - 100$$

$$\text{SGR} = \frac{[\ln(\text{Final weight}) - \ln(\text{Initial weight})] \times 100}{\text{time(days)}}$$

Tissue Collection

Skin mucus was gently collected from the skin surface of each fish using a cell scraper and transferred into 2 ml Eppendorf tube, and centrifuged at $400 \times g$ for 10 min at 4°C to remove possible cells and scales. Blood was collected from the caudal vessels using heparinized syringes. A subsample was used for hematological analysis and the remainder centrifuged at $10,000 \times g$ for 10 min at 4°C . After centrifugation, plasma was collected and immediately stored at -80°C for evaluation of innate immune parameters. Head-kidney tissues were excised from all fish and immediately stored at -80°C . The second gill arch of each fish was excised and immersion-fixed in 10% neutral buffered formalin (pH 7.4) for 24 h at 4°C and stored in 70% ethanol for later paraffin embedding.

Analysis of Hematological Parameters

The hematological profiling was conducted according to Machado et al. (2015) and consisted of measurements of hematocrit (Ht), hemoglobin (Hb; SPINREACT kit, ref. 1001230, Spain), and total white (WBC) and red (RBC) blood cells counts. Subsequently, the mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) were calculated. Also, blood smears were made, air dried, and after fixation as described by Machado et al. (2015), the absolute value ($\times 10^4 \text{ ml}^{-1}$) of each cell type (neutrophils, monocytes, lymphocytes and thrombocytes) was calculated based on total blood WBC count.

Plasma and Skin Mucus Innate Humoral Parameters

Plasma Lysozyme

Lysozyme activity was estimated in plasma samples and measured using a turbidimetric assay as described by Costas et al. (2011). Briefly, a solution of *Micrococcus lysodeikticus* (0.5 mg ml^{-1} , 0.05 M sodium phosphate buffer, pH 6.2) was prepared. To a microplate, 15 μl of plasma in triplicates and 250 μl of the above suspension were added to give a final volume of 265 μl . The reaction was carried out at 25°C and the absorbance (450 nm) was measured after 0.5 and 4.5 min in a Synergy HT microplate reader (BioTeck Instruments, Synergy HT). Lyophilized hen egg white lysozyme (Sigma) was serially diluted in sodium phosphate buffer (0.05 M, pH 6.2) and used to develop a standard curve. The amount of lysozyme in the sample was calculated using the formula of the standard curve.

Plasma and Mucus Peroxidase

Total peroxidase activity in plasma and skin mucus was measured following the procedure described by Quade and Roth (1997). Briefly, 15 μl of plasma or mucus in triplicates were diluted with 135 μl of Hank's Balanced Salt Solution (HBSS) without Ca^{+2} and Mg^{+2} in flat-bottomed 96-well plates. Then, 50 μl of 20 mM 3,3',5,5'-tetramethylbenzidine hydrochloride (TMB; Sigma) and 50 μl of 5 mM H_2O_2 were added. The color-change reaction was stopped after 2 min by adding 50 μl of 2 M sulfuric acid and the optical density was read at 450 nm in a Synergy HT microplate reader. Wells without plasma or mucus were used as blanks. The peroxidase activity (units ml^{-1} plasma or mg per protein mucus) was determined by defining one unit of peroxidase as that which produces an absorbance change of 1 OD. Mucus results were presented as units of peroxidase per milligram of protein.

Plasma and Mucus Total Protein and Plasma Immunoglobulin

Total protein concentrations were determined in 1:50 (v/v) diluted plasma and mucus samples using the bicinchoninic acid (BCA) Protein Assay Kit (Pierce #23225, Rockford, IL, United States) for microplates. Bovine serum albumin served as a standard. For immunoglobulins determination, the immunoglobulins were precipitated with polyethylene glycol in 1:2 (v/v) diluted plasma and mucus. After a 2 h incubation, samples were centrifuged for 10 min at $4472 \times g$ and 10 μl of the supernatant was then diluted 1:50 (v/v) in distilled water

and protein determined using the previous Protein Assay Kit. Total plasma and mucus immunoglobulins were determined by subtracting the values that result from the immunoglobulin precipitation by polyethylene glycol to the values obtained for total protein of the samples (Siwicki and Anderson, 1993).

Plasma and Mucus Antiprotease Activity

The anti-protease activity was determined as described by Ellis (1990) with modifications (Machado et al., 2015). Briefly, 10 μ l of plasma or 40 μ l mucus was incubated with 10 μ l of a trypsin solution (5 mg ml⁻¹ in 0.5% NaHCO₃, pH 8.3) for 10 min at 22°C in polystyrene microtubes. To the incubation mixture, 100 μ l of phosphate buffer (NaH₂PO₄, 13.9 mg ml⁻¹, pH 7.0) and 125 ml of azocasein (20 mg ml⁻¹ in 0.5% NaHCO₃, pH 8.3) were added and incubated for 1 h at 22°C. Finally, 250 μ l of 10% trichloroacetic acid (TCA) were added to each microtube and incubated for 30 min at 22°C. The mixture was centrifuged at 10,000 $\times$ g for 5 min at room temperature. Afterward, 100 μ l of the supernatant was transferred to a 96 well-plate containing 100 μ l of 1N NaOH per well. The OD was read at 450 nm in a Synergy HT microplate reader. Phosphate buffer in place of plasma and mucus and trypsin served as blank, whereas the reference sample was phosphate buffer in place of plasma and mucus. The inhibition percentage of trypsin activity was calculated based on the reference sample.

Plasma and Mucus Proteases Activity

For protease activity evaluation, 100 μ l of 0.5% NaHCO₃ (pH 8.3) and 125 μ l azocasein (20 mg ml⁻¹ in 0.5% NaHCO₃, pH 8.3) was added to 10 μ l of plasma or 100 μ l of mucus. After incubation during 24 h at 22°C in polystyrene microtubes, 250 μ l of 10% TCA was added and centrifuged (10,000 $\times$ g for 5 min) and the absorbance of 100 μ l of the supernatant, plus 100 μ l of 1N NaOH read at 450 nm in a Synergy HT microplate reader. The percentage protease activity compared to the reference sample (trypsin solution, 5 mg ml⁻¹ in 0.5% NaHCO₃, pH 8.3) was calculated (Ellis, 1990).

Plasma and Mucus Bactericidal Activity

Photobacterium damsela subsp. *piscicida*, *Phdp*, strain PP3, isolated from yellowtail (*Seriola quinqueradiata*; Japan) by Dr. Andrew C. Barnes (Marine Laboratory, Aberdeen, United Kingdom) was used in the bactericidal activity assay. Bacteria were cultured and exponentially growing bacteria were re-suspended in sterile HBSS and adjusted to 1 $\times$ 10⁶ CFU ml⁻¹. Plating serial dilutions of the suspensions onto TSA-1 plates and counting the number of CFU following incubation at 22°C confirmed the bacterial concentration of the inoculum. Plasma bactericidal activity was then determined following the method described by Graham and Secombes (1988) with modifications (Machado et al., 2015). Briefly, 20 μ l of plasma or mucus were added to duplicate wells of a U-shaped 96-well plate. HBSS was added to some wells instead of plasma and served as positive control. To each well, 20 μ l of *Phdp* (1 $\times$ 10⁶ CFU ml⁻¹) was added and the plate was incubated for 2.5 h at 25°C. Twenty-five μ l of 3-(4, 5 dimethyl-2-yl)-2, 5-diphenyl tetrazolium bromide (1 mg ml⁻¹; Sigma) was added to each well and incubated for

10 min at 25°C to allow the formation of formazan. Plates were then centrifuged at 2000 $\times$ g for 10 min and the precipitate was dissolved in 200 μ l of dimethyl sulfoxide (Sigma). The absorbance of the dissolved formazan was measured at 560 nm. Bactericidal activity was expressed as a percentage, calculated from the difference between bacteria surviving compared to the number of bacteria from positive controls (100%). All analyses were conducted in triplicates.

Gene Expression Analysis

Total RNA isolation was conducted with TRIzol Reagent (Invitrogen) following the manufacturer's specifications. RNA was treated with DNase I (GRIIP Research Solutions, Porto, Portugal) to remove genomic DNA, and first-strand cDNA was synthesized with NZY First-Strand cDNA Synthesis Kit (NZYTech, Lisbon, Portugal). Quantitative PCR assays were performed with an iQ5 Real Time PCR detection System (Bio-Rad) using 1 μ l of diluted cDNA (1:5 dilution) mixed with 10 μ l of iQ SYBR green 2x Supermix (Bio-Rad) and 0.4 μ l (10 mM) of each specific primer in a final volume of 20 μ l. The cDNA amplifications were carried out with specific primer pairs (Table 1) for genes of interest, which were designed using NCBI Primer Blast Tool according to known qPCR restrictions (amplicon size, T_m difference between primers, GC content and self-dimer or cross-dimer formation). The efficiencies of the primer pairs were analyzed in serial five-fold dilutions of cDNA by calculating the slope of the regression line of the cycle thresholds (Ct) versus the relative concentration of cDNA (Livak and Schmittgen, 2001).

Efficiency values, complete sequences and product length are presented in Table 1. Melting curve analysis was also performed to verify that no primer dimers were amplified. The standard cycling conditions were 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. All reactions were carried out as technical duplicates. The Ct values were compared through the comparative Ct method to the geometric mean derived from the expression of Senegalese sole 18S ribosomal (18S) (Livak and Schmittgen, 2001). Fold change units were calculated by dividing the normalized expression values of tissue from different treatments by the normalized expression values of normocapnia at 4 h treatment group.

Gills Immunofluorescence

The formalin fixed gill tissue was decalcified and processed for paraffin embedding. Gills were sectioned (5 μ m) using a Leica RM2235 RTS microtome. The sections were collected on APS coated glass slides, air-dried, de-waxed and rehydrated. Following a blocking step with BLØK blocking buffer (Merck KGaA, Darmstadt, Germany), immunohistochemical staining was accomplished using primary antibodies against key ion-acid-base transporters, which included antibodies against the Na⁺/K⁺ ATPase α subunit, Na⁺: K⁺: 2Cl⁻ cotransporter (NKCC), cystic fibrosis transmembrane regulator (CFTR), Na⁺/H⁺ exchanger 3b (NHE3b), cytosolic carbonic anhydrase I and II (CA I and CA II, respectively). See Table 2 for optimal dilutions, antigen retrieval and antibody incubation conditions. All antibodies were diluted in BLØK and further

TABLE 1 | Genes, real-time PCR primer information including gene names, symbols, reaction efficiencies, predicted product sizes, and primer sequences (5'-3').

Gene name	Symbol	Gene bank	Eff ^a	Product length ^b	Forward primer	Reverse primer
18S ribosomal	18S	EF126042.1	2.15	129	GGTCCGAAGCGTTTACTTTG	GCCCCAGTTTCAGAGAGAGAA
Interleukin 1 β	IL1- β	KU695545	2.06	127	ACTGAGGCCAGACCTGTAGC	TGACGGTTGACAGACAGCTT
Ciclo-oxygenase-2 ^c	COX 2	-	2.07	100	CCGACTTACAATGCGGATTATGGTT	TTGGGCAATCCTCTGGTACAG
Glucocorticoid receptor 1	GR1	AB614369.1	2.11	154	CATGACGACCTGAACCGAT	CCAGCCCAGACTGAAAGACA
Interleukin 10 ^c	IL10	-	2.06	78	CCGTCTTTGTGTTATTTCTCCAACAG	TGGAGTTCAGCTTTGTGATGTCA
Hypoxia Inducible factor 1	HIF-1	FF288855.1	1.96	140	TGCCACGCAGTAAATCAG	CTTCACCGCTTTGACTGTA

^aEfficiency of PCR reactions were calculated from serial dilutions of tissue RT reactions in the validation procedure. ^bAmplicon (nt). ^cTranscript sequences were retrieved from SoleaDB (http://www.juntadeandalucia.es/agriculturaypesca/ifapa/soleadb_ifapa/).

information on these antibodies can be found in **Table 2**. The following antibody pairs were used for double immunolabeling of the gill tissue sections as outlined in **Table 3**. Secondary antibodies, which included goat anti-mouse (Alexa555) and goat anti-rabbit (Alexa488), were diluted 1:500 in BLØK and incubated with sections for 1 h at 37°C. Sections were rinse in TPBS (0.05% Tween-20 in Phosphate Buffered Saline, pH 7.4) and counterstained with DAPI (4',6-Diamidine-2'-phenylindole dihydrochloride) and viewed with a Leica DM5500B photomicroscope using Leica LASX software and a Hamamatsu Orca Flash 4 camera. Images immunolabelled for NKA ($\alpha 5$) were analyzed using SigmaScan Pro software for cell average size (area), average (green or red) fluorescence area-intensity and shape-factor. Gill ionocyte counts were also made and expressed as either per length of the gill filament or per interlamellar space. The proportion of NHE3b positive NKA-IR cell was also made.

Respirometry

Metabolic rates were measured by intermittent flow respirometry as described by Svendsen et al. (2012). Static respirometer chambers (0.6 L) were used to measure oxygen consumption rate ($\dot{M}O_2$; mg O₂ kg⁻¹ h⁻¹) at the different pH values. Six fish were maintained in an ambient tank at the desired pH (pH 8.1, 7.9, 7.6, or 7.3) for 1 week acclimatization (Temp.: 19 ± 1°C; Salinity: 24 ppt; O₂ air saturation: 100%) and fasted for 24 h before being placed in the respirometer chamber.

Oxygen partial pressure (kPa) inside the respirometers was measured using galvanic sensor technology (Mini DO Probe; Loligo Systems; Denmark). The software AutoResp (Loligo Systems Aps, Tjele, Denmark) collected the data every 9 min, and calculated $\dot{M}O_2$ from measurements of oxygen content inside the respirometers, adjusted for the body mass of the individual fish. The respirometer was submerged in the ambient tank supplying water at the desired pH (pH 8.1, 7.9, 7.6, or 7.3) for the respirometer. A standard protocol was used to induce MMR where individual fish were handled for 3 min and then exposed to 2 min of air exposure. Following exhaustion, fish were transferred to the respirometers where $\dot{M}O_2$ recordings started immediately. MMR was estimated as the average of the highest three consecutive measures of $\dot{M}O_2$ recorded immediately after placement of the fish inside the respirometry chamber. Next, fish were left undisturbed in the respirometers for 24 h and $\dot{M}O_2$ values were logged continuously. SMR was estimated as the average of the 10% lowest $\dot{M}O_2$ values (Baktoft et al., 2016; Peixoto et al., 2016). AMS was calculated as the difference between MMR and SMR (Svendsen et al., 2014).

Statistical Analysis

All results are expressed as mean ± standard deviation (mean ± SD). Data were analyzed for normality and homogeneity of variance and, when necessary, transformed

TABLE 2 | Antibodies used for immunofluorescence microscopy.

Antibody	Origin
Na ⁺ /K ⁺ -ATPase α subunit (Rabbit Polyclonal α R1)	Wilson et al., 2007
NKCC (Mouse Monoclonal; T4 clone)	Developmental Studies Hybridoma Bank (Iowa, United States)
CFTR (cystic fibrosis transmembrane conductance factor)	R&D Systems
Na ⁺ /K ⁺ ATPase α subunit (Mouse Monoclonal; $\alpha 5$ clone)	Developmental Studies Hybridoma Bank (Iowa, United States)
NHE3b (Na ⁺ /H ⁺ exchanger)	Hirose
CA I (Carbonic Anhydrase I)	Abcam
CA II (Carbonic Anhydrase II)	Abcam
Alexa Fluor 488 (Goat anti rabbit)	Invitrogen (Carlsbad, CA, United States)
Alexa Fluor 568 (Goat anti mouse)	Invitrogen (Carlsbad, CA, United States)

TABLE 3 | Antibody combinations and dilutions used for immunofluorescence microscopy.

Combination	Primary antibodies	Dilution	Secondary antibodies	Dilution
NHE3b and $\alpha 5$	NHE3b	1:1000	Alexa 488	1:500
	$\alpha 5$	1:100	Alexa 568	1:500
NHE3b and NKCC	NHE3b	1:1000	Alexa 488	1:500
	NKCC	1:100	Alexa 568	1:500
α R1 and $\alpha 5$	α R1	1:500	Alexa 488	1:500
	$\alpha 5$	1:100	Alexa 568	1:500
α R1 and CFTR	α R1	1:500	Alexa 488	1:500
	CFTR	1:50	Alexa 568	1:500
CAI and $\alpha 5$	CAI	1:100	Alexa 488	1:500
	$\alpha 5$	1:100	Alexa 568	1:500
CAII and $\alpha 5$	CAII	1:100	Alexa 488	1:500
	$\alpha 5$	1:100	Alexa 568	1:500

before being treated statistically. All data expressed as percentage were arcsine transformed (Zar, 1999). Data were analyzed by two-way ANOVA, with time and pH as factors, followed by the Tukey *post hoc* test to identify differences in the experimental treatments. All statistical analyses were performed using the computer package STATISTICA 12 for WINDOWS. The level of significance used was $P \leq 0.05$ for all statistical tests. Finally, one-way ANOVAs were used to examine the effects of pH on growth estimates and the metabolic rate data.

RESULTS

Fish Growth Performance

After 4 weeks of exposure to the different pH, fish held at pH 7.3 displayed lower final body weight than those fish held at pH 8.1 and 7.9, indicating reduced growth among the fish exposed to elevated CO₂ levels (Table 4).

Hematological and Peripheral Leukocytes Responses

Data regarding fish hematological profiles and differential peripheral leukocyte counts can be found in Tables 5, 6. Total WBC, lymphocyte and monocyte concentrations increased in fish exposed to pH 7.3 compared to those exposed to pH 7.6, 7.9, and 8.1 regardless time. Similarly, fish exposed to the lowest pH revealed a higher concentration of thrombocytes compared to those under pH treatments 7.9 and 8.1. Lymphocyte numbers also augmented in fish held at pH 7.6 relative to the control pH (8.1). Finally, neutrophil concentration increased in fish held at pH 7.3 compared to those exposed to pH 7.6 and 8.1. Regardless of pH treatment, Ht, MCV, total WBC, thrombocytes, lymphocytes and neutrophils values showed an increase in time.

Plasma Humoral Responses

No differences were observed in plasma lysozyme and peroxidase activities (Table 7). Higher concentrations of plasma total proteins were observed after 4 weeks relative to 4 and 24 h, whereas total immunoglobulin levels increased at 4 h relative to 24 h, regardless pH. Senegalese sole exposed to pH 7.3 showed higher antiproteases activity at 24 h relative to the remaining treatments. At 4 weeks, fish reared at pH 8.1 and 7.9 showed increased levels of plasma antiprotease activity relative to those

sampled at 4 h, while fish held at pH 7.6 showed higher activity at 4 weeks relative to those sampled at 4 and 24 h. Plasma bactericidal activity decreased at 4 h in fish reared at pH 8.1 relative to those held at pH 7.3 while the opposite pattern was observed at 24 h. At pH 8.1, bactericidal activity was higher at 24 h and 4 weeks compared to 4 h, whereas fish under pH 7.9 and 7.6 presented higher bactericidal activities at 4 weeks relative to 4 h. Lastly, the bactericidal activity of fish from pH 7.3 treatment increased from 24 h to 4 weeks.

Mucus Humoral Responses

Mucus peroxidase activity decreased at 24 h relative to 4 h while the bactericidal activity increased at 24 h and 4 weeks relative to 4 h in fish exposed to pH 7.9 and at 4 weeks relative to 4 h in those reared at pH 7.6. Fish exposed to pH 8.1 and sampled at 24 h presented higher anti-protease activity compared to the remaining pH treatments and showed lower activity at 4 weeks than at 4 and 24 h (Table 8).

Gene Expression

The mRNA expression levels of *il1 β* (Figure 1A) and *cox2* (Figure 1B) increased in fish exposed to pH 7.3 compared to those exposed to pH 8.1 and 7.9 after 4 weeks. The lowest pH treatment (pH 7.3) also up-regulated *gr1* compared to pH 8.1 and 7.9, but this effect was present regardless of sampling time (Figure 1C). Also, at 4 weeks, mRNA expression levels of *il10* (Figure 1E) augmented in fish exposed to the lowest pH level (pH 7.3) relative to the remaining pH treatments. After 24 h, *hif1* transcripts were higher in fish under pH 7.6 relative to those exposed to pH 8.1 (Figure 1D). Fish held at the lowest pH (7.3) showed higher expression levels of *cox-2* and *il10* at 4 weeks compared to those sampled at 4 and 24 h. The expression of *il1 β* was also higher in the same fish group (7.3) but only compared to those sampled at 24 h. Finally, the mRNA expression of *hif1* was enhanced in fish sampled at 24 h compared to those sampled at 4 weeks and held at both pH 7.6 and 7.3.

Gills Immunofluorescence

The number of NHE3b positive gill ionocytes (NKA-immunoreactive cells) per gill filament section increased with lowest water pH (Figure 2). At 4 h of exposure, a higher amount of NHE3b positive cells was found in fish exposed to pH 7.3 relative to the remaining pH treatments, while after 24 h

TABLE 4 | Data on the initial weight (g) and final weight (g), weight gain (%) and specific growth rate (%) of Senegalese sole exposed to different water pH levels over 4 weeks.

Parameters	pH				One-way ANOVA (p-value)
	8.1	7.9	7.6	7.3	
Initial weight	82.48 $\pm$ 0.16	83.125 $\pm$ 0.77	83.67 $\pm$ 0.11	81.67 $\pm$ 0.42	ns
Final weight	89.68 $\pm$ 15.05 ^a	87.18 $\pm$ 17.28 ^a	84.32 $\pm$ 9.48 ^{ab}	76.37 $\pm$ 3.69 ^b	0.043
Weight gain	8.73 $\pm$ 18.24	4.87 $\pm$ 11.45	0.77 $\pm$ 11.38	-6.49 $\pm$ 4.51	ns
Specific growth rate	0.32 $\pm$ 0.77	0.12 $\pm$ 0.97	0.01 $\pm$ 0.50	-0.31 $\pm$ 0.22	ns

Values are presented as means $\pm$ SD (n = 6). P-values from one-way ANOVA ($p \leq 0.05$). If interaction was significant, Tukey *post hoc* test was used to identify differences in the experimental treatments. Different lowercase letters indicate differences among pH.

TABLE 5 | Hematocrit (%), hemoglobin (g dl), mean corpuscular volume (MCV; μm^3), mean corpuscular hemoglobin (MCH; pg cell^{-1}), mean corpuscular hemoglobin concentration (MCHC; $\text{g } 100 \text{ ml}^{-1}$), red blood cells [RBC; ($\times 10^6 \mu\text{l}$)], and white blood cells [WBC; ($\times 10^4 \mu\text{l}$)] of Senegalese sole submitted to different water pH during 4, 24 h and 4 weeks.

Parameters	pH											
	8.1			7.9			7.6			7.3		
	4 h	24 h	4 weeks	4 h	24 h	4 weeks	4 h	24 h	4 weeks	4 h	24 h	4 weeks
Hematocrit	8.27 $\pm$ 1.88	7.53 $\pm$ 0.93	9.17 $\pm$ 2.11	7.25 $\pm$ 1.35	8.17 $\pm$ 2.80	11.20 $\pm$ 2.23	7.72 $\pm$ 1.77	7.73 $\pm$ 1.16	9.00 $\pm$ 1.79	7.84 $\pm$ 1.46	9.01 $\pm$ 1.78	9.20 $\pm$ 2.23
Hemoglobin	1.78 $\pm$ 0.10	1.79 $\pm$ 0.10	1.79 $\pm$ 0.10	1.77 $\pm$ 0.14	1.81 $\pm$ 0.18	1.69 $\pm$ 0.11	1.80 $\pm$ 0.07	1.79 $\pm$ 0.17	1.69 $\pm$ 0.09	1.85 $\pm$ 0.15	1.88 $\pm$ 0.18	1.79 $\pm$ 0.18
MCV	74.72 $\pm$ 4.61	86.99 $\pm$ 21.17	126.17 $\pm$ 18.65	88.35 $\pm$ 35.12	91.94 $\pm$ 36.64	114.30 $\pm$ 5.62	94.06 $\pm$ 43.98	107.87 $\pm$ 42.28	126.05 $\pm$ 24.97	70.55 $\pm$ 55.76	101.57 $\pm$ 15.84	137.62 $\pm$ 31.42
MCH	17.02 $\pm$ 4.50	21.12 $\pm$ 6.24	25.68 $\pm$ 5.32	24.42 $\pm$ 10.67	20.43 $\pm$ 3.71	18.75 $\pm$ 4.22	21.63 $\pm$ 5.27	26.59 $\pm$ 6.22	23.56 $\pm$ 5.76	24.43 $\pm$ 4.94	21.13 $\pm$ 3.36	24.40 $\pm$ 5.92
MCHC	22.58 $\pm$ 4.93	24.14 $\pm$ 3.40	21.17 $\pm$ 7.20	25.16 $\pm$ 5.55	20.88 $\pm$ 3.48	15.68 $\pm$ 3.81	24.44 $\pm$ 4.96	23.37 $\pm$ 3.81	19.82 $\pm$ 5.17	24.65 $\pm$ 2.97	21.48 $\pm$ 3.33	20.00 $\pm$ 4.42
RBC	1.12 $\pm$ 0.29	0.92 $\pm$ 0.26	0.72 $\pm$ 0.11	0.82 $\pm$ 0.24	0.90 $\pm$ 0.11	0.94 $\pm$ 0.19	0.88 $\pm$ 0.21	0.72 $\pm$ 0.20	0.75 $\pm$ 0.14	0.78 $\pm$ 0.14	0.85 $\pm$ 0.29	0.76 $\pm$ 0.17
WBC	11.88 $\pm$ 1.15	12.75 $\pm$ 1.11	16.08 $\pm$ 2.52	13.68 $\pm$ 4.08	15.52 $\pm$ 3.68	19.18 $\pm$ 3.03	14.96 $\pm$ 3.22	16.19 $\pm$ 5.06	21.83 $\pm$ 2.63	16.49 $\pm$ 4.04	21.44 $\pm$ 7.38	27.37 $\pm$ 6.11

Two-way ANOVA				pH				Time		
Parameters	pH	Time	pH $\times$ Time	8.1	7.9	7.6	7.3	4 h	24 h	4 weeks
Hematocrit	ns	0.002	ns	–	–	–	–	B	B	A
Hemoglobin	ns	ns	ns	–	–	–	–	–	–	–
MCV	ns	<0.001	ns	–	–	–	–	A	A	B
MCH	ns	ns	ns	–	–	–	–	–	–	–
MCHC	ns	ns	ns	–	–	–	–	–	–	–
RBC	ns	ns	ns	–	–	–	–	–	–	–
WBC	<0.001	<0.001	ns	B	B	B	A	C	B	A

Values are presented as means $\pm$ SD ($n = 12$). P -values from two-way ANOVA ($p \leq 0.05$). If interaction was significant, Tukey post hoc test was used to identify differences in the experimental treatments. Different capital letters indicate differences among pH regardless time or among times regardless pH.

TABLE 6 | Absolute values ($\times 10^4/\mu$) of peripheral blood leukocytes (thrombocytes, lymphocytes, monocytes, and neutrophils) of Senegalese sole submitted to different water pH during 4, 24 h and 4 weeks.

Parameters	pH											
	8.1				7.9				7.6			
	4 h	24 h	4 weeks	4 h	24 h	4 weeks	4 h	24 h	4 h	24 h	4 weeks	4 h
Thrombocytes	7.14 $\pm$ 1.08	6.47 $\pm$ 1.23	9.32 $\pm$ 1.26	7.32 $\pm$ 1.86	7.33 $\pm$ 1.73	9.81 $\pm$ 1.22	7.65 $\pm$ 1.46	8.04 $\pm$ 2.05	11.15 $\pm$ 1.14	7.76 $\pm$ 1.20	9.09 $\pm$ 4.18	14.64 $\pm$ 3.96
Lymphocytes	4.31 $\pm$ 1.00	6.02 $\pm$ 1.24	5.28 $\pm$ 1.86	5.65 $\pm$ 2.58	6.99 $\pm$ 1.84	7.19 $\pm$ 3.16	6.49 $\pm$ 1.91	6.82 $\pm$ 2.17	9.69 $\pm$ 1.86	7.68 $\pm$ 3.18	10.06 $\pm$ 3.39	9.81 $\pm$ 3.04
Monocytes	0.14 $\pm$ 0.11	0.13 $\pm$ 0.14	0.23 $\pm$ 0.20	0.17 $\pm$ 0.14	0.36 $\pm$ 0.35	0.27 $\pm$ 0.21	0.21 $\pm$ 0.15	0.31 $\pm$ 0.41	0.17 $\pm$ 0.13	0.29 $\pm$ 0.15	0.41 $\pm$ 0.36	0.68 $\pm$ 0.65
Neutrophils	0.30 $\pm$ 0.17	0.13 $\pm$ 0.20	1.26 $\pm$ 0.56	0.53 $\pm$ 0.58	0.84 $\pm$ 0.99	1.95 $\pm$ 1.63	0.60 $\pm$ 0.52	0.92 $\pm$ 1.10	0.81 $\pm$ 0.37	0.75 $\pm$ 0.42	1.88 $\pm$ 0.73	2.24 $\pm$ 1.27

Parameters	pH							
	Time				pH x Time			
Thrombocytes	<0.001	<0.001	ns	ns	B	BC	B	AB
Lymphocytes	<0.001	0.008	ns	ns	C	B	B	B
Monocytes	0.008	ns	ns	ns	B	B	B	B
Neutrophils	<0.001	<0.001	ns	ns	B	AB	AB	B

Values are presented as means $\pm$ SD ($n = 12$). *P*-values from two-way ANOVA ($p \leq 0.05$). If interaction was significant, Tukey post hoc test was used to identify differences in the experimental treatments. Different capital letters indicate differences among pH regardless time or among times regardless pH.

all the CO₂ treatment groups (pH 7.3, 7.6 and 7.9) presented higher amounts of NHE3b positive cells compared to those observed with pH 8.1 control group. Moreover, after 4 weeks the number of NHE3b positive cells in fish exposed to both pH 7.3 and 7.6 continued enhanced relative to those observed in fish held at pH 8.1. Data for the shape factor of the whole cell (NKA immunoreactivity) (**Figure 3**) characterizes the degree of deviation from an ideal shape (a circle shape) normalized to a range of zero to one where one is closest to the ideal shape. The only difference observed was a lower shape factor in fish exposed to pH 7.9 compared to the pH 8.1 control group. No interactions were observed. There was no noticeable difference in area or intensity of immunofluorescence or in the number of immunoreactive cells (ionocytes) among the treatment for any other antibody tested. Example of gills immunohistochemical colocalization of NKA with NKCC, CA and NHE3b in the gills of Senegalese sole presented in **Figure 4**.

Respirometry

No differences were found in SMR or in MMR values of any experimental group whereas fish submitted to pH 7.3 presented a reduced AMS compared to those maintained at pH 8.1 (i.e., the control group) (**Table 9**). These findings indicated reduced aerobic performance among the fish exposed to the lowest pH.

DISCUSSION

Increasing water CO₂ in a RAS scenario or due to anthropogenic release leads to physiological adaptations in fish activating regulatory mechanism toward acidosis compensation. Findings of the present study pointed to a clear impact on immune mechanisms, gills and aerobic performance, mainly after chronic exposure to extreme hypercapnic water experienced in fish farming, with the outcome of negatively impacting Senegalese sole growth.

Acid-base regulation allows the well- functioning of basic metabolic mechanisms when homeostasis is challenged by environmental changes in the respiratory gases, carbon dioxide and oxygen. Small modifications of hematological and innate-immune mechanisms could allow fish to respond to this external stressor and ultimately restoring balance. Since the effects of short-term hypercapnic exposure are expected to be mitigated by slight physiological modifications (Cech and Crocker, 2002; Heuer and Grosell, 2014; Esbaugh, 2018), and the disturbance rapidly alleviated, few works have targeted this subject from an immunological perspective, assessing the direct effect on the immune mechanisms. The present study supports in fact this observation, since no differences were observed regarding hematological parameters nor peripheral leukocyte population after only 4 and 24 h of exposure to low water pH. Nonetheless, the increased plasma total bactericidal and antiproteases activities observed at 4 and 24 h of exposure, respectively, in Senegalese sole exposed to the lowest pH treatment (7.3) possibly reflected an immune response adjustment to the water acidification-induced stress. Also, skin mucus antiprotease activity decreased at 24 h in all hypercapnia treatments thus showing an effect

TABLE 7 | Plasma lysozyme ($\mu\text{g mg protein}^{-1}$), peroxidase (units ml^{-1}), total protein (mg ml^{-1}), total immunoglobulins (mg ml^{-1}), antiprotease (%), proteases (%) and bactericidal activities (%) of Senegalese sole submitted to different water pH during 4, 24 h and 4 weeks.

Plasma Parameters	pH											
	8.1			7.9			7.6			7.3		
	4 h	24 h	4 weeks	4 h	24 h	4 weeks	4 h	24 h	4 weeks	4 h	24 h	4 weeks
Lysozyme	1.86 $\pm$ 0.39	2.37 $\pm$ 0.58	1.09 $\pm$ 0.73	2.41 $\pm$ 0.24	2.25 $\pm$ 0.76	1.75 $\pm$ 0.60	2.38 $\pm$ 0.51	2.13 $\pm$ 0.75	1.77 $\pm$ 0.72	2.13 $\pm$ 0.63	1.82 $\pm$ 0.59	1.27 $\pm$ 1.15
Peroxidase	25.19 $\pm$ 2.78	38.53 $\pm$ 22.48	35.15 $\pm$ 21.62	29.02 $\pm$ 7.03	51.37 $\pm$ 22.42	49.64 $\pm$ 25.51	28.63 $\pm$ 2.44	124.56 $\pm$ 60.61	117.59 $\pm$ 63.43	31.07 $\pm$ 13.97	33.26 $\pm$ 9.32	34.75 $\pm$ 9.40
Total Protein	33.51 $\pm$ 3.06	30.88 $\pm$ 4.75	35.68 $\pm$ 2.22	31.49 $\pm$ 4.58	33.92 $\pm$ 3.60	35.84 $\pm$ 3.38	34.75 $\pm$ 3.12	32.34 $\pm$ 3.45	36.18 $\pm$ 4.15	31.00 $\pm$ 6.41	33.60 $\pm$ 2.18	34.72 $\pm$ 3.84
Total Immunoglobulin	17.38 $\pm$ 2.12	12.70 $\pm$ 6.69	12.72 $\pm$ 1.93	14.68 $\pm$ 3.57	10.50 $\pm$ 4.19	12.68 $\pm$ 2.81	18.65 $\pm$ 2.82	11.27 $\pm$ 4.35	15.06 $\pm$ 3.05	13.61 $\pm$ 7.68	11.11 $\pm$ 1.07	13.41 $\pm$ 2.05
Antiprotease activity	71.07 $\pm$ 0.32 ^{*§}	67.04 $\pm$ 2.13 ^{b*}	77.93 $\pm$ 0.81 [§]	68.56 $\pm$ 1.62 ^{*§}	65.79 $\pm$ 2.96 ^{b*}	76.66 $\pm$ 1.68 [§]	65.81 $\pm$ 4.43 [*]	64.11 $\pm$ 2.48 ^{b*}	77.16 $\pm$ 1.02 [§]	67.04 $\pm$ 3.46 [*]	76.25 $\pm$ 3.92 ^{a§}	70.39 $\pm$ 9.80 ^{*§}
Proteases activity	10.65 $\pm$ 0.59	10.67 $\pm$ 1.87	9.59 $\pm$ 0.74	10.08 $\pm$ 0.71	10.48 $\pm$ 0.71	9.74 $\pm$ 0.73	9.71 $\pm$ 0.39	9.49 $\pm$ 1.09	9.54 $\pm$ 1.00	10.29 $\pm$ 0.81	10.05 $\pm$ 0.73	9.19 $\pm$ 0.77
Bactericidal activity	31.14 $\pm$ 3.10 ^{b*}	47.63 $\pm$ 5.14 ^{a§}	44.74 $\pm$ 3.98 [§]	34.63 $\pm$ 5.61 ^{ab*}	43.34 $\pm$ 7.11 ^{ab*§}	46.93 $\pm$ 6.02 [§]	40.87 $\pm$ 4.06 ^{ab*}	41.13 $\pm$ 6.77 ^{ab*§}	52.35 $\pm$ 2.73 [§]	42.93 $\pm$ 4.85 ^{a*§}	34.71 $\pm$ 5.02 ^{b*}	50.76 $\pm$ 6.71 [§]

Two-way ANOVA					Time		
Parameters	pH	Time	pH x Time		4 h	24 h	4 weeks
Lysozyme	ns	ns	ns		–	–	–
Peroxidase	ns	ns	ns		–	–	–
Total Protein	ns	0.032	ns		B	B	A
Total Immunoglobulin	ns	0.003	ns		A	B	AB
Antiprotease activity	ns	<0.001	<0.001		B	B	A
Proteases activity	ns	ns	ns		–	–	–
Bactericidal activity	ns	<0.001	<0.001		C	B	A

Values are presented as means $\pm$ SD ($n = 12$). P -values from two-way ANOVA ($p \leq 0.05$). If interaction was significant, Tukey post hoc test was used to identify differences in the experimental treatments. Different lowercase letters stand for significant differences among pH treatments for the same time, while different symbols stands for significant differences between times for the same pH. Different capital letters indicate differences among pH regardless time or among times regardless pH.

TABLE 8 | Mucus peroxidase (units protein), total protein (mg ml⁻¹), antiprotease (%), proteases (%) and bactericidal activities (%) of Senegalese sole submitted to different water pH during 4, 24 h and 4 weeks.

Mucus Parameters	pH											
	8.1			7.9			7.6			7.3		
	4 h	24 h	4 weeks	4 h	24 h	4 weeks	4 h	24 h	4 weeks	4 h	24 h	4 weeks
Peroxidase	1.29 ± 0.31	1.14 ± 0.15	1.02 ± 0.13	1.57 ± 0.23	0.88 ± 0.06	1.22 ± 0.19	1.40 ± 0.57	1.17 ± 0.16	1.26 ± 0.10	1.05 ± 0.38	0.96 ± 0.33	1.06 ± 0.10
Total Protein	1.32 ± 0.41	1.28 ± 0.27	1.61 ± 0.34	0.89 ± 0.11	1.71 ± 0.15	1.19 ± 0.09	1.70 ± 1.29	1.41 ± 0.48	1.10 ± 0.09	1.55 ± 0.64	2.03 ± 1.43	1.35 ± 0.16
Antiproteases	62.53 ± 3.67*	71.28 ± 1.04 ^a *	57.98 ± 2.34 [§]	69.53 ± 4.18	57.93 ± 2.43 ^b	62.63 ± 3.30	64.88 ± 7.95	58.01 ± 3.41 ^b	62.82 ± 4, 24	61.75 ± 9.98	58.32 ± 10.02 ^b	62.14 ± 5.57
Proteases	6.52 ± 1.05	6.43 ± 1.72	7.28 ± 1.18	5.38 ± 0.42	7.66 ± 1.29	6.02 ± 1.19	7.57 ± 3.31	7.15 ± 2.05	8.02 ± 0.25	6.99 ± 1.87	9.30 ± 4.50	7.54 ± 2.15
Bactericidal activity	25.41 ± 5.90	44.05 ± 17.23	25.52 ± 9.28	10.43 ± 6.72*	35.50 ± 4.60 [§]	45.14 ± 23.90 [§]	19.45 ± 7.87*	28.37 ± 6.16* [§]	52.52 ± 16.93 [§]	27.63 ± 4.18	31.11 ± 3.18	34.64 ± 7.50
Two-way ANOVA												
Parameters	pH	Time	pH x Time	Time								
				4 h	24 h	4 weeks						
Peroxidase	ns	0.005	ns	A	B	AB						
Total Protein	ns	ns	ns	–	–	–						
Antiprotease activity	ns	ns	0.001	–	–	–						
Proteases activity	ns	ns	ns	–	–	–						
Bactericidal activity	ns	<0.001	0.002	B	B	A						

Values are presented as means ± SD (n = 12). P-values from two-way ANOVA ($p \leq 0.05$). If interaction was significant, Tukey post hoc test was used to identify differences in the experimental treatments. Different lowercase letters stand for significant differences among pH treatments for the same time, while different symbols stands for significant differences between times for the same pH. Different capital letters indicate differences among pH regardless time or among times regardless pH.

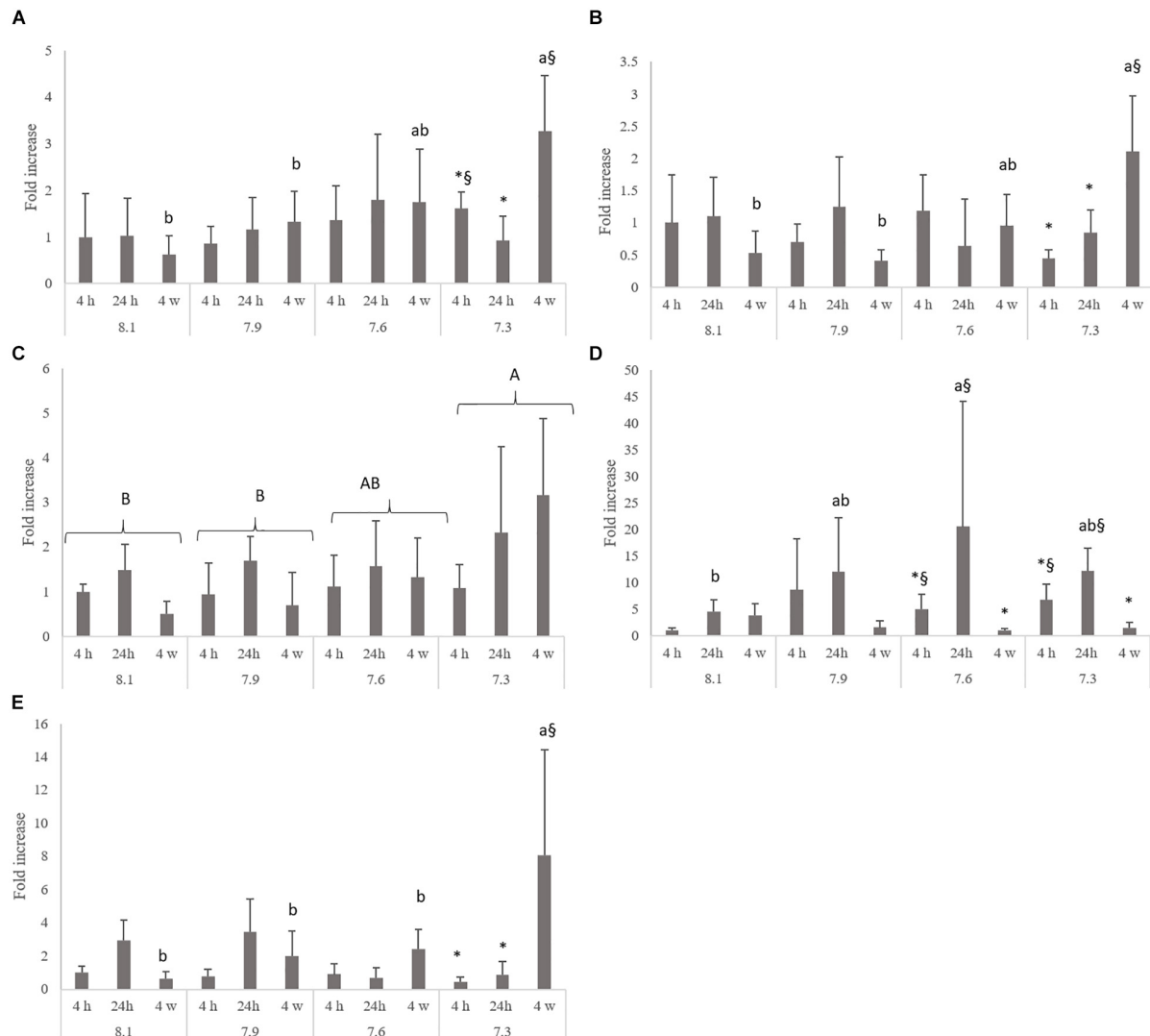


FIGURE 1 | Quantitative expression of interleukin-1 β (**A**; p -value = 0.032), cyclooxygenase-2 (**B**; p -value < 0.001), glucocorticoids receptor 1 (**C**; p -value = 0.007), hypoxia inducible factor-1 alpha (**D**; p -value = 0.0047) and interleukin 10 (**E**; p -value < 0.001) in the head-kidney of Senegalese sole juveniles submitted to pH levels of 8.1, 7.9, 7.6, and 7.3 for 4 h and 24 h and 4 weeks. Data are expressed as means $\pm$ SD (n = 6). Bars represent the fold increase in expression as compared to fish exposure to normocapnia (8.1 pH) at 4 h, previously normalized to endogenous 18S ribosomal (18 s). Different lowercase letters stand for significant differences among pH treatments for the same time, while different symbols stands for significant differences between times for the same pH. Different capital letters indicate differences among pH regardless time or among times regardless pH. (Two-way ANOVA; Tukey *post hoc* test; $p \leq 0.05$).

of acute hypercapnia exposure also on the external mucosal barrier. Mucosal surfaces (i.e., skin, gills and gut) provide fish the first line of defense, and skin mucus, similarly to peripheral blood, is armed with potent molecules, including lysozymes, complement proteins, lectins and antimicrobial peptides with important immune roles (Cabillon and Lazado, 2019). In this study, the modulation of humoral parameters found both in skin mucus and plasma seem to suggest some degree of adjustment to the acidic ambient water, even after a short period.

By being in direct contact with the external environment, CO₂ is rapidly sensed by chemoreceptors in fish gills, which then trigger compensatory mechanisms toward the acidosis by transporting acid and base equivalents in and out the body

through branchial ionocytes (Heuer and Grosell, 2014). In fact, extreme hypercapnia for short-periods can severely disrupt acid-base balance and gas transfer across fish gills in the same way as a long-term exposure to intermediate hypercapnia levels (Randall and Daxboeck, 1984). In the present work, the gills of fish exposed to the lowest water pH (7.3) for only 4 h displayed an increased number of Na⁺/H⁺ exchanger (NHE3b) positive ionocytes which are likely involved in acid secretion as a physiological adaptation acidosis compensation (Evans et al., 2005). Though these cells progressively decreased with time at pH 7.3, their value is nonetheless higher than the control treatment group. Moreover, fish reared in the remaining hypercapnia treatments (pH 7.9 and 7.6) presented even higher frequencies of NHE3b

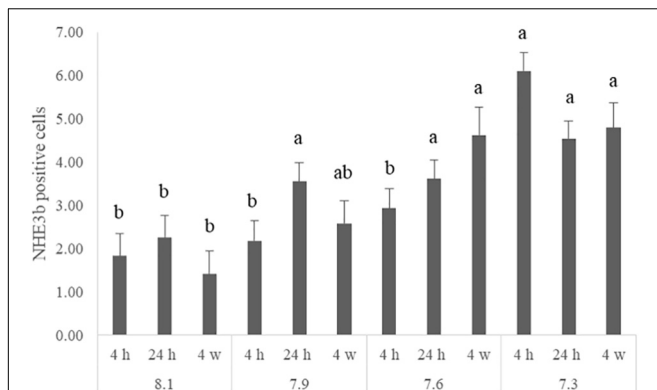


FIGURE 2 | Number of NHE3b positive cells for each quantified filament in the gills of Senegalese sole juveniles submitted to pH levels of 8.1, 7.9, 7.6, and 7.3 for 4 and 24 h and 4 weeks. Data are expressed as means $\pm$ SD ($n = 6$; p -value = 0.029). Different lowercase letters stand for significant differences among pH treatments for the same time. (Two-way ANOVA; Tukey *post hoc* test; $p \leq 0.05$).

positive cells than the control at 24 h. NHE3 is involved in the system that mediates transepithelial pH regulation which makes use of the inward Na^+ gradient to drive the excretion of H^+ in ionocytes (Claiborne et al., 2002). Seidelin et al. (2001) reported that its mRNA expression and protein abundance is consistent with a need for acid excretion upon exposure to hypercapnia. However, at the lower levels (pH 7.9) of hypercapnia typical of ocean acidification conditions, acidosis can be compensated by increased HCO_3^- retention without an increase in NHE expression (Heuer and Grosell, 2014).

In the present study, the gene coding for the hypoxia inducible factor-1 α (*hif1*) was found up-regulated at 24 h in the head-kidney of fish held at pH 7.6 relative to the control while being maintained for the remaining levels of hypercapnia. HIF

is the prime regulator of the transcriptional response to oxygen availability and is also responsible for regulating inflammatory genes transcription (Schofield and Ratcliffe, 2004). As the product of oxidative metabolism, CO_2 shares an inverse relationship with O_2 levels. Moreover, in a normoxic situation HIF-1 is constitutively marked for degradation by prolyl hydrolases (Semenza, 1999), which in turn targets HIF-1 α subunit for ubiquitination and degradation (Schofield and Ratcliffe, 2004). In contrast, under hypoxic conditions and when CO_2 is higher, HIF-1 α degradation is diminished (Selfridge et al., 2016). In the present study, water oxygen levels were stable in all hypercapnic treatments, which could explain the general lack of clear results found in HIF-1 α mRNA expression since this mechanism is controlled by oxygen. Furthermore, Costas et al. (2011) observed that HIF-1 α expression could be augmented in response to a stress, induced by repeated handling, which could elucidate the observed *hif1* up-regulation in fish exposed to pH 7.6. HIF-1 is described by Cramer et al. (2003) as essential for the maintaining the ATP pool and proper functioning of myeloid cells activity, as neutrophils and monocytes. Therefore, the slight increase of *hif1* expression in the hypercapnic treatments (only significantly higher at pH 7.6 at 24 h) together with higher plasma bactericidal and anti-proteases activities could be a result of an acute stress recognition and response.

In response to the prolonged exposures (4 weeks), all hypercapnic treatments displayed a higher plasma antiproteases activity relative to the control group. Additionally, mRNA levels of the pro-inflammatory cytokine IL1 β and cyclooxygenase 2, COX2, an enzyme involved in the production of prostaglandins in response to inflammation, were found up-regulated in the highest hypercapnic treatment (pH 7.3) relative to pH 8.1 and to those exposed to the lowest hypercapnic treatment, pH 7.9. Also, gene expression levels of the anti-inflammatory cytokine IL10 increased at pH 7.3 compared to all other treatments. In contrast to that observed at 24 h in response to water acidification, the

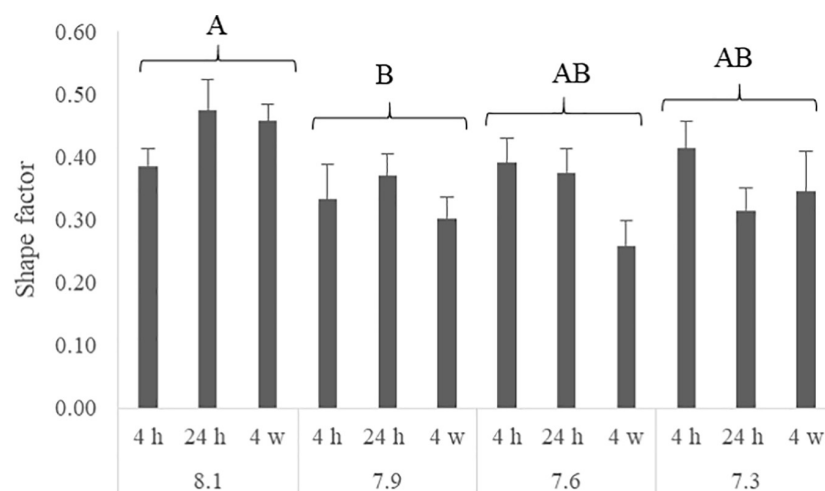


FIGURE 3 | Shape factor of each $\alpha 5$ positive cells in the gills of Senegalese sole juveniles submitted to pH levels of 8.1, 7.9, 7.6, and 7.3 for 4 and 24 h and 4 weeks. Data are expressed as means $\pm$ SD ($n = 6$; p -value = 0.001). Different capital letters indicate differences among pH regardless time or among times regardless pH. (Two-way ANOVA; Tukey *post hoc* test; $p \leq 0.05$).

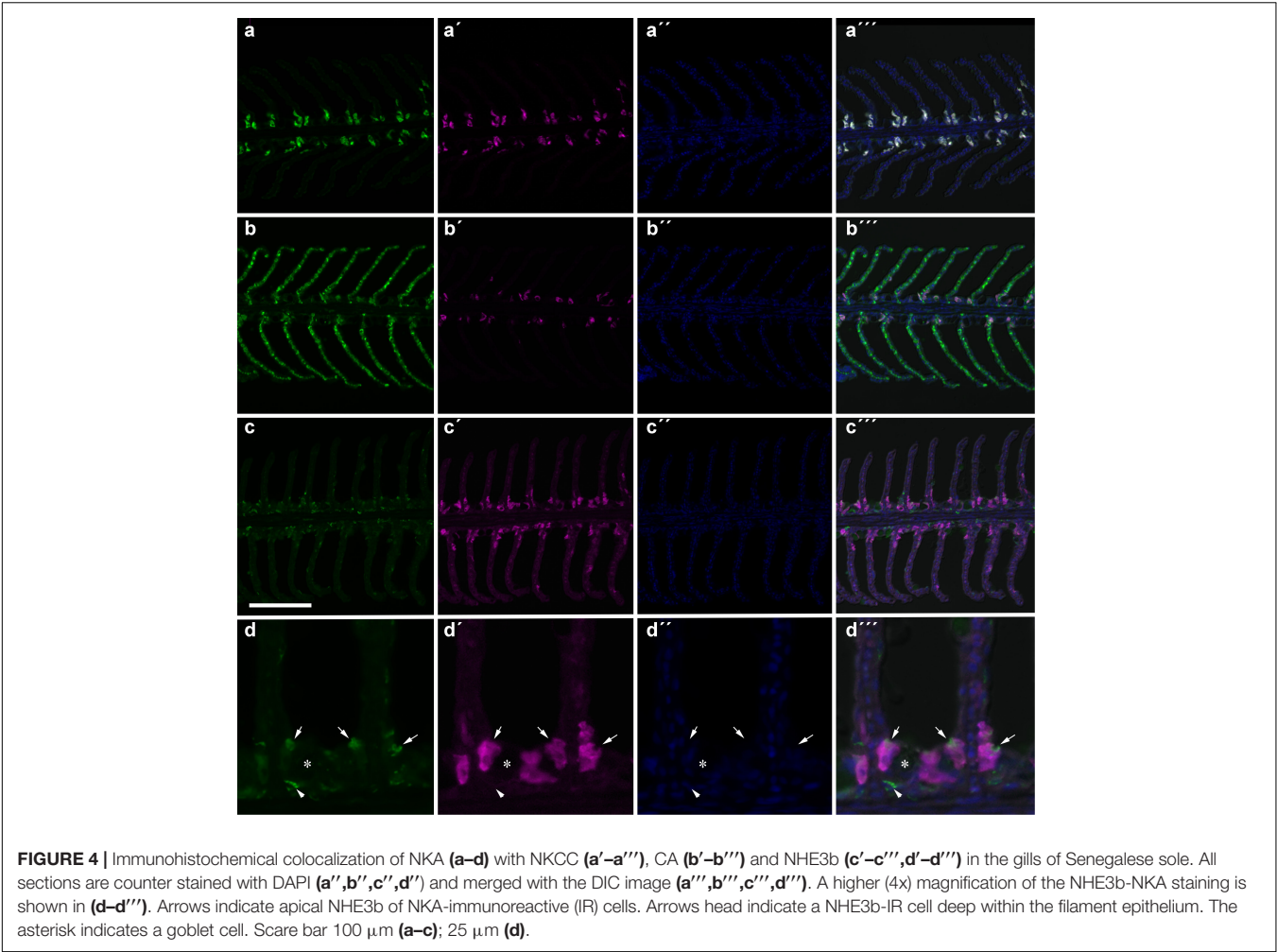


TABLE 9 | Standard metabolic rate (SMR), maximum metabolic rate (MMR), and aerobic metabolic scope (AMS) (mg O₂ kg⁻¹ h⁻¹) in Senegalese sole submitted to different water pH (8.1, 7.9, 7.6, and 7.3).

Parameters	pH				One-way ANOVA (p-value)
	8.1	7.9	7.6	7.3	
SMR	60.24 ± 10.18	73.27 ± 17.95	77.09 ± 10.63	55.58 ± 12.19	ns
MMR	119.47 ± 14.55	120.95 ± 19.68	122.53 ± 16.03	94.51 ± 9.59	ns
AMS	59.231 ± 4.97 ^a	47.68 ± 7.56 ^{ab}	45.44 ± 11.86 ^{ab}	38.93 ± 11.02 ^b	0.050

Values are presented as means ± SD (n = 6). P-values from one-way ANOVA (p ≤ 0.05). If interaction was significant, Tukey post hoc test was used to identify differences in the experimental treatments. Different lowercase letters stand for significant differences among pH.

expression of HIF-1α decreased in fish exposed to the two lowest pH treatments. These observations revealed a marked effect of water CO₂ increase in the fish immune mechanisms with the up-regulation of important pro- and anti-inflammatory mediators and stress-related genes. In fact, gill NHE3b positive ionocyte numbers remained high under hypercapnic treatments even after a 4 weeks period. It is therefore suggested that, in response to severe hypercapnia, the physiological compensatory mechanisms are activated toward a chronic stress response, possibly resulting in the diverting of energy away from growth. In fact, a clear reduction of sole final body mass was observed in response to the

decrease of water pH, particularly at pH 7.3. This is supported by Foss et al. (2002) and Mota et al. (2019) who showed a reduced growth in juvenile spotted wolfish (*Anarhichas minor* Olafsen) and Atlantic salmon, respectively, with an increase of water CO₂. Moreover, Cech and Crocker (2002) found impaired growth of white sturgeon (*Acipenser transmontanus*) reared at pH 7.0 compared to normocapnia (pH 8.0).

Finally, SMR and MMR provide information regarding lower and upper limits for aerobic energy metabolism. Despite the lack of clear effects of the increase water CO₂ on the standard and MMRs, fish AMS was found to be diminished in the lowest pH

treatment (pH 7.3). The progressive decrease of AMS shows a gradual reduction of the aerobic performance available for the normal metabolism in extreme hypercapnic situations, possibly as a result of the expenditure of metabolic energy toward the immune system combined with decreasing trends in the MMR as the pH declined. Supporting this hypothesis, and as previously demonstrated by Claireaux et al. (2000) for Atlantic cod (*Gadus morhua*), a positive correlation between final growth and aerobic metabolic scope ($r^2 = 0.896$) was found. Moreover, the observed reduction of the aerobic performance (i.e., AMS) by the low pH exposure could have reduced the energy availability for meal digestion and assimilation, or the specific dynamic action (SDA) as has been measured in eels (Methling et al., 2013). This could also have contributed to the reduced growth observed in the low pH treatments.

The present results showed a marked effect of increased water CO₂ on Senegalese sole with changes in gills physiology, immune mechanisms and aerobic performance. The increase of humoral immune parameters and up-regulation of important inflammatory mediators and stress-related genes after both acute and prolonged exposure to low pH pointed to a clear physiological adaptation of fish toward the acidic water. This physiological adaptation together with lower AMS and growth impairment could be the result of energy deviation toward the immune response to water hypercapnia.

DATA AVAILABILITY STATEMENT

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

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ETHICS STATEMENT

The animal study was reviewed and approved by this study was directed by trained scientists (following FELASA category C recommendations) and conducted according to the guidelines on the protection of animals used for scientific purposes from the European directive 2010/63/UE.

AUTHOR CONTRIBUTIONS

MM, FA, JS, and BC conceived the experiments. MM and FA conducted the experimental trial. LP and JW performed the gill immunohistochemistry. MM, RA, and JS performed the respirometry work. RA assisted with analytical procedures. MM directed most laboratory techniques and wrote the manuscript under the supervision of BC. All authors contributed to and approved the manuscript.

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LPS Modulates the Expression of Iron-Related Immune Genes in Two Antarctic Notothenoids

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The non-specific immunity can induce iron deprivation as a defense mechanism against potential bacterial pathogens, but little information is available as to its role in Antarctic fish. In this study the response of iron metabolism related genes was evaluated in liver and head kidney of the Antarctic notothenoids *Notothenia coriiceps* and *Notothenia rossii* 7 days after lipopolysaccharide (LPS) injection. Average plasma Fe^{2+} concentration was unaffected by treatment in any of the species. The gene expression response to LPS varied between tissues and species, being stronger in *N. coriiceps* and more prominent in the head kidney than liver. The reaction to LPS was marked by increased individual variability in most genes analyzed, even when the change in expression was not statistically significant, suggesting different individual sensitivity and coping responses in these wild fish. We found that iron related genes had an attenuated and homogenous response to LPS but there was no detectable relationship between plasma Fe^{2+} and gene expression. However, overall in both tissues and species LPS exposure set a multilevel response that concur to promote intracellular accumulation of iron, an indication that Antarctic Notothenoids use innate nutritional immunity as a resistance mechanism against pathogens.

Keywords: *Notothenia coriiceps*, *Notothenia rossii*, iron metabolism, Antarctic fish, nutritional immunity

INTRODUCTION

The vertebrates, including fishes, have developed innate and an adaptive immune systems composed both cellular and humoral components to respond to pathogens (Barandica and Tort, 2008). Both systems can be communicated through soluble mediators known as cytokines that regulate the magnitude of the immune response, controlling pathogen growth, promoting inflammation and triggering the adaptive immune response (Sompayrac, 2012). The main organs with immunological function in fish are the thymus, spleen, liver, head kidney and mucosa-associated lymphoid tissue (MALT). They can be grouped into primary (thymus and head kidney) and secondary (spleen, liver, and MALT) immune organs according to their participation in the production, maturation, activation and proliferation of immune components (Soulliere and Dixon, 2017).

During inflammation, the innate immune system can induce several antimicrobial mechanisms, including the depletion of iron available to the pathogen at the systemic and cellular levels (Johnson and Wessling-Resnick, 2012). This defense mechanism, known as nutritional immunity, consists of the removal of iron from the circulation and its subsequent sequestration inside the cell (Collins, 2008). Proinflammatory cytokines such as IL-6 stimulate the transcription of hepcidin, triggering and potentiating the hypoferric response of inflammation (Nemeth et al., 2004a). Several bacterial pathogens obtain the iron they need for their survival and replication from an external source (Ratledge and Dover, 2000; Pulgar et al., 2015; Bethke et al., 2016, 2018; Calquín et al., 2018). For this reason, eukaryotic organisms must effectively control iron homeostasis, through the regulation of the proteins involved in its metabolism (Skaar, 2010).

In fish, iron is mainly taken up by the intestine (Bury and Martin, 2003) where it can enter as heme iron, Fe^{2+} and Fe^{3+} (Bury and Martin, 2003). Duodenal cytochrome B is necessary to convert Fe^{3+} to Fe^{2+} (Bury and Martin, 2003). The pool of Fe^{2+} interacts with the divalent metal transporter present on the apical side of the enterocytes (Gunshin et al., 1997; Andrews, 1999). Once inside the cell, the iron can be stored in the cytoplasmic ferritin (the portion of iron that is not for immediate use) (Torti and Torti, 2002), directed toward the mitochondria (for biosynthesis of the Fe-S cluster and heme group) or used in other routes of iron metabolism (Hentze et al., 2004).

The exit of iron from the enterocytes is regulated by ferroportin (Donovan et al., 2000). However, hepcidin can induce the degradation of ferroportin, controlling the release of iron from the basolateral membrane into the bloodstream (Nemeth et al., 2004b). The export of iron also depends on the presence of a copper-associated oxidase, hephaestin in intestinal cells or of ceruloplasmin in non-intestinal cells (Harris et al., 1999; Vulpe et al., 1999). The ferroxidase allows the iron to be loaded on to the cytoplasmic transferrin to be directed to other tissues that need it (Hentze et al., 2004). Iron uptake can be then mediated by transferrin receptor 1 (TfR1) that is expressed in all tissues (Cheng et al., 2004) or by transferrin receptor 2 (TfR2), yet not described in fish, that in mammals is found in hepatocytes, duodenal crypt cells and erythroid cells, suggesting a more specialized role of this receptor in iron metabolism (Kawabata et al., 1999). In fish, such as *Salmo salar* and *Eleginops maclovinus*, the expression of the main proteins involved in iron metabolism appears to be modulated by the presence of live bacterial pathogens (Pulgar et al., 2015; Martínez et al., 2017a).

Research on Antarctic fish immune responses has mainly addressed the effect of thermal stress on the metabolism of carbohydrates and antioxidant defense system (Machado et al., 2014; Klein et al., 2017; Souza et al., 2018), or analyzed the level of transcriptional response of immune genes after stimulation with Poly I:C and with heat-killed cells, *E. coli* (Ahn et al., 2016) and *Psychrobacter* sp. (Buonocore et al., 2016). Furthermore, cDNAs of iron metabolism proteins have been cloned, e.g., ceruloplasmin (*Cp*), transferrin (*Tf*), ferritin (*Fth1*) and divalent metal transporter 1 (*Dmt1*) (Scudiero et al., 2007, 2009), but no specific information is available on how they are regulated before an infectious process. Furthermore, there is no information

on nutritional immunity associated with iron in Antarctic fish or if this type of immune response could be activated by the detection of pathogen-associated molecular patterns (PAMPs), which activate the immune system, but do not require iron. Therefore, the objective of the present study was to evaluate the transcriptional response of immune genes associated with iron in Antarctic Notothenoid fish (*Notothenia coriiceps* and *Notothenia rossii*) stimulated with a bacterial PAMPs (specifically LPS, a lipopolysaccharide that integrates the cell wall of gram-negative bacteria).

MATERIALS AND METHODS

Sample Collection

Notothenia coriiceps (30 ± 2.4 cm of length and 384 ± 93 g of weight) and *N. rossii* (30 ± 4.2 cm of length and 312 ± 124.7 g of weight) were captured by hook-and-line from a boat, from 5–20 m deep in the waters near the Chinese Great Wall Station, at King George Island (GPS coordinates: $62^{\circ}13'S$, $58^{\circ}58'W$), in the Antarctic Peninsula, during late January 2017. Upon fishing fish were initially randomly placed in six 200 L flow-through plastic tanks for up to 3 days and then measured, weighed, tagged with opercular metal tags and allocated in groups with similar biomass and size distribution in the same flow-through system (two tanks per species; $n = 7-8$ per tank). Fish were anesthetized in phenoxyethanol (0.02 ml/L) before all experimental manipulations and left undisturbed for 1 week before the experiments to acclimate. At the beginning of the assay (day 0), fish were anesthetized as above and one group per species was injected intraperitoneally (IP) with saline (1.1% NaCl, 0.2 vol.% body weight) and acted as control, while the other group was IP-injected with LPS (1.5 mg/ml LPS in 1.1% NaCl, 0.2% vol body weight, to an effective dosage of 3 mg/kg). This procedure was repeated in day 2, when fish received a second injection of either saline (control) or LPS. At day 7 all fish were lethally anesthetized, blood was collected in heparinized syringes (ammonium salt heparin, 1000 U/ml, Sigma-Aldrich), and fish were sacrificed by cervical section to collect tissues. The head kidney and liver were dissected aseptically, immediately placed in RNAlater (Sigma-Aldrich), placed at 4°C for 24 h and then stored at -20°C . The blood was centrifuged at 10,000 g for 5 min at 4°C to obtain the plasma, which was subsequently stored at -80°C . Water parameters in tanks were monitored twice daily and were approximately constant throughout the experiment – temperature ($2.1 \pm 0.5^{\circ}\text{C}$), salinity (29 ± 0.5 ppt) and oxygen (10.5 ± 1.0 ppm). Two *N. coriiceps* died in the LPS group and no mortality was observed for *N. rossii*.

Blood Plasma Iron

For determination of iron, 1 ml of 5% nitric acid (Sigma-Aldrich) was added to the blood plasma (110 μL), followed by sonication for 10 min and centrifugation at 16,000 g for 5 min at room temperature. The supernatant was removed to a new 15 ml falcon tube and the Fe^{2+} fraction was measured at 259.940 nm in an Agilent Microwave Plasma Atomic Emission Spectroscopy (4200 MP-AES, Agilent Technologies). Only Fe^{2+}

was measured due to limitations of plasma volume and of the spectrometer which could not measure the Fe^{3+} emission wavelength. The Agilent MP-AES Expert software was used to calculate concentration based on a 14 points standard curve (range 4.47E-05 mM–4.47E-08 mM) with automatic background subtraction provided by a blank (5% nitric acid), in accordance with the manufacturer's instructions. The limits of detection (LOD) and limit of quantification (LOQ) were calculated from three and ten times the standard deviation of 15 consecutive blank measurements, respectively (Li et al., 2013). The values obtained were 3.77E-05 mM to LOD and 1.26E-04 mM to LOQ. The presence of interference was analyzed through spiking samples with 8.95E-04 mM (50 ppb) and 1.79E-03 mM (100 ppb) standards to the plasma samples and was found not to be significant. Recoveries were calculated from the same spiked plasma samples and estimated at 105%.

RNA Processing and Quantification

Total RNA was extracted from 50 mg of tissue using Trizol (Ambion), following the manufacturer's instructions. The RNA pellets were dissolved in diethylpyrocarbonate water (DEPC, Sigma-Aldrich) and stored at -80°C . RNA was quantified spectrophotometrically at 260 nm (NanoDrop Technologies) and quality was checked using electrophoresis in 1% agarose gels. Total RNA (2 μg) was used as a template for reverse transcription reactions to synthesize cDNA using MMLV reverse transcriptase (Promega) and the oligo-dT primer (Invitrogen).

The cDNAs were diluted to 100 ng (2 μL) and used as a template for quantitative polymerase chain reaction (qPCR) of *ferritin heavy chain* (*Fth1*), *ferritin middle chain* (*Fm*), *ferroportin* (*Fp*), *transferrin* (*Tf*), general identification as *transferrin receptor* (*TfRC*) on NCBI database, however, with higher similarity with *transferrin receptor type 1* (*TfRC1*) based in ENSEMBL and CCMAR Sea Genomics databases, general nomenclature as *hepcidin-like* (*Hamp*) identified on public NCBI database, however, has more similarity to *hepcidin type 2 variant 4* (*Hamp2*) based on CCMAR Sea Genomics database, *ceruloplasmin* (*Cp*), *interleukin-6 receptor alpha chain* (*IL-6R α*), *IL-6 receptor beta chain* (*IL-6R β*), and *18s ribosomal RNA* (*18S*). The primers for interleukin-6 were not considered because they did not work very well. These primers were designed based on *N. coriiceps* species because is the only genome partially published and available on public NCBI database¹ and further confirmed by blast against of several known genome of fish species that are phylogenetically similar such as *Danio rerio*, *Oryzias melastigma*, *Oreochromis niloticus*, *Gasterosteus aculeatus*, *Tetraodon nigroviridis*, *Xiphophorus maculatus* by public ENSEMBL database² and *Dicentrarchus labrax* and *Sparus aurata* fish species by public CCMAR Sea Genomics database³. The qPCR reaction was performed according to a published protocol (Martínez et al., 2017b), specifically: 95°C for 10 min, followed by 40 cycles at 90°C for 10 s, 60°C for 15 s, and 72°C for 15 s. Melting curve analysis of the amplified products

was performed at the end of each PCR to confirm that only one PCR product was amplified and detected. Expression levels were analyzed using the comparative Ct method ($2^{-\Delta\Delta\text{CT}}$) (Livak and Schmittgen, 2001). Data were expressed as the fold change in gene expression normalized to an endogenous reference gene (housekeeping 18s) and relative to the untreated control. The primers used are listed in Table 1. PCR efficiencies were calculated according to the equation: $E = 10^{[-1/\text{slope}]}$ (Rasmussen, 2001).

Statistical Analysis

Data is shown as mean $\pm$ standard error (SE) of the mean. Normality and homogeneity of the variances were verified using the Shapiro-Wilk test. The logarithmic transformation was used if data did not follow a normal distribution. The Student's *t*-test was used to compare the effect of LPS versus control for iron and gene expression data. The level of statistical significance was 5%. Because several comparisons did not achieve statistical significance and an increase in variability was observed in the LPS treated samples, a Principal Component Analysis (PCA) with rotation based on the Varimax equation with Kaiser Normalization was carried out order to determine the global sample distribution between control and LPS mean centered expression data in liver and head kidney of the two species. The software SPSS (IBM SPSS version 25) was used for the statistical analysis, applying univariate analysis of variance (ANOVA), means, box's M and Fisher's transformation to produce a correlation and covariance matrix for discriminant analysis. A Principal Component Regression based on a linear regression on principal components score was applied considering the component score coefficient matrix and component score covariance matrix to discriminate between control and LPS treatment groups. The RStudio software (version 1.0.143) was used to confirm PCA data and produce the confidence ellipses (95%), starting from centroid position for each tissue and species based on component regression of two dimensions (Principal components 1 and 2).

RESULTS

Plasma Iron and Expression of Iron-Related Immune Genes

The concentration of plasma iron (Fe^{2+}) varied between 1.53 and 10.8 μM in *N. coriiceps* and 1.55–23.8 μM in *N. rossii*, with no statistical difference between control and LPS treatment (Figures 1A,B).

The iron-related immune gene expression response to LPS varied with tissues and species. *N. coriiceps* was more responsive to LPS than *N. rossii*. In *N. coriiceps* of the nine genes analyzed, 4 were significantly upregulated ($p < 0.05$) in the liver and 7 in the head kidney, 3 of which were common to both tissues – *Fth1*, *Tf* and *Hamp2* (Figures 2A–I). In contrast, in *N. rossii* only *Hamp2* was significantly upregulated in the liver and 3 genes were significantly upregulated in head kidney – *Fth1*, *Tf* and *Cp* (Figures 2A–I). *Fp* was significantly downregulated

¹<https://www.ncbi.nlm.nih.gov/>

²<https://www.ensembl.org/index.html>

³<https://146.193.226.37/>

TABLE 1 | qPCR primer pairs, product sizes and efficiency.

Primer	Nucleotide sequences (5'3')	PCR product size	GenBank	N. coriiceps		N. rossii	
				Efficiency Liver (%)	Efficiency Head-kidney (%)	Efficiency Liver (%)	Efficiency Head-kidney (%)
Ferritin heavy chain (Fth1) Fw	AGTGGAGGCCCTTGAATGTGC	130pb	FM210467.1	97.4	109.4	95.8	94.2
Ferritin heavy chain (Fth1) Rv	GTCAGGTTAGTGAGTCTCGATGAA						
Ferritin middle chain (Fm) Fw	GGACACAGGATGCGGACATAA	121pb	XM_010792901.1	105	102.2	95.3	104.6
Ferritin middle chain (Fm) Rv	AACGCTACTCCACTTCCCCA						
Ferroportin (Fp) Fw	GCCATGGTCACGTCATGTA	127pb	XM_010788639.1	97.4	107.2	96.6	92.7
Ferroportin (Fp) Rv	GTTAGACGGTGGTGGGAAGG						
Hepcidin type 2 (Hamp2) Fw	GAGCCGATGAGCGTTGAAAG	139pb	XM_010774309.1	105.9	105.9	94.9	99.9
Hepcidin type 2 (Hamp2) Rv	AGGACAATCCGCGACACACC						
Ceruloplasmin (Cp) Fw	GTTTCCAGCCACCTTTCAGACAGT	104pb	XM_010778042.1	109.6	98.7	100.9	101.3
Ceruloplasmin (Cp) Rv	TCGCCTCCATGCCACCTTTAAT						
Interleukin 6-receptor α chain (IL6R α) Fw	TGGCAGCTTAAGCCAGAAAG	148pb	XM_010787044.1	109.7	97.6	106.3	96.7
Interleukin 6-receptor α chain (IL6R α) Rv	GGCAATTGGAGCGTAGTCT						
Interleukin 6-receptor β chain (IL6R β) Fw	GGACAATGAGATCGCCATGC	131pb	XM_010787241.1	100.7	98.3	101.5	100.8
Interleukin 6-receptor β chain (IL6R β) Rv	TGTCAACCCCTTGATAAAGCCC						
Transferrin (Tf) Fw	CAGTGGTCAGGCGTTGAAGA	146pb	NM_001303296.1	107.3	105.9	99.1	95.8
Transferrin (Tf) Rv	TAGTAAGGCTCGGTGTGGGA						
Transferrin receptor type 1 (TfRC1) Fw	GTCCCCCAGAGAGAGTCCAT	127pb	XM_010774827.1	103.5	109.2	94.2	107.1
Transferrin receptor type 1 (TfRC1) Rv	TCGGAACAGGTTGAAGTCGG						
Housekeeping (18s) Fw	GTCGGGAAACCAAGTC	116pb	AF518193.1	107.9	101.6	108.6	99.7
Housekeeping (18s) Rv	TTGAGTCAAAATTAAAGCCGCA						

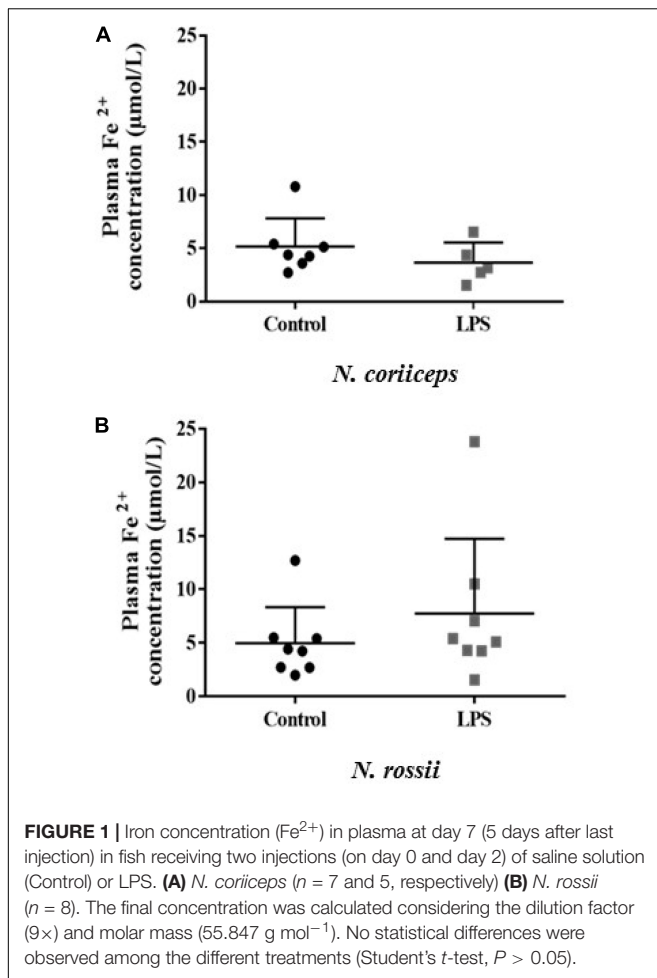


FIGURE 1 | Iron concentration (Fe^{2+}) in plasma at day 7 (5 days after last injection) in fish receiving two injections (on day 0 and day 2) of saline solution (Control) or LPS. **(A)** *N. coriiceps* ($n = 7$ and 5 , respectively) **(B)** *N. rossii* ($n = 8$). The final concentration was calculated considering the dilution factor ($9\times$) and molar mass ($55.847 \text{ g mol}^{-1}$). No statistical differences were observed among the different treatments (Student's t -test, $P > 0.05$).

in *N. coriiceps* liver while *Cp* and *TfRC1* were significantly downregulated in *N. rossii* liver (**Figures 2C,E,G**).

Multivariate Analysis

Since the response to LPS was relatively weak but an increase in gene expression variability was apparent, even when not statistically significant, we sought to determine if there was an association of the gene expression responses with the experimental groups using a PCA for each tissue and species. In *N. coriiceps* liver, 78% of the gene expression variance was explained by the two first principal components (PC), 42% for PC1 and 36% for PC2. In head kidney, the two first PC explained 85% of the gene expression variance, 60.5% for PC1 and 25.2% for PC2. In *N. rossii* liver, 3 PC were required to explain almost 75% of the gene expression variance, 34.1% for PC1, 22.9% for PC2, and 18.3% for PC3. In head kidney, 3 PC explained 80% of the gene expression variance, 40.8% for PC1, 23% for PC2, and 15.7% for PC3.

From the component coefficient matrix and the rotated component matrix (**Table 2**), the differentiation between genes and treatment groups occurred mainly along PC1 for both tissues and species. However, the load factors of the different genes, regardless of treatment, vary widely for each PC, depending

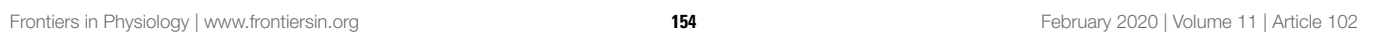
on tissue and species. The PCA analysis of gene expression in *N. coriiceps* liver shows that PC1 was strongly and positively correlated (>0.75) with *Fth1*, *Hamp2*, *IL6R α* and moderately (>0.5) with *Tf*, and inversely correlated with *Fp*, while PC2 directly correlates with *Cp*, *IL6R β* , *TfRC1*, *Fm* and less with *Fp*. In *N. coriiceps* head kidney, PC1 was strongly or moderately correlated (>0.75 or >0.5) to most genes tested with exception *IL6R α* , and PC2 correlated positively with *IL6R α* and less with *Cp*, and inversely with *Fm*. As indicated, above, in *N. rossii* dispersion was higher and three components were required to explain at least 75% of the distribution, but the same three genes appear to be responsible for determining the PC1 in both liver and head kidney. Thus, in liver, *Fm*, *Fp* and *IL6R β* were the most loading factors on PC1, *Fth1* and *TfRC1* on PC2, while PC3 was mostly positively correlated with *IL6R α* (>0.5) and inversely with *Hamp2*. In head kidney PC1 correlates strongly (>0.75) with *Fm*, *Fp* and *IL6R β* and moderately (>0.5) with *Fth1* and *Cp*. PC2 was mostly determined by *Tf* and *IL6R α* and PC3 directly by *Fth1* and *TfRC1* (>0.5) and inversely by *Hamp2* (<0.5).

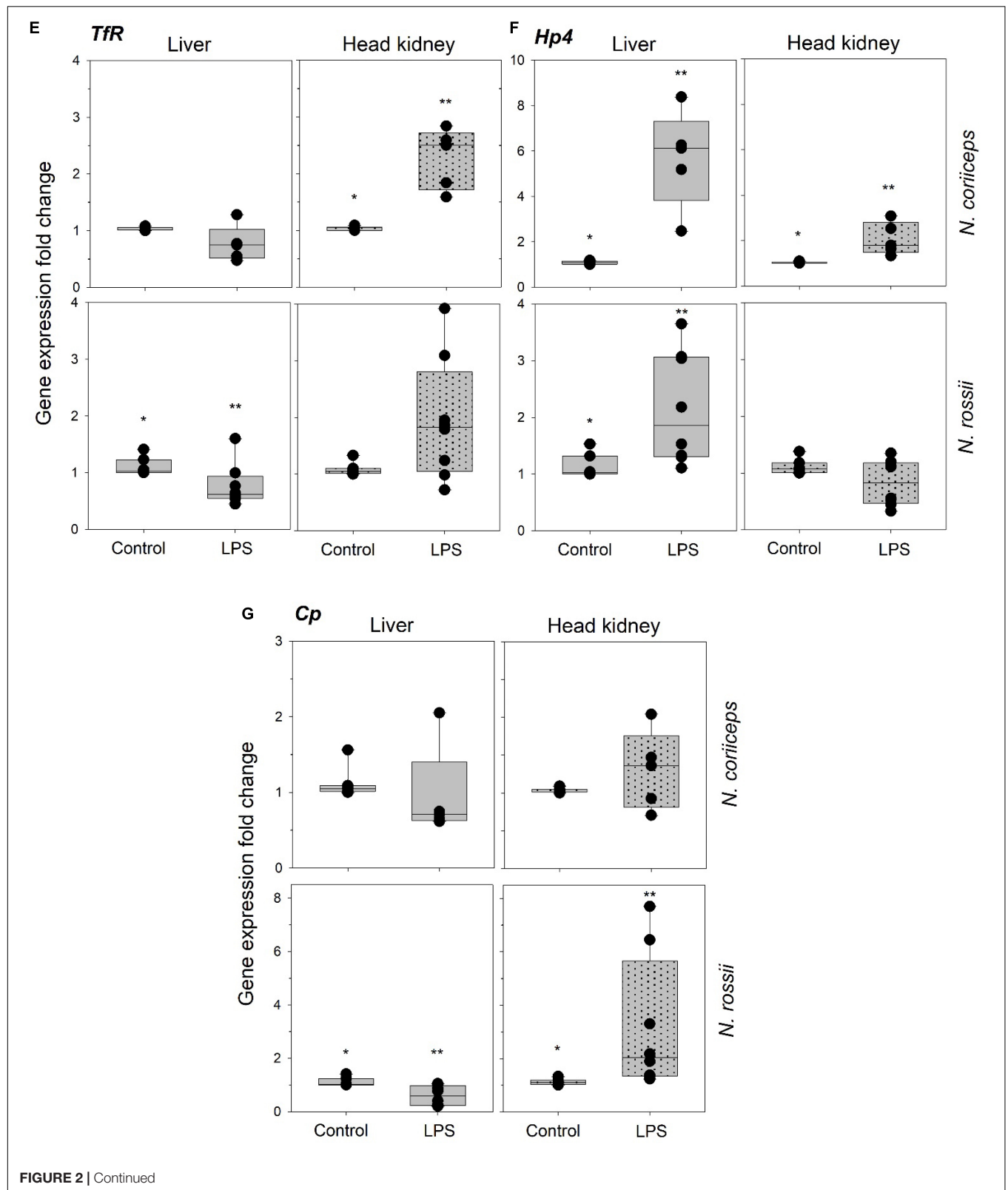
A Principal Component Regression used the first 2 PC variables as regressors to determine the variance between the group clusters. For both immune tissues of *N. coriiceps* the clusters of control and LPS groups aggregated wide apart and the variance of control individuals was low contrasting with the LPS treated fish which showed a much higher variance, spreading mostly along the PC2 axis (**Figures 3A,B**). In *N. rossii* the clusters of the control and LPS groups were closer together, including a slight overlapping, in both liver and head kidney expression distribution, with control centroids on the edge of the LPS 95% confidence ellipses. Nonetheless, and as for *N. coriiceps*, individuals of the control group clustered close together, as in *N. coriiceps*, while individuals of LPS group show high dispersion, along both PC1 and PC2 axis (**Figures 3C,D**). Overall, the analysis explains the allocation of all control individuals into the same treatment group for both species, whereas for LPS 82% of *N. coriiceps* and 85% of *N. rossii* were correctly classified as belonging to the same treatment group.

DISCUSSION

This study found that stimulation with lipopolysaccharide, a gram-negative cell wall component, had no effect on circulating Fe^{2+} levels, of two closely Notothenioid species, *N. coriiceps* and *N. rossii*, but caused modifications in the expression of iron metabolism related immune genes, both in liver and in head kidney. This gene response showed important differences in magnitude between species, and the effects of LPS were more attenuated and variable in *N. rossii* when compared to *N. coriiceps*.

In the present study no significant changes were observed in plasma Fe^{2+} between the two *Notothenia* species and in response to LPS. Since Fe^{2+} concentrations were within the range of measurements made in other teleosts (Congleton and Wagner, 1991; Welker et al., 2007; Kopp et al., 2011, 2014; Eslamloo et al., 2012; Hedayati et al., 2016), it suggests potentially similar requirements and homeostatic mechanisms





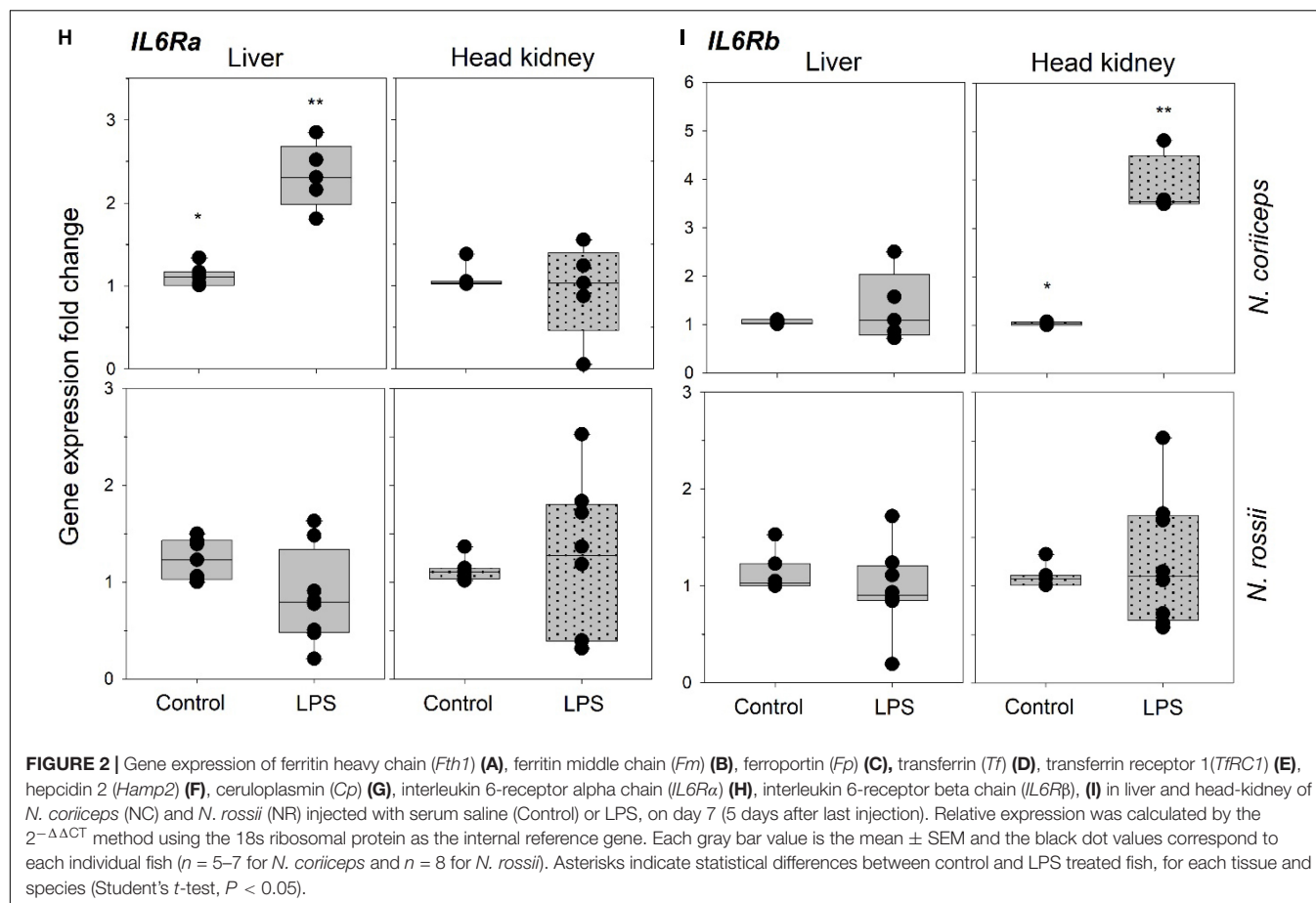
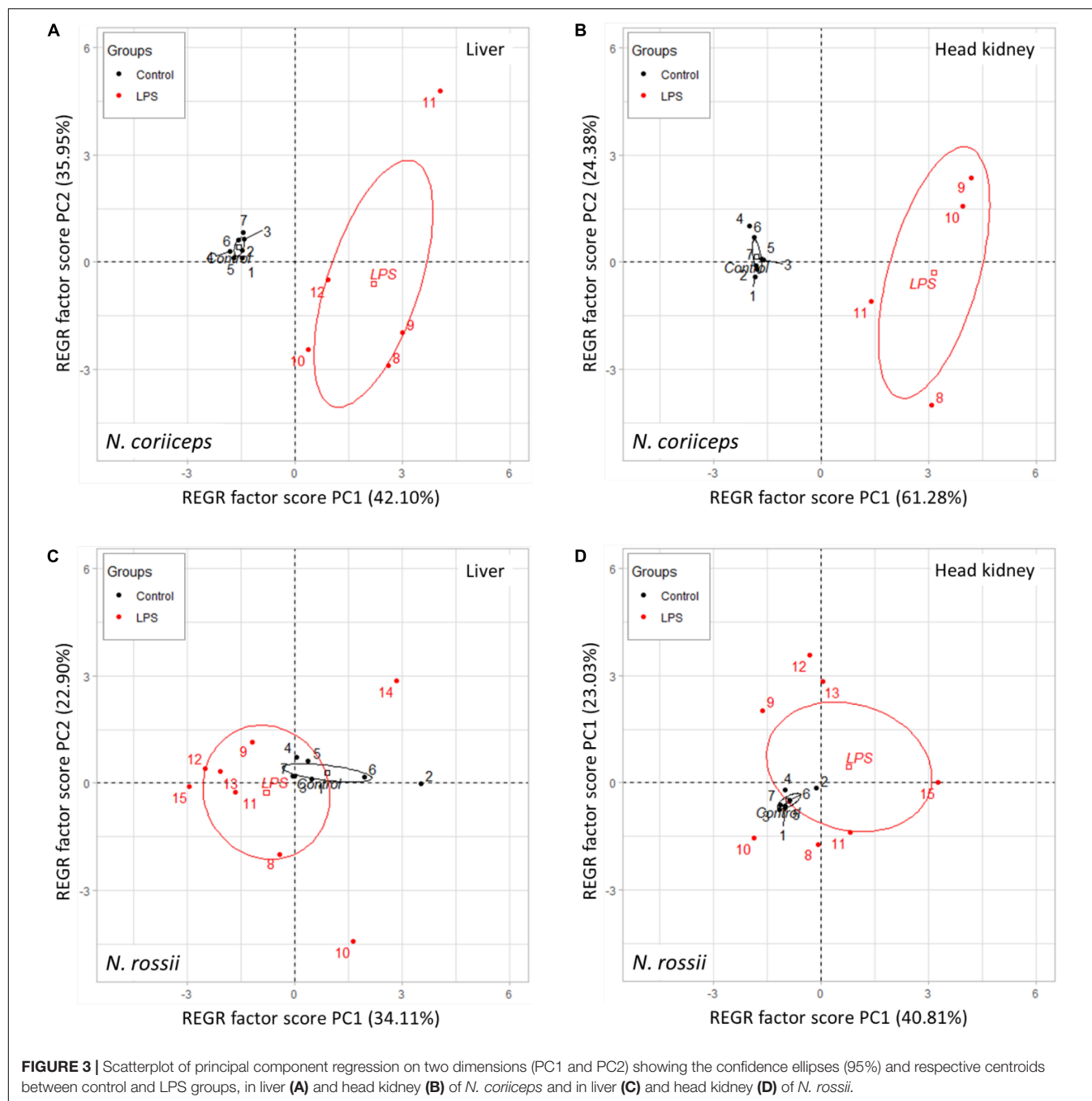


TABLE 2 | Principal Component Analysis by rotation method (Varimax with Kaiser Normalization) applied in different iron-related genes in liver and head kidney of *N. coriiceps* and *N. rossii*.

Tissue - Species	Rotated component matrix									
	Liver – <i>N. coriiceps</i>		Head kidney – <i>N. coriiceps</i>		Liver – <i>N. rossii</i>			Head kidney – <i>N. rossii</i>		
	Component		Component		Component			Component		
	1	2	1	2	1	2	3	1	2	3
Ferritin heavy chain (<i>Fth1</i>)	0.924	−0.66	0.972	−0.002	−0.078	0.861	−0.371	0.639	0.089	0.673
Ferritin middle chain (<i>Fm</i>)	0.228	0.847	0.689	−0.676	0.906	−0.117	0.027	0.985	−0.001	0.069
Ferroportin (<i>Fp</i>)	−0.766	0.589	0.917	−0.281	0.922	0.177	−0.036	0.951	0.071	−0.094
Transferrin (<i>Tf</i>)	0.547	0.428	0.966	0.030	0.089	0.737	0.172	0.035	0.928	−0.009
Transferrin receptor type 1 (<i>TfRC1</i>)	−0.428	0.865	0.930	−0.023	0.559	0.764	0.012	0.301	0.550	0.571
Hepcidin type 2 (<i>Hamp2</i>)	0.962	0.221	0.917	0.226	−0.225	0.217	−0.808	−0.208	0.349	−0.686
Ceruloplasmin (<i>Cp</i>)	−0.47	0.901	0.603	0.692	0.022	0.643	−0.113	0.546	0.490	0.169
Interleukin 6-receptor α chain (<i>IL6Ra</i>)	0.968	−0.18	0.039	0.915	−0.023	0.619	0.674	−0.085	0.905	−0.069
Interleukin 6-receptor β chain (<i>IL6Rb</i>)	0.424	0.847	0.875	−0.460	0.886	0.118	−0.062	0.972	−0.082	0.121
Cumulative Variance Explained (%)	42.1	78.1	61.3	85.7	34.1	57.1	75.3	40.8	63.8	79.5

between Antarctic and temperate fish. Although it has been shown in many vertebrate species that bacterial infection leads to a depletion of circulating iron (reviewed by Skaar, 2010; Johnson and Wessling-Resnick, 2012), most studies in fish showed either acute or week-lasting effects of LPS administration

over the expression of iron-chelating or iron-transporting mechanisms but did not measure the iron itself (Prieto-Álamo et al., 2009; Liu et al., 2012; Martínez et al., 2017a) and thus the effectiveness and dynamics of LPS alone in lowering plasma iron is far from resolved. In rainbow trout, *Oncorhynchus mykiss*,



LPS injection (1 mg/kg) caused a significant decrease in plasma iron within 48–72 h post-injection but circulating levels returned to normal by 96–120 h (Congleton and Wagner, 1991) and similar kinetics were observed in Atlantic salmon, *Salmo salar*, injected an identical dosage (Langston et al., 2001). It is therefore also possible that an effect in Fe^{2+} plasma concentrations may have been missed in our time-frame and dosage, which followed previous studies on temperate fish (Nayak et al., 2011; Guzman-Villanueva et al., 2014; Seppola et al., 2015; Chen et al., 2016) but may be less adequate in these Antarctic species. In the present study

fish were injected twice, at 0 and 48 h, and sampled at 120 h after the last injection, a design chosen taken in consideration that low temperature habitats may contribute to a slower innate immune system response, as it has been described in cold-water fish when compared to temperate fish (Tort et al., 2003; MacKenzie et al., 2008; Magnoni et al., 2015; Bonneaud et al., 2016; Chen et al., 2016; Abram et al., 2017; Martínez et al., 2017a).

The overall gene expression response, albeit not as vigorous as in other vertebrates (Collins, 2008; Johnson and Wessling-Resnick, 2012; Bethke et al., 2016), also suggests that an iron

related immune response is active, much alike what it was seen in the sub-Antarctic Notothenioid *Eleginops maclovinus* (Martínez et al., 2017a), the Senegalese sole, *Solea senegalensis* (Prieto-Álamo et al., 2009) or the roughskin sculpin, *Trachidermus fasciatus* (Liu et al., 2012), among other fish species.

Several genes with important roles in iron metabolism responded in the liver and head kidney in one or both species. Interestingly, there were differences in responsiveness between the two species, with more genes responding and at a higher level in *N. coriiceps*. The reason for this is not immediately apparent but the two species, although captured in the same general area, tend to segregate with *N. rossii* in open and deeper channels and *N. coriiceps* usually under rocks in shallower waters (Jones et al., 2009; Kandalski et al., 2018; Barrera-Oro et al., 2019), which could lead to different exposure to potential pathogens and different immune responsiveness. Some *N. coriiceps* specimens can be found near the surface in low tide, hiding in rock crevices surrounded by algal debris, thus possibly more exposed to microorganisms. Additionally, while *N. coriiceps* lives in these coastal shallow waters throughout its life, in the case of *N. rossii* only the juveniles appear to use these areas, with adults moving into deeper and open waters (Jones et al., 2009), and a life stage difference can thus account for some of the variance in the responses.

The liver is a major organ for iron storage and the site of synthesis for many proteins involved in iron metabolism. Although we have not determined iron levels in liver, the overall changes in gene expression in both species seem to favor iron accumulation in liver cells although this is more evident in *N. coriiceps*. In *N. coriiceps* liver upregulation of *Fth1* and *Tf* may promote binding of intracellular iron while upregulation of *Hamp2* and downregulation of *Fp* should inhibit its export (Zahringer et al., 1976; Torti and Torti, 2002; Neves et al., 2009). In *N. rossii* liver the export machinery seemed to be downregulated (increased *Hamp2* and *Cp* and reduced *TfR1* transcription) but the net effect is expected to be in the same direction and would reduce iron availability for extracellular bacteria.

The fish head kidney is involved in the immune response, but it is also the major haematopoietic organ, thus a likely responder to LPS and an important site for iron trafficking. In fact, expression of head kidney hepcidin genes has been described as a crucial regulator of erythropoiesis during anemia in fish (Joao et al., 2016). In our experiment, the tendency for intracellular accumulation of iron in the head kidney is apparent in *N. rossii* (by upregulation of *Tf*, *Fth1* and *Cp*) and also in *N. coriiceps* (upregulation of *Tf*, *Fth1*, *Fm*, and *Hamp2*) although in the later species iron turnover rate may be increased (by upregulation of *TfR1* and *Cp*, possibly involved in extracellular transport). Overall this is consistent with previous reports which suggested an iron deficiency condition after LPS stimulation of the European sea bass, *Dicentrarchus labrax* (Neves et al., 2009) and the sub-Antarctic *Eleginops maclovinus* (Martínez et al., 2017a) and up-regulation of the same iron-related genes during experimentally induced anemia in fish (Joao et al., 2016).

Interleukins are modulators of haematopoiesis, inflammation and immune responses. It has been further shown in fish that IL-6 is induced in macrophages during sepsis, and suggested that this may concur to reduce iron availability by induction of hepcidin, as a means to limit the spread of infection (Costa et al., 2011). Interleukin-6 could not be analyzed despite several attempts to isolate the IL-6 sequence in both species, based on the *N. coriiceps* genome (available in NCBI database, under ID: GCF_000735185.1) (Shin et al., 2014) and in our transcriptomic data on *N. rossii* (unpublished). However, the two interleukin-6 receptor genes were analyzed, *IL-6R α* was highly expressed in liver and *IL-6R β* was highly expressed in head kidney of *N. coriiceps*. Interestingly, no significant change in gene expression response occurred in *N. rossii*. The differential response of IL-6R genes between the two species is consistent with iron metabolism-related genes response which was higher in *N. coriiceps*. This may suggest that also in these species IL-6 cytokines are involved in the induction of the synthesis of the iron regulatory hepcidin during hypoferrremia inflammation (Berczi and Szentivanyi, 2003; Nemeth et al., 2004a; Shi and Camus, 2006; Roy et al., 2016). As indicated above, either the timing or a lower general immune response may explain the observations of low or poor immune responses. Although unlikely because of their close phylogeny, it is also possible that the two species may employ alternative pathways in iron metabolism defense. Indeed, species differences in physiology seems to exist. *N. coriiceps* appear to have lower thermal tolerance than *N. rossii* based on endocrine, metabolic and antioxidant parameters (Kandalski et al., 2018). Also, the more active *N. rossii* seems to have higher antioxidant defense system (ROS scavenge) enzyme activity than *N. coriiceps*, which could be related to their habits and different metabolic rates (Klein et al., 2017).

The PCA analysis also reflects the differences on the response among species, with a clearer separation between control and LPS-treated fish in *N. coriiceps* than in *N. rossii*. However, the large variability in responses between individuals, and specific genes in each tissue, especially in the LPS groups makes it difficult to identify which are the main genes reacting to challenge, and thus determining the different PC. These differences, however, show that overall, in both species, the weight of genes determining PC1 appears to be stronger for head kidney than for in liver, where PC2 has a relatively larger weight, which may show that more factors can be involved in the response in the latter tissue. Still, in both tissues of *N. coriiceps*, the PC1 is mostly determined by *Fth1* and *Hamp2*, while *Fm* and *Cp* appear relevant for PC2. In *N. rossii* PC1 in liver and head kidney is mainly dependent on the weight of *Fm*, *Fp* and also *IL6R β* , while the determination of PC2 is shared by more genes but with lower relative importance. Thus, it seems that iron-binding proteins and transmembrane iron-transporters are responding but that magnitude, time-frame and the repertoire of mechanisms vary widely between species and individuals upon challenge.

Indeed, regardless of the changes in the mean expression level of the genes tested, a striking effect of the LPS injection was

the increase in the variability of the individual response, when compared to the fish injected with saline. This is observable when looking at the individual gene expression variation and becomes obvious when a PCA was performed. From the analysis it is obvious that some individuals clearly responded to LPS by mounting an immune/iron-related reaction, but not others, although control and LPS-treated fish generally group together in the respective 95% confidence cluster. This lack of synchronicity in response to a seemingly stressful process has been observed in many species, especially when using wild-caught specimens, with likely variations in their life-history experiences (Robertson et al., 2016), nutrition state (Ahola et al., 2017), physiological conditions (Killen et al., 2017) or stress response norms (Balasch and Tort, 2019). In our case, fish were acclimated before the experiments and treated equally thereafter, but changes in size, age and possibly on social condition (Filby et al., 2010) still existed. Additionally, this variability was also observed on the nature of the genes that responded and not only on the magnitude of the response. This could reflect differences in sensitivity or differences in baseline iron, resulting in an asynchronous response. As indicated above for plasma Fe^{2+} our initial assumption was that a slower metabolism at low temperature could delay the response to LPS compared to temperate teleosts, but this assumption may have been proven wrong. It is possible that an acute response to LPS may have been missed early in the relatively long timeframe of this experiment. Further studies, using several shorter time points, and including iron transport should help to understand innate nutritional immunity in Antarctic fish.

In summary, despite a less than robust immune response of Antarctic Notothenioids marked by considerable individual variability we have shown that overall LPS promotes the mobilization of genes important for iron retention in liver and head-kidney which should contribute to lower iron levels in extracellular fluids and fight bacterial infection through iron starvation.

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.

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ETHICS STATEMENT

The animal collection and experimentation were approved by the Portuguese Environment Agency, under the regulations set by the Treaty of Madrid for scientific investigation on Antarctica. The experiments performed were complied with the EU and Portuguese regulations for animal experimentation and the Guidelines of the Comisión Nacional de Investigación Científica y Tecnológica de Chile (CONICYT) and the Universidad Austral de Chile for the use of animals.

AUTHOR CONTRIBUTIONS

DM, CS, PG, AC, DP, JP, RO, and LV-C contributed to analyzing the data, working on the original manuscript and several revisions, and final version. PG, LV-C, and AC designed the experimental assay. CS and PG carried out the experiments and collected the samples. DM, CS, JP, and RO performed the lab analysis.

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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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Erratum: LPS Modulates the Expression of Iron-Related Immune Genes in Two Antarctic Notothenoids

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Air Exposure Affects Physiological Responses, Innate Immunity, Apoptosis and DNA Methylation of Kuruma Shrimp, *Marsupenaeus japonicus*

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Air exposure stress is a common phenomenon for commercial crustacean species in aquaculture and during waterless transportation. However, the antioxidant responses to air exposure discussed in previous studies may be insufficient to present the complexities involved in this process. The comprehensive immune responses, especially considering the immune genes, cell apoptosis, and epigenetic changes, are still unknown. Accordingly, we investigated the multifaceted responses of *Marsupenaeus japonicus* to air exposure. The results showed that the expression profiles of the apoptosis genes (e.g., *IAP*, *TXNIP*, *caspase*, and *caspase-3*) and the hypoxia-related genes (e.g., *hsp70*, *hif-1 α* , and *HcY*) were all dramatically induced in the hepatopancreas and gills of *M. japonicus*. Heart rates, T-AOC (total antioxidant capacity) and lactate contents showed time-dependent changes upon air exposure. Air exposure significantly induced apoptosis in hepatopancreas and gills. Compared with the control group, the apoptosis index (AI) of the 12.5 h experimental group increased significantly ($p < 0.05$) in the hepatopancreas and gills. Most individuals in the experimental group (EG, 12.5 h) had lower methylation ratios than the control group (CG). Air exposure markedly reduced the full-methylation and total-methylation ratios (31.39% for the CG and 26.46% for the EG). This study provided a comprehensive understanding of the antioxidant responses of *M. japonicus* considering its physiology, innate immunity, apoptosis, and DNA methylation levels, and provided theoretical guidance for waterless transportation.

Keywords: *Marsupenaeus japonicus*, air exposure, oxidative stress, non-specific immunity, apoptosis, DNA methylation

INTRODUCTION

The Kuruma shrimp, *Marsupenaeus japonicus*, is an important commercial species that is widely distributed in the Indo-Western Pacific (Tsoi et al., 2014). Compared to other shrimps, *M. japonicus* have numerous advantages, such as fast growth rates, good reproductive capability, and survival during waterless transportation (Duan et al., 2016a). Air exposure stress is a common phenomenon

for commercial crustacean species in aquaculture and during live transport (Lorenzon et al., 2008; Fotedar and Evans, 2011; Romero et al., 2011). Upon air exposure, aquatic animals are subject to water shortage and hypoxia stress, accompanied by the generation of reactive oxygen species (ROS) (Romero et al., 2007; Paital and Chainy, 2010; Paital, 2013; Abasubong et al., 2018). Air exposure affects cellular damage and metabolic capacity for *Mytilus galloprovincialis* (Andrade et al., 2019) and causes a significant but reparable immunological response in *Litopenaeus vannamei* (Xu et al., 2019). Excessive ROS have an obvious oxidation effect on lipids, nucleic acids, and proteins that leads to an imbalance of homeostasis and physiological metabolism (Romero et al., 2011; Lennicke et al., 2015; Sies, 2017). Continuous oxidative stress triggers caspase-independent cell death and induces apoptosis or necrosis (Simon et al., 2000; Wang et al., 2012, 2019; Nathan and Cunningham-Bussell, 2013; Holze et al., 2018; Ondricek and Thomas, 2018).

Crustaceans mainly rely on non-specific innate immunity, including physical defense, and cellular and humoral immunity (Sadaaki and Bok Luel, 2005; Li and Xiang, 2013). To eliminate ROS-mediated damages, organisms protect themselves with enzymatic and non-enzymatic antioxidant defenses (Storey, 1996). The antioxidant enzyme systems mainly include superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) (Ighodaro and Akinloye, 2018). Duan et al. (2016a) investigated the effect of air exposure on antioxidant enzyme activities of Kuruma shrimp and found that the activities of SOD and CAT increased significantly. T-AOC (total antioxidant capacity), including enzymatic and non-enzymatic antioxidants, reflects the comprehensive antioxidant capacity of an organism (Jia et al., 2011; Liu H.L. et al., 2015). Air exposure induced significant oxidative and antioxidant responses in *L. vannamei* (Xu et al., 2018). Other research has detected significant changes in antioxidant enzyme activities in *Lithodes santolla* (Schvezov et al., 2019), *Paralomis granulosa* (Romero et al., 2011), *Apostichopus japonicus* (Huo et al., 2018), *Penaeus monodon* (Duan et al., 2016b), *Babylonia areolate* (Liu et al., 2017), and *Oryzias melastigma* (Cui et al., 2019). Hypoxia exposure had significant effects on the expression profiles of apoptosis and hypoxia-related genes, such as *p53*, *Hif-1 α* , *Hsp70*, *GSH-Px* (glutathione peroxidase) and *CAT* (catalase) (Xiao, 2015; Sun et al., 2016a; Cai et al., 2018; Ondricek and Thomas, 2018; Camacho-Jiménez et al., 2019). Hypoxia-inducible factors (HIFs) are recognized as master regulators of the cellular response to hypoxia stress by binding HRE (hypoxia-responsive element) (Schofield and Ratcliffe, 2004; Choudhry and Harris, 2018). The role of sHIF-1 α in the regulation of the HIF signaling pathway and the immune response was confirmed in mandarin fish (*Siniperca chuatsi*) (He et al., 2019). *Cancer magister* responds to hypoxia by increasing Hc concentrations and regulating subunit compositions (Head, 2010). Heat shock protein 70 (*Hsp70*) plays a vital role in preventing protein aggregation, folding and refolding of proteins and the degradation of unstable and misfolded proteins (Daugaard et al., 2007). The thioredoxin system, including TRX, TRXR, and NADPH, plays a critical role in resisting

oxidative stress, which has been suggested to have complex regulatory functions in shrimp (Garcia-Orozco et al., 2012; Lu and Holmgren, 2014; Liu P.-F. et al., 2015; Zuo et al., 2019). DNA methylation, a conservative method of epigenetic regulation in eukaryotes, regulates many biological processes such as transcription, DNA repair and cell differentiation (Zemach et al., 2010; Sahu et al., 2013; Wang et al., 2015). DNA methylation levels are usually influenced by various biotic and abiotic environmental stresses, including oxidative stress (Franco et al., 2008; Murgatroyd et al., 2009; Downen et al., 2012).

In general, air exposure is a comprehensive stimulation to aquatic animals. However, most previous studies have mainly focused on antioxidant enzyme activities. The comprehensive immune responses, especially immune genes, cell apoptosis, and epigenetic changes, are still unknown. Our objectives in the study were to determine the effects of air exposure on the physiological responses, non-specific immunity, apoptosis and DNA methylation levels of *M. japonicus*.

MATERIALS AND METHODS

Animals

All healthy *M. japonicus* individuals (weight: 6.25 ± 1.3 g) were obtained from an aquaculture farm in Zhangzhou (Fujian, China) and were acclimated in environmentally controlled cement pools for 2 weeks (25°C, 29‰ salinity and under continuous aeration). The filtered seawater was renewed every other day and the shrimps were fed twice daily with moderate amounts of commercial pelleted food. Shrimps were fasted for 24 h before the stress treatment.

Air Exposure and Sampling

After the conditioning period, two hundred individuals were transferred into an air-conditioned room at 25°C, and an air-exposure challenge was performed as follows. For the air-exposure treatment, the water on the surfaces of all individuals was sucked up using gauze and all individuals were then placed in six flat-bottomed rectangular tanks (80 cm $\times$ 50 cm $\times$ 30 cm). Every 20 min, these shrimp were sprayed with seawater to keep the skin moist. During the air exposure period, the hepatopancreas, gills and muscles from nine individuals were sampled at control (unstressed), 2.5, 5, 7.5, 10, and 12.5 h after exposure. Further, we recorded the numbers of dead individuals and counted the cumulative mortality. The criterion determining death was the loss of swimming ability after resubmersion. The heart rates of six individuals were determined by direct counting for 2 min at each sample point. The hepatopancreas and gills were immediately preserved in RNAfixer (BioTeck, China) for RNA extraction. Parts of the gill and hepatopancreas were rinsed with normal saline and then immediately preserved in 4% paraformaldehyde for detecting cell apoptosis. Part of the muscle and hepatopancreas tissues were immediately frozen at -80°C for lactate content determination and

methylation-sensitive amplification polymorphism (MSAP) analysis, respectively.

Homology Cloning and Sequence Analysis

Total RNA was extracted using RNAiso Plus (TaKaRa, Japan) following the specification. The purity and integrity were assessed. First-strand cDNA was synthesized using TransScript II First-Strand cDNA SuperMix (TransGen Biotech, China) according to the manufacturer's protocol. Primers were designed based on our transcriptome unigenes and sequences of other species that have been uploaded to NCBI (Table 1). PCR amplification products were ligated into the pMD19-T simple vector and sequenced in both directions. The open reading frames (ORFs) of *IAP* (inhibitor of apoptosis) and *TXNIP* (thioredoxin interacting protein) were predicted using the ORF finder¹. The amino acid sequence was deduced by the EMBOSS Transeq². ExPASy³ was used to analyze the protein molecular mass and isoelectric point (pI). The protein domains were predicted by SMART software⁴. Sequence alignments and phylogenetic trees were performed using MEGA 7.0 software (Kumar et al., 2016).

¹<https://www.ncbi.nlm.nih.gov/orffinder/>

²https://www.ebi.ac.uk/Tools/st/emboss_transeq/

³<https://www.expasy.org/>

⁴<http://smart.embl.de/>

The Quantitative Real-Time PCR Analysis

The expression profiles of apoptosis and hypoxia-related genes in the hepatopancreas and gills were measured by qRT-PCR, which was implemented on the QuantStudio 6 Flex Real-time PCR System (Applied Biosystems, United States) using SYBR® Premix ExTaq II (2×) (TaKaRa, Japan) following the protocols. All samples were run in triplicate and the *M. japonicus* elongation factor 1- α (*EF1- α*) was served as the reference gene (Table 1). Gene expression change was calculated by the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen, 2001). Expression data were shown with the mean $\pm$ standard deviation.

Total Antioxidant Capacity (T-AOC) and Lactate Content Determination

The total antioxidant capacity (T-AOC) of the hepatopancreas tissues was determined spectrophotometrically at 520 nm according to the instructions of a commercial assay kit (Jiancheng, China). Muscle tissues were used for lactate content determination at 530 nm using a lactic acid assay kit (Jiancheng, China). The determinations were conducted in triplicate for each sample using the Infinite M200 Pro system (Tecan, Switzerland) following the protocols. One unit of T-AOC was defined as the amount of protein per mg needed to increase 0.01 of the absorbance values every minute under the assay conditions (U/mg protein). The lactate content was expressed as mmol/g protein.

TABLE 1 | Primer sequences for qRT-PCR and MSAP analysis.

Name	Primer sequence (5'–3')	Name	Adaptor and primer sequence (5'–3')
IAP_F1	ATTGGAAATACAAGGAAG	Adaptors	
IAP_R1	CAGAATACTGAGGGAGGC	HMA1	CGAGCAGGACTCATGA
IAP_F2	CCAGTGACGGTTTCTATT	HMA2	GATCATGAGTCCTGCT
IAP_R2	GTGTCATCCGACTGTAGTG	EA1	CTCGTAGACTGCGTACC
IAP_F3	CAGTAAAGCCCAGTTCCA	EA2	AATTGGTACGCAGTC
IAP_R3	CAAAACTGCCTATGATAAAAAAT		
TXNIP5_F	ATGGCACGGAAGCTACAAAAG	Pre-amplification	
TXNIP3_R	TTACTCAAAGGTCTTCTCCTC	HM_Y	ATCCATGAGTCCTGCTCGG
Real-time PCR		EcoR_Y	GACTGCGTACCAATTCA
IAP-qRTF	CAATACCAGCACCCAGAG		
IAP-qRTR	GCTTCAGCCAAATCACAT	Selective amplification	
TXNIP_qRTF	GTCACGGACCGCAGGCAGCAG	HM1	ATCCATGAGTCCTGCTCGGCTCT
TXNIP_qRTR	CTCACTGGCGTCTTCGGACTC	HM2	ATCCATGAGTCCTGCTCGGCAT
Hsp70_qRTF	TCATCAACGAGAGCACGAA	HM3	ATCCATGAGTCCTGCTCGGTAC
Hsp70_qRTR	TCCGCAGTTTCTTCATCT	HM4	ATCCATGAGTCCTGCTCGGTTT
Hif1 α _qRTF	CTCGCAGGACAGGATGACT	HM5	ATCCATGAGTCCTGCTCGGCTCC
Hif1 α _qRTR	GACGCACTCGCTTGAACAA	HM6	ATCCATGAGTCCTGCTCGGTAC
HcY_qRTF	ACCAACAGCGAAGTCATT	EcoR1	(FAM)GACTGCGTACCAATTCAAC
HcY_qRTR	GATGTCCTCACCGAAGTAG	EcoR2	(FAM)GACTGCGTACCAATTCAAC
Caspase_qRTF	TTCCGGAACCCCTCAAAC	EcoR3	(FAM)GACTGCGTACCAATTCAAC
Caspase_qRTR	GGTTTGTGTGCTTTCGGAA	EcoR4	(FAM)GACTGCGTACCAATTCAAC
Caspase3_qRTF	ATTGTATTTATTGAGCGTGC	EcoR5	(FAM)GACTGCGTACCAATTCAAC
Caspase3_qRTR	TTGGAGGGGGATAACATAGTCTT	EcoR6	(FAM)GACTGCGTACCAATTCAAC
EF1 α _qRTF	GGAAGTGGAGGCAGGACC		
EF1 α _qRTR	AGCCACCGTTTGCTTCAT		

DAPI Staining and TUNEL Assay

The hepatopancreas and gills from five individuals in each group (control and 12.5 h) were sampled and preserved in 4% paraformaldehyde for 24 h. The fixed tissues were embedded in paraffin following ethanol dehydration and vitrification in dimethylbenzene. The paraffin sections were dewaxed, repaired and ruptured. The number of apoptotic cells in hepatopancreas and gills was determined using a One-Step TUNEL apoptosis assay kit (Beyotime, China) following the manufacturer's instructions. Apoptotic cell nuclei and total cell nuclei were quantitated by TUNEL staining (green fluorescence) and DAPI staining (blue fluorescence), respectively. The double-stained sections were examined using an inverted fluorescence microscope (NIKON Eclipse TI-SR, Japan). For each sample, four visual fields were selected randomly and were photographed by an inverted fluorescence microscope. Image-Pro Plus 6.0 software was used to count the number of apoptotic and common cells. The apoptosis index (AI) was defined as the proportion of apoptotic cells in the total cells.

DNA Methylation Levels and Patterns Under Air Exposure

Using the phenol-chloroform method, total genomic DNA was extracted from the hepatopancreas tissues of samples from the control and at 12.5 h after air exposure. The DNA integrity was visualized by agarose gel electrophoresis (1%) and concentration was measured using the Qubit 2.0 fluorometer (Life Technologies, CA, United States). DNA samples with clear bands were diluted to 100 ~ 200 ng/ μ L for the downstream analysis. The MSAP analysis was performed according to Xiong et al. (1999) and He et al. (2015) with modifications. The enzyme-digestion products of *Eco*RI/*Hpa*II and *Eco*RI/*Msp*I were ligated to adapters with T4 DNA ligase (TaKaRa) and performed pre-amplification and selective amplification with fluorescent-labeled primers (Table 1).

The selective amplification products were detected by capillary electrophoresis (CE) on an ABI 3730XL auto DNA sequencer (Shanghai Sangon, China). The MSAP fragments were analyzed using GeneMapper v3.2 software. Different enzyme digestion combinations of the same DNA sample were denoted as EH/EM. Three kinds of bands were detected and counted, including EH + /EM + (type I), EH + /EM- (type II) and EH-/EM + (type III). The methylation ratio was calculated as the following formula: Hemi-methylation ratio = Type II/(Type I + II + III) and Full-methylation ratio = Type III/(Type I + II + III).

Statistical Analysis

The data were analyzed using SPSS Statistics version 22 (IBM, United States). The results were presented as the means $\pm$ SD. The significant difference levels were detected by one-way analysis of variance (ANOVA) followed by Tukey's test, and a *p*-value less than 0.05 (<0.05) is statistically significant.

RESULTS

Sequence Analysis of *TXNIP* and *IAP*

The open reading frame (ORF) of MjTXNIP was 1044 bp (GenBank accession number: MN265397), encoding a polypeptide of 347 amino-acid residues with a calculated molecular weight of 39.97 kDa and a theoretical pI of 8.61. SMART analysis revealed that the predicted *TXNIP* protein contained an arrestin C domain located at 184-315 residues with an expected value of 2.44e-23 (Figure 1A). Homology comparisons showed that *TXNIP* shared amino acid identities with *TXNIP* from *L. vannamei* (98.85%), *Daphnia magna* (75.43%), *Limulus polyphemus* (65.99%), *Drosophila melanogaster* (61.73%), *Danio rerio* (22.67%), and *Homo sapiens* (21.52%). The open reading frame (ORF) of MjIAP (GenBank accession number: MN265396) was 2136 bp, encoding a polypeptide of 711 amino-acid residues with a calculated molecular weight of 78.48 kDa and a theoretical pI of 5.71. The SMART analysis revealed that the predicted *IAP* protein contains three BIR domains and a RING domain, which are located at 17-82, 108-172, 257-322, and 664-699 residues, respectively (Figure 1B). Multiple sequence alignment showed that MjIAP shared high amino-acid identity with *IAP* from *P. monodon* (85.96%, ABO38431.1), *L. vannamei* (84.83%, ADH03018.1) and *Scylla paramamosain* (59.73%, AMW91738.1). Phylogenetic analysis showed that the MjIAP clustered with the *IAP* protein of other crustaceans with a bootstrapping value of 100.

Expression Profiles of *MjIAP*, *MjTXNIP*, *hsp70*, *hif-1 α* , *HcY*, *caspase*, and *caspase3* Under Air Exposure Stress

The expression profiles of the apoptosis- and the hypoxia-related genes are shown in Figures 2, 3. Overall, these genes showed time-dependent changes after 5 h post-exposure in the hepatopancreas and after 2.5 h in the gill tissues. The mRNA expression levels of MjIAP gradually increased with time during air exposure. The expression levels in the hepatopancreas showed 2.34-, 4.3-, and 9.88-fold increases ($p < 0.05$) at 7.5, 10, and 12.5 h post-exposure, respectively (Figure 2A). In the gills, the mRNA expression levels of MjIAP showed 2.4- and 3.8-fold increases ($p < 0.05$) at 2.5 and 5 h, respectively (Figure 3A). In the hepatopancreas, the transcriptional level of MjTXNIP at the 10 h time point showed a significant incremental change ($p < 0.05$) (Figure 2B), while the expression levels in the gills peaked at 12.5 h with a 1.38-fold increase ($p < 0.05$) (Figure 3B). In the hepatopancreas, the mRNA expression levels of *caspase* increased 1.79-fold at the 5 h time point ($p < 0.05$) and peaked at the 7.5 h time point with a 1.91-fold increase (Figure 2C). In the gills, the mRNA levels showed a significant incremental change at the 10 h time point with a 1.44-fold increase (Figure 3C). The *caspase-3* levels in the hepatopancreas were significantly up-regulated at 7.5 h post-exposure and peaked at 10 h post-exposure with a 2.51-fold increase (Figure 2D). In the gills, the expression levels were significantly elevated at 5 and 7.5 h with 1.36-fold and 1.94-fold increases, respectively (Figure 3D).

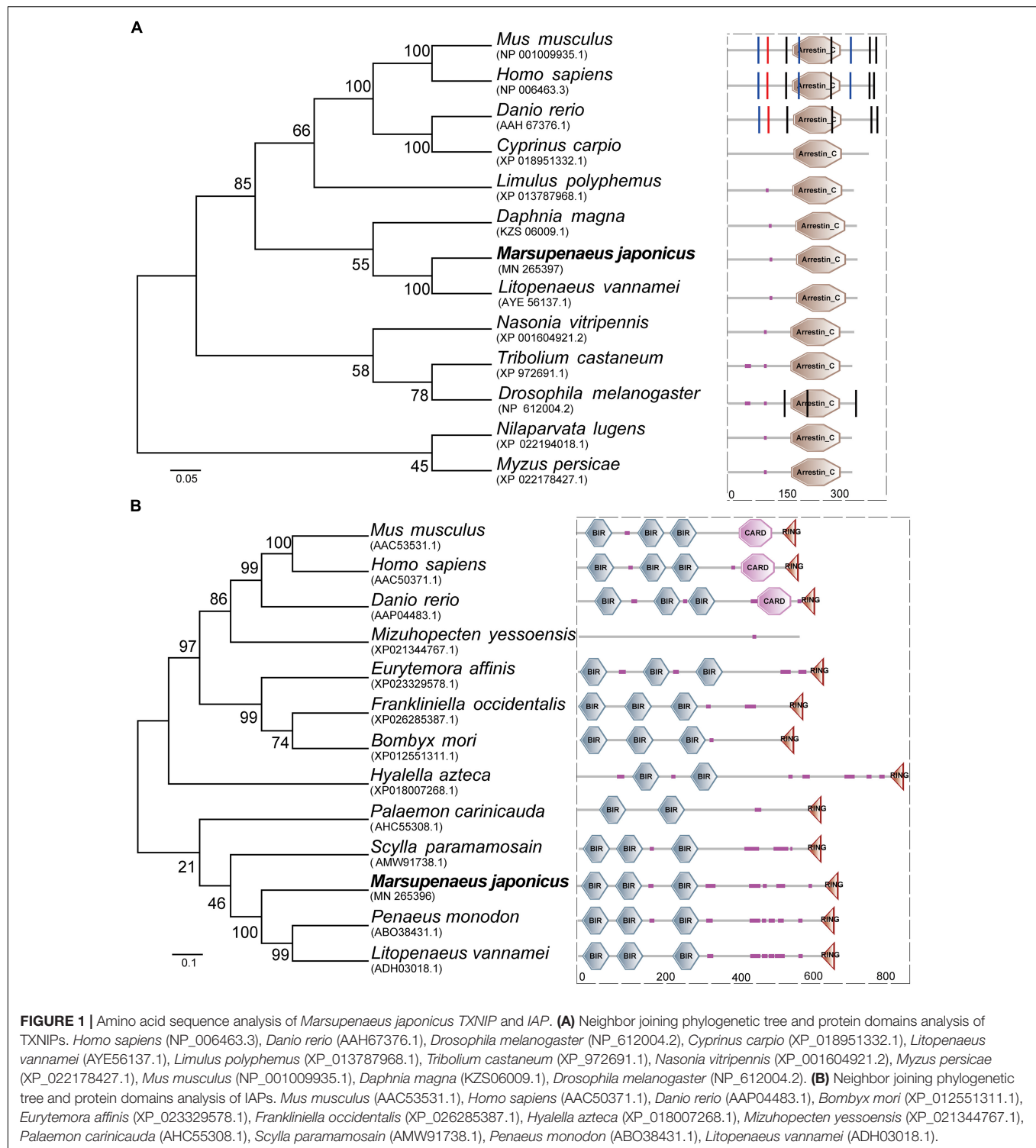


FIGURE 1 | Amino acid sequence analysis of *Marsupenaeus japonicus* TXNIP and IAP. **(A)** Neighbor joining phylogenetic tree and protein domains analysis of TXNIPs. *Homo sapiens* (NP_006463.3), *Danio rerio* (AAH67376.1), *Drosophila melanogaster* (NP_612004.2), *Cyprinus carpio* (XP_018951332.1), *Litopenaeus vannamei* (AYE56137.1), *Limulus polyphemus* (XP_013787968.1), *Tribolium castaneum* (XP_972691.1), *Nasonia vitripennis* (XP_001604921.2), *Myzus persicae* (XP_022178427.1), *Mus musculus* (NP_001009935.1), *Daphnia magna* (KZS06009.1), *Drosophila melanogaster* (NP_612004.2). **(B)** Neighbor joining phylogenetic tree and protein domains analysis of IAPs. *Mus musculus* (AAC53531.1), *Homo sapiens* (AAC50371.1), *Danio rerio* (AAP04483.1), *Bombyx mori* (XP_012551311.1), *Eurytemora affinis* (XP_023329578.1), *Frankliniella occidentalis* (XP_026285387.1), *Hyalella azteca* (XP_018007268.1), *Mizuhopecten yessoensis* (XP_021344767.1), *Palaemon carinicauda* (AHC55308.1), *Scylla paramamosain* (AMW91738.1), *Penaeus monodon* (ABO38431.1), *Litopenaeus vannamei* (ADH03018.1).

After 5 h post-exposure, the *hsp70* transcriptional levels in the hepatopancreas showed significant up-regulation that peaked at the 7.5 h time point, and was 3.14-fold higher when compared with the control group ($p < 0.05$), these levels then decreased significantly back to the baseline levels (Figure 2E). In the gills, the mRNA expression of *hsp70* was significantly elevated

at 2.5 and 5 h with 2.13- and 3.5-fold increases ($p < 0.05$), respectively (Figure 3E). The transcriptional levels of *hif-1α* in the hepatopancreas increased significantly by 1.87- and 2.83-fold ($p < 0.05$) at 10 and 12.5 h post-exposure, respectively (Figure 2F). In the gills, the *hif-1α* transcriptional levels showed a significant incremental change at the 10 h time point with

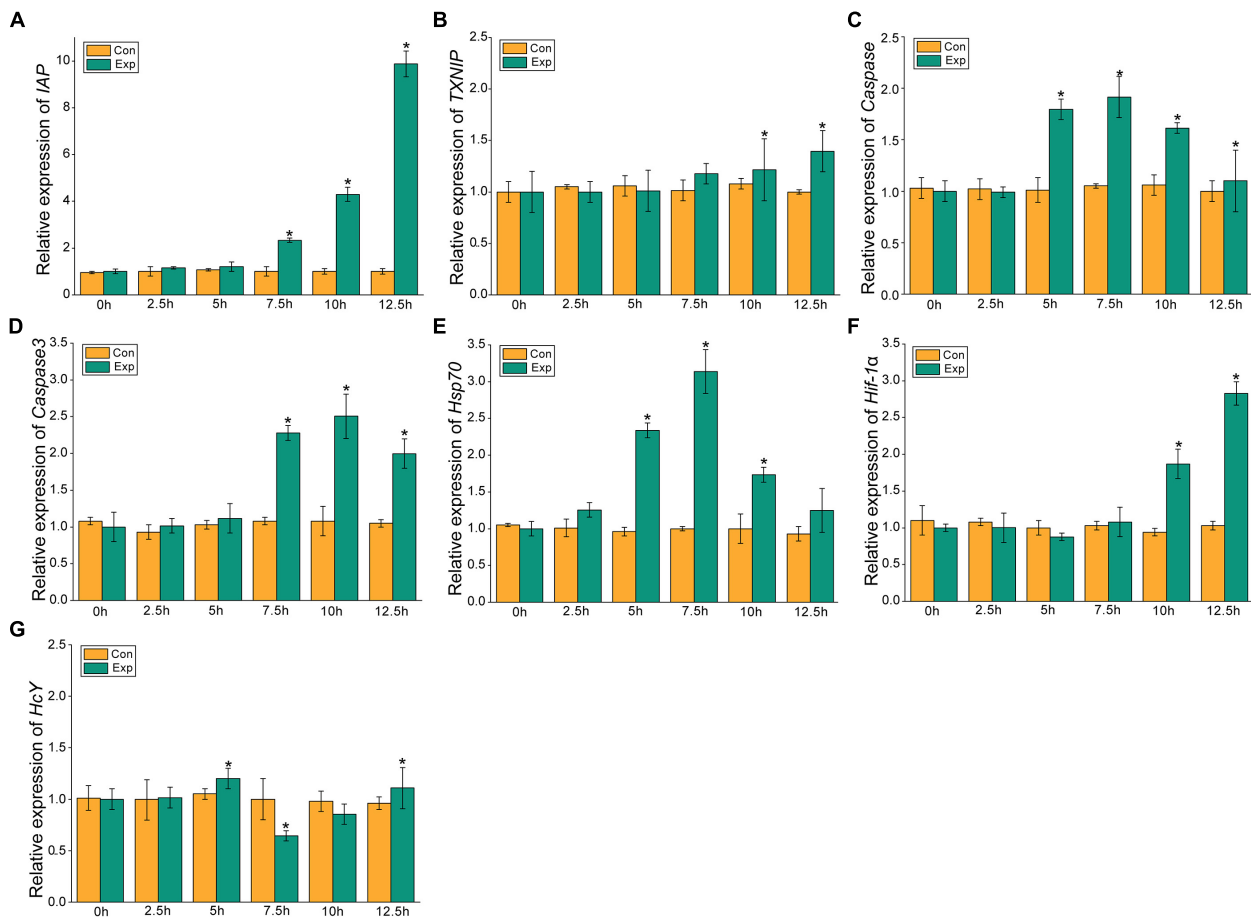


FIGURE 2 | Expression profiles of several apoptosis and hypoxia-related genes in the hepatopancreas. The relative mRNA expression levels of IAP (A), TXNIP (B), Caspase (C), Caspase3 (D), Hsp70 (E), Hif-1α (F), HcY (G). Each bar represents the mean ± SD ($n = 6$). A significant difference between groups at $p < 0.05$ (ANOVA) is indicated by ***.

a 1.95-fold increase ($p < 0.05$) (Figure 3F). The obvious up-regulation of the *HcY* expression in the hepatopancreas was detected at the 5 h time point with a 1.2-fold increase (Figure 2G). The *HcY* expression levels in the gills were significantly elevated at 2.5 and 5 h with 1.34- and 1.88-fold increases ($p < 0.05$), respectively (Figure 3G).

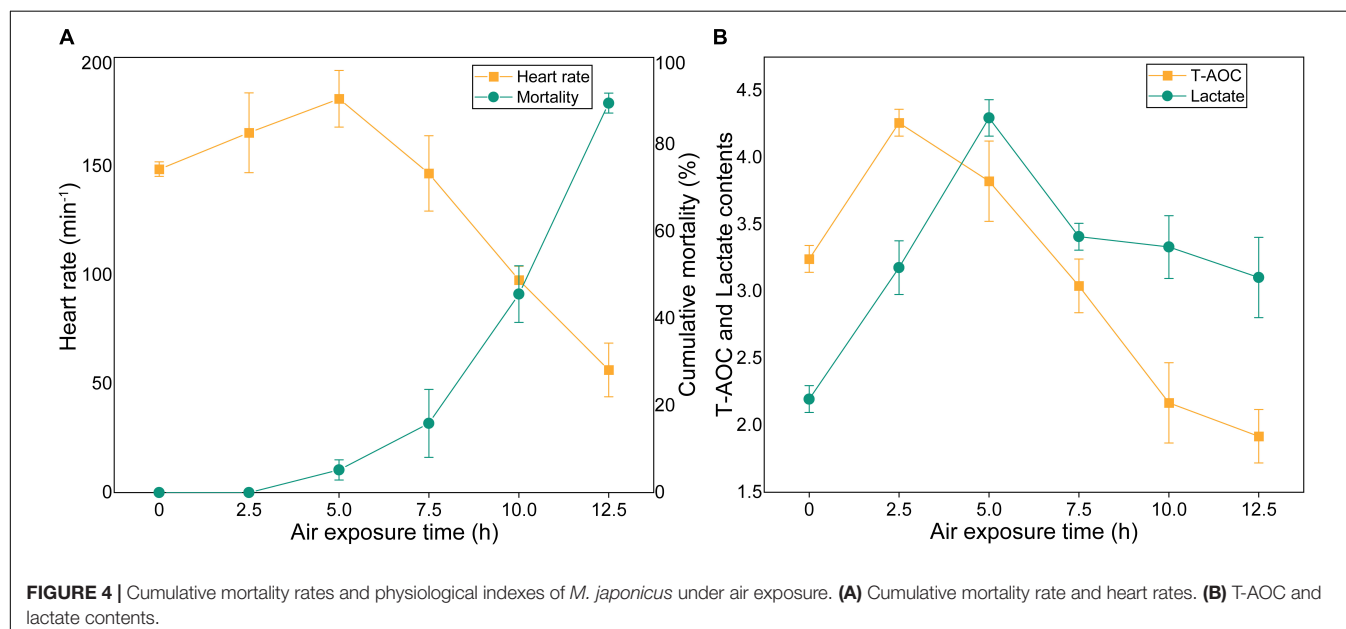
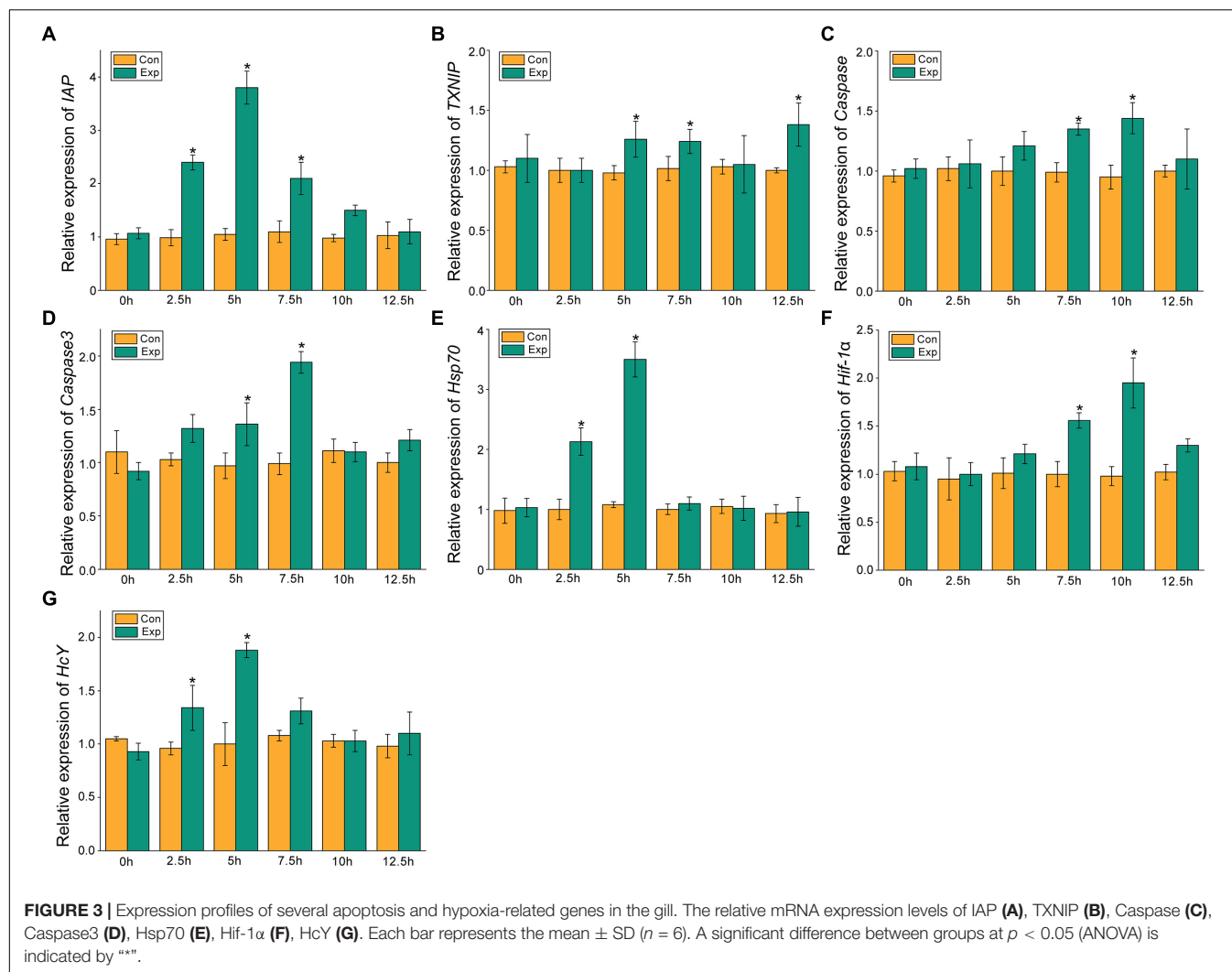
The Cumulative Mortality and Physiological Indexes

The cumulative mortality and physiological indexes showed time-dependent changes upon air exposure (Figure 4). Several individuals showed stress symptoms, and nine individuals died at the 5 h time point (the mortality rate was 4.5%) (Figure 4A). As the exposure time progressed, the cumulative mortality rate was 15% at 7.5 h post-exposure, and then increased markedly to 89.5% at 12.5 h. The heart rates exhibited an initial increase and a subsequent decrease (Figure 4A). The heart rates significantly up-regulated at the 2.5 h time point and then peaked at the 5 h time point at 181 beats/min. The heart rates then quickly decreased until faint beating was observed at the 12.5 h time point. T-AOC significantly increased at 2.5 h post-exposure,

which was a 1.3-fold increase ($p < 0.05$) compared with the control group (Figure 4B). After the peak, T-AOC decreased rapidly to the lowest level at the 12.5 h time point (1.9 U/mg protein). The lactate concentrations remained elevated through the 2.5 and 5 h post-exposure points, which were increased 1.72- and 2.1-fold, respectively, compared with the control group and then decreased rapidly (Figure 4B). At the 12.5 h time point, the lactate concentrations were still higher than those of the control group.

Detection of Apoptosis in the Hepatopancreas and Gills via TUNEL

In this study, air exposure significantly induced apoptosis in the hepatopancreas and gill cells (Figure 5). TUNEL-positive cell nuclei appeared green and common cells appeared blue. For each sample, four visual fields were randomly selected for counting the apoptosis index (AI). In the hepatopancreas, the apoptosis index (AI) values of the control and the 12.5 h post-exposure group were 10.5 and 30.5%, respectively. Compared with the control group, the AI for the 12.5 h experimental group increased significantly ($p < 0.05$). In the



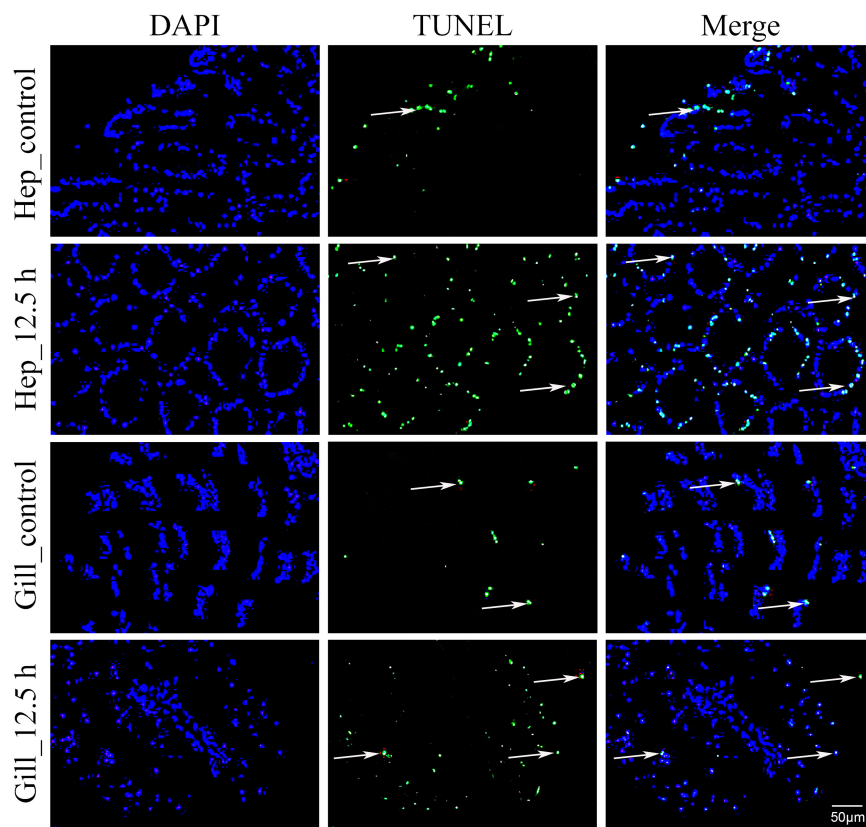


FIGURE 5 | Apoptosis analysis of *M. japonicus* hepatopancreas and gill (200 $\times$). TUNEL-positive cell nuclei appear green, and parts are indicated by the arrows.

gills, the AI for 12.5 h post-exposure group was significantly higher than that for the control group ($p < 0.05$), at 24.5 and 9.5%, respectively. Compared with the gill tissues, the hepatopancreas showed a higher mean AI at 12.5 h post-exposure. However, no significant changes ($p > 0.05$) were observed between the two groups.

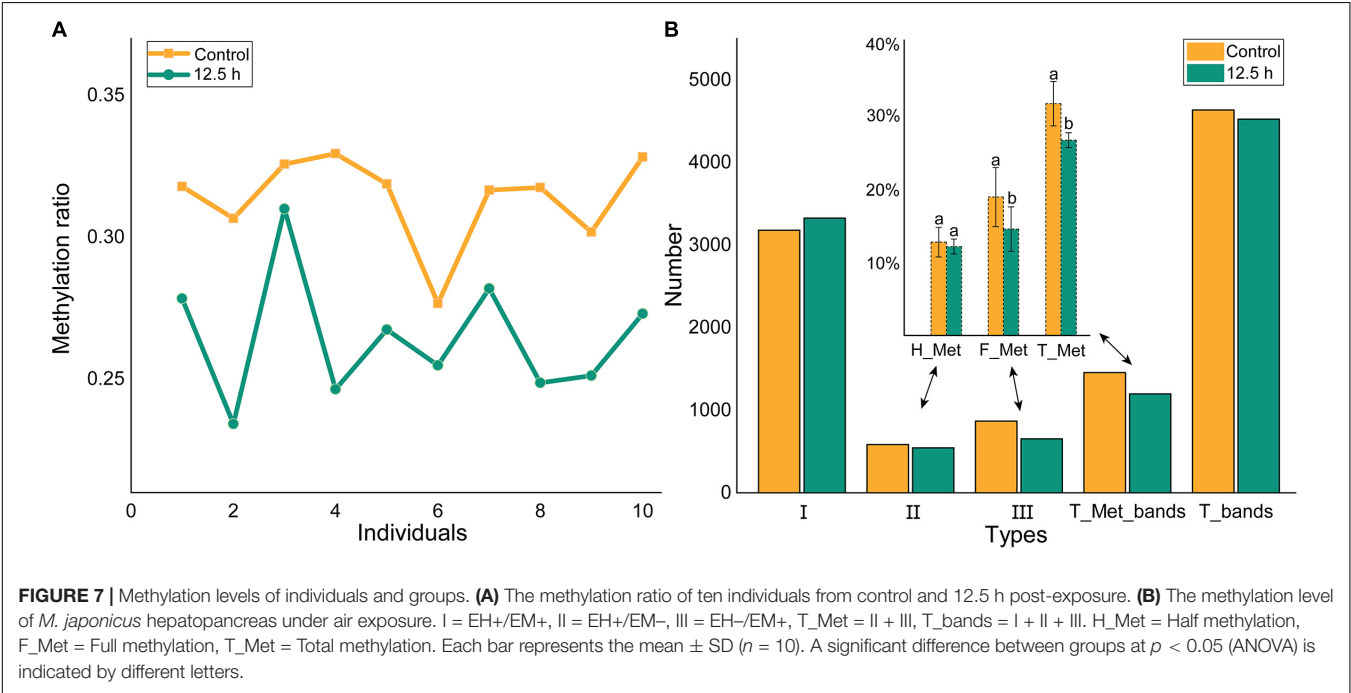
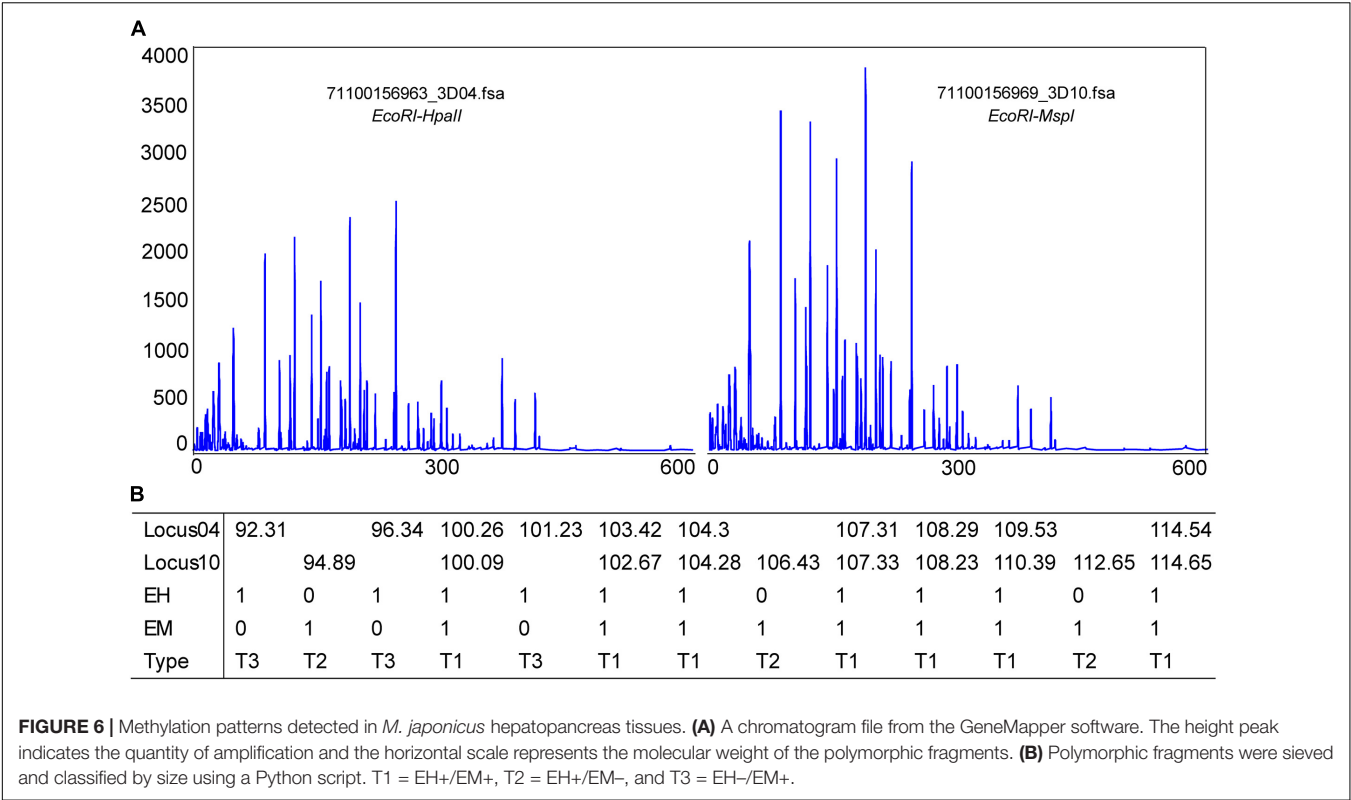
Changes in DNA Methylation Patterns in the Hepatopancreas

Methylation-sensitive amplification polymorphism fragment gel files were transformed into chromatogram files by GeneMapper (Figure 6A and Supplementary Table S1). All fragments were sieved and classified by size using a Python script (Figure 6B). The methylation ratios of ten individuals from the control group and from 12.5 h after exposure are presented in Figure 7A. Most individuals in the control group (CG) had higher methylation ratios than those in the experimental group (EG). A total of 4635 fragments for the CG and 4524 fragments for the EG were obtained (Figure 7B). The numbers of fragments of types I, II, and III were 3180, 586, and 869 for CG and were 3327, 545, and 652 for EG, respectively. The computational results showed a significant difference in the full-methylation levels ($p < 0.05$). However, there was no significant difference between the hemi-methylation levels. For both CG and EG, the full-methylation ratios were higher than the hemi-methylation ratio

(Figure 7B). The average methylation ratio was 31.39% for CG and 26.46% for EG.

DISCUSSION

As a swimming and burrowing species, *M. japonicus* is usually subjected to aerial exposure during the processes of packing and transportation (Hewitt and Duncan, 2001; Abe et al., 2007; Fotadar and Evans, 2011). In our study, some individuals showed stress symptoms and nine individuals died at 5 h post-exposure. The cumulative mortality rate rapidly increased after 7.5 h post-exposure. At the 12.5 h time point, the remaining individuals were only sufficient for sampling. The aerial stress tolerance was consistent with the results of Duan et al. (2016a). Heart rate, a direct measure of the physiological state, is correlated with oxygen consumption and the metabolic rate (Green, 2011; Claireaux and Chabot, 2016; Lund et al., 2017). In the early stages, the heart rate significantly increased for oxygen supply and ATP production. Because the almost transparent heart is covered with a carapace, there are still no reliable tools to record heart rates. The lower heart rates were a sign of a decline in overall bodily function. During the experiment, we monitored the gill ventilation frequency, which reflected individual vitality levels. The gill ventilation frequencies exhibited an initial increase and a



subsequent decrease, and could not be observed directly at the final stage.

During air exposure, T-AOC showed time-dependent changes. In the early stages of exposure, the antioxidant defense system increased T-AOC. As the exposure time progressed, the increased activity of the antioxidant enzymes was not sufficient to effectively remove ROS, however, the body needs to constantly consume antioxidants to reduce oxidative damage. Liu H.L. et al. (2015) showed that T-AOC in the hepatopancreas of *L. vannamei* decreased after 10 min of air exposure and then increased.

During hypoxia, crustaceans utilize anaerobic glycolysis to satisfy their energy requirements, thus accumulating lactate (Soñanez-Organis et al., 2010; Dunbar et al., 2017; Valère-Rivet et al., 2019). For the early stages of air exposure, the lactate concentrations remained elevated through 2.5 and 5 h post-exposure, which were consistent with the results of Abe et al. (Abe et al., 2007; Aparicio-Simón et al., 2018). Subsequently, the comprehensive metabolic system of the shrimp was severely affected, and part of the lactic acid was converted into glucose to maintain homeostasis and the energy supply, thus reducing the lactic acid content.

The duration of air exposure significantly influenced the expression profiles of the hypoxia-related genes in the hepatopancreas and gills. Hepatopancreas, a multifunctional organ, integrate metabolism and immune functions. Gills, a crustacean respiratory organ, participate in ion transport, acid-base balance, ammonia excretion, and heavy metal accumulation (Henry et al., 2012). Overall, gill tissues may be more sensitive to desiccation stress than the hepatopancreas tissues, which makes sense given their direct exposure to the environment. In this study, the *Hif-1α* expression levels in the hepatopancreas and gill tissues significantly increased at the 10 and 7.5 h time points, which is in accordance with most research results (Sun et al., 2016b; Okamura et al., 2018). In the hepatopancreas, the transcriptional level of *Hif-1α* peaked at the 12.5 h time point, while the expression levels in the gills exhibited an initial increase and a subsequent decrease. As an important organ of respiration, the microvascular cavity at the edge of the gill filaments lobule is filled with a large amount of hemocyanin for oxygen transport. The *HcY* expression increased gradually within 2.5 h of air exposure in the gills, but was not obvious in the hepatopancreas. With a change in dissolved oxygen, crustaceans increase the hemolymph pH or change the composition of the Hc molecules to enhance the hemocyanin oxygen affinity (Coates and Nairn, 2014). At the initial stage of air exposure, the up-regulation of lactate concentrations might affect pH values, which weakened the binding capacity between hemocyanin and oxygen (Whiteley et al., 1997). Further studies are needed to investigate hemocyanin concentrations during air exposure.

Continuous oxidative stress triggers caspase-independent cell death and induces apoptosis or necrosis (Simon et al., 2000). Several stress conditions, such as high temperatures, hypoxia exposure, and ultraviolet (UV) irradiation, have induced extensive apoptosis in the larval embryos of the Japanese flounder (Yabu et al., 2003; Williams et al., 2017). In this study, air exposure significantly affected the percentage of TUNEL-positive cells. Compared with the control group, the apoptosis index (AI) for the 12.5 h experimental group increased significantly ($p < 0.05$) in the hepatopancreas and gills. A higher apoptosis index was detected in the hepatopancreas than in the gills, which was possibly because to the hepatopancreas is the major metabolic center of ROSs production in crustaceans (Arun and Subramanian, 1998; Moore et al., 2007). The MjIAP showed different expression patterns in the hepatopancreas and gills. An earlier upward trend was presented in gills at 2.5 h, which might suppress the early apoptosis of the gill cells. Further

studies are needed to investigate the histological symptoms and ultrastructure of the hepatopancreas and gills.

As the major factors for apoptosis, the caspases are a family of structurally related cysteine proteases and contain inflammatory mediator (1,4,5,11), activator (2,8,9,10) and executioner (3,6,7) subfamilies (Shalini et al., 2015; Julien and Wells, 2017). Chronic exposure of croaker to hypoxia increased caspase-3/7 activity, as well as the apoptosis of ovarian follicle cells (Ondricek and Thomas, 2018). Hypoxia ischemia can result in a significant increase in the *caspase-3* expression levels and neuronal apoptosis in the brains of neonatal mice (Deng et al., 2019). In this study, the *caspase* and *caspase-3* expression levels increased initially and then decreased, and the *caspase* gene was more sensitive than the *caspase-3* gene. The *caspase* gene showed sequence similarity to the *caspase-8* gene of other species (Wang et al., 2008). The caspase protein was essential for virus-induced apoptosis in *M. japonicus*. As an activator, *caspase-8* activation can induce direct cleavage of downstream caspase-3/7 (Kaufmann et al., 2012; Feltham et al., 2017). Caspase-3/8 activities will be detected in further studies. As an endogenous inhibitor of caspases, IAP does not bind to *caspase-8* but inhibits its substrate, *caspase-3*, to execute anti-apoptotic roles (Wei et al., 2008). The MjIAP expression levels in the hepatopancreas gradually increased, reaching an increase of 9.88-fold ($p < 0.05$) at 12.5 h post-exposure, which may induce a decrease in *caspase* and *caspase-3* expression in the final stage. *TXNIP*, an inhibitor of the major ROS scavenger *TRX*, was positively associated with antibacterial responses but was negatively correlated with antiviral responses in shrimp (Zuo et al., 2019). In the hepatopancreas and gills, the transcriptional level of MjTXNIP exhibited similar variation trends.

DNA methylation is an important epigenetic modification, which may contribute to environmentally induced phenotypic variations by regulating gene transcription (Angers et al., 2010; Roberts and Gavery, 2012). Zhang et al. (2017) showed that the total methylation levels of *Crassostrea gigas* gills and adductors significantly increased at 12 h post-exposure. The methylation levels were different for different tissues of the same individual or for different states of the same tissue, even in the offspring of full-sibling families. The high methylation diversity among individuals may be related to the high genome heterozygosity of shrimp (Yuan et al., 2018; Zhang et al., 2019). The control group and the experimental group all had one abnormal individual, but this did not affect the overall outcome. The air exposure led to a lower methylation ratio in the experimental group than in the control group. There are varying degrees of methylation in invertebrates, which are often below the rate for vertebrates (Gavery and Roberts, 2010; Roberts and Gavery, 2012). The methylation rates in this study were higher than those of *Fenneropenaeus chinensis* (He et al., 2015). DNA methylation has an obvious tissue-specific pattern (Sun et al., 2014; Zhao et al., 2015). Except for the hepatopancreas, the DNA methylation changes in other tissues still need further verification. It is well known that DNA methylation in promoter regions normally represses gene expression (Suzuki and Bird, 2008; Maunakea et al., 2010; Jones, 2012; Zhou et al., 2019). Wan et al. (2015) determined the existence of two different

modes of DNA methylation-dependent gene regulation. The DNA methylation of *IAP*, *TXNIP*, *caspase*, and *caspase-3* genes may play a key role in the differential expression patterns, which needs further verification.

CONCLUSION

This study provided a relatively comprehensive understanding of the desiccation and oxidative stress responses of *M. japonicus* in terms of physiology, innate immunity, cell apoptosis, and DNA methylation levels, and facilitated further investigation into the molecular mechanisms of shrimp under oxidative stress. In the coming centuries, aquatic animals will face more severe challenges, including ocean warming and the expansion of the death zone (Keeling et al., 2010; Altieri and Gedan, 2015; Vinagre et al., 2019). This research can serve as a case study for future research on hypoxia adaptation and the antioxidant mechanisms of other marine groups.

DATA AVAILABILITY STATEMENT

The data of MjTXNIP (MN265397) and MjIAP (MN265396) are publicly accessible in the NCBI (<https://www.ncbi.nlm.nih.gov/>).

ETHICS STATEMENT

All experimental procedures were conducted in conformity with institutional guidelines for the care and use of laboratory animals in Xiamen University, Xiamen, China.

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AUTHOR CONTRIBUTIONS

PW, YM, JW, and YS designed the experiments. PW and ZL conducted computational and statistical analyses. PW and YM drafted the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fphys.2020.00223/full#supplementary-material>

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Differential Proteomic Analysis of Chinese Giant Salamander Liver in Response to Fasting

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Chinese giant salamander *Andrias davidianus* has strong tolerance to starvation. Fasting triggers a complex array of adaptive metabolic responses, a process in which the liver plays a central role. Here, a high-throughput proteomic analysis was carried out on liver samples obtained from adult *A. davidianus* after 3, 7, and 11 months of fasting. As a result, the expression levels of 364 proteins were significantly changed in the fasted liver. Functional analysis demonstrated that the expression levels of key proteins involved in fatty acid oxidation, tricarboxylic acid cycle, gluconeogenesis, ketogenesis, amino acid oxidation, urea cycle, and antioxidant systems were increased in the fasted liver, especially at 7 and 11 months after fasting. In contrast, the expression levels of vital proteins involved in pentose phosphate pathway and protein synthesis were decreased after fasting. We also found that fasting not only activated fatty acid oxidation and ketogenesis-related transcription factors PPARA and PPARGC1A, but also activated gluconeogenesis-related transcription factors FOXO1, HNF4A, and KLF15. This study confirms the central role of lipid and acetyl-CoA metabolism in *A. davidianus* liver in response to fasting at the protein level and provides insights into the molecular mechanisms underlying the metabolic response of *A. davidianus* liver to fasting.

Keywords: Chinese giant salamander, fasting, liver, metabolism, proteome

INTRODUCTION

Groundwater biotopes are characterized by poor and discontinuous food supplies. The organisms faced with reduced food intake have evolved efficient physiological and metabolic adaptations to extend their survival (Issartel et al., 2010). Several hypogean species are able to survive for long periods of food deprivation—nearly 1 year in invertebrates, and up to several years in cave fishes and salamanders (Hervant et al., 1999; Hervant et al., 2001). A study report proposed that the hypogean species had a lower metabolic rate, higher amounts of energy reserves (glycogen, triglycerides, and proteins) and reduction in utilization rates than epigeal ones, enabling them to fuel energy metabolism for much longer without food (Hervant and Renault, 2002; Issartel et al., 2010).

The liver is the central organ for metabolic activity in vertebrates and is also an important storage depot for energy reserve (glycogen and lipids). Urodele amphibians not only have large amounts of glycogen in hepatocytes, but also have larger amount of lipids than other vertebrates

(Bizjak Mali et al., 2013). Hepatic fat serves as energy reserve in many vertebrates including fishes and amphibians during starvation (Groom et al., 2013). The utilization of the energy reserves varies in different species with successive periods of glycogen, lipid, and finally lipid-protein-dominant catabolism. For hypogean salamander *Proteus anguinus* that can tolerate fasting for between 18 and 96 months, its liver glycogen was decreased by 50%, lipids by 25%, and proteins by 35% after 18 months of starvation (Hervant et al., 2001; Bizjak Mali et al., 2013). Reduced hepatocyte size, scarce lipid droplets, and numerous mitochondria and peroxisomes were observed in the hepatocytes of *P. anguinus* after 18 months of starvation (Bizjak Mali et al., 2013). For the frog *Rana esculenta*, its liver glycogen concentration decreased by 95% after 18 months of starvation at 20°C (Grably and Piery, 1981). For the South African clawed toad *Xenopus laevis*, its liver glycogen levels were reduced by 80–90% after 12 months of starvation at 20°C (Merkle and Hanke, 1988). For other vertebrates, several studies showed that genes involved in ketogenesis, gluconeogenesis, and fatty acid beta-oxidation, were up-regulated in liver of chicken after 16 h of starvation (Desert et al., 2008), in liver of mice after 24 h of starvation (Kinouchi et al., 2018; Rennert et al., 2018), in liver of fishes after 21 days of starvation (Drew et al., 2008; Qian et al., 2016). Compared to other vertebrates, the hypogean salamanders exhibited remarkable resistance to long periods of food deprivation, this may be due to the remarkable accumulation of lipid and glycogen deposits in the liver, and glycogen level maintenance during food deprivation.

The giant salamanders are aquatic urodeles including Cryptobranchidae, Amphiumidae, Sirenidae, and Proteidae. The Cryptobranchidae include the hellbender, Japanese giant salamander, and Chinese giant salamander. They are largely water-breathers with reduced lung function. The lungs can function during hypoxia events, but routine gas exchange is aquatic, and almost entirely integumentary (Ultsch, 2012). Chinese giant salamander *Andrias davidianus* is the largest extant amphibian in the world and the only species of cryptobranchidae in China. It lives and breeds in large hill streams, normally in forested areas of central and southern China (Turvey et al., 2019). Wild species is threatened with extinction due to habitat loss, water pollution, and over-hunting, and thus is listed as a class II protected species of China and in the Appendix I of Convention on International Trade of Endangered Species (CITES, 2014). Over the past decade, the increased number of the artificially cultivated *A. davidianus* provide the potential resource for scientific research (He et al., 2018). However, owing to little genomic data was available previously for *A. davidianus*, it has hindered the understanding of molecular mechanisms underlying stress response and physiological adaptation of *A. davidianus*. Currently, complete transcriptome analysis reports of *A. davidianus* (Geng et al., 2017; Huang et al., 2017) pave the way for research on physiological adaptations to extreme environments. Giant salamanders can survive for long periods without food up to several years. However, it is unclear what molecular mechanisms underlie their survival. Therefore, to characterize the physiological and metabolic response of *A. davidianus* to prolonged fasting, we investigated protein

expression changes in the liver from adult *A. davidianus* after 3, 7, and 11 months of fasting by iTRAQ-coupled RPLC-MS/MS. This study lays the foundation for understanding better how aquatic amphibians to survive in their food limited habitats.

MATERIALS AND METHODS

Animals, Sample Preparation

Adult healthy female Chinese giant salamanders weighing about 2 kg, were obtained from an artificial breeding farm at Chongqing Kui Xu Biotechnology Incorporated Company in Chongqing, China. The giant salamanders were maintained in aerated freshwater tanks at 20°C under weak lighting conditions and fed daily with diced silver carp. The giant salamanders were acclimatized to laboratory conditions for 2 weeks prior to fasting. A total of 20 salamanders were randomly divided into 4 groups with 5 salamanders per group: three fasting groups and one normal control group. The salamanders in the fasting groups were subjected to fasting for 3, 7, and 11 months. The salamanders were heavily anesthetized by MS-222 and sacrificed by decapitation and liver tissues were collected from control group and fasting groups.

Then, protein extraction was performed according to a method described by Isaacson et al. (2006) with some modification. The liver tissues were ground into power with liquid nitrogen and precipitated by cooled acetone containing 10% TCA. It was then centrifuged by 1,5000 g for 15 min at 4°C and dried by vacuum freeze dryer. The deposit was suspended in cold phenol extraction buffer, and an equal volume of phenol saturated with Tris-HCl (pH 7.5) was added and centrifuged at 5000 g for 30 min at 4°C to collect the upper phenolic phase. Next, cold 0.1 M ammonium acetate in methanol was added for precipitation followed by centrifugation at 1,0000 g for 10 min at 4°C. Then, the deposit was washed by cold methanol and acetone and centrifuged at 1,0000 g for 10 min at 4°C. The dried deposit was dissolved in lysis solution at 30°C for 1 h, and centrifuged by 1,5000 g for 30 min. The supernatant was collected and protein concentration in the extracts were determined by the BCA method (Smith et al., 1985).

Protein Digestion and iTRAQ Labeling

A total of 100 µg proteins from each sample were reduced and alkylated as described in the iTRAQ protocol (Applied Biosystems), followed by digestion with 50 ng/µL sequencing-grade trypsin solution at 37°C overnight. The digested peptides were dried by vacuum centrifugation. The peptides from control, 3, 7, and 11 months of fasting samples were labeled with 117, 118, 119, and 121 iTRAQ tags, respectively, according to the manufacturer's protocol (Applied Biosystems, United States). The labeled samples were pooled and vacuum dried.

RPLC-MS/MS Analysis

The dried sample was re-suspended in 100 µL buffer A (10 mM KH₂PO₄ pH 3.0, 25% acetonitrile), and separated on the Agilent 1200 HPLC System (Agilent). The parameters of RPLC were set as follows: analytical guard column (4.6 mm × 12.5 mm,

5 μm); narrow-bore (2.1 mm $\times$ 150 mm, 5 μm) with 215 and 280 nm UV detection; flow rate of 0.3 mL/min using a non-linear binary gradient starting with buffer A and transitioning to buffer B (10 mM KH_2PO_4 pH 3.0, 500 mM KCl, 25% acetonitrile). Each segment was collected at 4.5 min interval between 8 and 52 min. A total of 10 segments were collected and dried by a vacuum freeze dryer.

The online Nano-RPLC was employed on the Eksigent nanoLC-Ultra 2D System (AB SCIEX). The dried peptides from each segment were re-suspended in Nano-RPLC buffer A (0.1% formic acid, 2% acetonitrile) and loaded on C18 nanoLC trap column (100 μm $\times$ 3 cm, C18, 3 μm , 150 $\text{\AA}$) and washed by Nano-RPLC buffer A at 2 $\mu\text{L}/\text{min}$ for 10 min. An elution gradient of 5–35% acetonitrile (0.1% formic acid) run for 70 min was used on an analytical ChromXP C18 column (75 μm $\times$ 15 cm, C18, 3 μm 120 $\text{\AA}$) with spray tip. Data acquisition was performed with a Triple TOF 5600 System (AB SCIEX, United States) fitted with a Nanospray III source (AB SCIEX, United States) and a pulled quartz tip as the emitter (New Objectives, United States). Data were acquired at an ion spray voltage of 2.5 kV, curtain gas of 30 psi, nebulizer gas of 5 psi, and an interface heater temperature of 150°C. For information dependent acquisition (IDA), survey scans were acquired at 250 ms, and as many as 35 product ion scans were collected if they exceeded a threshold of 150 counts/s with a 2+ to 5+ charge-state. The total cycle time was fixed to 2.5 s. A rolling collision energy setting was applied to all precursor ions for collision-induced dissociation (CID). Dynamic exclusion was set to 1/2 of peak width (18 s). The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (Perez-Riverol et al., 2019) partner repository with the dataset identifier PXD014924.

Data Analysis

Data was processed with Protein Pilot Software v. 5.0 (AB SCIEX, United States) against the protein database obtained from transcriptome data of *A. davidianus* using the Paragon algorithm (Shilov et al., 2007). The following search parameters were utilized to analyze MS/MS data: trypsin as the digestion enzyme with a maximum of two missed cleavage allowed, fixed modifications of Carbamidomethyl (C) and iTRAQplex modification (K and N-terminus), variable modifications of Oxidation (M), peptide mass tolerance of ± 20 ppm, fragment mass tolerance of ± 0.1 Da, and peptide false discovery rate (FDR) ≤ 0.01 .

The quantification of iTRAQ labeled peptides was carried out based on reporter ion intensity using Proteome Discoverer 1.4 software. Only unused score > 1.3 and unique peptide ≥ 1 were utilized to determine protein quantification. The frequency distribution histogram was developed to analyze iTRAQ quantitative data (Cox and Mann, 2008). Firstly, protein ratio was calculated as the relative expression level in fasting group to that in control group. Next, the *P*-value for log protein ratios was calculated using previously published method (Cox and Mann, 2008; Geng et al., 2016). Based on the above analysis, fold change > 1.5 and *P*-value < 0.05 were the selection criteria for the significantly changed proteins.

Bioinformatics Analysis

To characterize the expression patterns of the proteins, Cluster 3.0/TreeView was utilized for hierarchical clustering of the differentially expressed proteins in the liver samples after fasting (Eisen et al., 1998). DAVID database was used to perform functional and pathway enrichment analyses of the differentially expressed proteins according to a modified Fisher's exact test in combination with FDR method as described in detail previously (Huang da et al., 2009). In addition, the differentially expressed proteins were analyzed by Ingenuity Pathway Analysis (IPA) version 9.0 software for transcription regulators, canonical pathways and biological functions (Kramer et al., 2014).

Western Blot Analysis

Antibodies raised against salamander proteins were not available, thus mammalian antibodies were used in this study. Briefly, 60 μg of proteins were separated by 12% SDS-PAGE and transferred to a nitrocellulose membrane. The membranes were incubated with rabbit anti-ADH1, anti-FBP1, anti-GOT2, and rabbit anti-YWHAE (Bioss, 1:1000) overnight at 4°C. The membrane was incubated with horseradish peroxidase (HRP)-conjugated secondary goat anti-rabbit IgG (Sigma, 1:2500). Finally, the protein band was visualized with Amersham enhanced chemiluminescence (ECL) substrates. Exposure imaging was performed on the ImageQuant LAS 4000 Chemiluminescence Imager (GE Healthcare), and the band density was measured using ImageQuant TL software (GE Healthcare). β -actin (Sigma, 1:1000) was used as an internal reference.

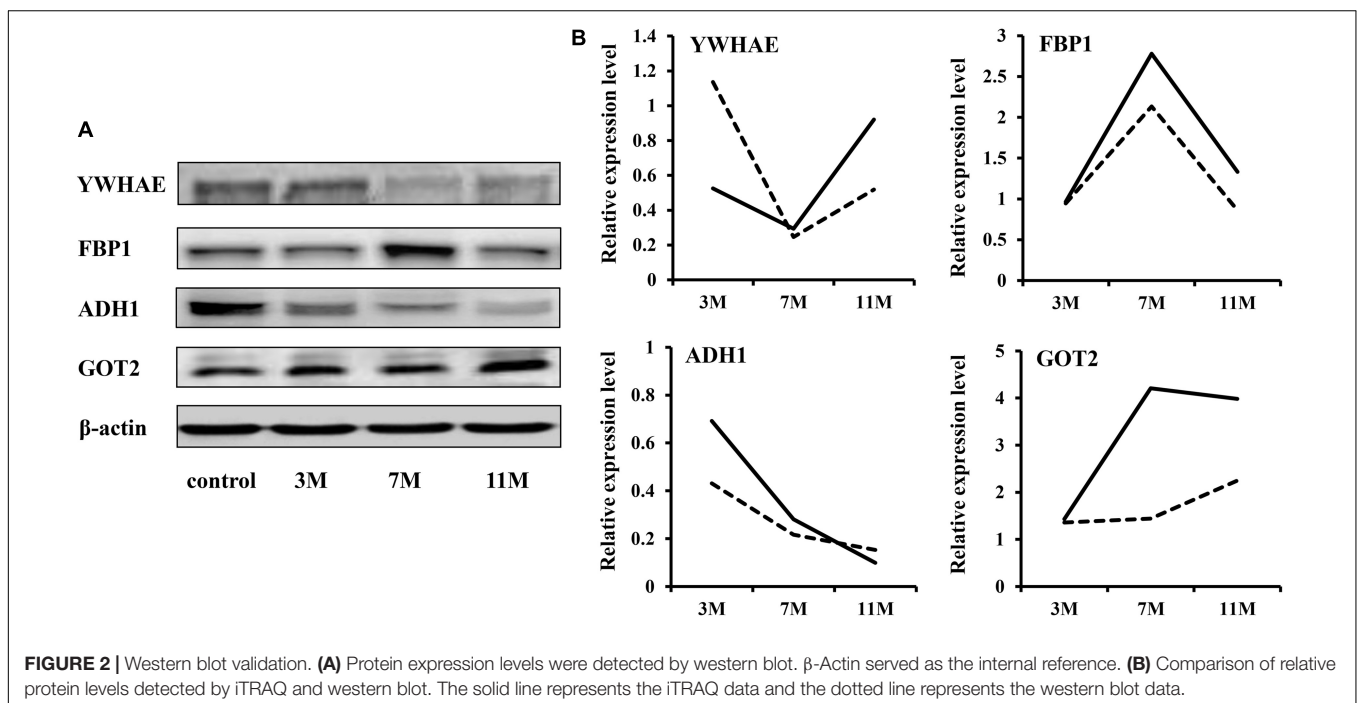
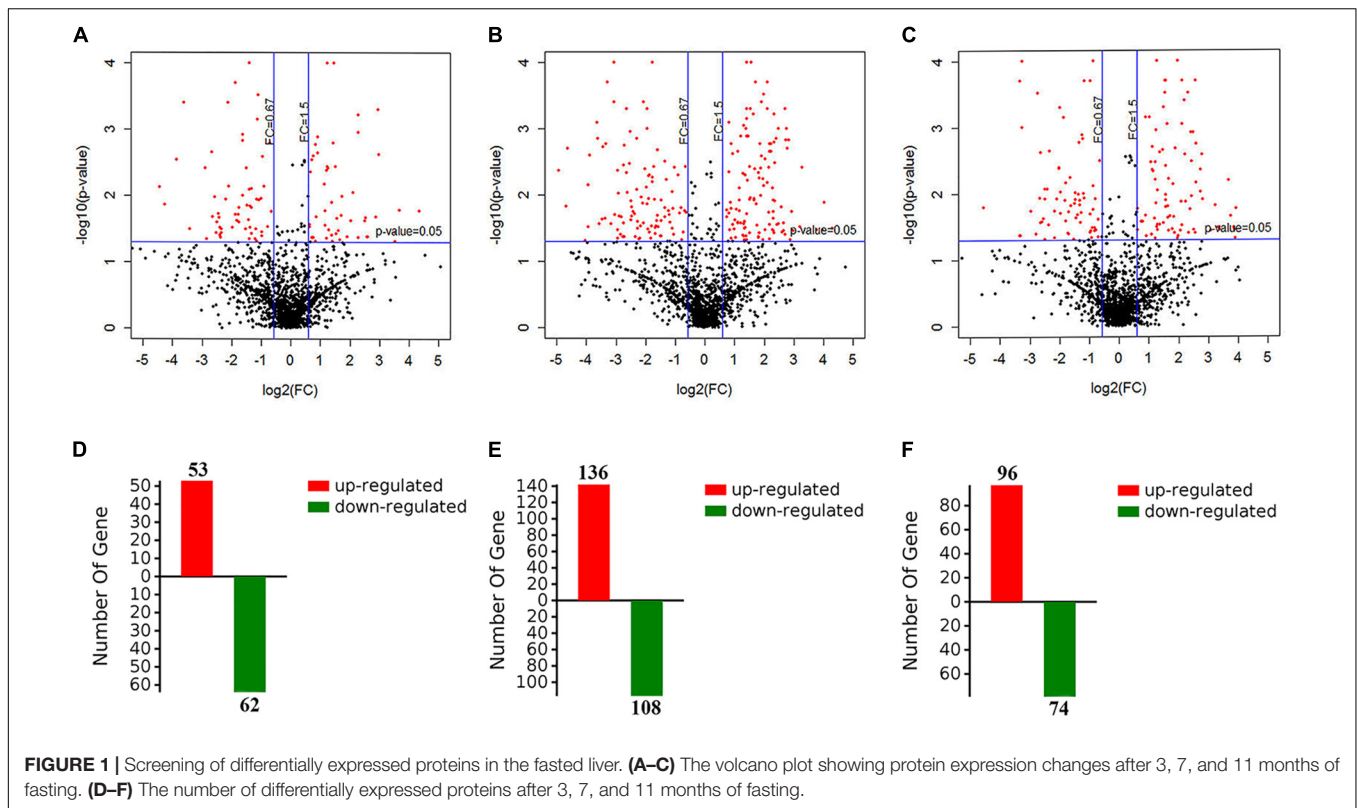
RESULTS

Quantitative Proteomics Analysis Revealed Alterations in Liver Proteins in Fasted *Andrias davidianus*

To evaluate the metabolic response of *A. davidianus* liver to fasting, iTRAQ coupled with LC-MS/MS technology was employed to assess proteome changes in the liver after 0 (control), 3, 7, and 11 months of fasting. A total of 1820 potential proteins were identified based on the screening criteria of unused score > 1.3 and unique peptide ≥ 1 . Based on the screening criteria of fold change > 1.5 and *P*-value < 0.05 , 364 differentially expressed proteins were identified in the fasted liver (**Supplementary Table S1**), and the results are displayed by a volcano plot (**Figure 1**). Compared to control, more proteins had their levels changed after 7 months of fasting than 3 or 11 months of fasting.

Western Blot Analysis

To validate the iTRAQ results, four abundantly expressed proteins were randomly selected and subjected to western blot: 14-3-3 epsilon (YWHAE), fructose-1,6-bisphosphatase 1 (FBP1), alcohol dehydrogenase 1 (ADH1), and aspartate aminotransferase 2 (GOT2). The results showed that ADH1 was significantly down-regulated during fasting; YWHAE was significantly down-regulated after 7 months of fasting; FBP1 was



significantly up-regulated after 7 months of fasting (Figure 2). Up-regulation expression of GOT2 was detected only after 11 months of fasting by western blot, but both after 7 and 11 months of fasting by iTRAQ, maybe due to that there is great

randomness for acquiring fragmentation spectra of a precursor ion in the Triple TOF 5600. Overall, the protein expression trends between western blot and proteomic analysis were basically similar, suggesting that the results of iTRAQ were reliable.

Clustering of Protein Expression Patterns in the Liver of the Fasted *Andrias davidianus*

Dynamic expression patterns of differentially expressed proteins in the liver of *A. davidianus* after 3, 7, and 11 months of fasting were categorized by K-means clustering, and the results showed that 364 differentially expressed proteins were categorized into two clusters (Figure 3A). Cluster 1 contained proteins which showed an increasing trend, and the average expression level of 182 proteins in the liver after 7 and 11 months of fasting was higher than that at 3 months of fasting (Figure 3B). Cluster 2 contained proteins which showed a decreasing trend and the average expression level of 182 proteins in the liver after 7 months of fasting was lower than that at 3 and 11 months of fasting (Figure 3B).

Functional and Pathway Enrichment Analysis of Differentially Expressed Proteins in the Liver of the Fasted *Andrias davidianus*

GO and KEGG pathway enrichment analyses of differentially expressed proteins were carried out by DAVID database. Up-regulated expression proteins in cluster 1 were significantly enriched in fatty acid beta-oxidation, tricarboxylic acid cycle, gluconeogenesis, urea cycle, glycogen catabolic process,

and amino acid metabolism (Table 1). Among them, the expression of glycogen phosphorylase (PYGL), a key enzyme involved in glycogenolysis, was significantly increased after 3 and 7 months of fasting. Fructose 1,6-bisphosphatase (FBP1) and phosphoenolpyruvate carboxykinase (PCK1), key enzymes involved in gluconeogenesis, were significantly up-regulated after 7 and 11 months of fasting. Acyl CoA dehydrogenase (ACAD9, ACADL, and IVD) and 3-hydroxyacyl-CoA dehydrogenase (HADHA and HADHB) that participate in fatty acid beta-oxidation, were significantly up-regulated after 7 and 11 months of fasting. The expression of citrate synthase, a key enzyme involved in tricarboxylic acid cycle, was significantly enhanced after 7 and 11 months of fasting. The expression of hydroxymethylglutaryl coenzyme A synthetase (HMGCS1), a key enzyme involved in ketogenesis, was increased after 11 months of fasting. Aspartate aminotransferases (GOT1 and GOT2) involved in malate-aspartate shuttle, were significantly up-regulated after 7 and 11 months of fasting. The expression of urea cycle enzymes was increased after 7 and 11 months of fasting, such as argininosuccinate synthase (ASS1), ornithine carbamoyltransferase (OTC), and arginase (ARG1). The expression of cytochrome c oxidase (COX4I1), an electron transport chain terminal oxidase, was enhanced after 7 and 11 months of fasting. The expression levels of antioxidant enzymes were increased after 7 and 11 months of fasting, such as superoxide dismutase (SOD1 and SOD2), thioredoxin peroxidase (PRDX3) and glutathione peroxidase (GPX4).

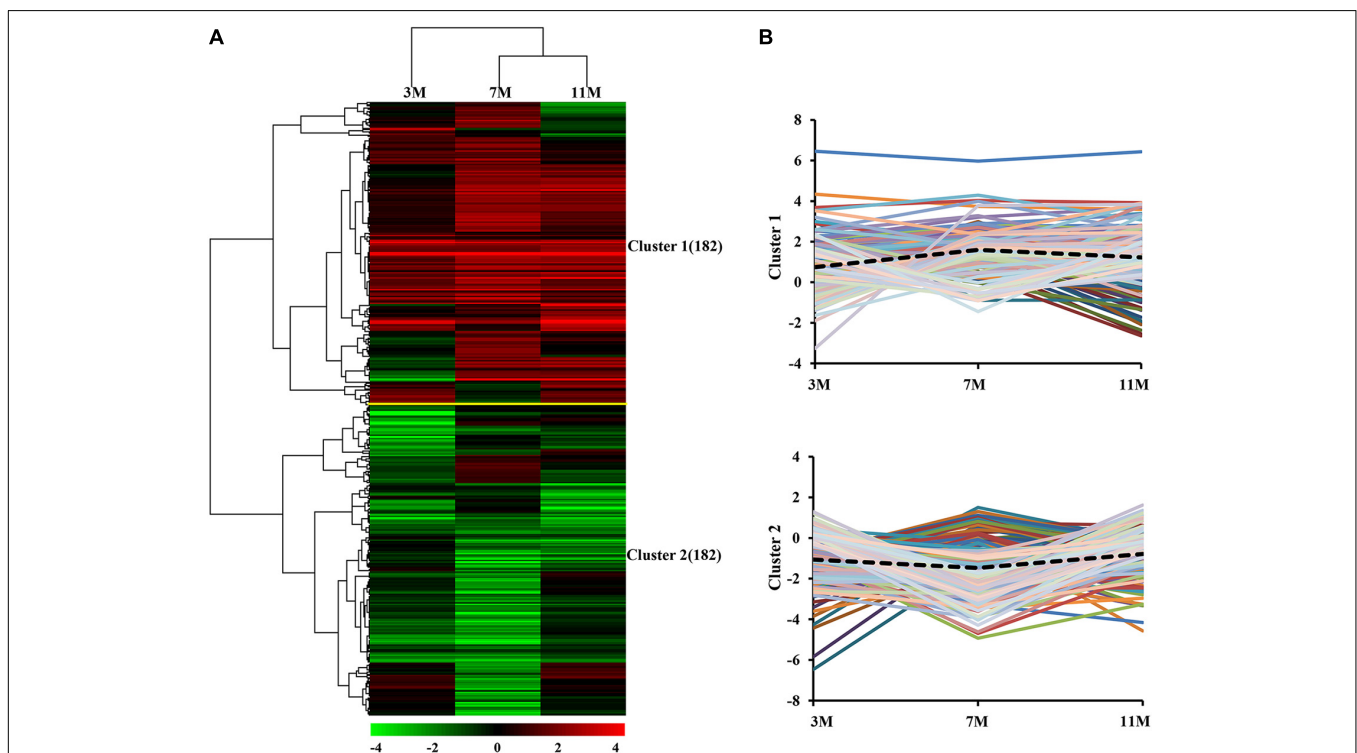


FIGURE 3 | Global comparison of protein expression patterns in the fasted liver. **(A)** Hierarchical clustering of expression patterns of differentially expressed proteins. Red and green colors denote a higher and lower expression level than the control, respectively. **(B)** Average expression patterns in each cluster. Dotted line represents average fold changes of proteins in each cluster.

TABLE 1 | Function enrichment analysis of differentially expressed proteins.

Enriched biological functions	P-value	Genes
Cluster 1		
Fatty acid beta-oxidation	5.88E-04	ACOX1, IVD, ACAD9, ACADL, ACAT1, HADHA, HADHB (7)
Tricarboxylic acid cycle	1.50E-03	ACO2, CS, MDH2, FH, MDH1 (4)
Carbohydrate metabolic process	1.60E-03	GLA, PYGL, NAGA, HEXB, UGDH, MDH2, MDH1 (7)
Lipid homeostasis	2.02E-03	ACOX1, IVD, ACAD9, ACADL (4)
Urea cycle	2.16E-03	ARG1, ASS1, OCT (3)
Gluconeogenesis	1.06E-02	GPI, TPI1, FBP1, PCK1 (4)
Cellular amino acid metabolic process	2.00E-02	GOT2, GOT1, OCT (3)
Glycogen catabolic process	2.43E-02	PYGL, AGL (2)
Cluster 2		
Protein folding	1.50E-06	HSP90AB1, P4HB, HSP90B1, CCT5, TXNDC5, CCT8, PDIA6, CCT2, CCT6A, CALR (10)
Translation	2.78E-04	RPS18, RPL6, RRPB1, RPL13A, SLC25A5, RPL3, SLC25A1, RPL24, RPS2, RPS3 (10)
Response to endoplasmic reticulum stress	4.14E-04	P4HB, EIF2S1, TXNDC5, PDIA6 (4)
Glycolytic process	1.22E-03	ALDOA, ALDOB, PKM, PGK1, ENO1 (5)
Pentose-phosphate shunt	3.28E-03	G6PD, TALDO1, PGD (3)

Down-regulated expression proteins in cluster 2 were significantly enriched in protein folding, translation, response to endoplasmic reticulum stress, glycolytic process, and pentose-phosphate shunt (Table 1). Among them, the expression of pyruvate kinase (PKM), a key enzyme involved in the glycolytic pathway, was significantly decreased after 3 and 11 months fasting. 6-Phosphogluconate dehydrogenase (PGD) and glucose-6-phosphate dehydrogenase (G6PD), the key enzymes of pentose phosphate pathway, were significantly down-regulated after 3 and 7 months of fasting. The decreased expression of translational elongation factor (EEF2), threonine tRNA ligase (TARS), and most ribosomal proteins was found in the fasted *A. davidianus* liver. The expression levels of chaperone proteins were reduced in the fasted *A. davidianus* liver, such as HSP90AB1 and HSP90B1.

To further clarify which signaling pathways played important roles in the liver of *A. davidianus* after fasting, IPA analysis was carried out to connect differentially expressed proteins with canonical pathways. The results of pathway analysis showed that glutathione-mediated detoxification and endoplasmic reticulum stress pathway were significantly enriched after 3 months of fasting; protein ubiquitination pathway and fatty acid oxidation were significantly enriched after 7 months of fasting; while citric acid cycle, gluconeogenesis, and ketogenesis were significantly enriched after 11 months of fasting (Figure 4). Moreover, pentose phosphate pathway was only enriched after 3 and 7 months of fasting, whereas amino acid metabolism (isoleucine degradation I, valine degradation I, and aspartate degradation II) were enriched after 7 and 11 months of fasting (Figure 4).

Activation Analysis of Upstream Regulators and Biofunctions in the Liver of the Fasted *Andrias davidianus*

Upstream Regulator Analysis in IPA is a novel function which can predict which transcriptional regulators are involved and whether they are likely activated or inhibited by analyzing linkage to differentially expressed proteins through coordinated expression.

The results of Upstream Regulator Analysis demonstrated that transcription factors CCAAT-enhanced binding protein α (C/EBP α) and hepatocyte nuclear factor 4 α (HNF4 α) were likely activated after 7 months of fasting, and Krüppel-like factor 5 (KLF15), forkhead box protein FOXO1 and peroxisome proliferator-activated receptor γ coactivator 1 α (PGC-1 α) were likely activated after 11 months of fasting, whereas transcription factors MYCN and E2F1 that participate in protein synthesis were likely inhibited in the fasted liver (Figure 5A). Downstream Effects Analysis in IPA identifies downstream biological functions that are expected to be increased or decreased based on the gene expression changes. The results of Downstream Effects Analysis showed that necrosis and death of liver cells as well as endoplasmic reticulum stress response of cells were likely increased after 3 months of fasting; flux of lipid and synthesis of reactive oxygen species (ROS) were likely increased after 7 months of fasting; transmembrane potential of mitochondrial and synthesis of ATP were likely increased after 11 months of fasting (Figure 5B).

DISCUSSION

Hypogean salamanders are able to survive for several years without food, and they can adjust their metabolism to food deprivation by utilizing metabolites stored during times when food is abundant (Hervant et al., 2001). The metabolic reserves and their utilization vary with species. The metabolic response of animals to fasting is primarily coordinated by the liver. The molecular mechanisms that underlie their survival remain unclear. Although transcriptome analysis of mouse liver (Bauer et al., 2004; Li et al., 2006; Sokolovic et al., 2008) and chicken liver (Desert et al., 2008) in response to fasting has been reported, no investigation has explored the metabolic response of Urodela amphibian liver to prolonged fasting. Therefore, this study examined the expression levels of proteins in the liver of *A. davidianus* after 3, 7, and 11 months of fasting. We found

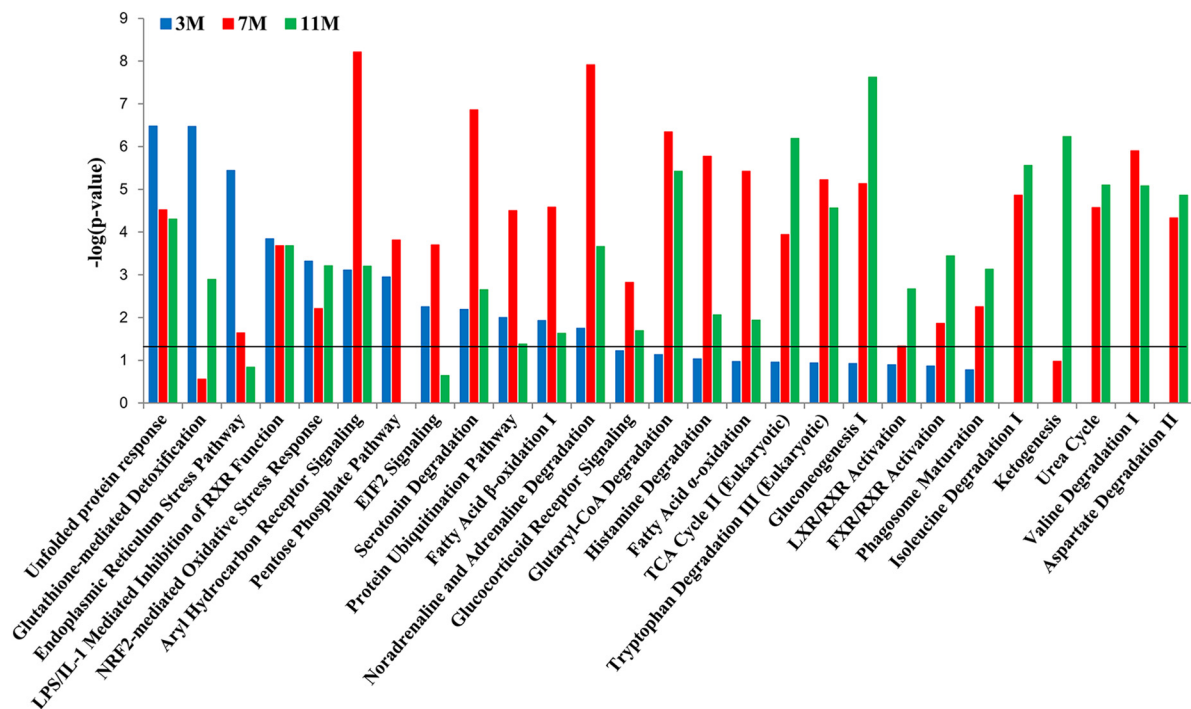


FIGURE 4 | Pathway enrichment analysis of differentially expressed proteins in the fasted liver. Dark line represents the threshold of $P < 0.05$.

that the expression levels of proteins associated with fatty acid β -oxidation, amino acid metabolism, tricarboxylic acid cycle, gluconeogenesis, ketogenesis, and antioxidant system were up-regulated in the fasted *A. davidianus* liver.

Up-Regulation of Liver Gluconeogenesis After Prolonged Fasting

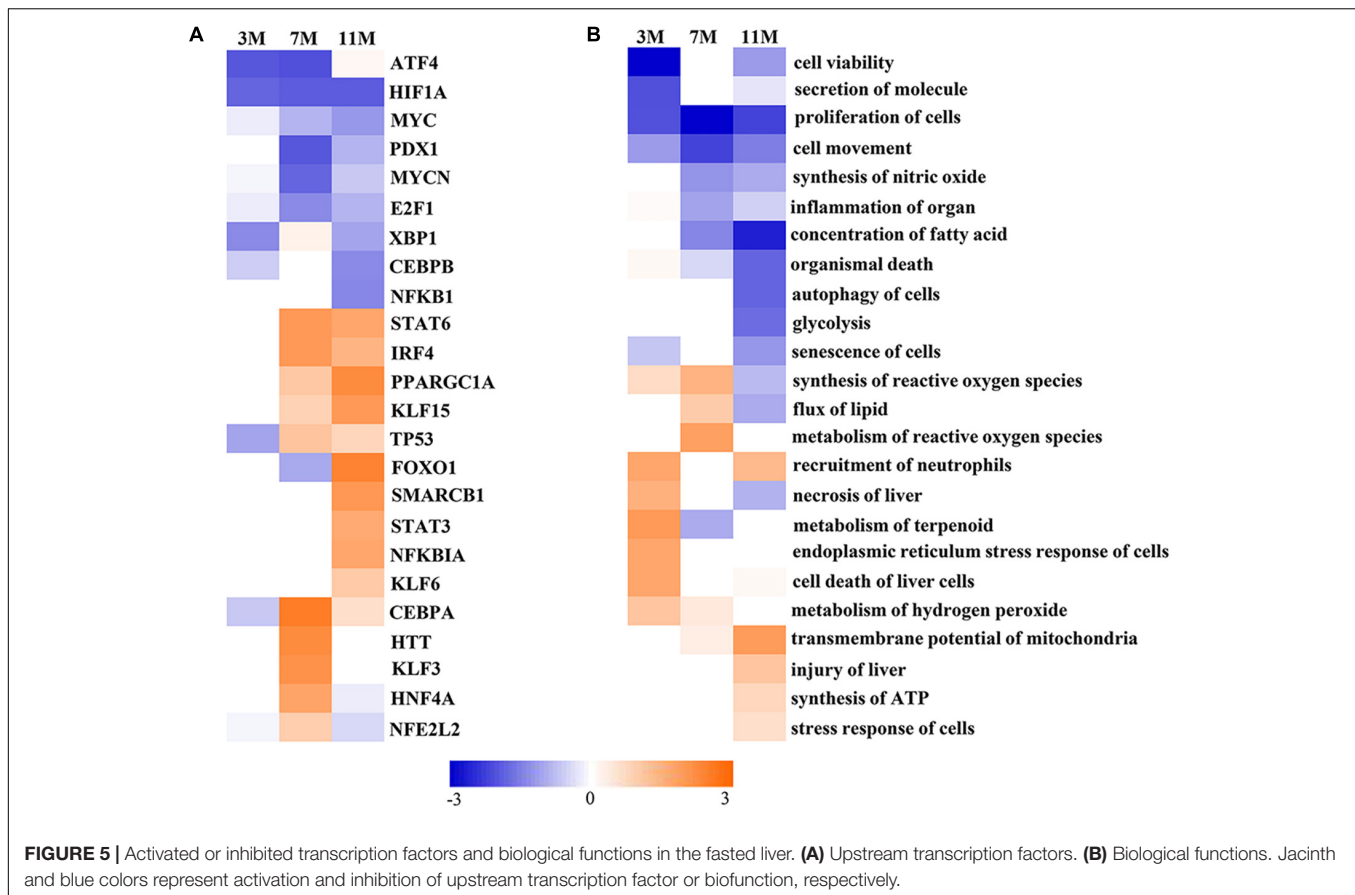
At the early stages of fasting, hepatic glycogen degradation is enough to provide glucose to the extrahepatic tissue, but as the fasting continues, gluconeogenesis becomes more pronounced. The body mainly relies on gluconeogenesis to maintain a relatively constant blood glucose concentration. In this study, the expression levels of key proteins (FBP1 and PCK1) that participate in gluconeogenesis were found to be increased in the liver of *A. davidianus* after prolonged fasting. Transcription factors (FOXO1, HNF4 α , KLF15, and C/EBP α) were likely activated in the liver after prolonged fasting by Upstream Regulator Analysis in IPA. FOXO1 is a key transcription factor that regulates hepatic gluconeogenesis. Under fasting conditions, FOXO1 expression is regulated by the coactivator p300 (Wondisford et al., 2014). FOXO1 synergizes with HNF4 α to activate the expression of the gluconeogenesis rate-limiting enzyme G6Pase (Hirota et al., 2008; Dankel et al., 2010; Jitrapakdee, 2012). During fasting, KLF15 not only promotes gluconeogenesis by inducing expression of gluconeogenesis-related genes (Teshigawara et al., 2005; Takashima et al., 2010), but also induces alanine aminotransferase gene expression, thereby increasing amino acid-derived gluconeogenesis precursors supply, and hence

gluconeogenesis (Gray et al., 2007). Under fasting conditions, C/EBP α induces the expression of the pyruvate carboxylase gene and promotes gluconeogenesis (Louet et al., 2010). These results indicate that liver gluconeogenesis was enhanced to maintain blood glucose levels of *A. davidianus* after prolonged fasting.

Studies have reported that the activity of key enzymes of glycolysis and lipogenesis was reduced in the *Sparus aurata* liver after 22 days of fasting (Meton et al., 1999). This study also found that the expression levels of key proteins (PKM, PGD, and G6PD) that modulate glycolysis and pentose phosphate pathway were decreased in the liver of *A. davidianus* after 3 and 7 months of fasting, revealing the decrease of glycolysis and pentose phosphate pathway in the fasted *A. davidianus*. The pentose phosphate pathway provides a variety of materials for biosynthetic metabolism, such as NADPH for fatty acid and cholesterol synthesis. These results indicate that biosynthesis was inhibited to save energy for the *A. davidianus* after prolonged fasting.

Fatty Acid β -Oxidation and Ketogenesis Are Up-Regulated During Prolonged Fasting

Fasting inhibits lipogenesis and promotes fat mobilization, resulting in β -oxidation of fatty acids to provide energy for the body (Goldstein and Hager, 2015). This study found that the expression levels of key proteins that regulate fatty acid β -oxidation, tricarboxylic acid cycle, ketogenesis, and electron transport chain, were enhanced after 7 and 11 months of fasting. Downstream Effects Analysis by IPA predicted that the



function of fatty acid oxidation and transmembrane potential of mitochondrial was likely enhanced after 7 and 11 months of fasting while fatty acid synthesis was likely inhibited after 11 months of fasting. These results confirm that the liver synthesizes large amount of ketone bodies from acetyl-CoA through β -oxidation of fatty acids to fuel extrahepatic tissues during prolonged fasting (Cahill, 2006; Newman and Verdin, 2014). In addition, transcription factor PPAR α was likely activated in the liver of *A. davidianus* after prolonged fasting by Upstream Regulator Analysis in IPA. The activated PPAR α by fasting promoted the expression of fatty acid oxidation and ketogenesis-related genes in synergy with other fasting-related transcription factors, such as CREB3L3 (Nakagawa et al., 2016), p300 (Zhang et al., 2016), and PGC-1 α (Rodgers and Puigserver, 2007). These results indicate that the enhanced fatty acid oxidation is needed to provide energy for the *A. davidianus* after prolonged fasting and can also explain why *A. davidianus* can survive for long periods of food deprivation.

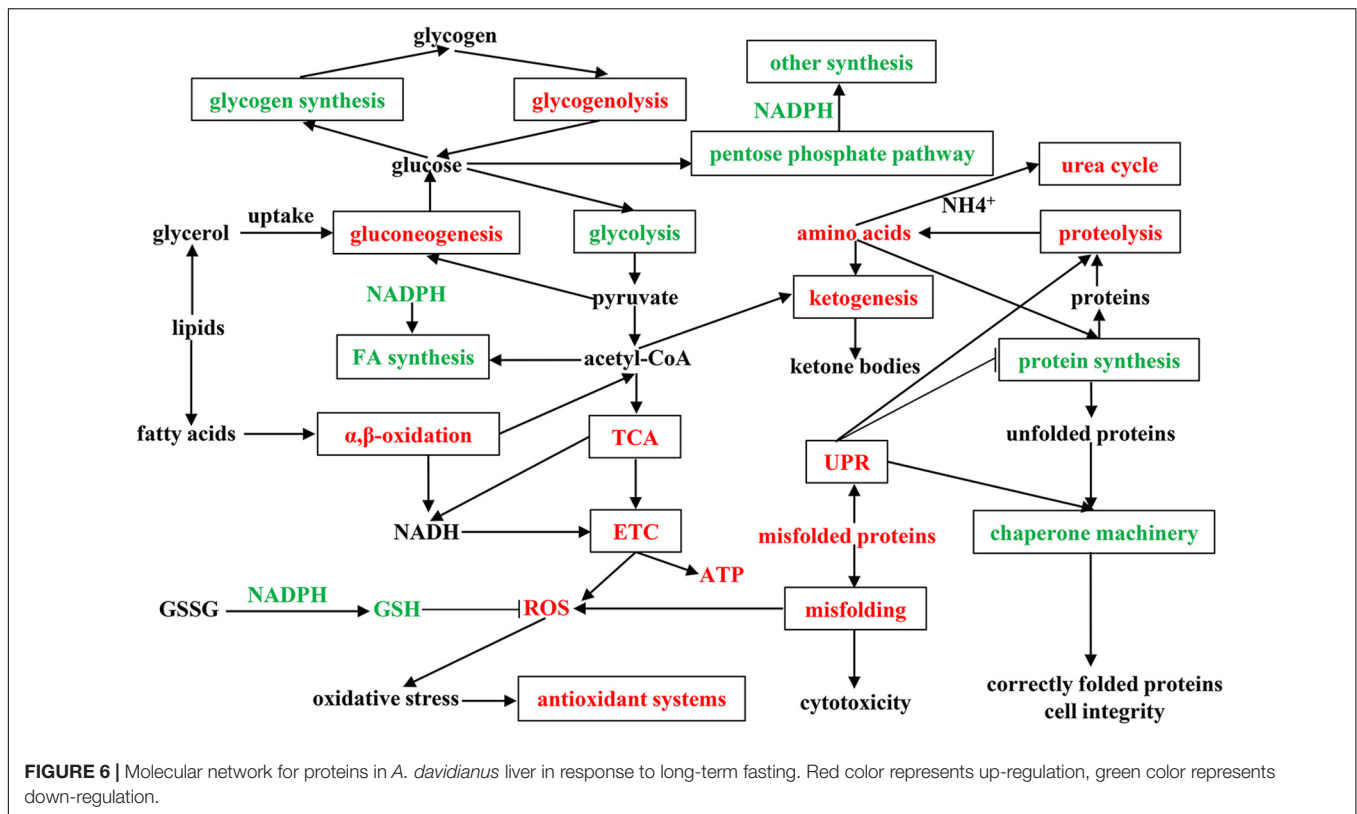
Prolonged Fasting Is Accompanied by Increase of Proteolysis and Inhibition of Protein Synthesis

In the situation of limited energy supply, the organisms adapt by adjusting the rate of protein synthesis and degradation. A study showed that protein synthesis in the endoplasmic

reticulum was significantly decreased and proteolysis was significantly increased in the liver of yellow croaker after 21 days of fasting (Qian et al., 2016). The increased expression of aspartate aminotransferase that participates in malate-aspartate shuttle in the fasted *A. davidianus* liver indicates that prolonged fasting induces tissue protein degradation, thus promoting gluconeogenesis of glucogenic amino acids. However, the reduced expression of ribosomal proteins, EEF2, MYCN, and E2F1 in the fasted *A. davidianus* liver may be involved in decreased protein synthesis function. Overall, prolonged fasting is accompanied by the increase of proteolysis and inhibition of protein synthesis in the *A. davidianus* liver.

Under stress conditions, molecular chaperones prevent protein aggregation and promote refolding of denatured proteins (Young et al., 2004). The expression levels of chaperone proteins were found to be reduced in the fasted *A. davidianus* liver, indicating that the decreased protein folding will help the organism to save energy under fasting conditions. Once protein folding is inhibited, the amount of misfolded proteins increases, resulting in ROS synthesis and unfolded protein response (UPR) (Goldberg, 2003). Collectively, it can be concluded that fasting inhibits protein folding in the *A. davidianus* liver, thereby inducing UPR.

During prolonged fasting, a large amount of NH_4^+ is produced by combined deamination in the liver, thereby



generating urea through ornithine circulation. The up-regulation expression of vital proteins modulating urea cycle and arginine synthesis in the fasted *A. davidianus* liver indicates that amino acid oxidation and urea cycle were enhanced after prolonged fasting. Taken together, this study first reports that fasting induces amino acid oxidation and urea cycle in the amphibian liver.

Prolonged Fasting Up-Regulates ROS Production and Antioxidant System

Oxidative stress can arise from overproduction of ROS by fatty acid β -oxidation and is defined as an imbalance between ROS production and antioxidant defense system. During fasting, many animals enhance their antioxidant defenses to cope with oxidative stress (Moreira et al., 2016). For example, lipid peroxidation and oxidative stress were strengthened in the liver of 72 h-fasted rats (Sorensen et al., 2006); the expression levels of antioxidant enzymes were up-regulated in the liver of 24 h-fasted mice (Sokolovic et al., 2008); antioxidant defense mechanism was activated in the liver of fish after 5 weeks of fasting (Morales et al., 2004). We also found that the expression levels of antioxidant enzymes were up-regulated in the liver of *A. davidianus* after prolonged fasting, due to that antioxidant defense system must minimize the levels of most harmful ROS. From these results, it can be concluded that the antioxidant defense system was enhanced to reduce oxidative damage caused by ROS overproduction, thus improving the tolerance of *A. davidianus* to prolonged fasting.

CONCLUSION

To fully understand the physiological and metabolic response of *A. davidianus* liver to prolonged fasting, a molecular network was established according to our results (Figure 6). The expression changes of proteins in the *A. davidianus* liver during long-term fasting was stage-dependent and closely related to fuel distribution. The expression levels of proteins involved in fatty acid β -oxidation, protein decomposition, tricarboxylic acid cycle, gluconeogenesis, ketogenesis, and urea cycle were increased after 7 and 11 months of fasting, while the expression levels of proteins involved in protein synthesis were inhibited. The enhanced fatty acid β -oxidation produced a large amount of ROS during fasting, and the organisms must up-regulate antioxidant defense system to reduce oxidative damage caused by ROS overproduction, thus improving the tolerance of *A. davidianus* to prolonged fasting. These results confirm the central role of lipid metabolism in *A. davidianus* liver in the response to fasting.

LIMITATIONS OF THE STUDY

One potential limitation of our study is that it was carried out in the liver, and further studies should be done in the small intestine and stomach which also play key roles during fasting in the future. Another potential limitation is that the study is lack of “refeeding group” and is out of consideration of the influence of aging in the experimental design. Further studies are needed to verify these results in the future.

DATA AVAILABILITY STATEMENT

The proteomic data generated in this study can be found in the Proteomics Identifications Database (accession number: PXD014924).

ETHICS STATEMENT

The animal handling and sampling procedures were performed in strict accordance with the guidelines of the Animal Care and Use Committee of Henan Normal University and were approved by the Animal Care and Ethics Committee of Henan Normal University.

AUTHOR CONTRIBUTIONS

CX conceived the project. XG carried out most of the experiments. JG and XZ conducted sample

preparation. LZ and JS assisted in data analysis. ZQ assisted in animal feeding. XG and CX wrote the manuscript. All authors approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fphys.2020.00208/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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Expression and Regulation of *pde6h* by Thyroid Hormone During Metamorphosis in *Paralichthys olivaceus*

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PDE6H is a cone cell-specific inhibitory subunit that plays a critical role in the adaptation of the photosensitive system to bright and dark phases of the light environment. Thyroid hormone (TH) is one of the most important factors that control development and metabolism in animals, composed mainly of triiodothyronine (T3), and thyroxine (T4). TH also plays a key role in the metamorphosis of the flounder (*Paralichthys olivaceus*), wherein exogenous TH can accelerate the behavioral changes of larvae from the pelagic to benthic type accompanying changes in the light environment from bright to dark. In this study, transcriptional analysis showed that *pde6h* is expressed in adult eye, that its expression peaks at the climax of metamorphosis, and that it can be significantly up-regulated to the highest level by exogenous T4 in the early stages of metamorphosis but is inhibited by thiourea (TU). The rescue experiment showed that metamorphic inhibition of larvae and expression inhibition of *pde6h* gene in TU groups can be rescued by removing TU. Further, dual-luciferase reporter assay indicated the putative regulatory effect of TH on *pde6h* expression, mediated directly on the gene promoter by the TR α A gene. Together, we speculated that TH may control physiological adaptation of the photosensitive system to light changes during metamorphosis by acting directly on *pde6h*. This study can help us further study the physiological function of *pde6h* during flounder metamorphosis in the future.

Keywords: *Paralichthys olivaceus*, metamorphosis, thyroid hormone, *pde6h*, dual-luciferase

INTRODUCTION

Phosphodiesterases 6 is a photoreceptor cell-specific subfamily of phosphodiesterases (PDEs), consisting of α , β , and α' catalytic subunits encoded by the *PDE6A*, *PDE6B*, and *PDE6C* genes, and γ and γ' inhibitory subunits encoded by the *PDE6G* and *PDE6H* genes, respectively (Cote, 2004; Conti and Beavo, 2007). PDE6s are distinctively expressed in vertebrate rod and cone photoreceptor

cells. Rods express the *PDE6A* and *PDE6B* genes, which form a catalytic heterodimer, and the *PDE6G* inhibitory subunit gene, whereas cones express *PDE6C*, which forms a catalytic homodimer, and the *PDE6H* inhibitory subunit gene (Cote, 2004; Larhammar et al., 2009; Lagman et al., 2016). The PDE6 enzymes include two catalytic subunit proteins, which have two GAF domains and one catalytic domain, and two accessory inhibitory subunits (Guo et al., 2006; Conti and Beavo, 2007). Under dark conditions, the accessory inhibitory subunits interact with a GAF domain and the catalytic domain of the catalytic subunits and thus block PDE6 activity (Guo et al., 2006). In contrast, under light conditions, photon-activated opsins promote a GTP molecule to replace GDP at the active site of the α subunit of the heterotrimeric G-protein transducin, thereby resulting in the dissociation of transducin into an activated α subunit and a heterodimer of β and γ subunits (Lagman et al., 2016). The α subunit then activates PDE6, which hydrolyzes cGMP into GMP. The reduction in cGMP levels leads to the closure of cyclic nucleotide-gated channels and results in hyperpolarization of the photoreceptor cell (Arshavsky et al., 2002; Zhang et al., 2012; Lagman et al., 2016). Despite the cloning of cone cell-specific inhibitory subunit *PDE6H* more than 20 years ago, its regulation in vertebrate development remains unknown.

Flounder (*Paralichthys olivaceus*), an important marine fish, undergoes a dramatic metamorphosis from larval to juvenile stage (Inui and Miwa, 1985). The metamorphosis is accompanied by drastic morphological, physiological, and behavioral changes. Particularly, the right eye moves to the left and the lifestyle changes from pelagic to benthic, which may be closely related to the development of a retinal photosensitive system. It is known that exogenous thyroid hormone (TH) can accelerate the metamorphic process of *P. olivaceus*, while thiourea (TU), a TH synthesis inhibitor, blocks metamorphosis (Schreiber and Specker, 1998). TH includes two main hormones, namely triiodothyronine (T3) and thyroxine (T4). T4 is a pro-hormone, whereas T3 is the hormone that binds to thyroid hormone receptor (TR) *in vivo* (Harvey and Williams, 2002; Carvalho and Dupuy, 2017). TRs are nuclear receptors comprising two main classes, alpha, and beta (Flamant et al., 2006). TRs act as transcription factors, ultimately affecting the regulation of gene expression by binding to T3 (Flamant et al., 2006; Ortega-Carvalho et al., 2014).

Although some studies in recent years have shown that TH regulates cone photoreceptor differentiation in the retinas (Kelley et al., 1995; Applebury et al., 2000; Ng et al., 2001; Mader and Cameron, 2006; Roberts et al., 2006; Srinivas et al., 2006; Trimarchi et al., 2008), there is surprisingly little known about the relationship between TH and visual signal transduction in vertebrate development. In this study, we analyzed the expression of *pde6h* gene during metamorphosis and in adult tissues and identified the regulatory relationship between TH and *pde6h* in *P. olivaceus*. The results showed that *pde6h* was mainly expressed in adult eye, was highly expressed at the metamorphic climax, and can be directly regulated by T3 binding to TR α A. This study will fulfill a need to address the currently deficient understanding of the expression and regulation of *pde6h* in the flounder and its roles in development.

MATERIALS AND METHODS

Ethics Statement

Our study was performed in strict accordance with Laboratory Animals—Guidelines for ethical review of animal welfare of China (GB/T 35892-2018). All experimental procedures were approved by the Animal Ethics Committee of Shanghai Ocean University (SHOU-DW-2017-039).

Fish Samples

Flounder (*P. olivaceus*) were collected from the Beidaihe Central Experiment Station (Chinese Academy of Fishery Sciences, Hebei Province, China). Larvae at 15 days post-hatching (dph) were randomly divided into three groups (2000 fish per tank): the NC group (normal control) was cultured with natural seawater, the TH group was exposed to seawater with 130 nM of exogenous T4 (Sangon, Shanghai), and the TU group was exposed to seawater containing 30 mg/L of exogenous TU (thiourea; Sangon, Shanghai) (Inui and Miwa, 1985). These three groups of larvae were cultured with *Artemia nauplii* from 15 dph till the end of the experiment. According to Minami (1982) (Minami, 1982), whole larvae ($n = 6$ pools in each group, 3 specimens/pool) from NC, TH, and TU groups were periodically collected at 16 dph (Early metamorphosis I, the stage prior to the start of eye migration), 21 dph (Early metamorphosis II, when the right eye has started to shift and six coronal fins begin to elongate), 25 dph (Metaphase metamorphosis I, when the right eye has become visible from the ocular side but has not reached the dorsal midline), 28 dph (Mid-metamorphosis II, climax metamorphosis, when the right eye has become visible from the ocular side and reached the dorsal midline and coronal fins assume the greatest length), 31 dph (Late metamorphosis I, the right eye has just become located on the overhead and starts to move to the left side of the body and coronal fins are significantly shortened), 36 dph (Late metamorphosis II, the right eye is on the left side of the body, coronal fins still have remnants, and body surface melanin increases), and 41 dph (juvenile, the right eye has completely moved to the left side of the fish body, the coronal fins disappear, and the pigment is well developed). All larvae and juveniles were anesthetized with MS-222 (3-Aminobenzoic acid ethyl ester methanesulfonate, Sigma-Aldrich) for observation under a microscope, washed with DEPC water, and stored with RNAlater (Invitrogen, Life Technology, Carlsbad, CA, United States) at -80°C .

Paralichthys olivaceus adults were firstly anesthetized with MS-222 and were killed by decapitation. Tissues containing the heart, liver, stomach, kidney, brain, gill, muscle, eye, and intestine were then collected by dissecting six fish ($n = 6$) and directly frozen in liquid nitrogen. All samples were stored at -80°C for RNA extraction until subsequent RNA isolation.

Rescue Experiment

To investigate whether the metamorphosis-inhibited larvae in the TU-treated group can be rescued, TU-treated larvae at 35 dph were divided into three groups: the TU group (TU-treated larvae cultured in seawater containing 30 mg/L TU),

the TU + NC group (TU-treated larvae cultured in natural seawater), and the TU + TH group (TU-treated larvae cultured in seawater containing 130 nM T4). These three groups of larvae were cultured until 41 dph. Larvae ($n = 6$ pools in each group, 3 specimens/pool) at 36 dph and 41 dph were anesthetized with MS-222, placed in RNAlater, and stored at -80°C for RNA extraction.

Quantitative Real-Time PCR

Total RNAs were isolated from the whole body of the collected fish samples using TRIzol Reagent (Invitrogen, Life Technology, Carlsbad, CA, United States) according to the manufacturer's instructions. Total RNAs were treated with RQ1 RNase-free DNase (Promega, Madison, WI, United States) to remove genomic DNA contamination. RNA integrity was assessed by agarose gel electrophoresis, RNA concentration was examined by NANODROP 2000C spectrophotometer (Thermo, Waltham, MA, United States), and $2.0 > A_{260}/A_{280}$ ratios > 1.8 was considered for RNA purity.

Total RNAs were reverse-transcribed using a RT-PCR kit (Promega, Madison, WI, United States) according to the manufacturer's instructions. The qRT-PCR (quantitative real-time PCR) primers (Table 1) were designed using Primer Premier 5.0 software for the *pde6h* gene sequence (GenBank: XM_020083982.1). qRT-PCR was conducted using PowerUpTM SYBRTM Green Master Mix on a CFX96TouchTM Real-Time PCR Detection System (Bio-Rad, United States). The total reaction volume was set to 20 μL , comprising 10 μL PowerUpTM SYBRTM Green Master Mix (Applied BiosystemsTM, United States), 1 μL cDNA template, forward primer (0.2 μM), and reverse primer (0.2 μM) (Table 1). The PCR conditions used are as follows: initial denaturation for 3.0 min at 94°C , followed by 40 cycles at 94°C for 20 s, and at 60°C for 30 s. Each experiment was repeated twice. Samples were run in parallel with the reference gene β -actin. All melting curves were plotted to confirm amplification specificity, and the corresponding efficiencies (E) of qRT-PCR were found to be 0.90–0.99. The relative mRNA expression was determined using the $2^{-\Delta\Delta\text{CT}}$ method (Schefe, 2006).

Cell Culture

Flounder embryonic cells (FECs), obtained from the Yellow Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences, were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, CA, United States) supplemented with 20 mM HEPES, pH 7.5, antibiotics (100 U/mL penicillin and 100 $\mu\text{g}/\text{mL}$ streptomycin; Gibco BRL), 15% fetal bovine serum (Gibco), sea perch serum (SPS, 0.5%), and basic fibroblast growth factor (bFGF, 2 ng/mL; Gibco BRL). Cells were maintained at 24°C in an ambient air incubator according to Chen's method (Chen et al., 2004).

Dual-Luciferase Assay

A ~ 2300 bp sequence upstream to the *pde6h* start codon was selected from NCBI GenBank (GenBank: XM_020083982.1) and used to predict the binding site of TRs according to the known sequences of thyroid-hormone responsive elements [TREs: 5'-(A/G)GGT(C/A/G)A-3'], which bind to TRs (Ortiga-Carvalho

TABLE 1 | Primers used in the study.

Primer	Primer sequence (5' $\rightarrow$ 3')	Application
TR α A1252-F	GGAATTCAATGGAGCCAATGTCCAACAA	TR α A CDS
TR α A1252-R	GGGGTACCTCAGACTTCTGCTGCTCG	
Pro- <i>pde6h</i> -F	GGGGTACCGATTCCACCATTATCCGTCA	<i>pde6h</i> promotor
Pro- <i>pde6h</i> -R	GGGAGCTCCACTGCGCTGTTCCGTA	
q- <i>pde6h</i> -F	AGTAAGGCACCTAAACCA	qRT-PCR
q- <i>pde6h</i> -R	AGGAATACATGAGCGACTA	
β -actin F	GGAAATCGTGCCTGACATTAAG	qRT-PCR
β -actin R	CCTCTGGACAACGGAACTCTCT	

F and R, forward and reverse primers, respectively; qRT-PCR, quantitative real-time PCR. The underline represents restriction enzyme sites.

et al., 2014). The promoter sequence of *pde6h* was PCR-amplified from *P. olivaceus*, and its mutant sequence was synthesized by a biotechnology company (Sangon Biotech, Shanghai, China). They were cloned into pGL3 basic vector to serve as the reporter, respectively, and pRL-TK served as the control reporter. The full-length coding sequence of TR α A was PCR-amplified from *P. olivaceus* and cloned into p3XFLAG under the CMV promoter to serve as an effector, and triiodothyronine (T3) served as the other effector. The recombinant plasmids and T3 (75 nM) were transfected into FECs in the following six groups: pGL3 basic, Propde6h, Propde6h + T3, Propde6h + CMV: TR α A, Mutant + CMV: TR α A + T3 and Propde6h + CMV: TR α A + T3. After transfection for 24 h, the firefly luciferase (LUC) activity was measured with a dual-luciferase reporter assay system (Promega, United States), wherein the Renilla luciferase (REN) activity served as the internal control to evaluate the transfection efficiency. All cell culture experiments were carried out in triplicate. The primers used are listed in Table 1.

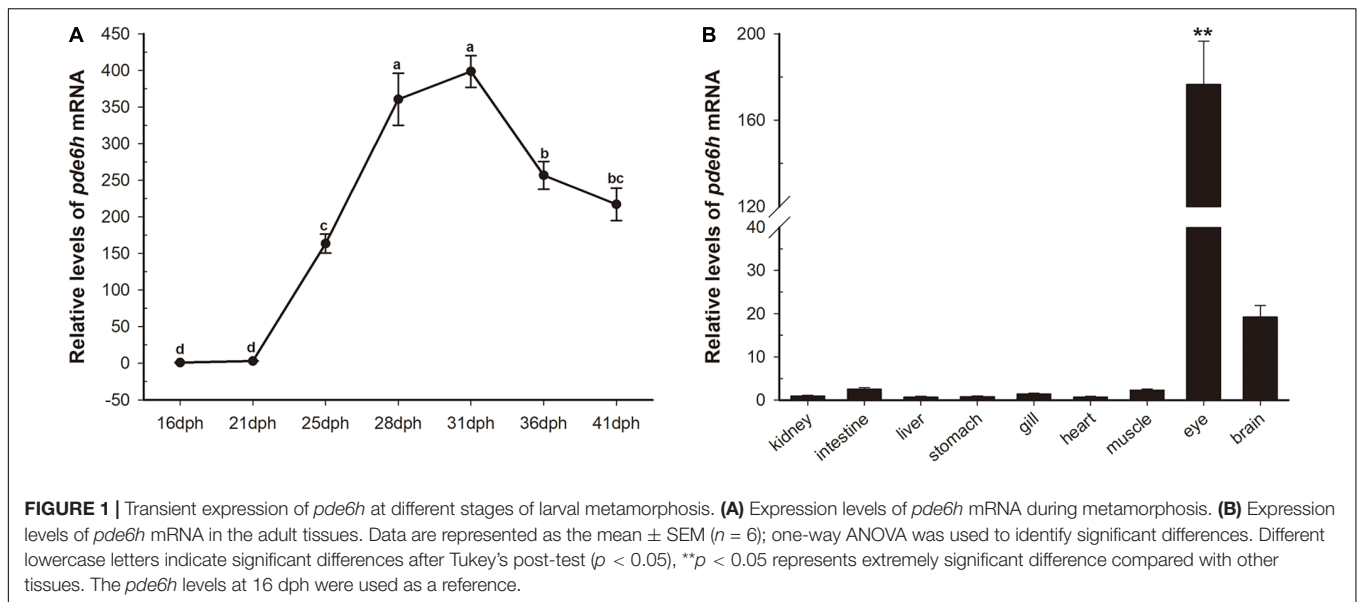
Statistical Analysis

All of the data on *pde6h* expression are represented as mean $\pm$ Standard Error (SEM, $n = 6$). Statistical significance was examined using one- or two-way analysis of variance (ANOVA), followed by Tukey's post-test using SigmaStat 3.5 software. P -values < 0.05 were considered to depict significant differences.

RESULTS

Temporal Expression of *pde6h* mRNA During Metamorphosis and in Adult Tissues

During metamorphosis, *pde6h* levels at 16 dph were used as a reference. As shown in Figure 1A, *pde6h* expression tends to increase and then decrease during metamorphosis and peaks at 31 dph. Specifically, its level rises slowly from 16 dph to 21 dph, increases sharply from 21 dph to 31 dph, and peaks at 31 dph. This is followed by a decrease from 31 dph to 36 dph and a gradual decline from 36 dph to 41 dph. These results show that high expression of *pde6h* gene is synchronous with the peak of metamorphosis.



In adult tissues, *pde6h* levels in the kidney were used as a reference. As shown in **Figure 1B**, *pde6h* gene was extremely highly expressed in the eye compared to other tissues, indicating that it may be an eye-specific gene in the flounder.

Effect of TH and TU on *pde6h* mRNA

As shown in **Figure 2**, exogenous TH and TU significantly affect the expression levels of *pde6h* mRNA. At 21 dph and 25 dph, the level of *pde6h* in the TH group larvae was significantly higher than that of the NC group ($p < 0.05$), while at other time points, there was no significant difference in the *pde6h* levels in the two groups. During metamorphosis, *pde6h* was significantly under-expressed in the TU group larvae compared with in the NC group from 16 dph to 41 dph ($p < 0.05$) and continues to remain at a very low level. These results show that expression of *pde6h* in the three groups of larvae correlates with the presence of exogenous TH regulating larval metamorphosis.

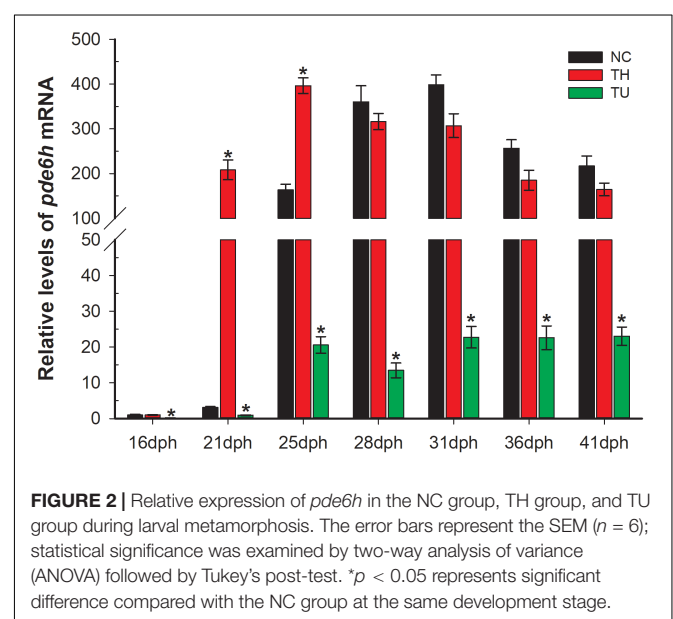
pde6h Expression in the Rescue Experiment of the Metamorphosis-Inhibited Larvae

In **Figure 3A**, the metamorphosis-inhibited larvae at 35 dph that were shifted into natural seawater (TU + NC group) and TH seawater (130 nM T₄, TU + TH group) and reared for 6 days were successfully rescued at 41 dph. However, the larvae maintained in TU until 41 dph were still found to have impaired metamorphosis. The metamorphosis-completed flounders in the 41 dph NC and 41 dph TH served as control. Alongside, the *pde6h* expression in the different larval groups was detected using qRT-PCR. As shown in **Figure 3B**, the *pde6h* level in the 41 dph TU + NC and 41 dph TU + TH groups was significantly higher than that of the 35 dph TU ($p < 0.05$) or 41 dph TU ($p < 0.05$) groups, and no significant difference was observed when compared with the 41 dph NC and 41 dph TH groups. The

above results indicate that TH might play a key role in regulating *pde6h* expression during metamorphosis.

Regulation of the *pde6h* Promoter by T3

Analysis of *pde6h* promoter showed the presence of six potential TREs, and 2180 bp of promoter sequence and 712 bp of mutant sequence of the promoter were cloned for dual-luciferase assay (**Figure 4A**). Dual-luciferase assay was carried out to verify the effect of TR α A and T3 on *pde6h* promoter activity. Our results show that LUC/REN in the Prop*pde6h* + CMV:Tr α A + T3 group was significantly higher than that in the pGL3-basic ($p < 0.05$), Prop*pde6h* ($p < 0.05$), Prop*pde6h* + T3 ($p < 0.05$), Prop*pde6h* + CMV:Tr α A ($p < 0.05$), and Mutant + CMV:



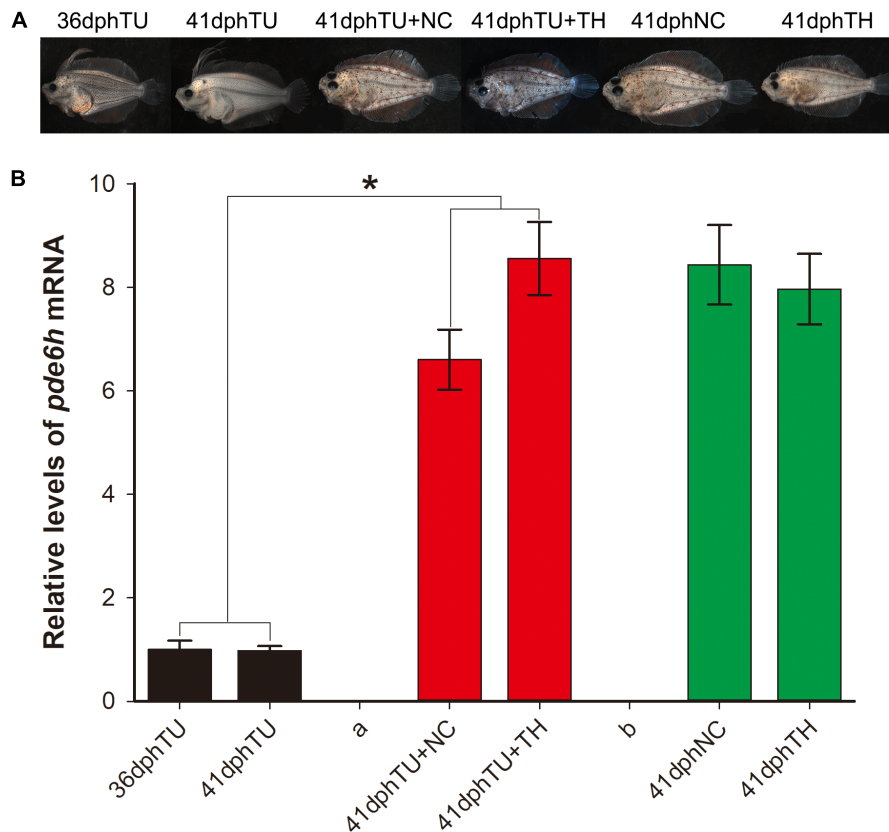


FIGURE 3 | Expression analysis of *pde6h* in the rescue experiment of the TU-inhibited metamorphosis larvae. **(A)** Larvae or juveniles in the rescue experiment of the TU-inhibited metamorphosis larvae. **(B)** Relative levels of *pde6h* upon rescue of the TU-inhibited metamorphosis larvae. The error bars represent the SEM ($n = 6$); statistical significance was detected by two-way analysis of variance (ANOVA) followed by Tukey's post-test. * $p < 0.05$ represents significant differences compared to the 36 dph TU group and 41 dph TU group.

TR α A + T3 ($p < 0.05$) group and that there are no significant difference between the pGL3-basic, Propde6h, Propde6h + T3, Propde6h + CMV:TR α A, and Mutant + CMV: TR α A + T3 groups (**Figure 4B**). These results indicate that *pde6h* promoter is responsive to both TR α A and T3.

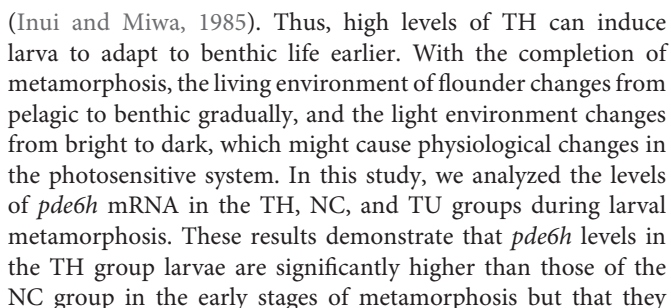
DISCUSSION

During *P. olivaceus* metamorphosis, their lifestyle changes gradually from pelagic to benthic, thereby changing the light environment gradually from brightness to dark with the increasing water depth. As the light environment changes, the visual system of the flounder may undergo adaptive physiological alterations. *PDE6H*, a cone cell-specific inhibitory subunit gene, belongs to the photoreceptor cell-specific PDE6 subfamily (Cote, 2004; Conti and Beavo, 2007). In terrestrial mammals, it can bind to the two domains of PDE6 enzyme, which are a GAF domain and the catalytic domain of the catalytic subunits, and block the activity of PDE6 enzyme in dark conditions (Guo et al., 2006). In light conditions, PDE6 enzyme can be activated in cascade by photon and opsins. Further, the activated PDE6 can effectuate the closure of cyclic nucleotide-gated channels

and mediate hyperpolarization of the photoreceptor cell by hydrolyzing cGMP into GMP, finally allowing the light signal to be transmitted (Arshavsky et al., 2002; Zhang et al., 2012; Lagman et al., 2016). In the flounder, the function of *pde6h* gene has not been reported so far.

In this study, we analyzed the expression and regulation of *pde6h* gene by exogenous TH signaling during metamorphosis. The high expression of *pde6h* at the peak of metamorphosis (**Figure 1**) indicated that it plays an important role in the regulation of flounder metamorphosis. Furthermore, the tissue distribution of *pde6h* gene showed that it is mainly expressed in the eye and hence that it may be an eye-specific gene. By referring to the function of *pde6h* in mammals, we speculated that *pde6h* plays a key role in the development of the eye photosensitive system in the flounder.

Thiourea, an effective TH -depleting drug, has been utilized to study the effects of TH on metamorphosis and can significantly inhibit levels of T4 and T3 *in vivo* during flounder metamorphosis (Davidson et al., 1979; Yu et al., 2017). Previous studies have shown that exogenous TH stimulates metamorphosis of pelagic larvae, producing miniatures of naturally metamorphosed benthic juveniles; in contrast, TU induces metamorphic stasis, resulting in giant pelagic larvae



are lower in the TU group during metamorphosis, indicating that *pde6h* gene can be directly or indirectly regulated by TH. The rescue experiment for TU group larvae showed that the metamorphosis-impaired larvae were rescued when TU was removed and that the levels of *pde6h* gene were correspondingly significantly up-regulated in the 41 dph TU + NC and 41 dph TU + TH groups compared to in 36 dph TU and 41 pdh TU groups. So, we conclude that TH can directly or indirectly regulate the expression of *pde6h* gene during flounder metamorphosis.

T4 is rarely active as a pro-hormone of T3; T3 has high activity and performs important physiological functions by acting on its target genes. The promoter regions of the target genes that are regulated by T3 contain thyroid-hormone responsive elements (TREs), which serve as the binding sites for THR α s (Cheng et al., 2010; Chiamolera et al., 2012). T3 binding to TRs can result in either a decrease or an increase of the transcription rates of T3 target genes (Oetting and Yen, 2007; Ortiga-Carvalho et al., 2014). TRs can constitutively bind to the TREs of T3 target genes and act in a ligand-independent manner, such that the transcription rate of target genes can change depending on whether or not the THR is bound to T3 (Ortiga-Carvalho et al., 2014). In this study, our dual-luciferase assay indicated that the *pde6h* promoter activity is induced synergistically by T3 and TR α A and that its mutant was not regulated by T3, TR α A, or T3 and TR α A. Therefore, we report that T3 binding to TR α A can promote transcriptional activity of *pde6h* promoter, indicating that *pde6h* is the target gene of T3 in the flounder. However, the specific sites bound by the TH receptors in the six alternative TREs require further investigation.

In summary, we conclude in this study that T3 can directly regulate transcription of *pde6h* gene by binding to TR α A in the flounder, *pde6h* may play an important role in physiological function and eye development during flounder metamorphosis. Furthermore, we speculate that TH may regulate the flounder photosensitive system to adapt to the light changes arising from a transition from pelagic to benthic life during metamorphosis by directly regulating *pde6h* expression. Further study on the function of *pde6h* in the flounder metamorphosis is needed in the future.

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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

This animal study involving fish was approved by the Institutional Animal Care and Use Committee in Shanghai Ocean University.

AUTHOR CONTRIBUTIONS

YF designed the study and analyzed the data. YC and JX performed all experiments in this manuscript. NH wrote the manuscript.

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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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High Temperature, pH, and Hypoxia Cause Oxidative Stress and Impair the Spermatic Performance of the Amazon Fish *Colossoma macropomum*

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The control of abiotic parameters is fundamental for fish survival, growth and reproduction. These factors have a direct effect on sperm quality. Thus, this study evaluated the effect of different temperatures (29, 31, 33, and 35°C), pHs (4 and 8), and hypoxia (1 mgO₂ L⁻¹) on sperm motility of *Colossoma macropomum* (tambaqui). The results indicated a longer duration of sperm motility at 29°C (50.1 ± 2.70 s) that progressively decreased when exposed to 35°C (31.2 ± 1.31 s) and hypoxia at pH 4 (27.4 ± 1.42 s) and pH 8 (30.44 ± 1.66 s; $p < 0.05$), respectively. Sperm oxygen consumption increased in hypoxia at both pH (pH 4 = 61.22; pH 8 = 54.74 pmol s⁻¹). There was an increase in the activity of glutathione-S-transferase (GST) and superoxide dismutase (SOD), as well as in lipid peroxidation levels (LPO) and DNA damage in sperm exposed to higher temperatures and hypoxia. The pH 4 and pH 8 under normoxia did not affect the quality of *C. macropomum* sperm. These results suggest that water warming and acidification, consequences of climate changes, significantly affect the reproduction of *C. macropomum*, reducing the quality of spermatozooids during fertilization.

Keywords: aquaculture, antioxidant enzymes, climate change, comet assay, fish breeding, motility, tropical fish

INTRODUCTION

Climate changes are universal and are causing unprecedented environmental and life damage. Among the changes are higher temperatures, increases in precipitation rates, extreme drought, and changes in the habitat of terrestrial and aquatic species, which can result in reallocation and adaptation to survival (Isaak et al., 2012). Freshwater species face particular challenges within this scenario, worsened by increased pollution and over-exploitation of commercial fisheries, leading to declining fish populations (Wenger et al., 2011), that is, elimination of sensitive species, through direct and indirect effects on the biology of these organisms (Jeppesen et al., 2010), as well as on sperm quality and viability (Jenkins et al., 2018). Thereby, in order to minimize the impacts caused by fishing and climate change in natural environments, aquaculture is becoming increasingly important for the maintenance of fish stocks and for

animal protein production. Therefore, targeted efforts to assess how fish in confined environments will be affected by the climate change in terms of reproductive efficiency are needed.

Dissolved oxygen (DO), pH, and temperature are important factors affecting survival, growth, and reproduction of fish (Caldwell and Hinshaw, 1994). In the Amazon region, these factors are influenced by diurnal variations of DO, seasonal flooding, thermal stratification, aquatic plants, and anthropogenic effects, such as eutrophication and global warming (Richards et al., 2009). The control of these factors is pivotal for the reproductive success of fish that have external fertilization (Dong et al., 2011), since sperm activity is only triggered when it comes into contact with water (Alavi and Cosson, 2006). Thus, sperm motility is strongly influenced by the activation medium (Dadras et al., 2016).

Sperm motility is a key factor determining semen quality being directly related to fertilization success, and is potentially used as a biomarker of environmental quality (Gallego et al., 2018). Negative effect of hypoxia, temperature, and pH on the spermatid quality of *Porichthys notatus*, *Sparus aurata* (Lahnsteiner and Caberlotto, 2012), and *Gonypterus blacodes* (Dumorné et al., 2018) have been documented. The duration of sperm motility of farmed fish species is approximately 1 min; for this reason, accurate assessments using rapid and sensitive methods are needed to increase the efficiency of artificial fertilization (Kime et al., 2001; Rurangwa et al., 2004).

The motility and fertilization capacity of sperm are dependent on energy stored in the form of ATP, which in turn is strongly influenced by the oxygen concentration (Bencic et al., 1999). However, molecular oxygen, although necessary, can induce the production of reactive oxygen species (ROS), causing oxidative stress. During periods of environmental stress to which sperm are subjected at the time of activation, several antioxidant enzymes act to neutralize ROS (Dadras et al., 2016). The antioxidant capacity of semen can vary among fish species (Slowinska et al., 2013) and the formation of ROS can cause different types of damage, in particular lipid peroxidation of membranes, due to the abundance of polyunsaturated fatty acids, and DNA damage, with consequent loss of motility and decreased fertilization (Aramli and Kalbassi, 2016).

The tambaqui, *Colossoma macropomum* (Cuvier, 1818), is a migratory tropical fish species native from the Amazon and Orinoco basins (Araujo-Lima and Goulding, 1998), belonging to family *Serrasalminidae* (Mirade, 2010). The spawning of tambaqui coincides with the beginning of the flood, occurring in October/

November and the species presents annual and total spawning. Mature individuals of *C. macropomum* have high fecundity, releasing more than one million oocytes. The fertilization is external and the species does not show parental care (Araujo-Lima and Goulding, 1998; Vieira et al., 1999). At the beginning of the flood season, individuals start their upstream migration into whitewater rivers, and when the gonads are fully developed they spawn along the grassy leaves that are being inundated with rising water. After spawning, the fish enter the floodplain areas where they feed mainly on fruits (Goulding and Carvalho, 1982; Araujo-Lima and Goulding, 1998).

This species is of great interest for researchers and producers due to its acclimatization facility, rapid growth, and great social and economic importance for the central region of the Amazon (Mendonça et al., 2009). Because it is a tropical species, *C. macropomum* is expected to be especially vulnerable to climate change, specifically to warming, because it has a narrower thermal sensitivity range compared to subtropical and temperate species and tends to live closer to its thermal limits (Lapointe et al., 2018). Therefore, even small temperature increases can lead to declining performance of individuals and populations (Tewksbury et al., 2008).

Considering that homeostasis of the sperm of *C. macropomum* is extremely important for the aquaculture industry, understanding how environmental parameters dependent on climate change affect sperm quality is critical in improving the methods and manipulations required for successful *in vitro* fertilization. Thus, the present study aims to investigate *in vitro* effects of temperature, pH and hypoxia on motility and antioxidant responses of the sperm of the Amazonian fish *C. macropomum*.

MATERIALS AND METHODS

Semen Collection

Males of *C. macropomum* ($n = 3$, 60.4 ± 0.36 cm, 4.13 ± 0.05 kg) were selected from 600 m² nurseries at the Center for Technology, Training and Production in Aquaculture (CTTPA; Balbina, Presidente Figueiredo, Amazonas – 1°55'54.4"S; 59°24'39.1"W). The fish were randomly selected for a light abdominal massage and in cranium-caudal direction, and those who released semen were chosen. The fish were recovered from this initial handling in 1000 L tanks with oxygen 5.54 ± 0.22 mg L⁻¹, temperature $29.2 \pm 0.07^\circ\text{C}$, and pH 7.73 ± 0.28 for a period of 6 h. Subsequently, spermiation was induced by intraperitoneal injection of crude extract of carp pituitary hormone (EBHC). The procedure was performed by injecting two boluses of EBHC. The first bolus was 0.5 mg/kg EBHC, and after an interval of 12 h, a second bolus of 1.0 mg/kg EBHC was injected. Six hours after injection of second hormone bolus, the semen was collected in graduated Falcon tubes, avoiding contact with water, blood, feces, or urine, thus preventing sperm activation. From each animal, 4 ml of semen was collected and immediately diluted 1:10 (50 μ l semen: extender 450 μ l) in Beltsville Thawing Solution (BTS-MINITUB®) and refrigerated at 4°C to be transported to the laboratory Ecophysiology and

Abbreviations: ADAPTA, Adaptations of aquatic biota of the Amazon; ATP, Adenosine triphosphate; BHT, Butylate hydroxytoluene; BSA, Bovine serum albumin; BTS, Beltsville thawing solution; CAPES, Coordination for the improvement of higher education personnel; CDNB, 1-Chloro-2,4-dinitrobenzene; CHP, Cumene hydroperoxide; CNPq, Brazilian National Research Council; CTTPA, Center for Technology Training and Production in Aquaculture; DDT, Dichloro-diphenyl-trichlorethane; DMSO, Dimethyl sulfoxide; EBHC, Crude extract of carp pituitary hormone; EDTA, Ethylene diamine tetra-acetic acid; ROS, Reactive oxygen species; FAPEAM, Amazonas State Research Foundation; FOX, Ferrous oxidation/xylenol orange; GST, Glutathione S transferase; HCL, Hydrochloric acid; INPA, National Institute for Research of the Amazon; LEEM, Laboratory of Ecophysiology and Molecular Evolution; LPO, Lipoperoxidation; NaOH, Sodium hydroxide; SEM, Standard error of mean; SOD, Superoxide Dismutase.

Molecular Evolution (LEEM) of the Brazilian National Institute for Research of the Amazon (INPA). All procedures and experimental manipulations used in this study were performed according to the Brazilian Guidelines for Animal Care and were approved by the Ethics Committee for the use of Animals of INPA, protocol number 004/2018.

Activation

For activation of sperm motility, the following treatments were tested: temperature (29, 31, 33, and 35°C), pH (4 and 8), and interaction between pH and hypoxia (1 mgO₂·L⁻¹) in controlled activator solution (distilled water-0 mOsm kg⁻¹). The different temperatures were maintained using thermostats HOPAR model (Aquarium Heater H-606). The pHs 4 and 8 were achieved by adding HCl 1% and NaOH 1%, respectively. The pH values were then monitored using a digital pH meter (Ohaus, Starter 3100). Hypoxia was adjusted with the addition of gaseous nitrogen until the concentration of 1 mgO₂ L⁻¹. The sperm were activated in the proportion 2:20 (v:v), in a closed environment with controlled temperature (23°C). Immediately after activation, sperm were observed under an optical microscope (Leica DM500; 40x), by the same observer to avoid the subject bias of the analyzes. The motility time was monitored with the aid of a timer (s) being evaluated from the start of movement until 100% sperm immobility. To identify the percent of sperm motility, a scale from 0 to 100% was used, according to Cosson et al. (2008), where: 1 = 0–5%, 2 = 5–25%, 3 = 25–50%, 4 = 50–75%, 5 = 75–100%. For each treatment, nine replicates were performed in triplicate.

Oxygen Consumption of Sperm

Oxygen consumption of sperm was monitored using a high-resolution respirometer (Oxygraph-2 K, Oroboros Instruments) equipped with a Peltier thermostat and an electromagnetic stirrer. The treatments (temperature, hypoxia, and pH) were controlled within two 2 ml closed Oxygraph chambers. With the aid of injection cannulas, 100 µl of semen were added, immediately activated inside the chambers. Oxygen consumption was recorded immediately after activation until cessation of sperm respiration. Recordings of new samples were performed at intervals of 5 min between exposures, to the complete stabilization of the equipment. Data are expressed as pmol·s⁻¹·cm⁻³.

Biochemical Analysis

For analysis of antioxidant enzymes, 200 µl of semen from each fish was activated, with five replicates in triplicate for each treatment and after motility cessation, it was immediately immersed in liquid nitrogen. The semen samples were homogenized in a buffer solution (Tris base 200 mM, ethylene diamine tetraacetic acid (EDTA) 1 mM, dichloro-diphenyl-trichlorethane (DDT) 1 mM, sucrose 500 mM, KCl 150 mM, and pH 7.6) and centrifuged at 9,000 g for 10 min at 4°C. The supernatant was used for analysis of the activity of glutathione S-transferase enzyme (GST), superoxide dismutase (SOD) and catalase (CAT), and for determination of lipid peroxidation (LPO) levels. GST activity was determined as described by Keen et al. (1976), considering absorbance changes at 340 nm using 1-chloro-2,4-dinitrobenzene

(CDNB) as the substrate. GST activity was calculated as nmol of conjugate CDNB·min⁻¹·mg⁻¹ protein, using the molar extinction coefficient 9.6 mM·cm⁻¹. SOD activity was quantified from the inhibition of the reduction rate of cytochrome c influenced by the xanthine/xanthine oxidase system at 550 nm (Flohé and Otting, 1984) and is expressed as U min⁻¹·mg of protein⁻¹. CAT activity was determined following the method of Beutler (1975) and is expressed as µmol H₂O₂·min⁻¹·mg⁻¹ protein. The inhibition rate of H₂O₂ decomposition was monitored by decreasing the absorbance at 240 nm. LPO levels were measured by the ferrous oxidation/xenol orange (FOX) method as described by Jiang et al. (1991) which consists of the oxidation of Fe⁺² to Fe⁺³ hydroperoxide in an acid medium and subsequent formation of Fe⁺³/xenol orange in the presence of the stabilizer butylate hydroxytoluene (BHT). The formation of this complex was quantified by increased absorption at 560 nm and is expressed in µM cumene hydroperoxide (CHP)·mg⁻¹ of sperm protein. Total sperm protein was determined spectrophotometrically at 595 nm, according to the colorimetric assay described by Bradford (1976) using bovine serum albumin (BSA) as standard. All assays were performed with a SpectraMax M2 spectrophotometer, Molecular Devices Inc., Sunnyvale, CA, USA.

Genotoxic Damage

To verify the DNA integrity of *C. macropomum* sperm cells, unicellular gel electrophoresis (comet assay) was used, according to Cabrita et al. (2005). After experimental exposures, 5 µl of semen was added to 300 µl of RPMI. Previously, the experimental units were covered with 0.5% agarose and all procedures were completed in triplicate. After agarose drying, the slides were placed in the lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, pH 10–10.5, 1% Triton X-100, and 10% DMSO) for 90 min at 4°C. The slides were then submerged in alkaline sodium hydroxide and EDTA buffer (300 mM NaOH and 1 mM EDTA, pH > 13) for 20 min. The slides were electrophoresed for 20 min at 25 V, 300 mA, and 4°C. Subsequently, the slides were neutralized (0.4 M Tris, pH 7.5 at 4°C) and stained with silver nitrate solution (5% sodium carbonate, 0.1% ammonium nitrate, 0.1% silver, 0.25% tungstosilicic acid, and 0.15% formaldehyde) for 15 min at 37°C. A total of 100 spermatozooids were randomly quantified per slide, using an optical microscope (Leica DM205) with a magnification of 100x. Damage was classified according the size of the tail that represents the degree of fragmentation of the DNA. DNA fragments of damaged nucleus migrate when exposed to an electrical field (electrophoresis). Fragments of different sizes migrate at different speeds, forming the typical figure of a comet. Thus, during the electrophoretic run, the more intense the breaks in the DNA molecule, the smaller the fragments generated and the greater the extent of migration and so the comet tail (Singh et al., 1988; Collins et al. 2008). The cells were divided into five categories, according to tail size, ranging from 0 to 4, i.e., class 0 corresponds to intact DNA, without tail; class 1, low damage index; class 2, intermediate damage; class 3, high damage; and class 4, extreme damage.

Statistical Analysis

Data are expressed as mean $\pm$ SEM. To compare the different treatments, an ANOVA one way, followed by Tukey's *post-hoc* test when differences ($p < 0.05$) between means were detected. Data that did not meet the premises of linear models were log transformed. All analysis was performed using the R-3.5.1 software (R Core Team, 2018), with additional car package (Fox and Weisberg, 2011).

RESULTS

Sperm Motility

The duration of *C. macropomum* sperm motility was 50.1 ± 2.7 s at 29°C and significantly reduced ($p < 0.05$) at 33°C (33.66 ± 2.58 s) and 35°C (31.22 ± 1.31 s; **Figure 1**). The percentage of mobile cells showed no differences at 29 and 31°C, but decreased significantly at 33 and 35°C (**Figure 1**). Exposure to pH 4 and pH 8 in hypoxia caused a significant reduction in motility time and percentage of mobile sperm ($p < 0.05$; **Figure 2**). Motility time and percentage of mobile sperm showed no differences when exposed to pH 4 and pH 8 in normoxia ($p > 0.05$; **Figure 2**).

Oxygen Consumption of Sperm

The oxygen consumption of sperm was not altered with increasing temperatures ($p > 0.05$; **Figure 3A**) or under different pHs within the same treatment (**Figure 3B**). However, exposure to hypoxia resulted in an increase in oxygen consumption at both pH 4 ($61.22 \text{ pmol s}^{-1}$) and pH 8 ($54.74 \text{ pmol s}^{-1}$) compared to normoxia at pH 4 ($14.20 \text{ pmol s}^{-1}$) and pH 8 ($10.59 \text{ pmol s}^{-1}$; $p < 0.05$; **Figure 3B**).

Biochemical Analysis

Activities of GST, SOD, and CAT and levels of LPO of *C. macropomum* sperm exposed to different temperatures are shown in **Figure 4**. GST activity was increased at 35°C, differing from sperm exposed to 33°C ($p < 0.05$), and similar to other treatments ($p > 0.05$; **Figure 4A**). SOD activity was significantly increased at 35°C compared to all other treatments ($p < 0.05$; **Figure 4B**). CAT did not differ in any of the treatments ($p > 0.05$; **Figure 4C**). Exposure to 35°C caused an increase in LPO levels compared to cells exposed to 29 and 33°C, while for sperm exposed to 33°C LPO levels were reduced compared to other groups ($p < 0.05$; **Figure 4D**). Low oxygen concentration exposure during sperm activation caused an increase of GST and SOD activities (**Figures 5A,B**). CAT activity at pH 8 in normoxia was equal to pH 4 normoxia ($p > 0.05$) and showed a similar not significant increase of sperm exposed to hypoxia at both pHs ($p < 0.05$; **Figure 5C**). Increased enzymatic activities in hypoxic environments contributed to increased LPO levels (**Figure 5D**). Normoxic environments at pHs 4 and 8 did not affect the activities of SOD and GST, as well as the LPO levels ($p > 0.05$; **Figure 5**).

Genotoxic Damages

Genetic damage to sperm of *C. macropomum* increased at higher temperatures (33 and 35°C) compared to sperm activated

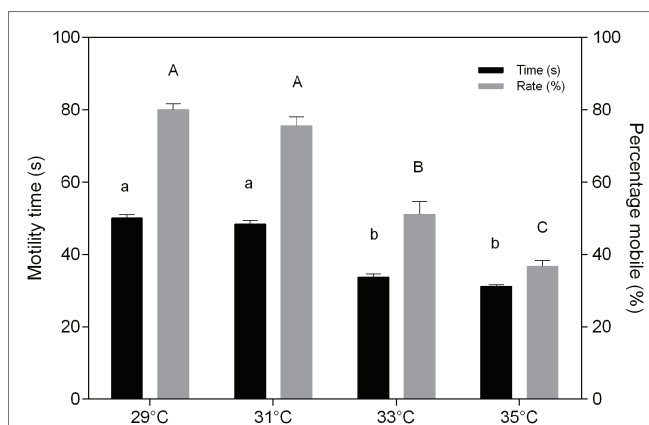


FIGURE 1 | *In vitro* effect of temperature on time (s) and percentage mobile (%) of sperm of *Colossoma macropomum*. Data are presented as mean $\pm$ SEM. Lowercase letters indicate difference in motility time between treatments. Uppercase letters indicate differences in motility rate between treatments ($p < 0.05$).

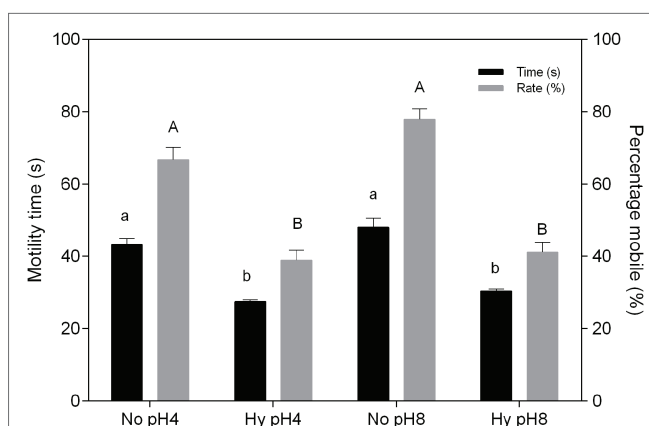


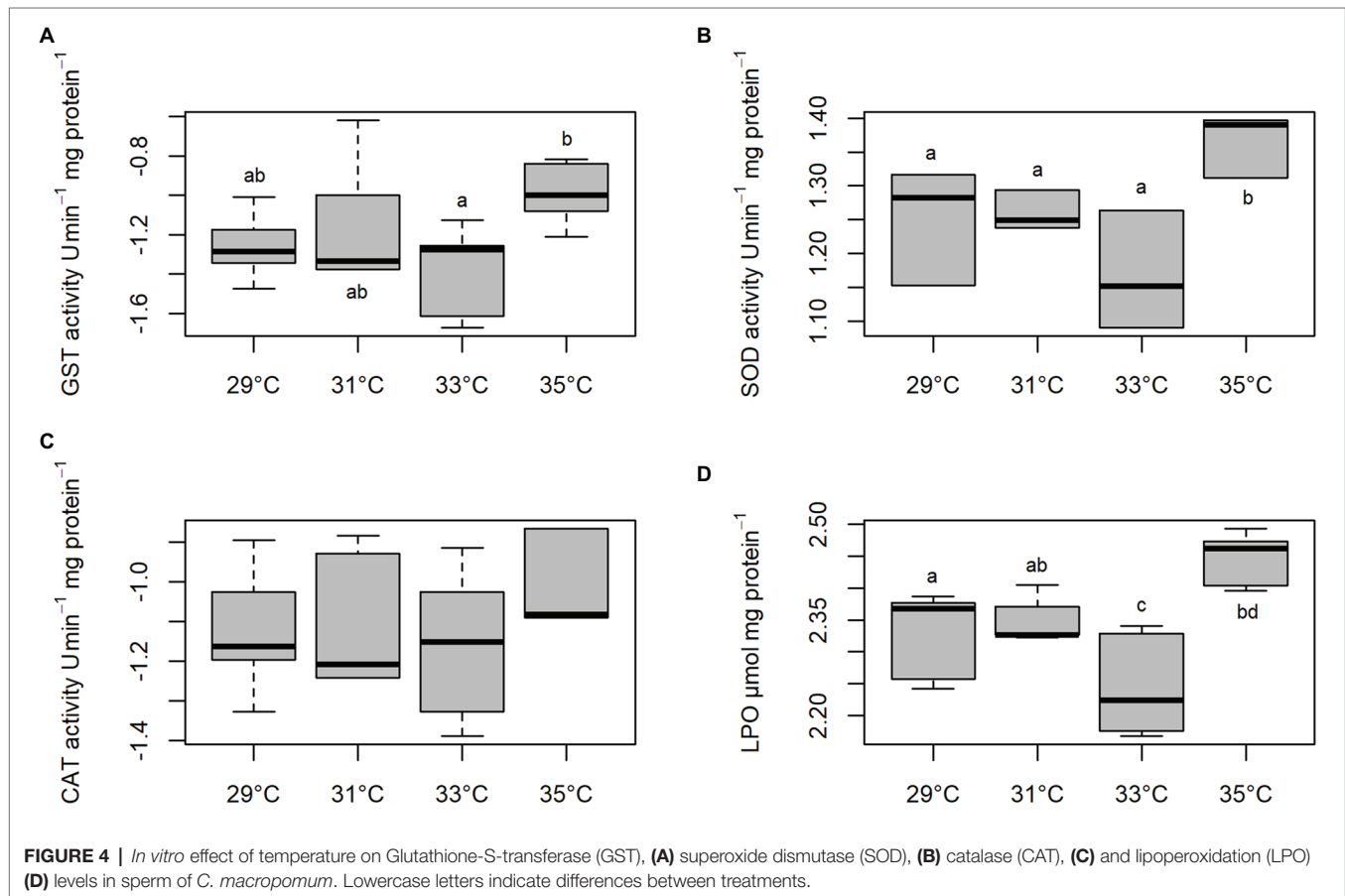
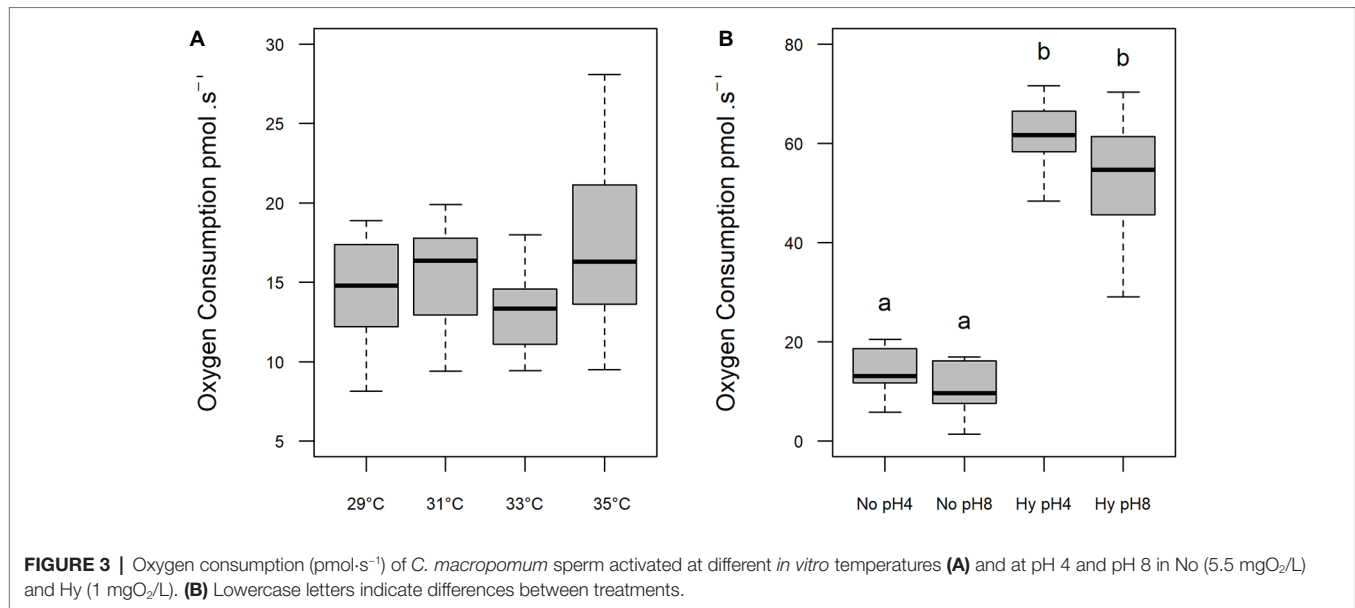
FIGURE 2 | *In vitro* effect of pH and hypoxia (Hy) on time (s) and percentage mobile (%) of sperm of *C. macropomum*. Data are presented as mean $\pm$ SEM. Lowercase letters indicate differences in motility time between Hy and normoxia (No). Uppercase letters indicate differences in motility rate between Hy and No ($p < 0.05$).

at 29 and 31°C (**Figure 6**). At 29 and 31°C, there was a prevalence of intact DNA with about 71 and 66%, respectively. At the highest temperature, 35°C, approximately 60% of the sperm cells had extreme damage, class 4 (**Figure 6**). Activated spermatozoa under normoxia showed the highest rate of intact cells (pH 4 = 43%, pH 8 = 57%), but low oxygen activation medium on both pHs caused an increased classes 2, 3, and 4 damages, considered intermediate, high, and extreme, respectively (**Figure 6**).

DISCUSSION

Temperature Effects

Sperm motility, a critical semen characteristic, depends on activation media (Williot et al., 2000) that strongly influence the fertilization



in teleosts (Bera et al., 2016). In the present study, sperm exposure to increased temperature (33 and 35°C) caused a reduction on time and percentage of mobile sperm (Figure 1) without altering the oxygen consumption (Figure 3A). These findings corroborate the report of Lahnsteiner and Caberlotto (2012) for *S. aurata*

and Dadras et al. (2016) for *Cyprinus carpio*, which showed that higher temperatures caused lower percentage of mobile sperm. According to Purchase et al. (2010), fish sperm has limited energetic resources; thus, temperature increase causes a motility decrease due to the acceleration of the metabolic processes.

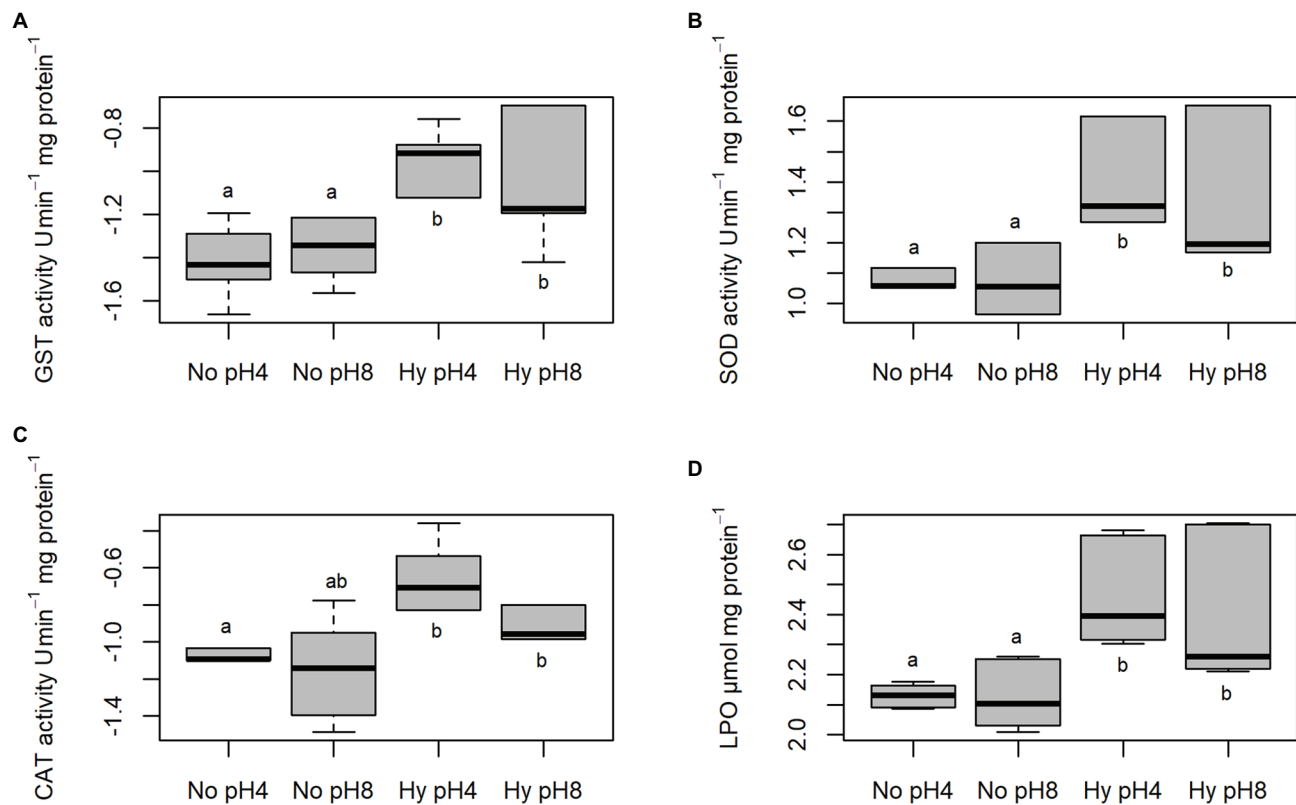


FIGURE 5 | *In vitro* effect of pH in No ($5.5 \text{ mgO}_2/\text{L}$) and Hy ($1 \text{ mgO}_2/\text{L}$) on GST, (A) SOD, (B) CAT, and (C) LPO, (D) in sperm of *C. macropomum*. Lowercase letters indicate differences between treatments.

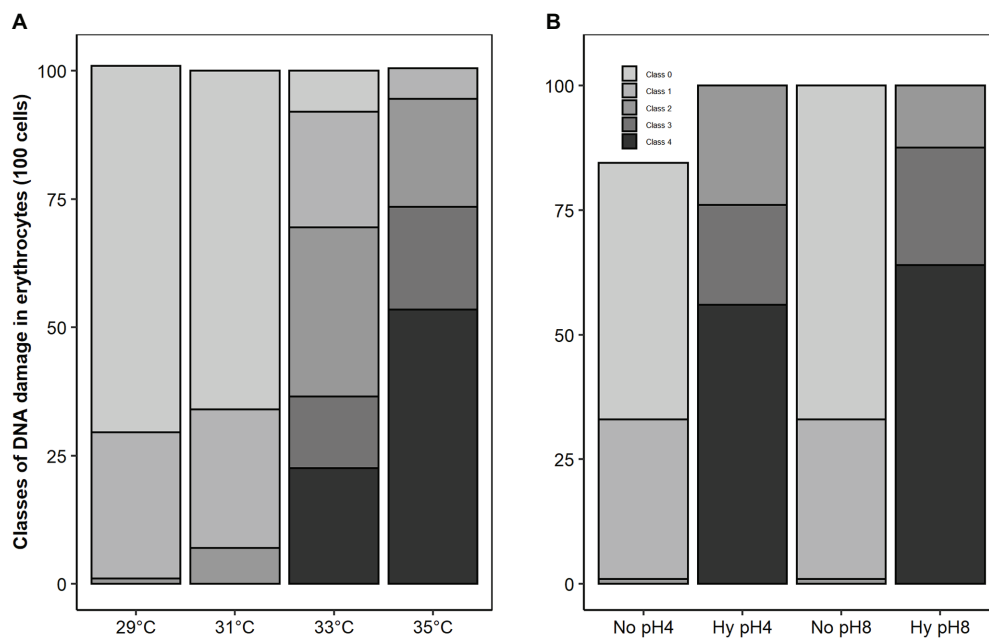


FIGURE 6 | Frequency of DNA damage of sperm of *C. macropomum* exposed to different temperatures (A) and exposed to pH 4 and pH 8 in No and Hy (B) *in vitro*.

Low temperatures reduce the intensity of flagellar movement significantly, prolonging motile duration of spermatozooids (Alavi and Cosson, 2006). In the present study, the high temperature accelerated the swimming speed of *C. macropomum* sperm cells, increasing the metabolic consumption of the cells and decreasing the motility time and the percentage of mobile cells. Activated sperm at low temperatures consume metabolic energies more slowly, increasing the duration of these cells, corroborating the report of Alavi and Cosson (2006), who found that low temperatures reduce the intensity of flagellar movement, significantly prolonging the mobile duration of sperm.

As a result, at lower temperatures, the consumption of ATP is reduced, which is directly related to the longer motility time (Lahnsteiner and Caberlotto, 2012). Dzyuba et al. (2019), studying the tropical species *Oreochromis niloticus*, showed that higher temperatures induce an increase in sperm motility velocity, which is accompanied by a reduction in motility time. The reduction in time and percentage of mobile sperm at the higher temperatures observed in the present study may be related to increased energy demands, which was not compensated for, since oxygen consumption remained constant at all temperatures. Therefore, our results suggest that semen from tambaqui has limited energy supply to deal with increased temperature, suggesting high vulnerability of this species under warming scenarios.

Another factor that may explain the lower percentage of mobile sperm observed at the higher temperature is related to the oxidative damages caused by ROS production, as observed in the present study. The exposure of sperm cells at 35°C resulted in an increase in the activity of antioxidant enzymes (SOD and GST; **Figure 4**); however, such increase was not enough to avoid damage to macromolecules, since there was an increase in lipoperoxidation and DNA damage (**Figures 4, 6A**). Oxidative stress can induce metabolic disorders that reduce sperm motility. As suggested by Aramli and Kalbassi (2016), oxidative stress causes disturbances in the sperm metabolism, which results in decreased sperm motility.

The oxidative damage observed in the present study suggests a inefficiency in the neutralization of ROS by the antioxidant defenses, since even with the increase of antioxidant enzyme activity, such as SOD, sperm presented high levels of lipoperoxidation and DNA damage. Aramli and Kalbassi (2016) also showed that the increase of the antioxidant defenses in the species *Acipenser persicus* was accompanied by the increase of oxidative damages. Fish sperm have characteristics that make them susceptible to damage related to oxidative stress, considering the high amount of polyunsaturated fatty acids of sperm membrane (Shaliutina et al., 2013).

Studies have shown that temperature increases potentially induce oxidative stress in different fish tissues (Wiens et al., 2017), including sperm cells, as presented in this paper with the increase in antioxidant enzymes, contributing to the increase in lipid peroxidation levels, as shown by Dadras et al. (2016) when evaluating the sperm of *C. carpio*,

identifying that the lipid peroxidation in these cells is temperature sensitive. Lipids are probably used as a source of energy by sperm to increase motility duration (Baeza et al., 2015) that plays an important role in fertilization capacity (Gholami et al., 2011). The lipid peroxidation of these cells is associated with deterioration of membrane function, decreased fluidity, and increased non-specific permeability to ions and other transmembrane transport processes (Wong-Ekkabut et al., 2007).

Although *C. macropomum* sperm cells were activated at the high temperature evaluated in this experiment, they showed a significant increase in tail damage (class 4). The evaluation of fish sperm DNA damage is important for fish farming, since the loss of genetic information or the appearance of larval deformities is detrimental to future generations (Cabrita et al., 2005; Lewis and Galloway, 2009). Due to the reduced motility time of freshwater fish spermatozoid, the activation environment is critical for the successful delivery of paternal DNA to the egg (Gazo et al., 2015). However, oxidative stress is highly detrimental to DNA integrity, since this damage is not limited to the direct effect on the fragmentation of chromatin; it can also alter gene expression or induce epigenetic deregulation (Chervona and Costa, 2012). The extreme DNA damage of *C. macropomum* sperm caused by activation at high temperatures is similar to the results found by Lacaze et al. (2011) in assessing DNA damage of the sperm of *Gammarus fossarum*. The authors explain that the increase in damage is associated with an overall stress caused by increased temperature and decreased oxygen concentration. Our results corroborate the findings of De Andrade et al. (2004), who found an increase in DNA damage of *Dreissena polymorpha* and *Netuma* sp. exposed to 37°C. Despite increased DNA damage in various species of fish exposed to various stressors, Pérez-Cerezales et al. (2010) reported that DNA damaged spermatozooids are capable of performing fertilization and that the oocyte can repair this damage to some extent in *Oncorhynchus mykiss*. These authors stated that when the DNA fragmentation rate is high, the oocyte repair capacity is insufficient, and infertility rate increases considerably.

Effects of pH and Hypoxia

In the present study, activation media pHs (pH 4 and pH 8) did not influence the sperm quality of *C. macropomum*. This may be related to the adaptation of this species to large pH range that drives resilience of sperm cell to pH changes and so, possibly, increasing reproductive success. Aride et al. (2004) reported that in confined environments *C. macropomum* is able to tolerate large variations in pH, which was confirmed by Wood et al. (2018). Cosson (2004) showed that the pH of activating medium usually affects motility, but in small proportions. In addition, the external pH can reduce or prolong motility, but it is not the main environmental factor affecting initial motility (Cosson et al., 2008). The results found in this study are similar to the findings by PerchecPoupard et al. (1997), who showed reduced pH impact on carp sperm motility, indicating that fish that inhabit

tropical/subtropical environments are possibly more resistant to pH variation. However, for species that inhabit temperate environments such as *Samotrutta*, variations in pH affect sperm motility (Dziewulska and Domagała, 2013).

Hypoxia exposure affected sperm quality of *C. macropomum*, suggesting that hypoxia, a common environmental condition in tropical waters and aquaculture, is highly detrimental to the reproduction of this tropical species. The results observed in the present study are in agreement with Wu et al. (2003), which suggested that hypoxia caused reduction of sperm motility in carp and other reproductive dysfunctions, such as the retardation of gametogenesis. Thomas et al. (2007) evaluated the formation of sperm cells of *Micropogonias undulates* and found that sperm production, gametogenesis and all stages of spermatogenesis were impaired in hypoxic environments.

The decrease in the time and percentage of mobile sperm of *C. macropomum* under hypoxic conditions was accompanied by an increase in oxygen consumption (Figure 3B). At low oxygen, sperm cells present an increase in the rate of flagellar motility, not measured in the present study, which resulted in decreased motility time due to the rapid consumption of ATP. As a result, oxygen consumption increased. Fitzpatrick et al. (2009) showed that sperm velocity of *P. notatus* exposed to hypoxia was positively related to oxygen consumption. Both species breed in hypoxic environments. During the breeding period, males of *P. notatus* are trapped in small pools facing drastic hypoxia, similar to *C. macropomum* in their natural environments. It is known that the mitochondria of fish sperm supply ATP demand aerobically (Dzyuba et al., 2017). According to Cosson (2010), as the ATP reserve is depleted along the spermatozoid activity, there is a gradual decrease of the flagellum movement, settling off the movement of the cell. In aquaculture, fish are commonly kept under artificial conditions, exposed to various adverse conditions, which may contribute to the establishment of hypoxic environments (Rurangwa et al., 2004). Therefore, sperm exposed in these environments consume more quickly ATP reserves, directly affecting the duration of motility and the efficiency of fertilization. In addition, recent studies suggest that changes in sperm quality may be related to changes at the molecular level, such as changes in gene expression or epigenetic factors. Wang et al. (2016) verified the occurrence of epigenetic changes in the sperm methyloma of *Oryzias melastigma* exposed to hypoxia, as well as differential expression of genes and proteins involved in spermatogenesis and gene silencing, which was associated with a reduction in sperm motility, even in generations that never had been exposed to hypoxic conditions.

We also observed that exposure to hypoxia caused an increase in antioxidant activity (SOD, CAT, and GST; Figure 5), which suggests an increased capacity to prevent ROS effects. However, despite the higher activity of antioxidant enzymes under hypoxia condition, there was an increase in lipoperoxidation levels and DNA damage. Thus, the oxidative damages observed in the present study may be related to loss of sperm quality under hypoxic conditions. It has been

shown that hypoxia induces oxidative stress in different fish tissues (Mustafa et al., 2011). The results of our study are similar to those of Bera et al. (2016), evaluating the activity of antioxidant enzymes in the testes and ovary of *C. carpio* in hypoxic environments. The authors reported an increase in CAT and SOD activities compared to tissues under normoxia. Other authors report the relationship between loss of sperm quality and oxidative damage in fish sperm under different stress conditions (Dadras et al., 2016; Wiens et al., 2017). Shaliutina et al. (2013) also showed a relationship between loss of sperm quality and oxidative stress in *Acipenser gueldenstaedtii* and *Acipenser bae*. Taken together, these results suggest that tambaqui reproduction is especially sensitive to disturbances caused by exposure to hypoxia.

CONCLUSIONS

The elevation of temperature and the decrease in DO, factors associated among others to climate change, will negatively affect sperm quality of *C. macropomum*. No pH effects on spermatozooids of *C. macropomum* were observed. This study provides basic information for future research on sperm quality of tropical fish in confined environments. However, further investigations are needed to describe the mechanisms involved in metabolism and sperm motility in *C. macropomum* under warm hypoxic environments. In general, the present results suggest that *C. macropomum* sperm are vulnerable to warm hypoxic waters, conditions associated with global warming, and may impair the reproduction of the species.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The animal study was reviewed and approved by Brazilian Guidelines for Animal Care and were approved by the Ethics Committee for the use of Animals of INPA, protocol number 004/2018.

AUTHOR CONTRIBUTIONS

AV and JC designed the work. JC performed the experiment, collected data, analyzed and drafted the original version of the manuscript. SS, SB-M, and DC helped with laboratory analyzes, discussed and reviewed the manuscript and AV supervised this work, obtained financial support, discussed and revised the manuscript. All authors contributed to the article and approved the submitted version.

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Effects of Elevated $p\text{CO}_2$ on the Survival and Growth of *Portunus trituberculatus*

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Identifying the response of *Portunus trituberculatus* to ocean acidification (OA) is critical to understanding the future development of this commercially important Chinese crab species. Recent studies have reported negative effects of OA on crustaceans. Here, we subjected swimming crabs to projected oceanic CO_2 levels (current: 380 μatm ; 2100: 750 μatm ; 2200: 1500 μatm) for 4 weeks and analyzed the effects on survival, growth, digestion, antioxidant capacity, immune function, tissue metabolites, and gut bacteria of the crabs and on seawater bacteria. We integrated these findings to construct a structural equation model to evaluate the contribution of these variables to the survival and growth of swimming crabs. Reduced crab growth shown under OA is significantly correlated with changes in gut, muscle, and hepatopancreas metabolites whereas enhanced crab survival is significantly associated with changes in the carbonate system, seawater and gut bacteria, and activities of antioxidative and digestive enzymes. In addition, seawater bacteria appear to play a central role in the digestion, stress response, immune response, and metabolism of swimming crabs and their gut bacteria. We predict that if anthropogenic CO_2 emissions continue to rise, future OA could lead to severe alterations in antioxidative, immune, and metabolic functions and gut bacterial community composition in the swimming crabs through direct oxidative stress and/or indirect seawater bacterial roles. These effects appear to mediate improved survival, but at the cost of growth of the swimming crabs.

Keywords: metabolomics, microbiota, nuclear magnetic resonance (NMR), ocean acidification, swimming crab

INTRODUCTION

Crustaceans face a range of variable environmental stressors during their complex life cycle. Temperature and salinity are commonly considered as the most important abiotic factors for the survival, growth, and reproduction of crustaceans (Ye et al., 2014; Prymaczok et al., 2016). However, ongoing ocean acidification (OA) may entail a new challenge for them. According to Sabine et al. (2004), the OA is a consequence of the absorption by oceans of atmospheric carbon dioxide (CO_2) due to anthropogenic activities such as cement production and fossil fuels utilization. A substantial rise in oceanic CO_2 partial pressure ($p\text{CO}_2$) has already led to a reduction of 0.1 units in the current pH of surface seawater compared to preindustrial levels. It is predicted that by 2100, there will be a further decrease of 0.3–0.5 pH units (Caldeira and Wickett, 2003; Orr et al., 2005).

The impacts of OA are raising increasing concerns with regard to crustaceans because of the sensitivity of calcifying animals (Hendriks et al., 2010). Among them, crabs present species-specific responses to OA, such as different impacts on calcification (Walther et al., 2010; Long et al., 2013a; Wei et al., 2015), and survival was shown to be reduced in crabs following a longer term exposure (months) to OA, although shorter term exposure (less than 1 month) did not have any apparent effects (Ceballos-Osuna et al., 2013; Long et al., 2013a; Ragagnin et al., 2018; Ramaglia et al., 2018). In addition, OA can also induce negative effects on crab fertilization, embryonic development, and behavior (de la Haye et al., 2011, 2012; Ceballos-Osuna et al., 2013; Long et al., 2013b; Dodd-Luke et al., 2015).

In general, such morphological and behavioral changes in crabs are associated with physiological changes. When exposed to OA, crabs sense the decreasing extracellular pH in tissues caused by OA (Wheatly and Henry, 1992; Pane and Barry, 2007). There is evidence that efficient acid-base, metabolic, and ionic regulation contributes to the compensation of extracellular pH changes, as shown in the velvet swimming crab, *Necora puber* (Spicer et al., 2007; Small et al., 2010), the dungeness crab, *Metacarcinus magister* (Hans et al., 2014), and the shore crab, *Carcinus maenas* (Maus et al., 2018). However, such osmoregulatory changes result in a rising energy cost and the reallocation of energy thereby compromises growth and behavior (Caldeira and Wickett, 2003; Dodd-Luke et al., 2015; Ragagnin et al., 2018). Extracellular anisotonic regulation and intracellular isotonic regulation have been observed in crustaceans in response to environmental osmolality changes (Ragagnin et al., 2018). However, OA has a depressing effect on oxygen consumption, which subsequently affects ATP production (Pörtner et al., 2004; Carter et al., 2013; Maus et al., 2018). The activity of energy-dependent osmoregulating enzymes such as Na⁺/K⁺-ATPase and V-type ATPase therefore decreases under acidified conditions (Whiteley et al., 2001; Hu et al., 2016). In this case, free amino acids are probably used as osmotic effectors. In addition, lactate has been shown to vary in spider crabs, *Hyas araneus*, exposed to elevated pCO₂ (Zittier et al., 2013). Overall, the underlying metabolic effects of OA have not been thoroughly evaluated in crabs.

The swimming crab, *Portunus trituberculatus* (Crustacea, Decapoda, Brachyura), is a widely cultured and consumed species in China with a yield of 617,540 tons in 2017 (China Fishery Statistical Yearbook, 2018). Our previous studies have shown that elevated pCO₂ (750 and 1500 μatm) has significant effects on the carapace of juvenile swimming crabs (e.g., a simplified arrangement of spinules, a reduced thickness, and an increased chitin content) (Ren et al., 2017) and their behavior (e.g., an increase in shoal average speed, a preference for dark environments and fast exploration) (Ren et al., 2018). Furthermore, previous studies have reported that OA can induce oxidative stress (Wang et al., 2009; Menu-Courey et al., 2019) and suppress immunity (Hernroth et al., 2012) in other crustaceans. Therefore, a holistic study is needed to advance our understanding of how OA exposure affects the swimming crab.

In this study, we subjected swimming crabs to increasing CO₂ levels for 4 weeks to simulate OA. Our aim was to extensively explore the effects of OA exposure on the survival,

growth, digestion, antioxidant capacity, immune function, tissue metabolites, and gut bacteria of swimming crabs as well as on seawater bacteria using biochemical assays, real-time quantitative polymerase chain reaction (RT-qPCR), 16S rRNA gene sequencing, and nuclear magnetic resonance (NMR)-based metabolomics analysis. This study provides new evidence that OA has a positive effect on the survival but a negative effect on the growth of swimming crabs through changes in crab digestion, antioxidant capacity, immune function, gut bacteria, and metabolites and the modulation of the seawater bacteria.

MATERIALS AND METHODS

Crabs and OA Exposure

Four hundred and twenty male juvenile stage VII swimming crabs (93.3 ± 10.0 mm carapace length [CL]) were purchased from a local aquaculture farm in Fenghua, Ningbo in Eastern China in late July 2018. Each crab was kept in a plastic box (210 × 170 × 80 mm) with small holes. These crabs were cultured in three tanks (1.5 × 1.2 × 0.6 m) containing 500 L of aerated and filtered local seawater per tank at ambient salinity (27.0 ± 0.4‰) for a 1-week acclimatization. To avoid heat stress to the crabs, a chiller (Guangli Cooling Equipment Company, Guangzhou, China) was used to maintain the seawater temperature at 28°C via the heat exchange between seawater and tap water. The crabs were fed with fresh clams (*Ruditapes philippinarum*) once daily between 5:00 and 6:00 p.m., and waste food was removed before feeding.

Three experimental OA groups were designed with seawater pCO₂ at current or predicted future levels: the ambient pCO₂ was 380 μatm (designated the 380 group), 750 μatm (designated the 750 group) was used to represent the scenario at the end of this century and 1500 μatm (designated the 1500 group) was used to represent the scenario in c. 2200 (Revi et al., 2014; Gattuso et al., 2015). To build the experimental systems, a simulated ocean acidification system (CN: ZL201520071087.1) (Ren et al., 2018) and an incubation system that was partially derived from the experimental system of Wang et al. (2018) were used. For each group, the incubation system per group consisted of a mixing tank (500 L) and three individual culture tanks (300 L), and seawater was recirculated and purified by using a water purification header tank connected to the culture tanks (200 L). The CO₂ gas was first mixed with air in the mixing tank using a gas flow adjustment system (AK Biotechnology Co., Ltd., Qingdao, Shandong, China), followed by bubbling into seawater via a nano-aeration pipe. Both the mixing tank and the header tank were continuously bubbled with the respective and stable air-CO₂ mixture. Each incubation system was stocked with 130 crabs with strong vitality and intact limbs with 43 crabs for two of the culture tanks and 44 crabs for the third culture tank. The flowing CO₂ was adjusted to maintain the designed pCO₂ level, and this gradually changed the pH from to the required level within 12 h. Three seawater inputs with different pCO₂ levels for the three incubation systems were obtained: 380 μatm (pH = 8.1) for the control group, 750 μatm (pH = 7.9), and 1500 μatm (pH = 7.6) (Figures 1A,B). The pCO₂ levels were read daily from the gas flow adjustment system. Measurements of free CO₂

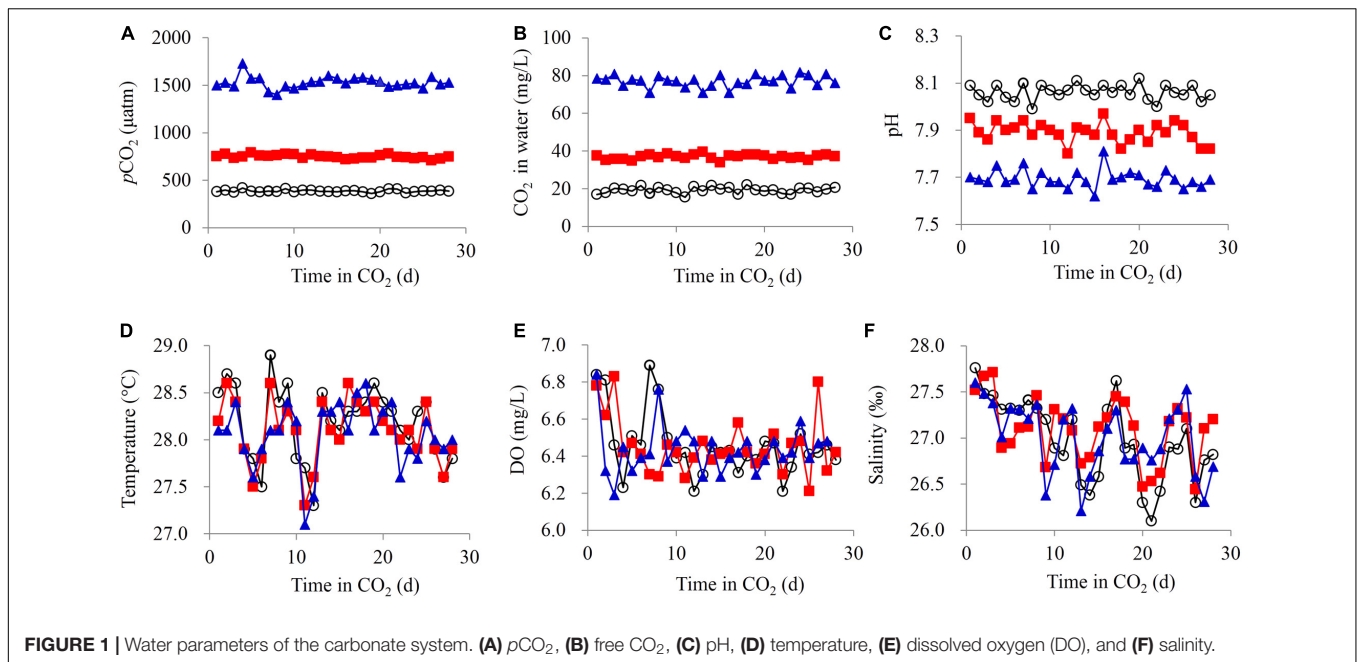


FIGURE 1 | Water parameters of the carbonate system. **(A)** pCO₂, **(B)** free CO₂, **(C)** pH, **(D)** temperature, **(E)** dissolved oxygen (DO), and **(F)** salinity.

concentrations in the seawater were determined daily by a base titration method following the protocol outlined in the Water Quality Analytical Methods (SL80-1994). The swimming crabs were checked daily for death or molting. The dead crabs and exuviae were removed from the tanks. Partial water exchange (25%) was performed every 2 days in each culture tank.

Water Quality, Sample Collection, Growth Performance, and Survival Rate

Water temperature, dissolved oxygen (DO), pH, and salinity were measured daily by using a handheld multiparameter water quality analyzer (YSI, Yellow Springs, OH, United States). The planktonic microbial biomass in the water was collected once a week during the experiment. For each group, approximately 500 mL of water sampled from the three culture tanks was filtered through a sterilized 100-μm pore nylon net, followed by a 0.22-μm pore polycarbonate filter (MilliporeSigma, Burlington, MA, United States). The filters were stored in sterilized tubes, immediately snap-frozen in liquid nitrogen, and stored at −80°C until further analysis. For each group, 20 crabs were randomly collected (6–7 crabs from each culture tank) at each sampling point (1, 2, 3, and 4 weeks) and sacrificed by being placed on ice. The CL, carapace width (CW), and body height (BH) were measured at the last sampling point (4 weeks). Their individual weights were determined for weight gain. Mortality was recorded during the experiment. Gut, muscle, and hepatopancreas tissues were collected from each sampled crab, immediately snap-frozen in liquid nitrogen, and stored at −80°C until further analysis. The gut samples were used for metabolite analysis ($n = 6$) and for bacterial analysis ($n = 6$). The muscle samples were used for metabolite analysis ($n = 6$). The hepatopancreas samples were used for metabolite analysis ($n = 6$) and enzymatic and gene expression analysis ($n = 6$).

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

Biochemical Assays

Hepatopancreas samples were homogenized using a TGrinder (TIANGEN, Beijing, China) in 5-mL centrifuge tubes with 9 volumes (v/w) of 0.9% physiological saline. The homogenates were centrifuged at 3750 rpm and at 4°C for 10 min. The resultant supernatants were collected and subsequently frozen at −80°C for biochemical assays. Trypsin, lipase, amylase, superoxide dismutase (SOD), glutathione-S-transferase (GST), malondialdehyde (MDA), total antioxidant capacity (T-AOC), acid phosphatase (ACP), and alkaline phosphatase (AKP) were analyzed using specific commercial assay kits (Nanjing Jiancheng Institute, Nanjing, Jiangsu, China).

mRNA Expression Analysis of Oxidative, Stress, and Immunity Genes

Total RNA from hepatopancreas samples was extracted using TRIzol Reagent (Invitrogen, Waltham, MA, United States) according to the manufacturer's protocol. The RNA quality and quantity were assessed using a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, United States) and the OD_{260/280} values of all the RNA samples ranged from 1.86 to 2.02. The isolated RNA was then treated with RQ1 RNase-free DNase (Promega, Durham, NC, United States) to eliminate DNA contamination. First-strand cDNA synthesis in reverse transcription was performed using an M-MLV reverse transcriptase (Promega, Durham, NC, United States) according to the manufacturer's instructions. RT-qPCR was performed on a Roche LightCycler

480 Real-time PCR System (Roche, Basel, Switzerland) using a Promega GoTaq® qPCR Master Mix (Promega, Durham, NC, United States). One microliter of the resultant dilution was added as a template in a final volume of 25 µL. The reactions were carried out on a quantitative thermal cycler using SYBR green I as a fluorescent dye. The PCR conditions were as follows: 95°C for 5 min for DNA denaturation, with the amplification lasting 40 cycles (95°C for 15 s, 60°C for 15 s, and 72°C for 15 s), and final extension for 10 min at 72°C. The specificities of the PCR products were detected by melting curve analysis and sequencing. In this study, β-actin was used as a reference gene. To ensure the specificity of the intended genes, all the primers for PCR were designed to span an intron. All the primer sequences used for the PCR analyses of the genes were synthesized by the Beijing Genomics Institute (Shanghai, China) and are listed in **Supplementary Table S1**. Each PCR run was performed in triplicate and reactions without cDNA were included as negative controls. The values obtained from PCR were analyzed using the $2^{-\Delta \Delta CT}$ method (Livak and Schmittgen, 2002).

Gut and Seawater Microbiota Analysis

The bacterial genomic DNA in the gut and seawater samples was extracted using a PowerFecal™ DNA Isolation kit (MO BIO Laboratories, Carlsbad, CA, United States). The 16S rRNA genes were partially amplified using bacterial universal V3–V4 primers 338F and 806R and sequenced using an Illumina MiSeq platform as previously reported (Shi et al., 2019).

PE reads were joined with FLASH using the default settings (Magoč and Salzberg, 2011). The joined pairs were then processed using QIIME v1.9.0 (Caporaso et al., 2010). In brief, the reads were quality filtered at Q20 using the script *split_libraries_fastq.py*. The sequences that passed the quality filtering were checked for chimeras using UCHIME (Edgar et al., 2011). After the chimeras were removed, the remaining sequences were binned into operational taxonomic units (OTUs, 97% nucleotide similarity) based on identification by UCLUST (Edgar, 2010). The most abundant sequence of each OTU was taxonomically assigned in the Greengenes database (release 13.8) using PyNAST (DeSantis et al., 2006a,b). The OTUs that were not affiliated with a bacterial domain were discarded. The OTU table was a 20 × randomly rarefied subset of 27,800 sequences per sample to avoid unequal sequencing depth. The original MiSeq 16S rRNA sequence data generated in this study have been deposited in the Sequence Read Archive of the DDBJ under accession number DRA008473.

To evaluate the overall differences in the bacterial community of the seawater and crab guts, non-metric multidimensional scaling (NMDS) and an analysis of similarity (ANOSIM) were performed based on Bray–Curtis distance metrics (Clarke, 1993). To identify the indicative bacteria that were associated with each group, the indicators at the OTU level were screened (Dufrêne and Legendre, 1997) when the relative abundance of the OTU in any group was greater than 1% and the indicator values (IndVal) were both significant between groups ($p < 0.05$) and greater than 0.4 using the package labdsv in R (Roberts, 2007). The OTU level

was selected because this level of characterization provides the finest taxonomical information.

NMR-Based Metabolomic Analyses of the Tissue Samples

Tissue NMR experiments were performed as described previously (Shi et al., 2019). For all three tissue ¹H NMR spectra, the residual water signal of δ 4.7–5.2 and methanol signal of δ 3.34–3.38 were discarded. The spectral region of δ 0.8–9.0 was binned with an equal width of 0.004 ppm (2.4 Hz). The integrated areas of all the bins were normalized to the wet weight of the corresponding tissue for each spectrum to compensate for the overall concentration differences.

The normalized NMR data were subjected to the software package SIMCA-P⁺ (12.0, Umetrics, Umeå, Sweden) for multivariate data analysis. Principal component analysis (PCA) was first performed with the data mean-centered to obtain an overview of group clustering and search for possible outliers. Subsequently, orthogonal projection to latent structure discriminant analysis (OPLS-DA) was conducted with unit-variance scaled data. The OPLS-DA models were validated using a fivefold cross-validation method with R^2X and Q^2 as the quality parameters (Trygg et al., 2007) and further evaluated using a cross-validation one-way analysis of variance (CV-ANOVA) approach (Eriksson et al., 2008).

To compare the metabolite effects caused by OA exposure, data are presented as metabolite concentrations in the OA groups relative to the control 380 group ($C_{750}-C_{380}$)/ C_{380} or ($C_{1500}-C_{380}$)/ C_{380} where C_{380} , C_{750} , and C_{1500} are the metabolite concentrations in the 380, 750, and 1500 groups, respectively.

Statistical Analyses

The survival rate was analyzed using a log-rank (Mantel–Cox) test (GraphPad Prism 5, GraphPad Software, Inc., La Jolla, CA, United States). The correlations between all the detected parameters were evaluated by performing a Pearson correlation analysis of the ANOVA results. A correlation was considered significant if $p < 0.05$. A Mantel test was employed in R software to evaluate the association between the digestive and immune enzyme activities; the seawater and gut bacteria; the stress genes, immune genes, and antioxidant genes; and the metabolites of the gut, muscle, and hepatopancreas. Subsequently, a structural equation modeling (SEM) was used to illustrate the interplay of these factors under OA exposure in Amos 22.0 (IBM, Chicago, IL, United States). The data matrix was fitted using SEM based on the maximum-likelihood estimation method. A favorable model fit was confirmed by χ^2 (Chi-square)/default model test ($\chi^2/DF < 2$), the root of the mean square residual (RMR < 0.1), a high goodness-of-fit index (GFI > 0.90), and the root-mean-square error of approximation (RMSEA < 0.05).

RESULTS

Water Quality After OA Exposure

The pCO_2 and aqueous CO₂ levels obviously increased with the input of CO₂ into the water (**Figures 1A,B**). Simultaneously, the

water pH decreased from 8.06 ± 0.03 to 7.89 ± 0.04 (750 μatm) and 7.69 ± 0.04 (1500 μatm) (**Figure 1C**). Therefore, CO₂-induced OA was simulated. During the experiment, the water temperature, DO, and salinity were similar across all the groups, with small variations (**Figures 1D–F**).

Effects of OA on the Survival Rate and the Growth Performance of the Swimming Crab

The survival rate of the swimming crabs increased from 75.2% in the 380 group to 76.1% in the 750 group and 82.3% in the 1500 group after the first week of OA treatment (**Figure 2A**). Mortalities occurred mainly during the first week of OA exposure. After 4 weeks of OA treatment, the survival rate appeared to be higher in crabs from the 1500 group (81.3%), followed by those in the 750 group (74.1%) and the control group (70.8%). At the end of the experiment, the weight gain in the crabs was significantly reduced in the 1500 group compared to that of the other two groups ($p < 0.05$), although there was no significant difference between the 380 and 750 groups ($p > 0.05$, **Figure 2B**). Weight gain depends mainly on successful molting. More swimming crabs molted in the 380 group than in the two acidified groups (**Figure 2C**). Concurrently, OA negatively impacted other growth parameters such as CL and BH (**Figure 2D**).

Effects of OA on Digestibility and Oxidative and Immunity Stress in the Hepatopancreas

The crabs exposed to 1500 μatm $p\text{CO}_2$ showed significantly decreased activity of lipase after two weeks ($p < 0.05$, **Figure 3A**). However, no significant effects on the hepatopancreas lipase were found in the crabs exposed to 750 μatm $p\text{CO}_2$ ($p > 0.05$, **Figure 3A**). No significant effects of elevated $p\text{CO}_2$ were observed for the activities of the hepatopancreas amylase or trypsin ($p > 0.05$, **Figures 3B,C**).

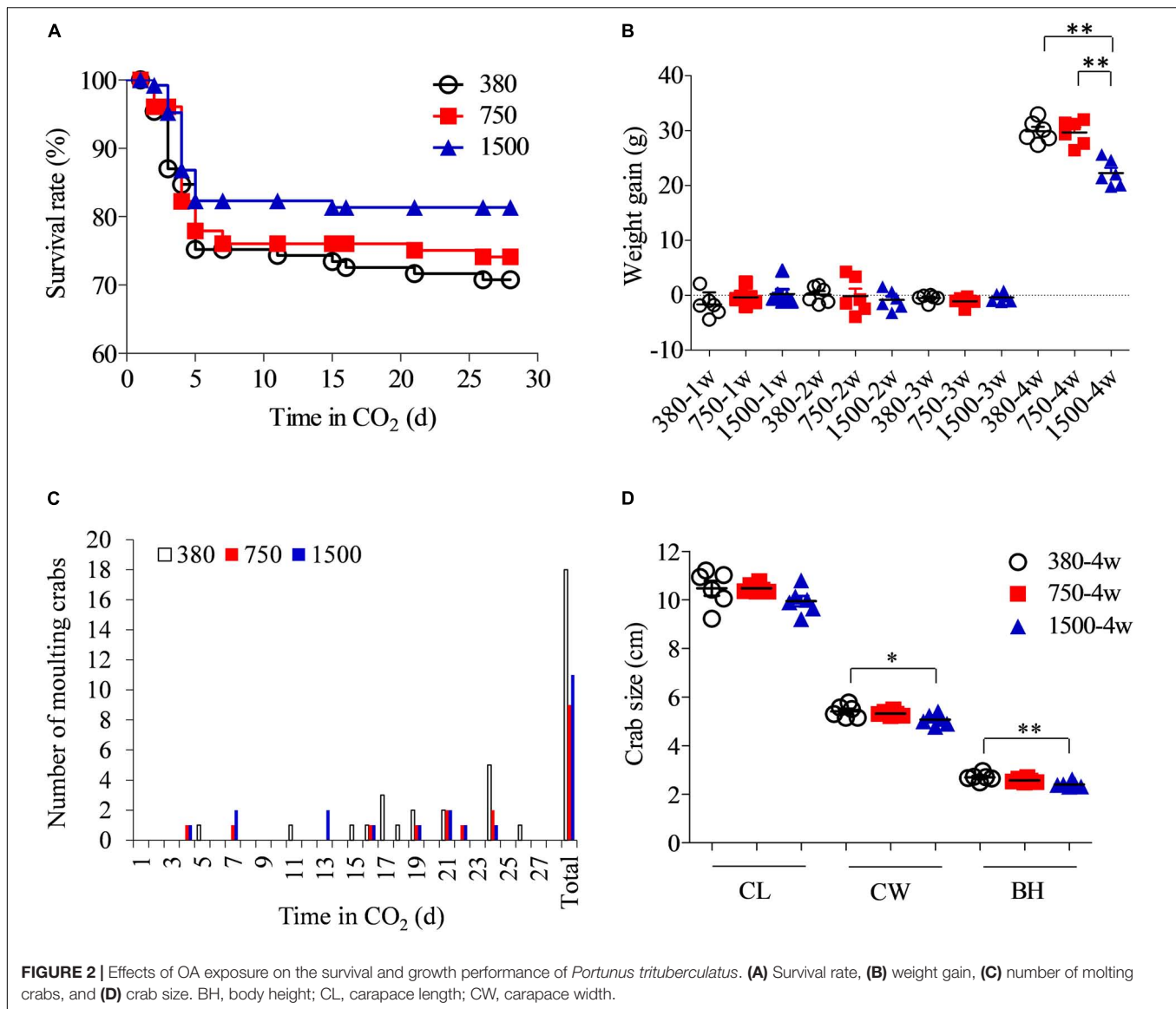
Environmental stress commonly induces oxidative stress in crustaceans; thus some oxidative stress markers were measured in the hepatopancreas at the biochemical and molecular levels. The 1500 μatm $p\text{CO}_2$ treatment markedly increased the activity of SOD at 1 week ($p < 0.05$, **Figure 3D**), with a recovery to the control level thereafter. Such a change in enzymatic activity contributed to the significantly altered mRNA expression levels of cMnSOD and ecCuZnSOD (**Figures 3E,F**). The levels of mRNA expression of cMnSOD significantly increased during the first 2 weeks of 1500 μatm $p\text{CO}_2$ exposure and there was a significant decrease at 3 weeks after exposure to 750 and 1500 μatm $p\text{CO}_2$ exposure ($p < 0.05$, **Figure 3E**). The mRNA expression levels of ecCuZnSOD significantly decreased at 1 and 3 weeks of 1500 μatm $p\text{CO}_2$ exposure and there was a significant increase at 4 weeks after exposure to 750 and 1500 μatm $p\text{CO}_2$ ($p < 0.05$, **Figure 3F**). Furthermore, 1500 μatm $p\text{CO}_2$ exposure significantly increased the activity of GST during the last three weeks ($p < 0.05$, **Figure 3G**). We also observed a marked increase in the MDA level after 3 weeks of 1500 μatm $p\text{CO}_2$ exposure ($p < 0.05$, **Figure 3I**). However, no significant

change in the T-AOC levels was observed after OA exposure ($p > 0.05$, **Figure 3H**). The expression levels of Hsp70 differed after exposure to 750 and 1500 μatm $p\text{CO}_2$, highlighted by the increased expression at 1 week and reduced expression at 3 weeks in the 750 group as well as an increased expression at 2 and 4 weeks in the 1500 group ($p < 0.05$, **Figure 3J**). We also noted that 1500 μatm $p\text{CO}_2$ significantly increased the expression of the *MT2* gene during the first 2 weeks ($p < 0.05$, **Figure 3K**). Furthermore, 750 μatm $p\text{CO}_2$ markedly stimulated the mRNA expression levels of MBD4 at 2 weeks, but 1500 μatm $p\text{CO}_2$ induced a significant change in the mRNA expression levels of MBD4 during the whole period of exposure ($p < 0.05$, **Figure 3L**).

The immune function is generally impaired when crustaceans are exposed to environmental stress; some immunity indices, therefore, were measured in the hepatopancreas. Our results showed that only the 1500 μatm $p\text{CO}_2$ treatment suppressed the activities of ACP and AKP at 4 weeks ($p < 0.05$, **Figures 3M,N**). Concurrently, both 750 and 1500 μatm $p\text{CO}_2$ markedly stimulated the mRNA expression level of CTL2 at 2 weeks ($p < 0.05$, **Figure 3O**). Only 1500 μatm $p\text{CO}_2$ markedly stimulated the mRNA expression level of TRAF6 at 1 and 3 weeks ($p < 0.05$, **Figure 3P**). In addition, 750 μatm $p\text{CO}_2$ markedly reduced the mRNA expression level of gC1qR during the latter 2 weeks of exposure, whereas 1500 μatm $p\text{CO}_2$ markedly reduced it only at 4 weeks ($p < 0.05$, **Figure 3Q**).

Effects of OA on the Seawater and Gut Bacteria

After quality control, a total of 8,490,028 high-quality sequences were obtained with an average of $51,144 \pm 13,106$ reads per sample (mean $\pm$ SD). Then, the unequal sequencing depths were reduced to 27,800 sequences per sample, resulting in 30,192 OTUs across all samples. The dominant phyla/classes represented in the gut at the 1-week samples included Tenericutes (46.32%), alphaproteobacteria (18.88%), and gammaproteobacteria (10.04%) (**Supplementary Figure S1a**). However, the dominant phyla/classes found in seawater at the 1-week samples included alphaproteobacteria (64.49%), Bacteroidetes (9.75%), and gammaproteobacteria (6.83%) (**Supplementary Figure S1b**). Furthermore, the composition of the bacterial community in both the control seawater and control crab gut changed over time. Time appears to have a greater effect than OA exposure on the bacteria (**Supplementary Figure S2**). However, bacterial community changes related to OA exposure were also observed when compared to the control, despite there being no significant difference in the Shannon index ($p > 0.05$, **Supplementary Figure S2**). Based on the Bray–Curtis distances of the OTUs detected across samples, an NMDS ordination biplot showed a separation between water and gut bacterial communities (**Figure 4A**). The NMDS biplot did not exhibit any clear clustering in the gut bacterial communities (**Figure 4B**) but did show a clear clustering in the aquatic bacterial communities (**Figure 4C**). The clusters identified from the aquatic bacterial communities were mainly based on developmental time, with subclusters based on $p\text{CO}_2$ concentrations. ANOSIM was used to further corroborate this pattern and revealed a similar bacterial



community composition between each of acidified gut tissues and its control counterpart (**Supplementary Table S2**). However, a significantly distinct bacterial community composition in some acidified seawater samples from their control counterparts was observed (**Supplementary Table S3**). For example, the bacterial community composition was significantly distinct between the W380 group and the W750 group at 4 weeks.

Given the discrete bacterial communities found in the gut and seawater, the assemblages that characterized such differences were studied. Here, the indicators of the dominant (average relative abundance > 1% at least in one group) bacterial OTU level were screened with significant differences between groups ($p < 0.05$) and IndVal values > 0.4. In the gut samples, only one OTU, belonging to *Sunxiuqinia*, was highly relative abundance in the 1500 group at 1 week (**Figure 5A**). At 2 weeks, two OTUs, belonging to *Robiginitalea* and *Oceanicola*, were the most abundant in the 1500 group. At 3 weeks, two OTUs, belonging

to *Salinicoccus roseus* and *Bacillaceae*, were most abundant in the 750 group, while *Sunxiuqinia* remained the most abundant in the 1500 group. At 4 weeks, *Candidatus bacilloplasma* was the most abundant in the control group, whereas *Alcanivorax* and *Rubritalea* were the most abundant in the 1500 group. In contrast, the water sample presented a substantial change in the composition of the bacterial communities, and many more OTUs were screened during the experiment (**Figures 5B–E**) (see details in *SI Text, Results: Effects of OA on the seawater bacteria*).

Effects of OA on the Metabolites of Crab Tissues

Typical ¹H NMR spectra from crab gut (**Supplementary Figures S3a,b**), muscle (**Supplementary Figures S3c,d**), and hepatopancreas (**Supplementary Figures S3e,f**) extracts showed a rich metabolite composition (**Supplementary Table S4**). PCA and OPLS-DA did not show any apparent clustering among

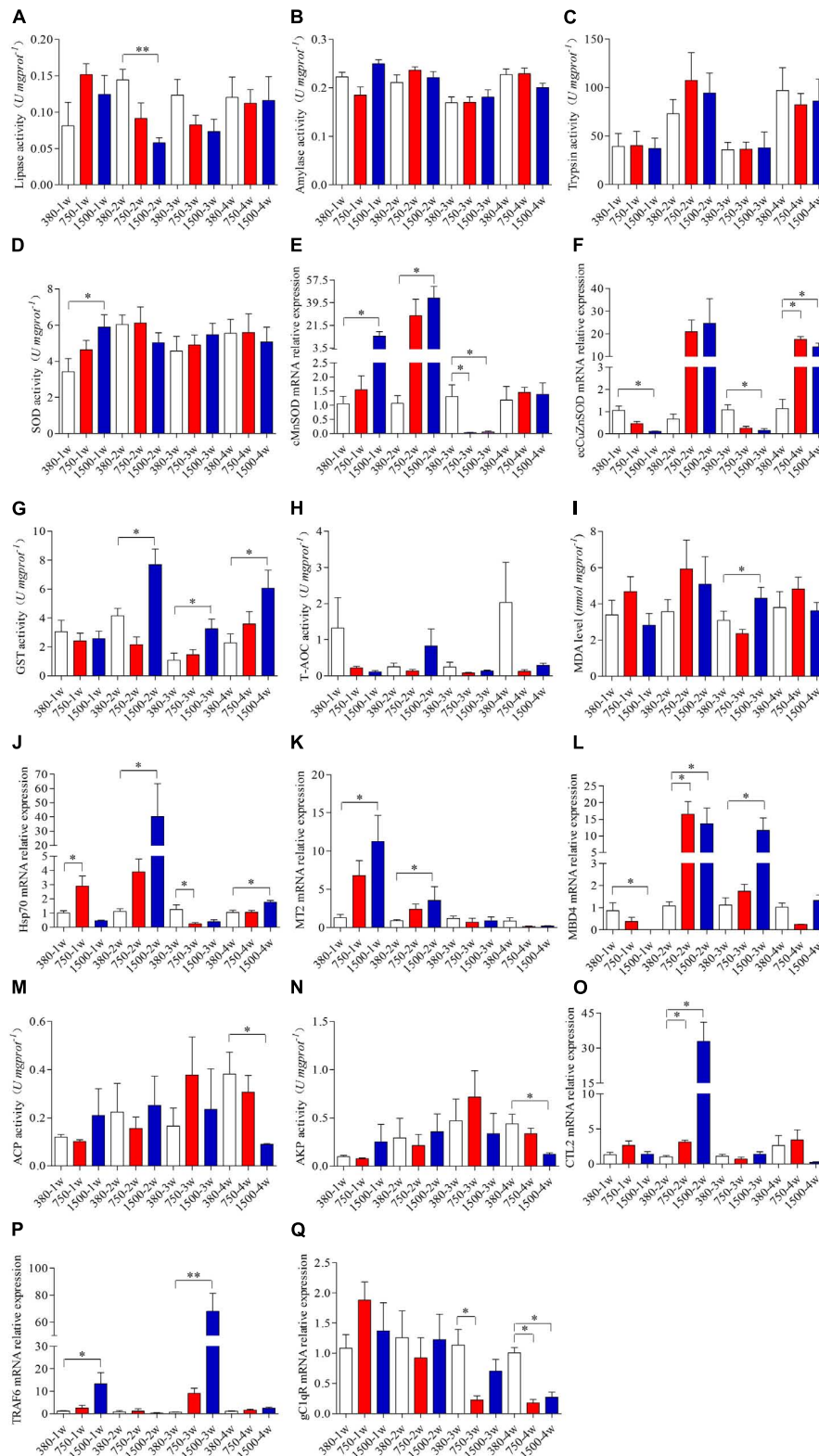
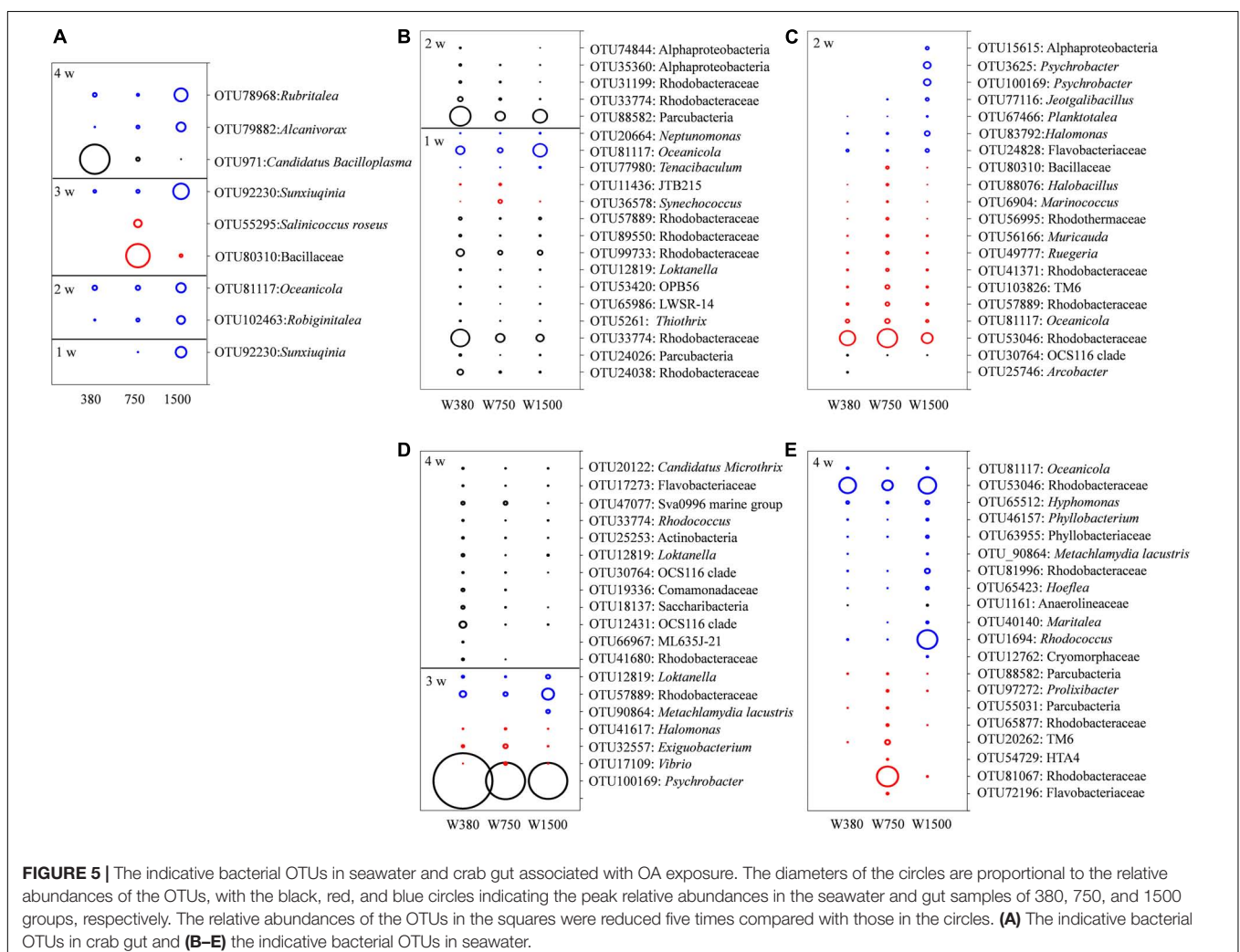
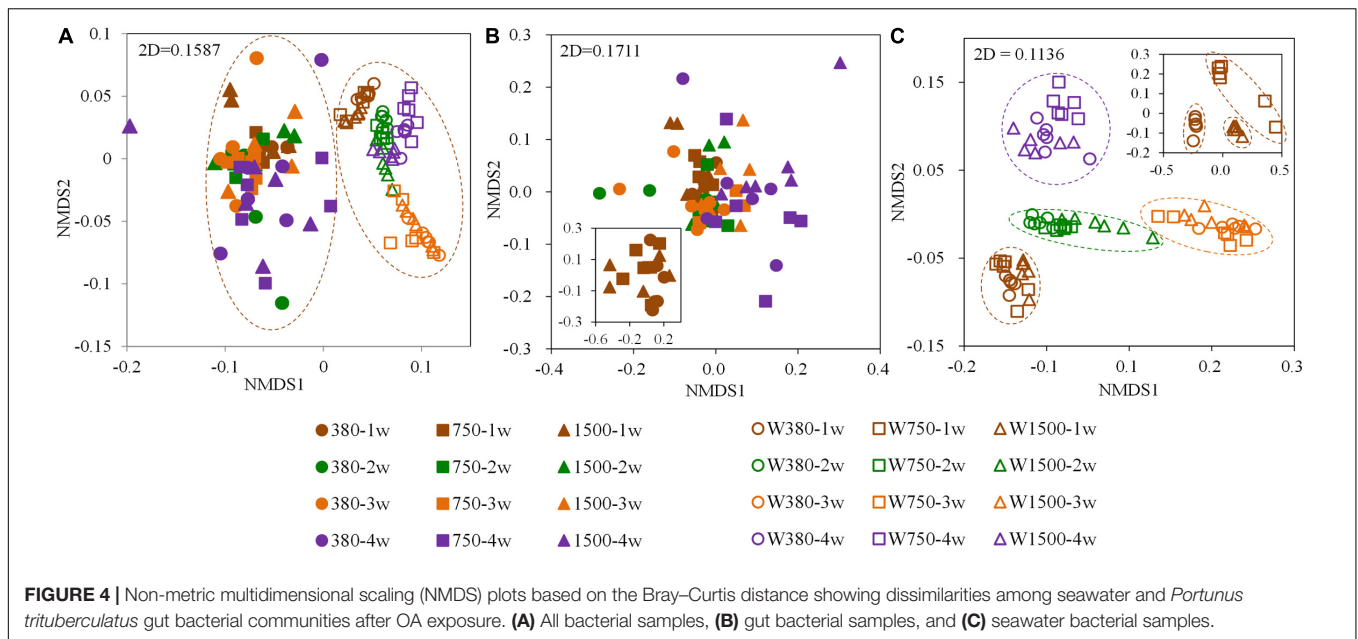


FIGURE 3 | Effects of OA exposure on the digestive enzyme activities, oxidative stress responses, and innate immunity responses of hepatopancreas of *Portunus trituberculatus*. **(A)** Lipase, **(B)** amylase, **(C)** trypsin, **(D)** superoxide dismutase (SOD), **(E)** cMnSOD, **(F)** ecCuZnSOD, **(G)** glutathione-S-transferase (GST), **(H)** total antioxidant capacity (T-AOC), **(I)** malondialdehyde (MDA), **(J)** Hsp70, **(K)** MT2, **(L)** MBD4, **(M)** acid phosphatase (ACP), **(N)** alkaline phosphatase (AKP), **(O)** CTL2, **(P)** TRAF6, and **(Q)** gC1qR.



the three groups at each sampling time, suggesting a lack of significant differences in metabolic phenotypes in the three tissues between the exposed and control crabs (**Supplementary Figure S4**). However, some metabolite changes appeared to be associated with OA exposure time (**Figure 6**). Overall, 1500 μatm $p\text{CO}_2$ caused a stronger effect than 750 μatm $p\text{CO}_2$ on the metabolite changes. Furthermore, the same $p\text{CO}_2$ concentration induced different metabolite changes in three tissues. Exposure to 750 μatm $p\text{CO}_2$ caused a less than 50% increase in the levels of 12 gut amino acids at 4 weeks. Exposure to 1500 μatm $p\text{CO}_2$ caused a higher increase than 750 μatm $p\text{CO}_2$ in the levels of these amino acids, as shown by an $\approx 150\%$ increase in tyrosine and tryptophan levels and a 73–86% increase in the levels of three branched chain amino acids and phenylalanine at 4 weeks. Some changes in the levels of muscle metabolites were observed due to elevated $p\text{CO}_2$. Exposure to 1500 μatm $p\text{CO}_2$ caused a 55–78% increase in the levels of seven amino acids at 4 weeks. It also caused a 42.0% increase at 1 week and a continuous decline thereafter in the ADP level, together with a 49.0% increase in the AMP level at 4 weeks. Furthermore, 750 μatm $p\text{CO}_2$ caused an 80% increase in the muscle ADP level together with a 69.5% decrease in the muscle AMP level at 2 weeks. The hepatopancreas showed the smallest metabolite changes following OA exposure. Exposure to 750 μatm $p\text{CO}_2$ caused a 41.2% increase in the COS level at 1 week as well as an approximately 45% decrease in the deoxyguanosine, uridine, and cytidine levels at 2 weeks. Exposure to 1500 μatm $p\text{CO}_2$ caused a 57.0% increase in the COS level at 1 week as well as a respective 42.3% and 69.4% increase in the tryptophan and glucose levels at 4 weeks.

Influencing Factors of Crab Survival and Growth

To further investigate which factor explains growth and survival effects in crabs exposed to elevated $p\text{CO}_2$, correlation analyses between all the variables were performed. Crab growth showed significant ($p < 0.05$ in all cases) positive correlations with the gut and seawater bacteria, three tissue metabolites, and crab survival, whereas crab survival was significantly and positively correlated with the digestive, antioxidant, and immune enzymes; the gut and muscle metabolites; and the gut and seawater bacteria (**Supplementary Table S5**). Furthermore, the seawater bacteria were positively correlated with the gut bacteria; three tissue metabolites; and the digestive, antioxidant, and immune enzymes. The gut metabolites had positive correlations with the hepatopancreas and muscle metabolites. The digestive enzymes were also positively correlated with the antioxidant and immune enzymes. An SEM model was constructed to further explore the direct/indirect effects of some physiological indices, microbiota, and metabolites on crab growth and survival. The SEM model fit the data well with $\chi^2/\text{DF} = 0.66$, $P = 0.95$, GFI = 0.95, RMSEA = 0.00, RMR = 0.05 and could explain 100% of the variance in both crab growth and survival (**Figure 7**). The muscle metabolites ($\lambda = 0.50$), gut metabolites ($\lambda = 0.48$), and hepatopancreas metabolites ($\lambda = 0.45$) contributed significantly to the positive direct effects on crab growth. The carbonate system ($\lambda = 0.80$), antioxidative enzymes ($\lambda = 0.24$), seawater bacteria ($\lambda = 0.16$), and gut bacteria ($\lambda = 0.14$) contributed

significantly to explaining the positive effects on crab survival, and were coupled with a significant negative effect from the digestive enzymes ($\lambda = -0.31$). We also observed comprehensive interplay among these indices (see details in *SI Text, Results: Interplays among detected indices*), indicating indirect effects on crab growth and survival.

DISCUSSION

Ocean acidification has comprehensive effects on the growth and development (Long et al., 2013a), physiology and metabolism (Maus et al., 2018), morphology (Ren et al., 2017), and behavior of a variety of marine crabs (Wang et al., 2018). However, the effects of OA have been reported only on the carapace morphology and behavior of juvenile *P. trituberculatus* (Ren et al., 2017, 2018). To the best of our knowledge, this is the first study to reveal the comprehensive effects of OA exposure on *P. trituberculatus*. Our results showed that OA has a mixed effect on swimming crabs, with increased survival and retarded growth. The negative effect on growth is in line with findings published for other crab species such as larvae of *H. araneus* (Walther et al., 2010; Schiffer et al., 2013, 2014; Wang et al., 2018), *Paralithodes camtschaticus*, and *Chionoecetes bairdi* (Long et al., 2013a,b), as well as embryos of *Petrolisthes cinctipes* (Carter et al., 2013; Ceballos-Osuna et al., 2013). Such a consensus between studies performed on different species, at different stages of development and with $p\text{CO}_2$ ranging from 710 to 3100 μatm , strongly supports crab sensitivity to OA. McLean et al. (2018) suggested that the reduction in growth would be due to metabolic suppression, reduced calcification, or energy reallocation. This study provides more information on this question through the analyses of the digestive physiology, antioxidant capacity, stress response, immune function, microbiome, and metabolome.

Influencing Factors of Retarded Crab Growth

Based on the SEM, three tissue metabolites and gut bacteria appeared to play an important role in crab growth. The elevated $p\text{CO}_2$ elicited an increase in the levels of lactate, glucose, and 12 amino acids in the crab gut, which strongly supports the metabolic depression hypothesis. Metabolic depression may be a beneficial strategy for crabs to respond to an acute stress (Langenbuch and Pörtner, 2004) suggested in other crabs such as *Carcinus maenas* juveniles (Maus et al., 2018) and *P. cinctipes* embryos (Carter et al., 2013), as well as other marine animals (Reipschläger and Pörtner, 1996; Pörtner et al., 1998; Michaelidis et al., 2005; Nakamura et al., 2011) under elevated $p\text{CO}_2$ conditions. OA is capable of enhancing the production of reactive oxygen species (ROS), which has been found to induce oxidative stress in shrimp, *Litopenaeus vannamei* (Wang et al., 2009), and American lobster, *Homarus americanus* (Menu-Courey et al., 2019). A significant increase in the hepatopancreas MDA level further supports the oxidative stress response induced by OA. Oxidative stress inhibited aerobic glycolysis and enhanced protein catabolism (Schock et al., 2010). The increased lactate and glucose levels with elevated $p\text{CO}_2$ are indicative of a change in the

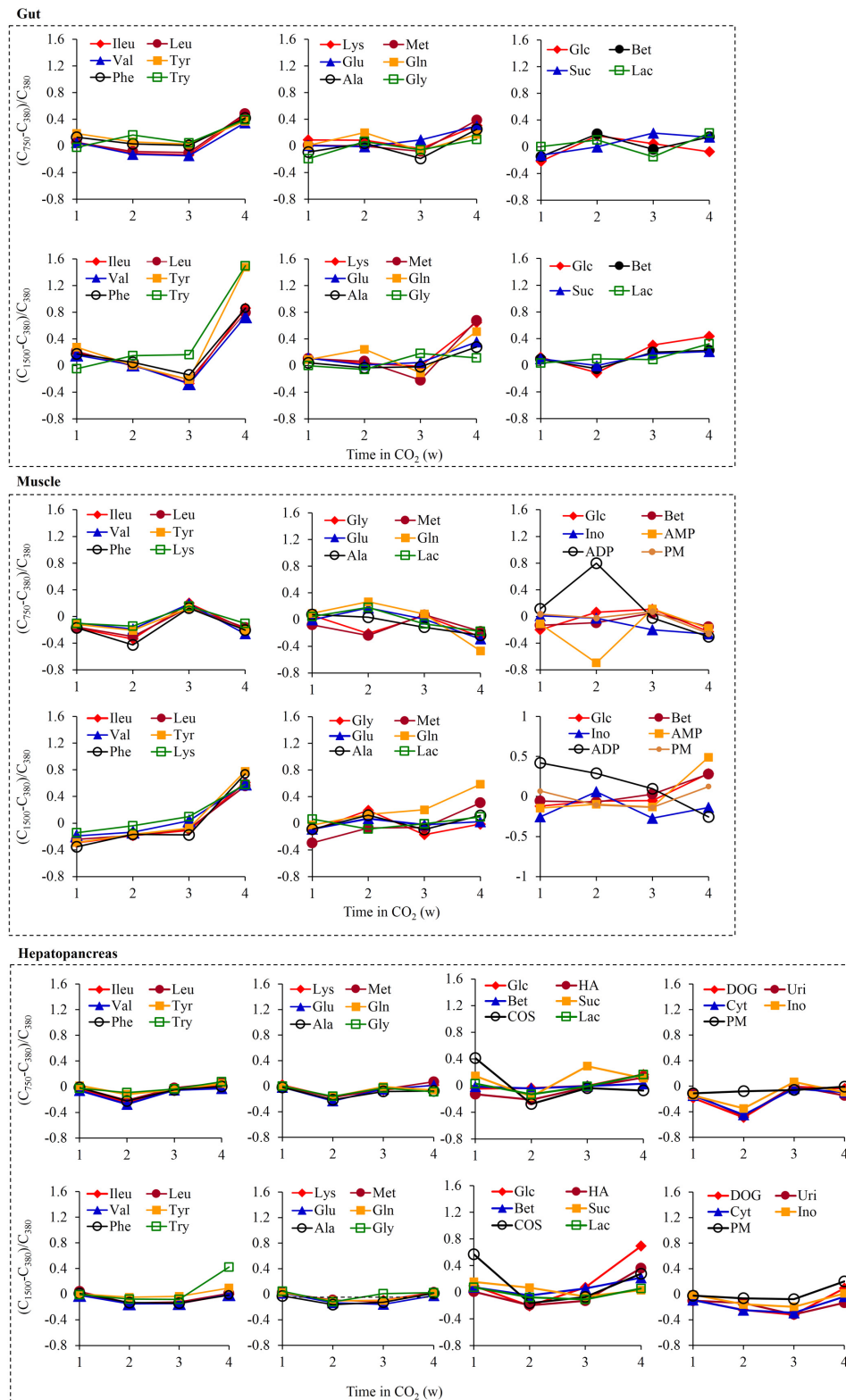
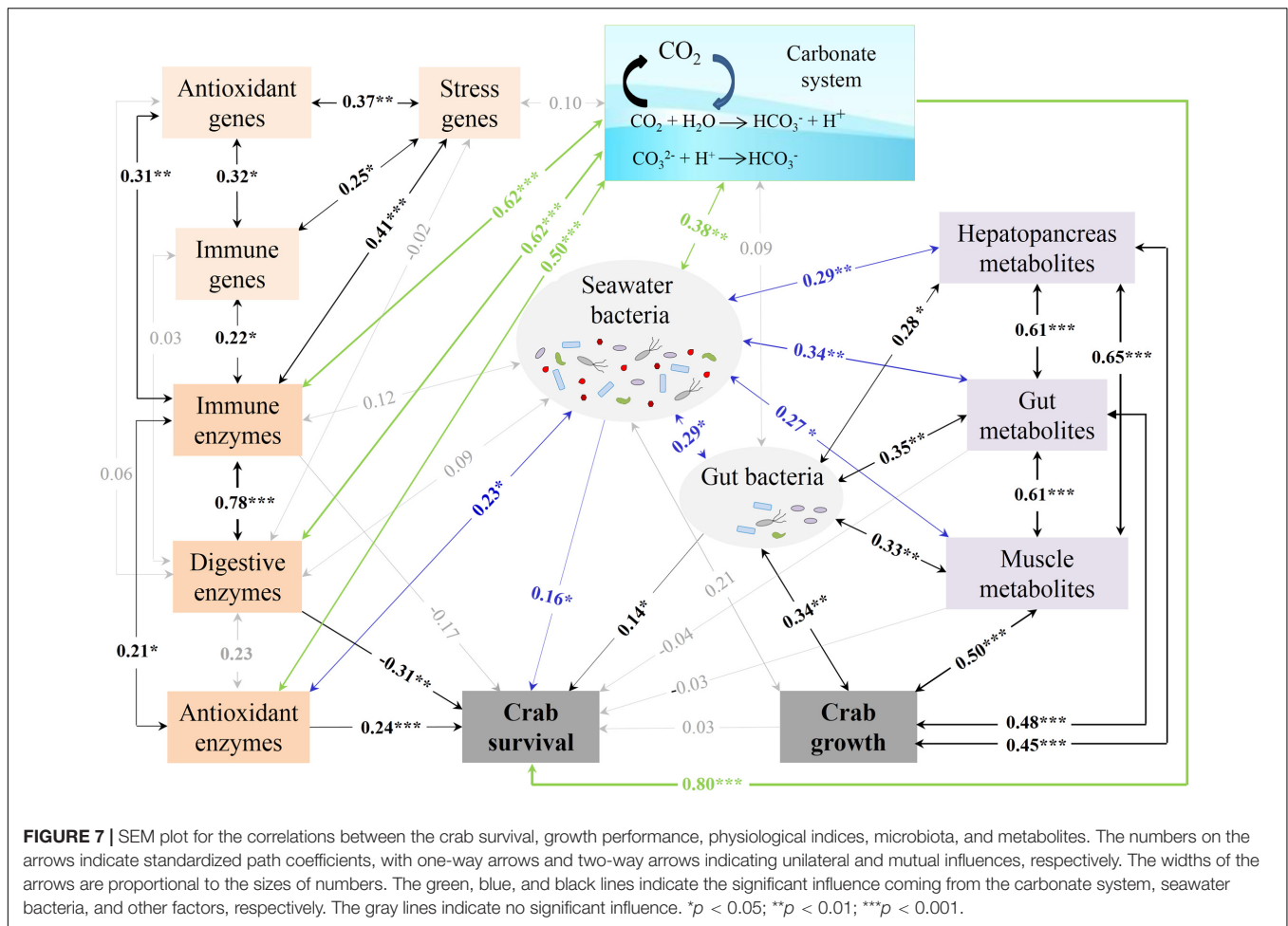


FIGURE 6 | Time dependence of the individual metabolite variations. The values of the y-axis were calculated relatively to the levels of the 380 group as $(C_{750}-C_{380})/C_{380}$ or $(C_{1500}-C_{380})/C_{380}$ where C_{750} and C_{1500} were the respective metabolite concentrations in the 750 group and 1500 group and C_{380} was the metabolite concentration in the 380 group. ADP, adenosine diphosphate; Ala, alanine; AMP, adenosine monophosphate; Bet, betaine; COS, choline-O-sulfate; Cyt, cytidine; DOG, deoxyguanosine; Glc, glucose; Gln, glutamine; Glu, glutamate; Gly, glycine; HA, histamine; Ileu, isoleucine; Ino, inosine; Lac, lactate; Leu, leucine; Lys, lysine; Met, methionine; Phe, phenylalanine; PM, 2-pyridinemethanol; Suc, succinate; Try, tryptophan; Tyr, tyrosine; Uri, uridine; Val, valine.



energy metabolism from aerobic to anaerobic respiration (Pane and Barry, 2007). The increased lactate level was also observed in dungeness crab (*Cancer magister*) (Pane and Barry, 2007) and in the warm-acclimated spider crab (*Hyas araneus*) (Zittier et al., 2013) subjected to short-term hypercapnia. Increased levels of both glucose and lactate were found in the blue crab (*Callinectes sapidus*) subjected to oxidative stress (Schock et al., 2010). However, our results are in contrast with the results of Hammer et al., who found no change in the glucose level and a significant decrease in the lactate level in the hemolymph of the green shore crab (*Carcinus maenas*) following exposure to elevated CO₂ (Hammer et al., 2012). One possible explanation for this discrepancy is that very high $p\text{CO}_2$ levels ranging between 2600 and 30,000 μatm were used in experiments on the green shore crab. This transition of respiration mode likely results in an inhibited glucose utilization. In addition, the increased levels of 12 amino acids in the crab gut probably result from protein catabolism. A shift to the catabolism of protein or amino acids occurred in *M. edulis* exposed to elevated CO₂ levels (Pörtner et al., 1998). Amino acid catabolism was observed in the green shore crabs exposed to elevated CO₂ (Hammer et al., 2012). Amino acid catabolism serves to produce ammonia to buffer surplus protons induced by OA (Lindinger et al., 1984). However,

amino acid catabolism was not measured in the swimming crabs in this study, likely indicating that a new acid–base equilibrium is established in the gut of surviving swimming crabs.

Muscle metabolites were also significantly correlated with crab growth. The elevated $p\text{CO}_2$ induced a transient rise followed by a continuous decline in free ADP levels and a rise in AMP levels, in combination with an increase in free amino acid levels. Decreased ATP/ADP levels were found in the gills of the green shore crab (Hammer et al., 2012) and in the mantles of oysters (*Crassostrea gigas*) (Lannig et al., 2010) exposed to elevated CO₂. These fluctuations in free ADP and AMP levels suggest an oscillating ATP turnover rate and a disturbed energy homeostasis in the muscle of swimming crabs. This view is supported by findings of the peanut worm (*Sipunculus nudus*) showing oscillations in high-energy phosphate levels during the hypercapnia (Pörtner et al., 1998). Furthermore, the increased AMP levels, which function as a direct agonist of AMP-activated protein kinase (Hardie, 2003), suggest net ATP conservation in the crab muscle at 4 weeks. It is well known that crabs need accumulating HCO₃[−] for compensate OA-induced extracellular and intracellular acidosis (Spicer et al., 2007; Tresguerres et al., 2008). Na⁺/K⁺-ATPase is responsible for establishing an ion gradient that can drive carbonic anhydrase, a

HCO₃[−] exchanger. These two enzymes both require a substantial amount of cellular energy (Pörtner et al., 2011). Thus, cellular energy is reallocated to the acid–base regulation. The gills are regarded as the major organ involved in extracellular ion homeostasis (Henry et al., 2012). The increased energy cost due to ion transport in the gills may elicit energy budget reallocation in other tissues of swimming crabs. Therefore, the energy oscillation in muscle might reflect the energy budget reallocation to reach a new acid–base equilibrium. We also found that 1500 μatm *p*CO₂ caused an increase in the levels of some amino acids in the muscle at 4 weeks, which may be related to the increased protein catabolism. However, the function of free amino acids in both the gut and muscle remains unknown. It is likely that the function is involved in osmoregulation (Hammer et al., 2012) that is directly linked to acid–base homeostasis (Whiteley et al., 2001). Nonetheless, OA-induced energy oscillation may come at the expense of the growth of swimming crabs.

Hepatopancreas metabolites were also significantly correlated with crab growth. An increase in glucose levels and an overall decrease in amino acid levels in the hepatopancreas provided more supporting information on the metabolism suppression of OA-exposed swimming crabs. These observations indicate amino acid catabolism in the hepatopancreas. We also hypothesized that protein catabolism occurred in this tissue because of a lack of energy needed for the energetically expensive protein synthesis under OA conditions. The enhanced protein and amino acid catabolism likely results in NH₄⁺ production (Pörtner et al., 1998). Another relevant metabolite is COS, which is produced by choline under hypoxic conditions and by betaine under aerobic conditions. The highly increased COS levels and almost unchanged betaine levels further support acute anoxia in the hepatopancreas under OA exposure. COS can function as an osmoregulatory solute (Hanson et al., 1991; Park and Gander, 1998). However, in this study, it may act to detoxify sulfate by the conjugation of sulfate with choline (Rivoal and Hanson, 1994). This is because elevated *p*CO₂ leads to the accumulation of sulfate as a counter ion for the elevated cation concentrations under acidified seawater, which has been observed in the hemolymph in Dungeness crabs, *Metacarcinus magister* (Hans et al., 2014). Taken together, the inhibited aerobic glycolysis, energy oscillation, and enhanced protein and amino acid catabolism could partly explain the retarded growth of swimming crabs exposed to elevated *p*CO₂.

The gut bacteria are usually closely associated with the metabolites of the host (Shi et al., 2019). Thus, it is not surprising to find that the gut bacteria were significantly associated with crab growth. However, only a minor impact was induced by OA exposure on the gut bacteria, suggesting that swimming crabs are capable of keeping the gut bacterial community steady. Nonetheless, OA still caused an overabundance of certain OTUs belonging to *Sunxiuqinia*, *Robiginitalea*, *Oceanicola*, *Alcanivorax*, and *Rubritalea*. These bacteria are residents in the marine environment and some have distinctive ecological functions. For instance, the genus *Alcanivorax* is well known for its ability to degrade *n*-alkanes (Wu et al., 2009; Lai et al., 2016; Konieczna et al., 2018). The genus *Oceanicola* is capable of

degrading polycyclic aromatic hydrocarbons (Yuan et al., 2009; Gutierrez et al., 2017). *Robiginitalea* and *Rubritalea* have been characterized as the producers of carotenoids (Song et al., 2018) with antioxidant activities (Shindo and Misawa, 2014). In contrast, OA caused a decrease in the relative abundance of *Candidatus bacilloplasma*. Such a development of bacterial growth and decline in the gut microecological environment indicates a slight disturbance to the healthy gut bacterial community composition. This might trigger the transition of crab fitness from healthy to sub-healthy. This assertion is supported by recent data showing that a dysbiosis in the gut microbial community happens in diseased shrimps and crabs (Xiong, 2018; Shi et al., 2019). The underlying reason for the change in gut microbial community composition is probably reallocation of energy to balance intracellular stress (Hsp70) induced by external pressure (OA pressure in this study), which in turn weakens the ability of the host to filter on alien bacterial species. The energy may be partly reallocated to antioxidant activity (SOD, GST, cMnSOD, and ecCuZnSOD) and immunological activity (ACP, AKP, CTL2, TRAF6, and gC1qR) under OA stress.

Influencing Factors of Enhanced Crab Survival

The enhanced survival of swimming crabs under OA was expected and observed in this study, despite the lack of enhanced survival in other crabs (Ceballos-Osuna et al., 2013; Long et al., 2013a; Ragagnin et al., 2018; Ramaglia et al., 2018). Based on the SEM, crab survival was mainly explained by the carbonate system, antioxidative enzymes, seawater bacteria, gut bacteria, and digestive enzymes (Figure 7). A substantial body of evidence has shown that OA is a stress to both crustaceans and other marine organisms (Ceballos-Osuna et al., 2013). In this study, the total population of seawater bacterial communities was rapidly and significantly affected by elevated *p*CO₂, strongly suggesting that seawater bacteria seem to be sensitive to elevated *p*CO₂ as found in reef biofilms and clam aquaculture water (Witt et al., 2011; Zha et al., 2017). In general, bacteria are flexible and show a potential to adapt to environmental stress. Regarding the short-term (only 4 weeks) exposure in this study, the shifts in the seawater bacterial community probably come from community succession rather than genetic variation. In other words, sensitive bacterial species are replaced by non- or less sensitive ones (Liu et al., 2010). We observed a significant increase in the relative abundance of 22 indicative OTUs, such as *Tenacibaculum* at 1 week and Flavobacteriaceae at 2 weeks, and a significant decrease in the relative abundance of 29 indicative OTUs after OA exposure. These changed OTUs may be the keystone species affected by OA in seawater. However, very few studies are related to the impact of OA exposure on seawater bacteria, except those in biofilms from the Australian Great Barrier Reef and seawater for blood clam farming (Witt et al., 2011; Zha et al., 2017). Nevertheless, a shift in the bacterial community composition means a changed microbial environment for swimming crab, which probably resulted in a changed seawater quality given the important roles played by bacteria in the biogeochemical cycles of marine ecosystems (Liu et al., 2010; Maas et al., 2013).

For example, a faster bacterial turnover of polysaccharides at a relatively low ocean pH has been found (Piontek et al., 2010). The global N₂ fixation potential of *Trichodesmium* could be reduced under acidified conditions (Hong et al., 2017; Luo et al., 2019). Although it is difficult to unravel the exact functions of the bacteria, which are indicative of the seawater status, in this study, their changes did have a significant direct contribution to the survival of swimming crabs. Furthermore, the significance of seawater bacteria may be beneficial not only for crab survival but also for its effects on the gut bacteria, tissue metabolites, and enzyme activity in swimming crabs (Figure 7).

Although the changed gut bacterial community retarded crab growth, it has a significant and beneficial effect on crab survival. One possible reason for this is that bacteria such as *Sunxiuqinia* and *Robiginitalea* are not pathogens. The overabundance of these bacteria can reduce empty niches for pathogen invasion, thus providing a positive contribution to crab survival.

Furthermore, antioxidative enzymes significantly and positively contributed to crab survival. A rapid significant increase in the activities of SOD and GST was observed as well as a quick significant regulation in the mRNA expression of cMnSOD and eCuZnSOD in the hepatopancreas after OA exposure, indicating a significantly improved antioxidative capacity of swimming crabs. This improved antioxidative capacity may help swimming crabs quickly respond and even eliminate OA-induced oxidative stress, which promotes crab survival.

Digestive enzymes also showed a significant and negative contribution to crab survival; however, among the three digestive enzymes detected, only lipase activity was decreased with the elevated pCO₂ at 2 weeks. The decreased lipase activity likely represents a possible switch from lipid to protein metabolism in the hepatopancreas as previously described in the larvae of the intertidal crab *P. cinctipes* (Carter et al., 2013). This metabolic transition covers the energetic cost in response to elevated pCO₂ and may limit crab survival.

CONCLUSION

Ocean acidification led to an antioxidative response, immune responses, metabolic depression, and changed gut bacteria in the swimming crabs via direct generalized oxidative stress and/or an indirect effect of seawater bacteria. These active responses effectively enhanced survival, but at the cost of the growth of swimming crabs. Furthermore, these active responses might endow swimming crabs with a faster response when first facing acidified seawater and improve the transgenerational flexibility of this species. This is because similar features have been found in the low-salinity tolerant swimming crabs (Ye et al., 2014). Importantly, seawater bacteria presented not only a direct contribution to crab survival and growth but also an indirect contribution through the significant interplay with physiological indices, gut bacteria, and tissue metabolites. However, the functions of seawater bacteria are complex and largely unknown and require further study in the future.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the DNA Data Bank of Japan, accession number DRA008473.

AUTHOR CONTRIBUTIONS

WL conceived and designed the experiments, analyzed the data, wrote the manuscript, prepared the figures and/or tables, and reviewed drafts of the manuscript. ZR performed the experiments, analyzed the data, and reviewed drafts of the manuscript. CM conceived and designed the experiments and reviewed drafts of the manuscript. YY conceived and designed the experiments, analyzed the data, wrote the manuscript, prepared the figures and/or tables, and reviewed drafts of the manuscript. CW conceived and designed the experiments and reviewed drafts of the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fphys.2020.00750/full#supplementary-material>

FIGURE S1 | Relative abundances of the dominant bacterial phyla within the crab gut (a) and seawater (b) groups after OA exposure. The bacterial phylotypes with relative abundance > 1% in at least one group were selected. Proteobacteria were grouped at the class level.

FIGURE S2 | Comparisons of crab gut (a) and seawater (b) bacterial α -diversity after OA exposure.

FIGURE S3 | Typical 600 MHz ¹H NMR spectra of gut, muscle, and hepatopancreas extracts of *Portunus trituberculatus* in the 380 and 1500 groups at 4 weeks. Resonance assignments are given in **Supplementary Table S4**. The regions of δ 5.2–9.2 in the three tissue spectra were vertically expanded eight times compared with the region of δ 0.8–4.8. Keys: 1, isoleucine; 2, leucine; 3, valine; 4, ethanol; 5, lactate; 6, threonine; 7, alanine; 8, methionine; 9, glutamate; 10, succinate; 11, glutamine; 12, β -alanine; 13, sarcosine; 14, succinimide; 15, aspartate; 16, choline-O-sulfate; 17, lysine; 18, phosphorylcholine; 19, arginine; 20, betaine; 21, taurine; 22, glycine; 23, β -glucose; 24, α -glucose; 25, maltose; 26, uracil; 27, cytidine; 28, uridine; 29, fumarate; 30, tyrosine; 31, histamine; 32, phenylalanine; 33, xanthine; 34, deoxyguanosine; 35, hypoxanthine; 36, inosine; 37, tryptophan; 38, adenosine monophosphate; 39, adenosine diphosphate; 40, 2-pyridinemethanol; 41, formate; 42, residual methanol; and 43, α -CH of glucose and amino acids.

FIGURE S4 | Principal component analysis plots of ¹H NMR spectra for the gut, muscle, and hepatopancreas extracts of *Portunus trituberculatus*

after 380 (open symbols), 750 (red symbols), 1500 (blue symbols) μatm pCO₂ exposure at 1 (dots), 2 (squares), 3 (triangles), and 4 (diamonds) weeks.

TABLE S1 | The primer sequences used in the present study.

TABLE S2 | Community dissimilarity test of the bacterial community structure in the crab gut samples based on analysis of similarity using the Bray–Curtis distance.

TABLE S3 | Community dissimilarity test of the bacterial community structure in the seawater samples based on an analysis of similarity using the Bray–Curtis distance.

TABLE S4 | ¹H NMR chemical shifts for metabolites from the gut, muscle, and hepatopancreas extracts of *Portunus trituberculatus*.

TABLE S5 | Mantel tests demonstrate the Pearson correlations of the crab survival, growth, physiological indices, microbiota, and metabolites.

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Effects of Sudden Drop in Salinity on Osmotic Pressure Regulation and Antioxidant Defense Mechanism of *Scapharca subcrenata*

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Salinity is an important ecological factor that impacts the growth and survival of aquatic organisms. The salinity of seawater in coastal and estuarine areas is often subject to dynamic changes because of seasonal rainfall and continental runoff. Thus, the current study investigated the effects of sudden changes in salinity on the survival rate and osmotic pressure regulation mechanisms of bottom-sowing seedlings of the economically important ark shell, *Scapharca subcrenata*. By simulating the sudden changes that occur in seawater salinity after rainstorms, the results showed that the osmotic pressure of the hemolymph and Na^+ , K^+ , Ca^{2+} , and Cl^- concentrations first decreased and then increased. When the salinity decreased from 30 to 14‰, hemoglobin, soluble total protein, taurine, and total free amino acid gradually increased; maximum levels of hemoglobin, soluble total protein, and taurine occurred once the salinity increased to 22‰ at 96 h. After 96 h, the total free amino acid content increased until 144 h. The reactive oxygen species (ROS) content and total antioxidant capacity (T-AOC) peaked at 96 h, whereas the expression levels of Mn-superoxide dismutase (MnSOD) and catalase (CAT) increased earlier, indicating that, with continuous ROS generation, antioxidant defense mechanisms were activated to avoid oxidative damage. Expression levels of cathepsin C (CTSC), cathepsin D (CTSD), heat shock protein 20 (HSP20), and heat shock protein 70 (HSP70) were significantly higher than in the control group at 48 h (salinity level 14‰); the expression levels of HSP20, heat shock protein 90 (HSP90), MnSOD, and glutathione peroxidase (GP_x) remained high, indicating that they were still required for osmotic pressure regulation to maintain the dynamic balance between the generation and removal of ROS as the salinity level increased. These results not only add to our basic understanding of the aquatic ecology of *S. subcrenata*, but also provide a theoretical ground for improving the survival rate of bottom-sowing, propagation, and release of *S. subcrenata* seedlings.

Keywords: *Scapharca subcrenata*, salinity, osmotic pressure, reactive oxygen species, antioxidant defense, heat shock proteins, cathepsin

INTRODUCTION

In coastal areas, salinity is subject to drastic changes in response to seasonal precipitation, tides, ocean currents, and seawater evaporation (Huong et al., 2010; Johnson et al., 2011). In response to changes in environmental salinity, organisms might perceive changes in osmotic pressure, responding metabolically to regulate the ion concentration and composition in cells, to maintain an optimal metabolic state. The formation of this optimal state is reflected in the dynamic equilibrium of internal osmotic pressure through physiological responses and the secretion and absorption of salt and water by the body (Kültz, 2012).

The ark shell *Scapharca subcrenata* is an economically important marine shellfish in China; it is vertically distributed in the sea between the low tide line and a 7-m depth. Most individuals live in fresh water-affected inner bay and neritic regions, showing a range of adaptations to salinity, and preferring to inhabit soft mud or muddy seafloors with a high sand content (Wang and Wang, 2008). Recently, overfishing and environmental changes have led to a decrease in available habitats. To restore the declining habitats of *S. subcrenata*, large-scale enhancements and release of seeds have occurred. In estuarine or intertidal zones, which are subject to significant changes in salinity, organisms with a fixed living mode or those only able to move slowly can grow well within an appropriate range of salinity; however, if the habitat salinity increases or decreases beyond that range, their growth is impacted and can even lead to a stress response, which in turn diminishes immune resistance (Wang and Chen, 2005; Sung et al., 2011). Therefore, selecting a suitable release area based on the salinity tolerance mechanisms of *S. subcrenata* would help improve the survival rate of *S. subcrenata* seeds.

Euryhaline organisms are better able to regulate their osmotic pressure compared with stenohaline organisms, enabling them to maintain a higher rate of food intake, absorption, transformation, and growth efficiency in a wider salinity range (Tseng and Hwang, 2008). However, the optimal growth salinity varies significantly from species to species. When the osmotic pressure of water is similar to the internal osmotic pressure, the energy consumption of the organism allocated to the regulation of internal osmotic pressure is the lowest, but is most beneficial to survival and growth (Verbalis, 2007; Seale et al., 2012). After *Litopenaeus vannamei* fed under a salinity of 35 psu was transferred to, and kept in 25, 20, or 15 psu environments for 1–6 h, the activity of granule cells, blood cells, phenol oxidase, and superoxide dismutase (SOD) significantly decreased. Subject to the dual-factor stress of *Vibrio alginolyticus* and salinity dip, the above immune indexes decreased more significantly, suggesting that the immunity level of *L. vannamei* also significantly decreased (Lin et al., 2012). When *Takifugu obscurus* was placed under a salinity of 30 psu, the number of chloride-secreting cells in the gill filament increased, as did its volume; in addition, hormones, such as growth hormone and cortisol, triggered the synthesis of more transport proteins to stimulate the proliferation and differentiation of chlorine cells, thereby changing the transmembrane transport capacity of ions and water molecules (Wang et al., 2016). Zhang et al. (2018) performed transcriptome

sequencing on *Apostichopus japonicus* grown under 20 and 30 psu and identified 109 differentially expressed genes. Gao et al. (2017a) simulated the falling then rising trend of seawater salinity after a rainstorm and found that the expression levels of *CAT*, *TP_x*, *GST_s*, *GST_m* tended to rise then fall, and these were significantly higher than groups with constant salinity.

Consequently, osmotic pressure regulation mechanisms not only involve the transport of ions and water and the regulation of the neuroendocrine system, but also depend on energy metabolism to maintain the activity of transport proteins and on the auxiliary functions of different kinds of osmotic pressure-regulating molecules, such as free amino acids. To date, few reports have focused on the salinity tolerance range of *S. subcrenata* and the osmotic pressure regulation mechanisms involved, despite the economic importance of this species. Therefore, the results of this study that shed light on the physiological adaptation mechanisms of *S. subcrenata* to changes in salinity will help increase not only our basic understanding of the biology of *S. subcrenata*, but also the survival rate of bottom-sown seedlings in habitats subject to regular changes in salinity.

MATERIALS AND METHODS

Source and Acclimation of *S. subcrenata*

Scapharca subcrenata (shell length: 32.85 ± 1.73 mm, body weight: 8.15 ± 1.03 g) were purchased from Fuyuan fisheries company (Rizhao, Shandong, China), and all experimental *S. subcrenata* were sourced from the same batch after artificial hatching. After purchasing the *S. subcrenatas*, they were acclimated in one culture container (length 1.2 m $\times$ width 1 m $\times$ height 1 m, water volume: 1200 L) for 15 days; water temperature was kept at 22°C, salinity at 30 ± 1 , pH at 7.9, dissolved oxygen concentration at > 6 mg/L, and the light cycle was set as the natural light cycle. Aquaculture water was obtained from the natural sea area and used after sedimentation and sand filtration. Two-thirds of the water was replaced with fresh seawater each day at 09:00 to ensure good water quality. During the period of acclimation, the food mixture of *Chlorella vulgaris*, *Isochrysis galbana*, and *Platymonas subcordiformis* was fed once a day at a volume ratio of 1:1:1 and then the food concentration was measured every 6 h.

Experimental Design

In total, 200 *S. subcrenatas* were randomly selected from the culture container and divided among five aquarium tanks (0.6 m $\times$ 0.5 m $\times$ 0.6 m, tank volume: 180 L), with 40 per tank. Four of the tanks were used to simulate four phases in the changes in seawater salinity after rainfall (Figure 1); four replicates were used. In Phase 1, the salinity was reduced from 30 to 14‰ at a rate of eight levels every 24 h; in Phase 2, the salinity was kept at 14‰ for 24 h; in Phase 3, the salinity was increased from 14 to 30‰ at a rate of eight levels every 24 h; and in Phase 4, the salinity was maintained at 30‰ for 24 h. Meanwhile, *S. subcrenata* cultured at a salinity of 30‰ in an aquarium was used as the control group, in which the salinity stayed at the same level throughout the study. The low-salinity seawater was prepared using tap water with 24 h

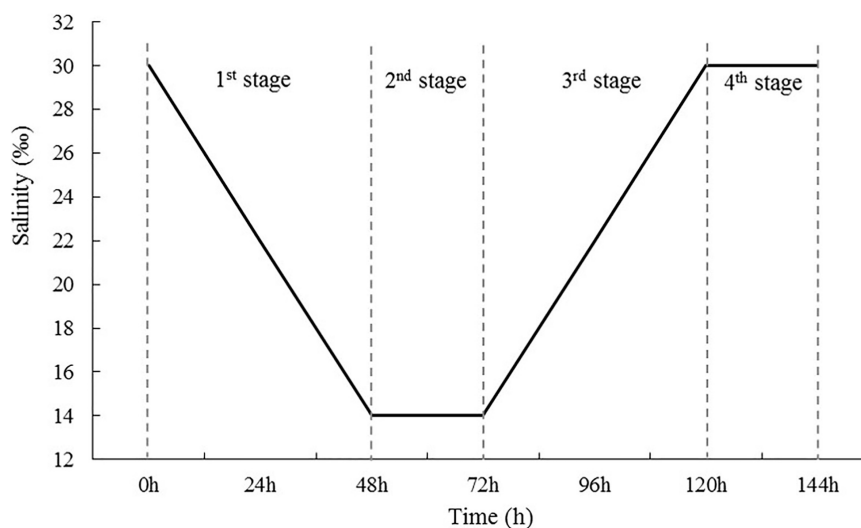


FIGURE 1 | Diagram of the salinity change mode used in the experiment. First, salinity decreased gradually from 30 to 14 psu at a rate of 8 psu every 24 h (first stage), and then was maintained at low levels for 24 h (second stage). After 72-h stress, salinity increased gradually to 30 psu at a rate of 8 psu 24 h⁻¹ (third stage) and was then maintained at 30 psu for 24 h (fourth stage).

aeration and seawater prepared through natural sand filtration; while the high-salinity seawater was prepared by adding sea salts (Blue Treasure Co., Ltd., Qingdao, China) to the experiment group in low-salinity seawater.

All *S. subcrenatas* were starved for 24 h prior to the experiment. During the experiment, no feed was given and the room temperature was kept at 22°C with air-conditioning control, with the same pH as it was in acclimation. Each aquarium was provided with continuous aeration to maintain the concentration of dissolved oxygen above 6 mg/L, and the ammonia concentration was always below 0.1 mg/L throughout the experiment.

Collection of Samples

Samples of gill tissue and hemolymph were collected at different time points in each phase: Phase 1, at 24 and 48 h after the start of the experiment, when salinity was reduced to 22‰ (DS1) and 14‰ (DS2); Phase 2, at 72 h after the beginning of experiment, when the salinity was maintained at 14‰ for 24 h (DL1); Phase 3, at 96 and 120 h after the start of experiment, when the salinity was increased to 22‰ (IS1) and 30‰ (IS2), respectively; and Phase 4, at 144 h, when the salinity was maintained at 30‰ for 24 h (IL1).

Before the experiment, 10 *S. subcrenatas* were randomly selected as the samples at 0 h for later analysis. The survival rate of *S. subcrenatas* in each aquarium was recorded at 24, 48, 72, 96, 120, and 144 h after the beginning of the experiment, and three *S. subcrenatas* were randomly selected from each aquarium, giving 15 *S. subcrenatas* in total, to measure the hemolymph osmotic pressure. The concentration of Na⁺, K⁺, Ca²⁺, Cl⁻, hemoglobin, soluble protein, and the concentration of taurine and total free amino acids were determined after centrifugation of hemolymph. Gill tissues were taken to analyze and determine the enzyme activity of Na⁺/K⁺-ATPase, the content of reactive

oxygen species (ROS), total antioxidant capacity (T-AOC), and the gene expression of Mn-SOD (*MnSOD*), Catalase (*CAT*), glutathione peroxidase (*GPx*), cathepsin C (*CTSC*), cathepsin D (*CTSD*), heat shock protein 20 (*HSP20*), *HSP70*, and *HSP90*.

Assay of Samples

Assay of Physiological Indices in Hemolymph

At the end of Phase 4, each *S. subcrenatas* was euthanized and dissected; the adductor muscle was removed and hemolymph was extracted from the blood sinus using a 10-mL medical syringe. It was then placed in a 1.5-mL sterile centrifuge tube in an ice bath. The time between the removal of any sample and the completion of sampling process was always < 10 min. Each centrifuge tube contained the hemolymph of five individuals. A Fiske 210 Micro-Sample Osmometer (Advanced Instruments, Norwood, MA, United States) was used for the determination of osmotic pressure. The remainder of the hemolymph was centrifuged at 4°C, 10,000 × g/min for 10 min, and the supernatant was used to determine the concentration of hemocyanin, total soluble protein, Na⁺, K⁺, Ca²⁺, Cl⁻, taurine, and total free amino acids.

The concentration of hemoglobin was measured using the hemiglobincyanide (HiCN) assay, and the contents of hemoglobin were measured with a hemoglobin instrument (WJX-1; Shanghai Touching Technology Co., Shanghai, China).

The concentration of total soluble protein in these samples was calculated using Bradford's (1976) Coomassie Brilliant Blue, with bovine serum albumin as the standard protein.

The contents of Na⁺, K⁺, Ca²⁺, and Cl⁻ in the supernatant of hemolymph under each salinity level were determined with a kit from the Nanjing Jiancheng Bioengineering Research Institute (Nanjing, China) (Gao et al., 2017a).

The mixed hemolymph supernatant was analyzed at Qingdao Institute of Bioenergy and Bioprocess Technology, Chinese

Academy of Sciences (Qingdao, China). After being treated with 8% trichloroacetic acid and sonication for 20 min, the supernatant was collected and tested under Sykam S-433D Automated Amino Acid Analyzer (Munich, Germany) to determine the concentration of taurine and total free amino acids in the hemolymph samples, respectively.

Na⁺/K⁺-ATPase Activity, ROS Content, and T-AOC Assays

After the hemolymph had been collected, the gill tissues were cut with scissors on an ice tray and evenly divided into two sections; each section was placed into a 1.5 mL centrifuge tube, quickly placed into liquid nitrogen, and then transferred to a -80°C refrigerator until use. Then 0.2–0.4 g gill tissues were extracted, and fully ground with the addition of 1.8 mL 0.86% saline in the ice-water bath. After grinding, the material was centrifuged at 3500 × g/min for 10 min until 10% tissue homogenates were obtained, to determine the content of ROS and T-AOC. The activity of Na⁺/K⁺-ATPase was determined using a kit supplied by Nanjing Jiancheng Bioengineering Institute and the enzyme activity was determined using the phosphorus determination method (Kaplan, 2005). One unit of enzyme activity was defined as the generation of 1 μmol inorganic phosphate from the decomposition of ATP per minute by ATPase in 1 mg of tissue protein (Peterson, 1978). The protein content in muscle homogenates was determined using Bradford's (1976) Coomassie Brilliant Blue, with bovine serum albumin as the standard protein.

Regarding the ROS content, three gill tissue homogenates from each aquarium were centrifuged at 1000 × g for 10 min, and then 1 mmol/L DCFH-DA (2',7'-dichlorofluorescein diacetate) was added to the supernatant. The mixture was incubated at 37°C for 30 min after being fully and evenly mixed. A fluorescence spectrophotometer (960MC, INESA, CC) was used to determine the fluorescence intensity at the optimum excitation wavelength of 500 nm and at the optimum emission wavelength of 525 nm, and the results were expressed as fluorescence arbitrary units (A.U.) (Gao et al., 2017b).

Total antioxidant capacity was determined using the ferric reducing ability of plasma method (Benzie and Strain, 1996). At 37°C, a unit (U/mg) of T-AOC was defined as the increase of 0.01 of optical density of all chemical reaction substances by 1 mg of tissue protein per minute.

Finally, the rest of the muscle tissues were absorbed with a filter paper, and the wet weight of muscle tissues for fifteen individuals was measured at each time point. These tissues were dried to constant weight in a 202-00S oven (Lichen Co. Ltd., Shanghai, China) at 105°C, their dry weight was measured, and the moisture content calculated. Muscle moisture = (wet weight of muscle tissues – dry weight of muscle tissues)/wet weight of muscle tissues × 100%.

Analysis of Gene Expression

A sample of gill tissue was added to a mortar and ground with liquid nitrogen. Then, 0.05 mg of the powder obtained was quickly mixed with 1 mL TRIzol (Invitrogen, United States) to extract the total RNA from the gill tissue. Total RNA was

extracted by removing the residual DNA from the sample using RQI RNase-Free DNase (TaKaRa, Kusatsu, Japan), and then RNA was reverse transcribed to cDNA using M-MLV reverse transcriptase (Promega, Madison, WI, United States). Real-time quantitative PCR was conducted using the SYBR® Premix Ex Taq™ II kit (Tli RNaseH Plus) (TaKaRa) and the TaKaRa Thermal Cycler Dice™ Real Time System TP800 instrument. The specific primers were designed based on cDNA complete sequences submitted to GenBank, and *MnSOD*, *CAT*, *GPx*, *CTSC*, *CTSD*, *HSP20*, *HSP70*, *HSP90*, and the reference gene β-actin were analyzed. Genetic information and primer sequences are presented in Table 1.

Statistical Analysis

Logarithmic transformation was used to satisfy the homogeneity test of variances and standard normal distribution. One-way ANOVA (SPSS18.0) and Tukey's test were utilized to analyze the difference of hemolymph osmotic pressure, ion concentration, activity of related enzymes, and gene expression in gill tissues under salinity changes at different sampling times, and *t*-test was performed to analyze the difference of associated physiological indexes in hemolymph and gill tissues between the salinity-changing group and the control group at the same sampling points, in which *P* < 0.05 was used as the significant difference level. All experimental data were indicated by mean ± standard error (mean ± SE) and the data after analysis were drawn using Sigmaplot.

RESULTS

Survival Rate

Throughout the experiment, no *S. subcrenata* were found dead in the control group. In the groups with sudden changes in salinity, the survival rate of *S. subcrenata* began to decrease at 96 h. The survival rate at the end of the experiment was 91.67%, although no significant difference was identified compared with 120 h (Figure 2, *P* = 0.281), which was significantly lower than that at any other time point during the study.

Osmotic Pressure of Hemolymph Fluid

The osmotic pressure of the hemolymph in *S. subcrenata* changed in accordance with the changes in seawater salinity. At 96 h, the osmotic pressure of the hemolymph was not significantly different from that at 120 h (Figure 3, *P* = 0.175), but was significantly lower than that at any other time point during the study. At 144 h, when the salinity had increased to 30‰, the osmotic pressure concentration remained significantly lower than at 0, 24, and 48 h. Except at 0 and 24 h, the osmotic pressure concentration in the treatment group was significantly lower than in the control group at all other time points (48 h, *P* = 0.001; 72 h, *P* < 0.001; 96 h, *P* < 0.001; 120 h, *P* < 0.001; 144 h, *P* < 0.001).

Concentrations of Na⁺, K⁺, Ca²⁺, and Cl⁻

At 72 h, the Na⁺ concentration was not significantly different compared with 96 h (Figure 4, *P* = 0.142), but was significantly

TABLE 1 | Real-time quantitative PCR primers for antioxidant enzymes, cathepsin, and heat shock proteins' genes of *Scapharca subcrenata*.

Gene	Sequence (5'–3')	Size (bp)	Efficiency (%)	References
Mn-SOD	F: CGGAACCACTCCTCCGTCA R: ACGCCGATTTTCTAACCGATT	167	100.15	Zheng et al., 2015
CAT	F: GCCCAGACCGAGACCTTAA R: AACTTAGCTAAGCGGGGTACC	183	102.64	Huang et al., 2016
GP _x	F: ACTCCGCGCGTCCACG R: GCCATTTACCTTTGGAT	149	97.04	Cheng et al., 2016
CTSC	F: CAACGGGTTGGCCGAGATCCCCAC R: ATTCTGGAAAACCAACGGAAGT	155	96.81	Designed by author
CTSD	F: CCTTACTACGGGGTTCCCAAC R: GCTTTACCGCGCGCAAAAGGT	213	104.22	Designed by author
HSP20	F: AACTTGCCAACCTCCTGACCTTA R: GCCAAACCCCTACCAGGCAAAT	173	100.82	Designed by author
HSP70	F: AACGGCTCTAAACGGAACCTGGG R: GGCCGTTTAACCCAGGGCTCT	189	97.05	Designed by author
HSP90	F: GTAAACCTCCAACAAAGGCCAGTT R: CGAAAGCGCGGGCAAATCCGCAAGC	134	99.31	Zheng et al., 2018
β-Actin	F: ACACGGTAAAGCAACCTACC R: GCGCCCCAAACTTCCGAA	207	102.92	Designed by author

Mn-SOD, Mn-superoxide dismutase; CAT, catalase; GP_x, glutathione peroxidase; CTSC, cathepsin C; CTSD, cathepsin D; HSP20, heat shock protein 20; HSP70, heat shock protein 70; HSP90, heat shock protein 90; F, forward primer; R, reverse primer.

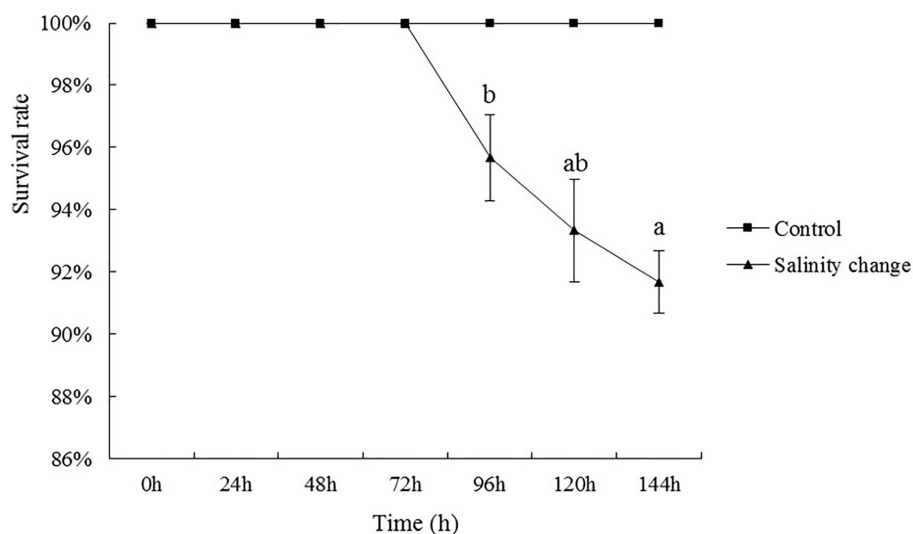


FIGURE 2 | Effects of salinity changes on the survival rate of ark shell (*Scapharca subcrenata*). Values are expressed as mean $\pm$ SE ($n = 4$). Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Tukey's test, using SPSS version 18.0. Means with the different lower case letters are significantly different at $P < 0.05$ level.

lower than that any other time point. Except at 0 h, the Na^+ concentration in hemolymph from the treatment group was significantly lower than in the control group at all other time points (24 h, $P = 0.003$; 48 h, $P < 0.001$; 72 h, $P < 0.001$; 96 h, $P < 0.001$; 120 h, $P < 0.001$; 144 h, $P < 0.001$). The minimum K^+ concentration occurred at 96 h, but was not significant different compared with 72 or 120 h (72 h, $P = 0.183$; 120 h, $P = 0.269$), although it was significantly lower than at any other point. The minimum concentration of Ca^{2+} also occurred at 96 h. At 144 h, the concentration of Ca^{2+} remained significantly lower than

that at 0, 24, or 48 h (0 h, $P < 0.001$; 24 h, $P = 0.001$; 48 h, $P = 0.001$). At 96 h, the Cl^- concentration was significantly lower than that at any other time point. At 72, 120, and 144 h, there were no significant differences in the Cl^- concentration in the treatment group, but each was significantly lower than that at 0, 24, and 48 h. Except at 0 h, the concentration of Cl^- in the treatment group was significantly lower than in the control group at all other time points (24 h, $P = 0.001$; 48 h, $P = 0.001$; 72 h, $P < 0.001$; 96 h, $P < 0.001$; 120 h, $P < 0.001$; 144 h, $P < 0.001$).

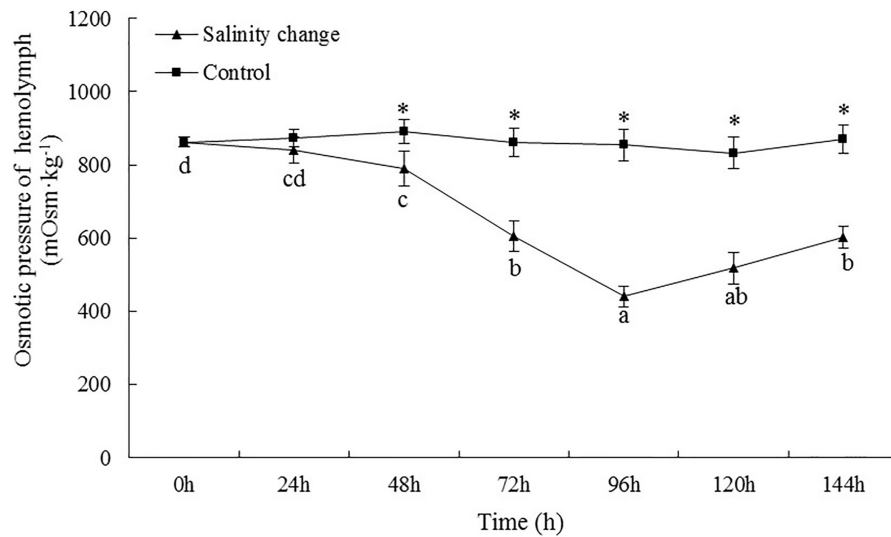


FIGURE 3 | Effects of salinity changes on the osmotic pressure of hemolymph in ark shell (*Scapharca subcrenata*). Values are expressed as mean $\pm$ SE ($n = 4$). Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Tukey's test, using SPSS version 18.0. Means with different lower case letters are significantly different at $P < 0.05$ level. Asterisks indicate significant differences between salinity change treatments and control treatment for the same time points, $P < 0.05$.

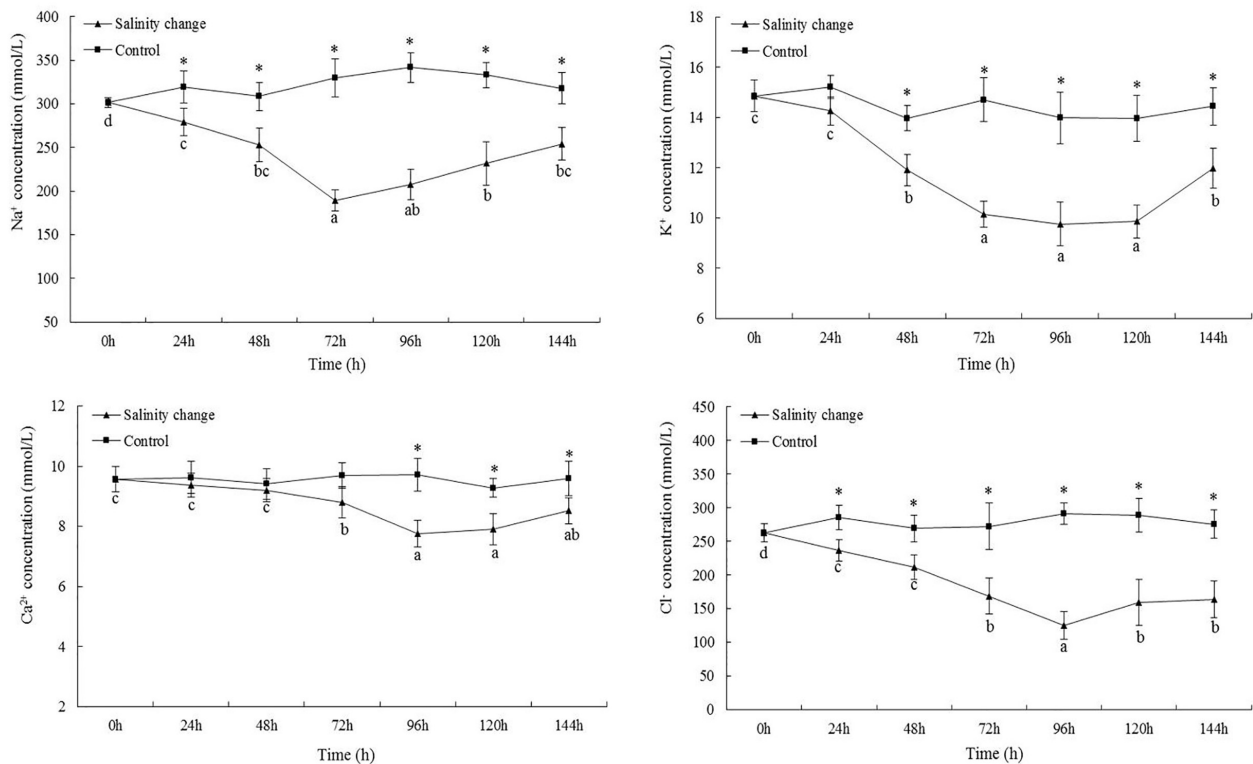


FIGURE 4 | Effects of salinity changes on the Na^+ , K^+ , Ca^{2+} , and Cl^- concentration of hemolymph in ark shell (*Scapharca subcrenata*). Values are expressed as mean $\pm$ SE ($n = 4$). Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Tukey's test, using SPSS version 18.0. Means with the different lower case letters are significantly different at $P < 0.05$ level. Asterisks indicate significant differences between salinity change treatments and control treatment for the same time points, $P < 0.05$.

Hemoglobin, Soluble Total Protein, Free Amino Acids, and Moisture Content

Sudden changes in salinity had a significant effect on the concentration of hemoglobin in *S. subcrenata* (Table 2, $df1 = 6$, $df2 = 21$, $F = 195.17$, $P < 0.001$). At 96 h, the hemoglobin concentration peaked, and was significantly higher than at 0, 24, or 48 h (0 h, $P < 0.001$; 24 h, $P < 0.001$; 48 h, $P < 0.001$). The concentration of soluble total protein also peaked at 96 h, followed by a gradual decrease until 144 h, when it was significantly lower than at 96 h ($P = 0.007$). From 48 h to the end of the experiment, the content of soluble total protein and taurine in the treatment group was also significantly higher than in the control group. At 96 h, taurine content also peaked, and was significantly higher than at any other time point. As the salinity level decreased and then increased, the total free amino acid content tended to gradually increase and peaked at 144 h; although no significant difference was identified compared with 96 and 120 h (96 h, $P = 0.258$; 120 h, $P = 0.327$), each was significantly higher than at any other time point. The moisture content also tended to increase then decrease with the changes in salinity, with the moisture content of gill muscle peaking at 72 h, which was significantly higher than that at 0, 24, 48, and 144 h. From 72 to 120 h, the moisture content of muscle in the treatment group was significantly higher than in the control group (72 h, $P < 0.001$; 96 h, $P = 0.001$; 120 h, $P = 0.001$).

Na⁺/K⁺-ATPase Activity, ROS Content, and T-AOC

At 48 h, the activity of Na⁺/K⁺-ATPase in the treatment group was significantly higher than in the control group and remained so until 144 h (Figure 5, 48 h, $P = 0.002$; 72 h, $P < 0.001$; 96 h, $P < 0.001$; 120 h, $P < 0.001$; 144 h, $P < 0.001$). Sudden changes

in salinity also had a significant effect on the content of ROS ($df1 = 6$, $df2 = 21$, $F = 163.26$, $P < 0.001$). At 96 h, the content of ROS was at its maximum and remained significantly higher at 144 h compared with at 0, 24, and 48 h. At 96 h, T-AOC reached its maximum, which was significantly higher than at 0, 24, 48, and 72 h. From 72 to 144 h, T-AOC in the treatment group was significantly higher than in the control group (72 h, $P < 0.001$; 96 h, $P < 0.001$; 120 h, $P < 0.001$; 144 h, $P < 0.001$).

Analysis of Gene Expression

Sudden changes in salinity had a significant effect on the expression levels of *MnSOD* (Figure 6, $df1 = 6$, $df2 = 21$, $F = 227.18$, $P < 0.001$). At 144 h, the expression level of *MnSOD* had peaked and was significantly different compared with 0, 24, 48, and 72 h. From 72 to 144 h, the expression levels of *MnSOD* in the treatment group were significantly higher than in the control group (72 h, $P < 0.001$; 96 h, $P < 0.001$; 120 h, $P < 0.001$; 144 h, $P < 0.001$). The expression levels of *CAT* tended to increase and then decrease with the sudden changes in salinity. At 120 h, no significant difference in the expression levels of *CAT* was identified compared with 144 h ($P = 0.197$), although it was significantly higher than at any other time point. At 144 h, the expression levels of *GP_x* were significantly higher than at any other time point. From 0 to 72 h, no significant difference in the expression levels of *GP_x* in the treatment group was identified compared with the control group; however, from 96 to 144 h, the expression levels of *GP_x* in the treatment group were significantly higher than in the control group (96 h, $P < 0.001$; 120 h, $P < 0.001$; 144 h, $P < 0.001$).

The maximum expression levels of *CTSC* occurred at 72 h, and were significantly lower at 96 h than at 72 h (Figure 6, $P = 0.005$), although both were significantly higher than at any other time point. From 48 to 144 h, the expression levels of *CTSC* and

TABLE 2 | Effects of sudden salinity changes on the contents of hemoglobin, soluble total protein, taurine, free amino acid, and tissue moisture in *Scapharca subcrenata*.

Time	Treatment	Content				
		Hemoglobin (mmol/L)	Soluble total protein (mg/mL)	Taurine (mg/100 g)	Free amino acid (mg/100g)	Tissue moisture (%)
0 h	Salinity change	1.42 ± 0.21 ^b	12.15 ± 1.18 ^d	56.52 ± 8.72 ^f	16.24 ± 2.52 ^b	73.09 ± 1.42 ^b
	Control	1.39 ± 0.14	11.74 ± 0.56	58.12 ± 7.38	14.94 ± 1.57	72.44 ± 1.19
24 h	Salinity change	1.36 ± 0.17 ^b	14.59 ± 1.26 ^c	71.33 ± 13.59 ^e	15.06 ± 1.87 ^b	72.26 ± 1.75 ^b
	Control	1.33 ± 0.10	12.98 ± 0.92	61.39 ± 5.06	16.73 ± 1.74	72.79 ± 0.93
48 h	Salinity change	1.48 ± 0.15 ^b	15.86 ± 1.15 ^c	75.18 ± 10.64 ^e	18.13 ± 1.95 ^b	74.51 ± 1.82 ^b
	Control	1.45 ± 0.25	11.29 ± 1.42 [*]	57.37 ± 12.15 [*]	16.11 ± 2.64	71.02 ± 0.75
72 h	Salinity change	2.37 ± 0.23 ^a	19.28 ± 1.49 ^{ab}	146.37 ± 15.26 ^c	20.64 ± 2.92 ^b	82.09 ± 1.94 ^a
	Control	1.41 ± 0.16 [*]	13.51 ± 1.18 [*]	60.59 ± 9.47 [*]	18.79 ± 1.38	71.89 ± 0.88 [*]
96 h	Salinity change	2.54 ± 0.26 ^a	22.35 ± 2.07 ^a	197.02 ± 16.90 ^a	33.92 ± 4.01 ^a	81.55 ± 1.43 ^a
	Control	1.36 ± 0.31 [*]	12.09 ± 0.63 [*]	64.75 ± 10.15 [*]	15.49 ± 2.05 [*]	73.14 ± 0.52 [*]
120 h	Salinity change	2.22 ± 0.31 ^a	20.14 ± 1.73 ^{ab}	166.42 ± 18.08 ^b	35.16 ± 3.87 ^a	80.64 ± 1.18 ^a
	Control	1.39 ± 0.20 [*]	14.17 ± 1.64 [*]	61.64 ± 7.97 [*]	17.09 ± 1.77 [*]	72.96 ± 1.03 [*]
144 h	Salinity change	2.09 ± 0.27 ^a	18.66 ± 2.13 ^b	113.92 ± 14.14 ^d	37.09 ± 3.50 ^a	75.92 ± 1.52 ^b
	Control	1.38 ± 0.16 [*]	12.85 ± 1.02 [*]	60.79 ± 10.74 [*]	16.52 ± 1.84 [*]	71.74 ± 0.72

Values are expressed as mean ± SE ($n = 4$). Different lower case letters indicate significant differences in the contents of hemoglobin, soluble total protein, taurine, free amino acid, and tissue moisture for the different time points in salinity change treatments, $P < 0.05$. Asterisks indicate significant differences in the contents of hemoglobin, soluble total protein, taurine, free amino acid, and tissue moisture for the same time points between salinity change treatments and control treatment, $P < 0.05$.

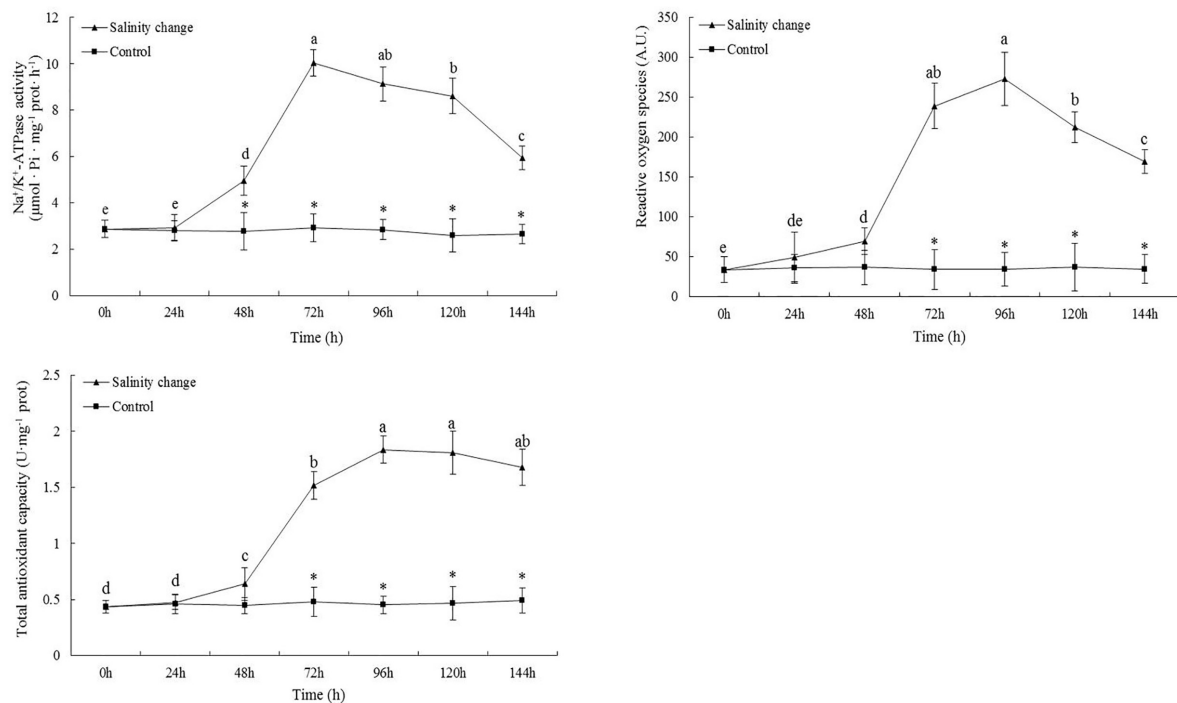


FIGURE 5 | Effects of salinity changes on the activity of Na^+/K^+ -ATPase, content of reactive oxygen species, and total antioxidant capacity in ark shell (*Scapharca subcrenata*). Values are expressed as mean $\pm$ SE ($n = 4$). Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Tukey's test, using SPSS version 18.0. Means with different lower case letters are significantly different at $P < 0.05$ level. Asterisks indicate significant differences between salinity change treatments and control treatment for the same time points, $P < 0.05$.

CTSD in the treatment group were significantly higher than in the control group. Sudden changes in salinity also had a significant effect on the expression levels of CTSD ($df1 = 6$, $df2 = 21$, $F = 283.52$, $P < 0.001$). At 72 and 96 h, the expression levels of CTSD were significantly higher than at any other time point.

With the sudden changes in salinity, the expression levels of HSP20 tended to increase and then decrease, with the pattern then repeating. At 72 h, the expression levels of HSP20 peaked and then decreased significantly after 96 h. From 48 to 144 h, the expression levels of HSP20 in the treatment group were significantly higher than in the control group (Figure 6, 48 h, $P < 0.001$; 72 h, $P < 0.001$; 96 h, $P < 0.001$; 120 h, $P < 0.001$; 144 h, $P = 0.014$). The expression levels of HSP70 also peaked at 72 h; although no significant difference was identified compared with 96 h, it was significantly higher than at any other time point. In the treatment group, the expression levels of HSP90 were significantly higher than in the control group at 24 h ($P < 0.001$), and remained so until the end of the experiment. At 48 h, the expression levels of HSP90 peaked, then decreasing, increasing, and then decreasing again, although the expression levels at each later time point were significantly lower than at 48 h.

DISCUSSION

The salinity level under which aquatic animals live varies greatly. For most aquatic animals, osmotic pressure regulation is a

basic physiological process that enables the body to adapt to differences in internal and external ion concentrations. However, osmotic pressure regulation is complex because the environment inhabited varies from organism to organism (Sokolova et al., 2012; Kültz, 2015; Urbina and Glover, 2015).

The gill is the first organ to come into contact with the external environment. It has multiple physiological functions: in addition, to gas exchange, ammonia nitrogen excretion, acid-base balance regulation, it is an important site and primary organ for osmotic regulation (Politis et al., 2018; Giffard-Mena et al., 2020). Previous studies showed that the gill epithelial cells have a crucial role in ion regulation, in which Na^+/K^+ -ATPase is vital to osmotic regulation by participating in the transport of ions (Abdel-Mohsen, 2009; Evans and Lambert, 2015). During the sudden changes in salinity in the current study, the Na^+/K^+ -ATPase activity peaked at 72 h, when *S. subcrenata* was in 14‰ water for 24 h. As the salinity gradually returned to 30‰, the enzyme activity also gradually declined. Furriel et al. (2000) considered that Na^+/K^+ -ATPase in the gills of crustaceans is the most important protease in osmotic regulation, Na^+/K^+ -ATPase accounts for 70% of the total ATPase activity, and can transport Na^+ out of the gill epithelial cells to the hemolymph, and transport K^+ in the hemolymph into the gill epithelial cells, thereby maintaining the equilibrium of Na^+ and K^+ in body and the osmotic pressure of the hemolymph. When the salinity was 24‰, the expression levels of NKA mRNA in *Pagrus pagrus* larvae were significantly higher than in a group at a salinity of 34‰,

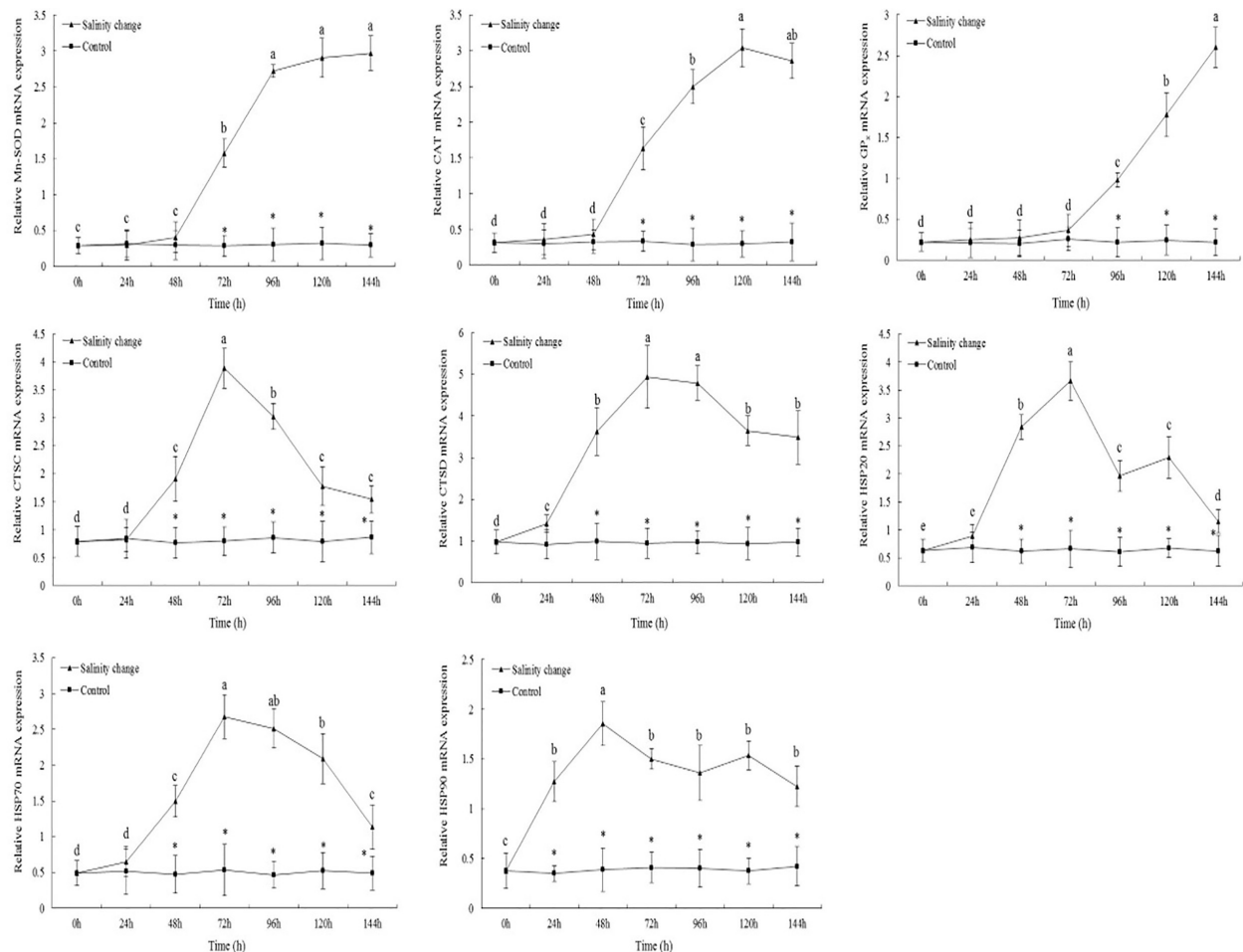


FIGURE 6 | Relative mRNA expression levels of antioxidant enzyme and heat shock proteins' genes in the gills of ark shell (*Scapharca subcrenata*). Values are expressed as mean $\pm$ SE ($n = 4$). Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Tukey's test, using SPSS version 18.0. Means with different lower case letters are significantly different at $P < 0.05$ level. Asterisks indicate significant differences between salinity change treatments and control treatment for the same time points, $P < 0.05$.

and the osmotic pressure of the hemolymph showed the same trend with the changes in salinity (Ostrowski et al., 2011). When *Gadus morhua* was transferred from a salinity of 33 to 9‰, the expression of NKA α in the gills and kidney significantly reduced; in contrast, when transferred from 9 to 33‰, its expression levels significantly increased (Larsen et al., 2012). The activity of Na $^{+}$ /K $^{+}$ -ATPase in the gill of *Poecilia latipinna* following adaptation to a salinity of 35‰ was significantly higher than in the 15‰ or freshwater group; the content of NKA α subunit protein in the 35‰ salinity group was 2.2 and 1.7 times that in the 15‰ salinity and freshwater groups, respectively, and the osmotic pressure of the plasma showed the same trends (Yang et al., 2009). Therefore, Na $^{+}$ /K $^{+}$ -ATPase participates in the active transport of Na $^{+}$ and K $^{+}$ across the cell membrane and, thus, is important in regulating the osmotic pressure level of the hemolymph.

In response to external changes in salinity, aquatic organisms regulate the permeability of water and inorganic ions, and

osmotic regulation involves changes in the content of osmotic effectors, such as inorganic ions and free amino acids, in the hemolymph. Inorganic ions have an important role in maintaining the osmotic pressure levels of the hemolymph, particularly Na $^{+}$ and Cl $^{-}$ (Cheng et al., 2002; McNamara and Faria, 2012). Crustaceans can actively absorb Na $^{+}$ and Cl $^{-}$ to compensate for salt loss from the body under low salinity levels, enabling them to adapt to environments with low or variable levels of salinity, such as estuaries (Flik and Haond, 2000). Inorganic ions are the main components affecting the osmotic pressure of the hemolymph in crustaceans. An increase in water salinity will increase the concentration of inorganic ions in the serum in *Eriocheir sinensis* and *Scylla serrate*, thus increasing its osmotic pressure (Romano et al., 2014; Long et al., 2017). In the treatment group, the concentrations of Ca $^{2+}$ and K $^{+}$ were significantly lower than in the control group at 96 and 48 h, respectively. These results also indicated that inorganic ions, such as Na $^{+}$, Cl $^{-}$, and K $^{+}$, acted as primary osmotic

effectors in the hemolymph and might have a greater impact on osmotic pressure compared with Ca^{2+} . K^+ plays a critical role in maintaining the osmotic pressure in neurons, maintaining the normal function of the nervous system. Intracellular changes of Na^+ concentration affect the osmotic pressure response of organism, seal the mantle cavity, and protect cells from extreme salinity (Berger and Kharazova, 1997). Natchin et al. (1979) studied *Mytilus edulis* and *Littorina littorea* and found that, in addition to Na^+ and K^+ pumps, these organisms also have a Na^+ exchange system related to Cl^- that regulates the cell volume and osmotic pressure. In this sense, the Na^+/K^+ pump and the Na^+/Cl^- pump might jointly be involved enabling marine shellfish to cope with salinity changes.

Free amino acids in marine shellfish are also important osmotic effectors, and are mainly sourced from the decomposition of cells or hemolymph proteins. In crustaceans, with increases in environmental salinity, the serum protein decreases, whereas the content of free amino acids increases (Fu et al., 2017). Similar results have been found in shellfish placed in low salinity environments: when the cells swell because of the moisture produced in response to osmotic pressure differences, changes in plasma membrane permeability can induce the outflow of specific amino acids, thus enabling the cells to remove excess moisture and restore cell volume by the efflux of low molecular weight free amino acids (Silva and Wright, 1992, 1994). Taurine, glycine, and alanine are the most commonly used free amino acids, and the changes in taurine account for most of the free amino acid changes (Neufeld and Wright, 1996). In this study, the hemoglobin concentration peaked at 96 h, followed by a decrease, although it was still significantly higher than in the control group. In addition to carrying oxygen, hemoglobin can store energy and maintain osmotic pressure and antibacterial activity (Wang et al., 2013; Kato et al., 2017). Therefore, with sudden changes of salinity, because *S. subcrenata* lacks a specific immune system, it has to rely on the innate immunity of blood cells and humoral factors to enhance its resistance in response to external environmental stress.

For *C. gigas* under low osmotic conditions, most free amino acids in the mantle cells showed a significant, synchronous decrease within 2–8 h; under high osmotic condition, glycine, alanine, and taurine significantly increased, with the rapid increase in alanine having a crucial role in the short-term adaptation of salinity changes, whereas taurine was involved in long-term adaptation (Hosoi et al., 2003). In the current study, taurine and total free amino acids increased gradually with the sudden drop of salinity, with taurine peaking at 96 h when the salinity level had increased to 22‰, whereas total free amino acids increased gradually, suggesting that taurine has a crucial role in the rapid response of *S. subcrenata* to short-term sudden changes in salinity, whereas total free amino acids are involved in responses to long-term changes in salinity. Lu et al. (2015) reported that, when the salinity level of the external environment decreased, the content of total free amino acids in *Scylla paramamosain* also decreased, whereby the extent of the decrease in the content of glutamate, glycine, proline, and arginine accounted for 50% of the decrease in the total free amino acid content. The internal accumulation of certain free

amino acids is a response to changes in external salinity. For osmotic pressure regulation, free amino acids can better stabilize macromolecules, such as proteins, compared with inorganic ions, without changing the structure and function of the enzyme or impacting the organism (Long et al., 2018). Therefore, free amino acids in the hemolymph are directly involved in the osmotic pressure regulation of *S. subcrenata*, and an effective means of maintaining the equilibrium of osmotic pressure is to increase the content of free amino acid.

Aerobic organisms continually produce ROS via metabolism. Excessive accumulation of ROS destroys macromolecular substances, such as proteins, carbohydrates, nucleic acids, and lipids (Finkel and Holbrook, 2000; Lushchak, 2011). As a result, maintaining the dynamic equilibrium between the production and removal of ROS is crucial to protect against oxidative damage and to maintain normal physiological functions (Martínez-Álvarez et al., 2005; Okoye et al., 2019). DCFH-DA shows sensitivity and specificity for the determination of ROS when used as a probe, but this type of compound is poorly selective against H_2O_2 . Furthermore, the fluorescent response of DCFH and its derivatives is based on the oxidation mechanism, which responds to not only intracellular H_2O_2 , but also other oxidation substances. It was also reported that an excessive concentration of DCFH can result in cytotoxicity, affecting the determination result (Miller et al., 2005; Wrona et al., 2005). In future studies, the method used to detect ROS should be selected by considering the low auto-oxidation, high optical stability, and low cytotoxicity of the method, in addition to its high selectivity and sensitivity.

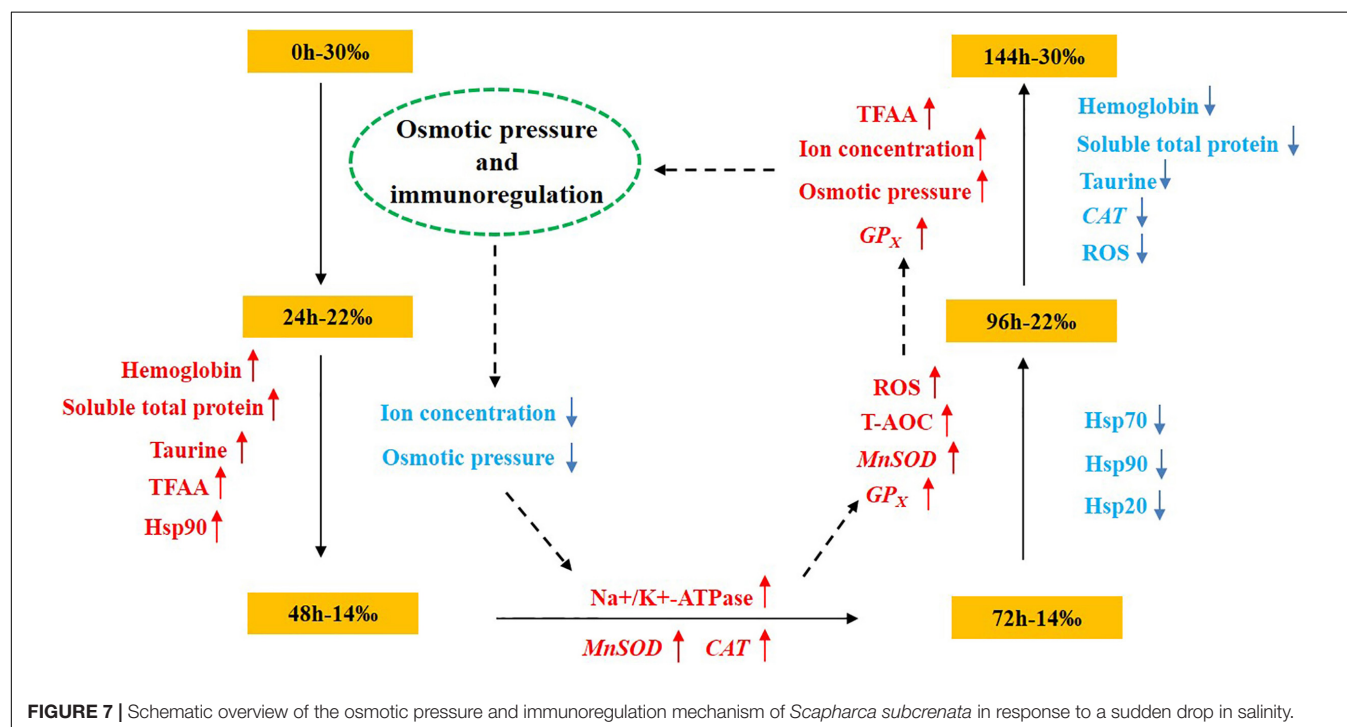
However, aquatic animals have an evolutionarily conserved antioxidant defense system that can remove excess ROS. In particular, as antioxidant enzymes, SOD and CAT are considered to be the first line of defense against oxygen poisoning (Bhagat et al., 2016; Wang et al., 2020). SOD activities in the zebra and white zebra strains of *Ruditapes philippinarum* increased significantly with decreasing salinity from 30 to 5‰, with the highest value at a salinity of 5‰. CAT activity in the white and white zebra strains decreased with decreasing salinity from 30 to 5‰, but increased slightly in the zebra strain at 15‰ and then increased slightly at 5‰ (Nie et al., 2020). The current results showed that the expression levels of *MnSOD* and *CAT* in the treatment group at 72 h (equivalent to 24 h under a salinity level of 14‰) were significantly higher than in the control group, suggesting that sudden changes in salinity led to oxidative damage internally, with the continuously accumulated ROS requiring the organism to activate its antioxidant defense mechanisms before the dynamic equilibrium between oxidation and oxidation resistance could be restored. The expression levels of *MnSOD* at 144 h were still increasing, whereas the expression levels of *CAT* were lower than that at 120 h; thus, the higher expression levels of *MnSOD* might lead to excessive accumulation of H_2O_2 , thereby inhibiting the function of *CAT*. Nevertheless, as the second line of defense against oxidative damage, GP_x also has an important role in cell metabolism and scavenging of free radicals; in cells, it can catalyze the reduction of hydroperoxides to hydroxy compounds (Cnubben et al., 2001; Peña-Llopis et al., 2003). From 96 h onward, the expression levels of GP_x in the

treatment group began to be significantly higher than in the control group. By the end of experiment, these were significantly higher than at any other point, whereas the expression levels of CAT tended to decrease; thus, it can be inferred that GP_x had a significant role in reducing toxic peroxides to non-toxic hydroxy compounds, thus shielding the structure and function of the cell membrane from interference and damage by the peroxide. However, when the expression levels of CAT decreased and that of MnSOD continued to increase, the scavenging of H₂O₂ was mainly dominated by GP_x. Regarding enzyme kinetics, GP_x in mammals and other vertebrates has a greater affinity for H₂O₂ compared with CAT (Reddy et al., 1998; Avanzo et al., 2001). GP_x is thus largely responsible for the removal of H₂O₂ in vertebrates, while CAT and GP_x play complementary roles in H₂O₂ removal (Mourante et al., 2002; Jo et al., 2008). As a result, in response to sudden changes in salinity, CAT and GP_x had not only a synergistic, but also a dominant role in mediating the scavenging of H₂O₂ to maintain the dynamic equilibrium between internal oxidation and reduction.

In invertebrates, cathepsins act as a primary component of the lysosomal proteolytic system and are responsible for the specific degradation of intracellular proteins (Knop et al., 1993). As the salinity level decreased and then increased, the expression levels of CTSC and CTSD tended to increase and then decrease, peaking at 72 h (under a salinity of 14‰). Then, the expression levels of CTSC and CTSD decreased, although each was still significantly higher than in the control group. These changes suggested that, in response to salinity stress, the higher expression levels of CTSC and CTSD contributed to enhancing the innate immune response of the organism and its resistance to invasion and infection by pathogenic bacteria. Nair et al. (2005)

studied *Strongylocentrotus purpuratus* and found that the expression levels of CTSL significantly increased subject to stimulation with lipopolysaccharide, thus inferring that CTSL is an important immune-related gene. Venier et al. (2006) found that, when *Mytilus galloprovincialis* was cultured in a stressful environment with heavy metals, the expression levels of CTSL were significantly higher than in the control group, suggesting that it is involved in the immune stress response. Therefore, our current findings indicated that cathepsins were involved in various physiological activities and had an important role in the regulation of innate immune response in invertebrates.

Heat shock proteins form a group of proteins with crucial physiological functions and a highly conserved structure; they are produced in large quantities in response to heat stress, tissue hypoxia, and osmotic pressure stress (Tine et al., 2010; Yang et al., 2019). In the current study, HSPs were induced with the sudden changes in salinity, although there were differences in the expression patterns of HSP20, HSP70, and HSP90. As a member of the small molecule HSP family, HSP20 might not refold non-denatured proteins on its own, but might combine with unfolded proteins and other HSPs to form complexes that are involved in biological processes, such as cellular stress resistance, formation of germ cells, and development of tissues and organs (Morrow and Tanguay, 2012; Liu et al., 2013). Two peaks occurred in the expression levels of HSP20, at 72 h (under a salinity of 14‰) and at 120 h, suggesting that HSP20 had an important role in enabling the organism to adapt to changing salinity stress. Hsp70 has an important role in regulating the effects of environmental stress and maintaining homeostasis in cells (Casas and La Peyre, 2020; Hong et al., 2020). In the current study, the expression levels of HSP70 peaked at 72 h, followed by a gradual decrease, although



it remained significantly higher than in the control group at 144 h. This suggested that the continuously accumulated ROS led to the denaturation and degradation of partial proteins. At this point, the increased expression levels of *HSP70* contributed to the repair of damaged proteins and the effective regulation of the cell cycle. Salinity stress facilitated the increased expression levels of *HSP70* in *A. japonicus*; especially under low salinity (20‰) stress, the higher expression levels of *HSP70* would help enhance the resistance of *A. japonicus* to salinity changes (Dong et al., 2008). *Hsp90* is a molecular chaperone involved in protein maturation and has a role in maintaining the stability of protein construction, participating in cell cycle regulation, organism immunity, and signal transduction (Galt et al., 2018). In the current study, the expression levels of *HSP90* peaked at 48 and 120 h, and were significantly higher than in the control group from 24 h onward; thus, *HSP90* might be more sensitive to sudden changes in salinity compared with *HSP70* and *HSP20*, and had a specific role during the initial salinity changes, participating in signal transduction and maintained the stability of cell function. Wu et al. (2011) reported that the content of *HSP90* mRNA stored in the cells for the maintenance of normal physiological function will be upregulated. It has also been shown that cysteine residues, methionine residues, and others in *HSP90* are vulnerable to oxidation. *HSP90* may be deprived of biological activity as molecular chaperone under long-term environmental stress. In the current study, the expression levels of *HSP90* also began to fall after 72 h, then increased again until 120 h, but no significant difference was identified compared with 72 h. Thus, during the initial stages of responding to sudden changes in salinity, *S. subcrenata* enhanced signal transduction by relying on the high expression levels of *HSP90*. After 72 h, *S. subcrenata* relied more on the synergy of *HSP20*, *HSP70*, and *HSP90* to better adapt to the environmental stress.

As an important economic shellfish in coastal and estuarine areas, seasonal heat and rainstorm often impact the survival of *S. subcrenata* after bottom sowing. In the current study, by simulating the sudden changes in salinity that occur after a rainstorm, it was found that the concentration of osmotic pressure of the hemolymph, and Na^+ , K^+ , Ca^{2+} , and Cl^- content decreased and then increased with the changes in salinity, whereas hemoglobin, soluble total protein, taurine, and total free amino acid tended to increase with the decrease in salinity, with the content of hemoglobin, soluble total protein, and taurine then gradually decreasing as salinity increased (Figure 7). ROS peaked at 96 h, whereas the activity of Na^+/K^+ -ATPase and the expression levels of *MnSOD*, *CAT*, *GPx*, *HSP20*, *HSP70*, and *HSP90* increased gradually. At 144 h, the expression levels of *MnSOD* and *GPx* gradually increased, indicating that, although the salinity level had increased to 30‰, ROS had accumulated internally during the initial sudden changes in salinity. Thus,

there was still a need for the antioxidant system to maintain the dynamic equilibrium between the production and removal of ROS, otherwise this would have impacted the cell structure and resulted in functional damage, which was an important cause of the reduced survival rate of *S. subcrenata* in the treatment group. Thus, there is a need to attempt to reasonably select the bottom-sowing and release area based on the osmotic pressure regulation mechanism of *S. subcrenata* and its tolerance to changing salinity levels and to avoid sowing and releasing after uninterrupted periods of rain, both approaches that will be instructive for improving the survival rate of *S. subcrenata* seedlings and, thus, the resource proliferation efficiency.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

All *S. subcrenata* in this study were handled in strict accordance with China legislation on scientific procedures on living animals. The protocol was approved by the ethics committee at University of Chinese Academy of Sciences (permit number: 399 20021109).

AUTHOR CONTRIBUTIONS

GX and LY conceptualized the study. ZM conducted research, collected the data, and wrote the manuscript. LL provided the materials and interpreted the data. GX had primary responsibility for the final content. All authors read and approved the final manuscript.

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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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A Comparative Study on Growth and Metabolism of *Eriocheir sinensis* Juveniles Under Chronically Low and High pH Stress

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This study elucidates the effects of chronic pH stress on the growth and metabolic response of juvenile Chinese mitten crab *Eriocheir sinensis*. Crabs were exposed under normal pH (control, pH = 8.0 ± 0.20), low pH (pH = 6.5 ± 0.20), and high pH (pH = 9.5 ± 0.20) in an 8-week trial. Both low and high pH suppressed weight gain but low pH had more adverse effects. No difference was observed on survival, crude lipid, and protein. Acidic stress significantly reduced protein efficiency. The malondialdehyde (MDA) content in hepatopancreas was highest at low pH. The superoxide dismutase (SOD) activity in hepatopancreas and total hemocyte counts (THC) in the stress groups were higher than that in the control. Crabs under high pH had the highest ACP and AKP activities, but there was no significant difference between the control and low pH groups. In the transcriptome analysis, 500.0M clean reads were obtained from the control, low pH, and high pH groups, and assembled into 83,025 transcripts. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways were analyzed to obtain the significantly changed pathways involving differently expressed genes. Ten and eight pathways in metabolism were significantly changed in low pH vs control and high pH vs control groups, respectively. According to the reported functions of these pathways, most of them participated in carbohydrate metabolism. The metabolism pathway analysis indicates the increases of stress resistance, glucose metabolism, and molting activities under chronically pH stress. This study suggests that low pH has more negative impact on crab growth, and oxidative phosphorylation is the main source of energy source under low pH stress, while aerobic glycolysis supplies most energy under high pH stress.

Keywords: *Eriocheir sinensis*, pH stress, antioxidant capacity, metabolic pathways, transcriptome

INTRODUCTION

Unlike seawater, freshwater has poor buffering capacity and can experience a wider range of pH fluctuation (Han et al., 2016). The level of pH in aquaculture pond fluctuates from 6.6 to 10.2 because carbon dioxide is removed by plants and algae through photosynthesis at daytime while carbon dioxide is released through respiration in the water at night (Li and Chen, 2008). The pH of

surface water may also be acidic either by the natural process of organic acidity or inorganic acids from atmospheric deposition (Spyra, 2017). The pH in aquaculture ponds can increase to above 9 during the red tide formed by the rise concentration of soluble organic substances (Wang and Wang, 1995) and photosynthesis at daytime (Hayashi et al., 2012).

Generally, the exposure of freshwater organisms to an abnormal pH can lead to mortality and reduction in growth and reproduction (Kim et al., 2015). An acidic environment can reduce the pH in blood or hemolymph in aquatic animals, resulting in low oxygen carrying capacity and physiological hypoxia (Han et al., 2018a). Alkaline stress would corrode the gills of aquatic animals, leading to the reduction of ion absorption and mortality (Qian et al., 2012). Though crustaceans have the ability to adapt to pH change in a certain range, it is also evident that extreme pH especially lower pH would trigger abnormal functions in physiological, molecular, and biochemical pathways (Chen et al., 2015; Kawamura et al., 2015; He et al., 2019).

Adaptability of animals to environmental stress largely depends on the capacity of transcriptome response and gene expression (Gracey, 2007; Xu et al., 2016). RNA sequencing (RNA-Seq) has been widely applied for the transcriptome research (Wang et al., 2009). This technology provides a platform to study the stress response and adaptative mechanism for a species even without its full genome referencing database. The hepatopancreas of crustaceans is a key organ involved in digestion and detoxification, and it is also a crucial metabolic center for eliminating excess ROS and plays an important role in the immune system (Vogt, 1994; Chen et al., 2017). Therefore, in this study, hepatopancreas transcriptome analysis was taken to investigate the effects of chronic pH stress on the metabolism of crabs. The Chinese mitten crab *Eriocheir sinensis* is an important species in aquaculture and its production reached 796,622 metric tons in 2014 with a value of over 5.5 billion US dollars (Wang et al., 2019). With the development of intensive farming, pH stress has become a major concern in crab farming. Therefore, a comparative study on the metabolic response to pH stress is necessary to develop a strategy to prevent crab mortality and subsequent economic loss under a chronic low or high pH stress. Our motivation to investigate the response of crab to pH stress was due to the development of saline-alkali water aquaculture in island saline water, which has been practiced worldwide, including in Thailand, Brazil, China, Mexico, Ecuador, the United States, and Vietnam (Dinh, 2015). However, most studies on crustacean aquaculture at low salinity are mainly focusing on shrimp *Litopenaeus vannamei* (Wang et al., 2015, 2017; Chen et al., 2019) and the cultivation of crabs has not been reported (Roy et al., 2010).

MATERIALS AND METHODS

Experimental Animals

Juvenile crabs (*E. sinensis*) were obtained from a local crab company in Shanghai, China. All crabs were acclimated in several plastic tanks (100 × 80 × 60 cm) for 2 weeks in the Biological

Station of East China Normal University. Healthy crabs (450) (2.10 ± 0.20 g) were randomly assigned into 15 tanks (300 L) with five corrugated plastic pipes (12 cm long and 25 mm in diameter) and five arched tiles as the shelters to avoid attacking. Three treatments included a control (pH = 8.0 ± 0.20), low pH stress (pH = 6.5 ± 0.20), and high pH stress (pH = 9.5 ± 0.20) with five replicates each and 30 crabs in each replicate. A commercial diet with 37% crude protein and 7% crude lipid was used in this study. All the crabs were hand-fed to apparent visual satiation thrice daily at 00:00, 09:00, 17:00, and 00:00 h for 8 weeks. The volume of 1/3 to 1/2 of the tank water was exchanged and the water pH was adjusted to the target levels by adding 1 mol L⁻¹ HCl or 1 mol L⁻¹ NaOH stock solution. The pH was measured and adjusted every 8 h. The water quality parameters across all feeding treatments were maintained at $24 \pm 1.0^\circ\text{C}$, dissolved oxygen > 7.5 mg L⁻¹, and ammonia-N < 0.05 mg L⁻¹.

Sample Collection

At the end of the 8-week trial, all crabs in each tank were counted and deprived of feed for 24 h before body weight was determined. Six crabs at the stage of intermolt from each tank were randomly collected and stored at -20°C for the analysis of whole-body composition. Other six crabs from each tank were anesthetized on ice for 10 min. The 1 mL syringe with 1:1 pre-cooled anticoagulant solution (510 mmol L⁻¹ NaCl, 100 mmol L⁻¹ glucose, 200 mmol L⁻¹ citric acid, 43.33 mmol L⁻¹ citric acid, 30 mmol L⁻¹ Na-citrate, 10 mmol L⁻¹ EDTA·2Na, pH 7.3) was used to collect the hemolymph from the third pereopod of each crab. Part of hemolymph was used for total hemocyte counts (THC), and the rest was centrifuged at $4000 \times g$ for 20 min at 4°C to collect the serum. Crabs were dissected to collect the hepatopancreas, and all hepatopancreas and serum samples were stored at -80°C for further analyses. The protocols for using animals in this study were approved by the Committee on the Ethics of Animal Experiments of East China Normal University (f20190201).

Growth Performance Evaluation and Whole-Body Composition Analysis

Survival rate (SR), weight gain (WG), and protein efficiency rate (PER) were calculated using the following formulae:

Survival rate (SR, %) = $100 \times (\text{final crab number}/\text{initial crab number})$;

Weight gain (WG, %) = $100 \times (\text{final weight} - \text{initial weight})/\text{initial weight}$;

Protein efficiency rate (PER) = wet weight gain/dry weight of protein intake.

The body composition of crabs and the proximate composition of diets were determined according to the standard methods (AOAC) (Chem, 1992). The whole-body proximate composition was measured in four crabs from each tank and then the average per tank of the same set was used for statistical analysis. Moisture was determined by oven dry at 105°C to a constant weight. Crude protein was measured by the Kjeldahl method using Kjeltac™ 8200 (Kjeltac, Foss, Sweden).

Lipid was quantified by the method of Bligh and Dyer (Folch et al., 1951) using a vacuum drying oven (DZF-6050, Jinghong, Ltd., Shanghai, China). Samples were digested with nitric acid and incinerated in a muffle furnace (PCD-E3000 Series, Peaks, Japan) at 550°C overnight for ash determination.

Total Hemocyte Counts (THC)

Total hemocyte counts were obtained by using a hemocytometer. Each hemolymph sample was repeated three times and the mean value was recorded for statistical analysis.

Biochemical Analysis

Superoxide dismutase (SOD) and malondialdehyde (MDA) of hepatopancreas were measured by using the iodine-starch colorimetric method with the commercial assay kits (Cat. No. A001-1, A003-1 and A005, Jiancheng, Bioengineering Institute, Nanjing, China). The acid phosphatase (ACP) and alkaline phosphatase (AKP) activity of serum were measured by the disodium phenyl phosphate hydrate method with commercial assay kits (Cat. No. A060-1 and A059-1, Jiancheng, Bioengineering Institute, Nanjing, China) according to the manufacturer's protocols.

RNA Extraction, cDNA Library Conduction, and Sequencing

The hepatopancreas samples were ground in liquid nitrogen, and total RNA was extracted using TRIzol® Reagent in accordance with the manufacturer's instruction (Invitrogen, United States). Extracted RNA was treated with DNase I (Takara, Japan) to remove genomic DNA. The quality and quantity of total RNA were assessed using a Nano Drop 2000 spectrophotometer (Thermo, Wilmington, DE, United States).

The RNA-seq transcriptome library was prepared following the TruSeq™ RNA sample preparation kit from Illumina (San Diego, CA, United States) using 1 µg of total RNA. mRNA was isolated according to the poly A selection method using Oligo (dT) beads and then was fragmented using the fragmentation buffer.

Single-stranded cDNA was synthesized with random hexamers using RNA as a template. Double-stranded cDNA was synthesized with the effect of dNTPs, DNA polymerase I, RNase H, and buffer, and it was purified by AMPure XP beads. A single (A) was added using Klenow buffer.

Adaptor-modified fragments were selected by AMPure XP beads, and PCR amplification was performed for 15 cycles. After quantification by Qubit 2.0, the sequencing library was diluted to 1.5 ng/µL. The insert size of the library was tested by Agilent 2100, and was quantified by the Q-PCR method to guarantee the quality of the sequencing library. RNA-seq sequencing library was sequenced using Illumina HiSeq 4000. The raw sequencing data were evaluated by FAST-QC¹ to remove low-quality reads (i.e., Q value < 20), adapter sequences, reads with ambiguous bases ("N"), and fragments < 20 bp in length. As there was no reference genome for Chinese mitten crab, the

sequenced reads was spliced using Trinity first (Grabherr et al., 2011), and the hierarchical cluster analysis was used with Corset² (Davidson and Oshlack, 2014).

Gene Expression Analysis and Functional Enrichment

To identify differential expression genes between the two different treatments in two tissues, RSEM³ was used to quantify gene abundance. The expression level of each transcript was calculated according to the method of fragments per kilobase of transcript sequence per millions base pairs (FRKM). Differential expression analysis was conducted using DESeq2 with p -value ≤ 0.05 . Gene Ontology (GO) analysis⁴ was performed to facilitate elucidating the biological implications of unique genes in the significant or representative profiles of the differentially expressed genes; 19 significantly changed genes were randomly chosen for validation by real-time quantitative PCR (qRT-PCR). Kyoto Encyclopedia of Genes and Genomes (KEGG) was performed for functional-enrichment analysis in the metabolic pathways at FDR ≤ 0.05 . KEGG pathway analysis was carried out using KOBAS⁵.

Statistical Analysis

All results were tested for normality and homogeneity of variance by Levene's equal variance test. Data were presented as means $\pm$ standard error (SE). Each variable was analyzed by one-way analysis of variance (ANOVA) followed by Duncan's multiple range test (SPSS 19.0 package; SPSS Inc., New York, NY, United States). The levels of statistical difference were set at $P < 0.01$ as extreme difference and $P < 0.05$ as significant difference.

RESULTS

Growth Performance and Whole-Body Composition

The weight gain of the crab was significantly affected by pH stress. The crabs at pH 9.5 gained more weight than those at pH 6.5 (Figure 1A). There was no significant difference in survival rate, crude lipid, and crude protein (Figures 1B,C). The PER at low pH was lower than the control and the high pH group (Figure 1D), but there was no difference between the control and high pH group.

Antioxidant Capacity and Immune Status

The MDA content in hepatopancreas was highest in the pH 6.5 group and there was no significant difference between the control group and high pH group (Figure 2A). The SOD activity in hepatopancreas was higher in the high pH group than the other two groups (Figure 2B). THC in the pH stress groups were higher

¹<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>

²<https://code.google.com/p/corsetproject/>

³<http://deweylab.biostat.wisc.edu/rsem/>

⁴<http://www.geneontology.org/>

⁵<http://kobas.cbi.pku.edu.cn/home.do>

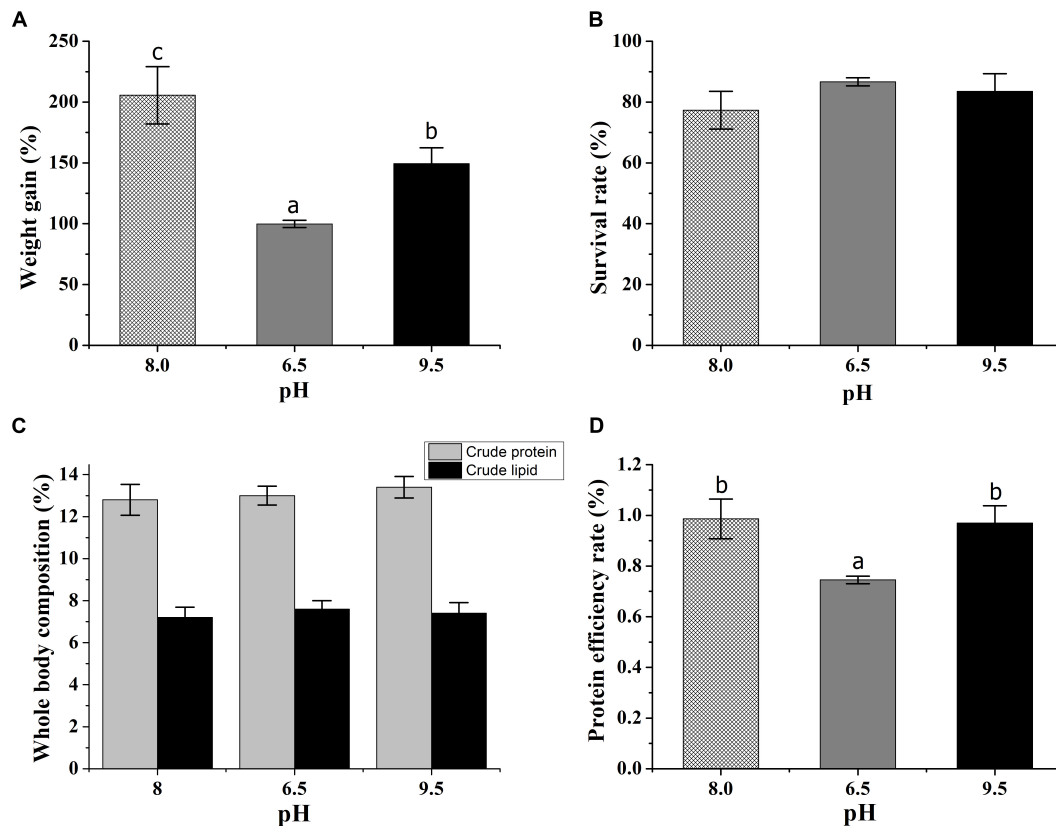


FIGURE 1 | Effects of acidic and alkaline stress on the weight gain (A), survival rate (B), whole-body composition (C), and protein efficiency rate (D) of *E. sinensis*. The results were presented as mean $\pm$ SE and different lowercase letters mean significant differences by Duncan's test ($P < 0.05$).

than those in the control group (Figure 2C). Crabs at pH 9.5 had the highest ACP and AKP activities (Figures 2D,E). There was no significant difference between the control group and low pH group in the ACP and AKP activities.

Transcriptome Sequencing and *de novo* Assembly

A total of 175.7M, 156.2M, and 168.1M clean reads were obtained from the control, low pH, and high pH group, respectively, after the removal of low-quality reads. The mean GC (%) of these three groups was 56.80, 55.79, and 55.92%, respectively. In total, 83,025 transcripts were obtained and analyzed by *de novo* assembly (Table 1). The summary of the RNA-Seq results is shown in Table 1 and the accession number of *de novo* was PRJNA554226.

Analysis of Gene Expression

The mean mapping ratio of the control, low pH, and high pH groups was 82.77, 82.27, and 82.56%, respectively. The expression of 2459 genes (1344-up and 1115-down) was significantly different in the hepatopancreas between the low pH and the control groups (L pH vs Control) ($P < 0.05$, Figure 3A). The expression of 1645 genes (775-up and 870-down) was significantly different between the high pH and the control groups (Figure 3B). Log FCs from qRT-PCR were compared with

the RNA-seq expression analysis results and these two results had a correlation coefficient of 0.8 demonstrating the credibility of the RNA-Seq results.

GO and KEGG Analysis

Based on the GO analysis, the functions of the differently expressed genes could be classified into three main categories: biological process, molecular function, and cellular component. KEGG pathways were analyzed to obtain significantly changed pathways involving the differently expressed genes. Ten out of 23 significantly changed pathways were related to metabolism in the low pH vs control group, while there were 19 significantly changed pathways in the high pH vs control group and eight of them were in metabolism. According to the reported functions of these pathways, most of them participated in carbohydrate metabolism (Tables 2, 3).

DISCUSSION

A pH value between 6.5 and 9.0 is satisfactory for most freshwater animals in long-term of farming practice (Alabaster and Lloyd, 1980). Moreover, a high pH value of 9.5 is quite common in ponds for crab farming (Kong et al., 2012). In this study, there was no significant difference in growth performance parameters between

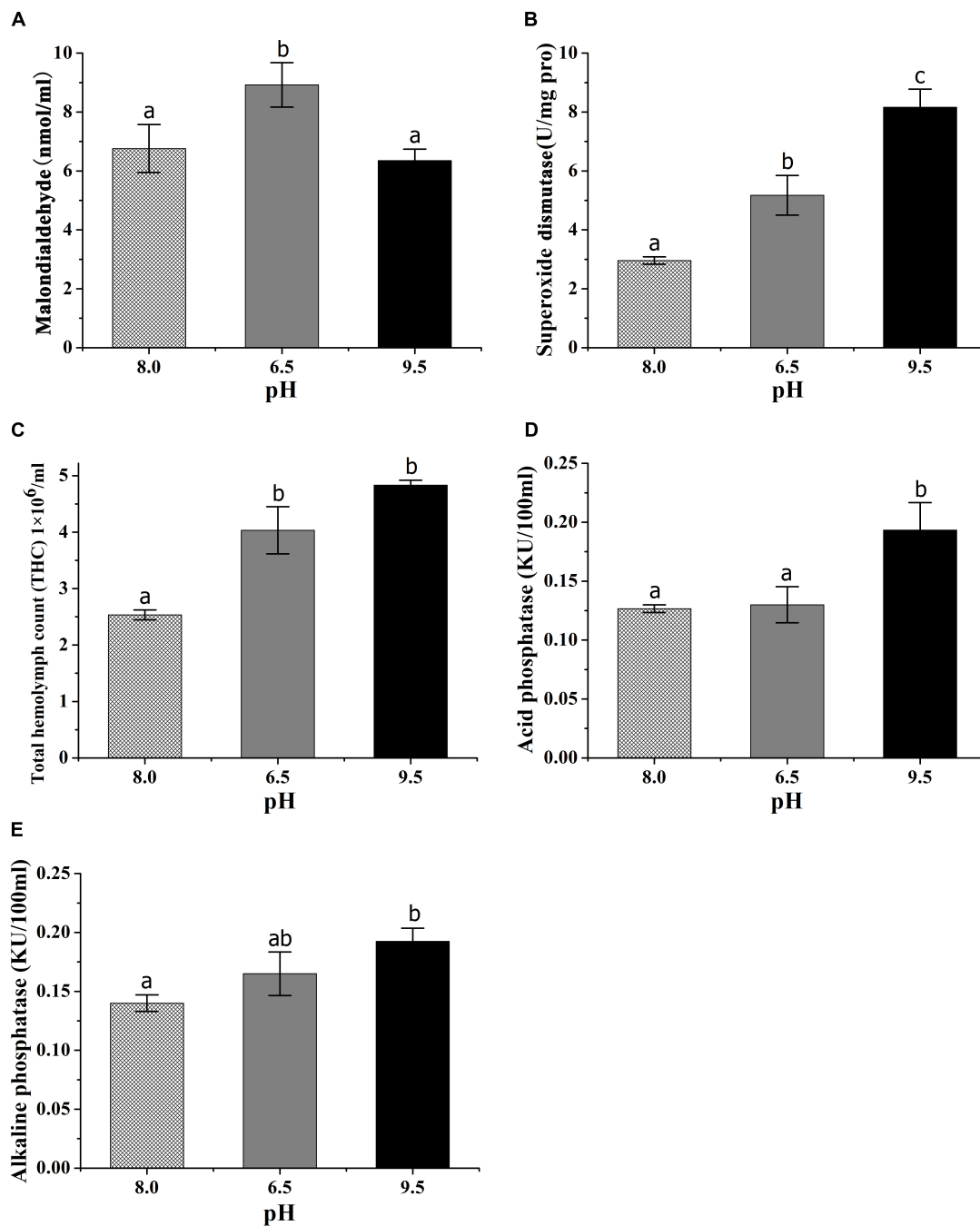
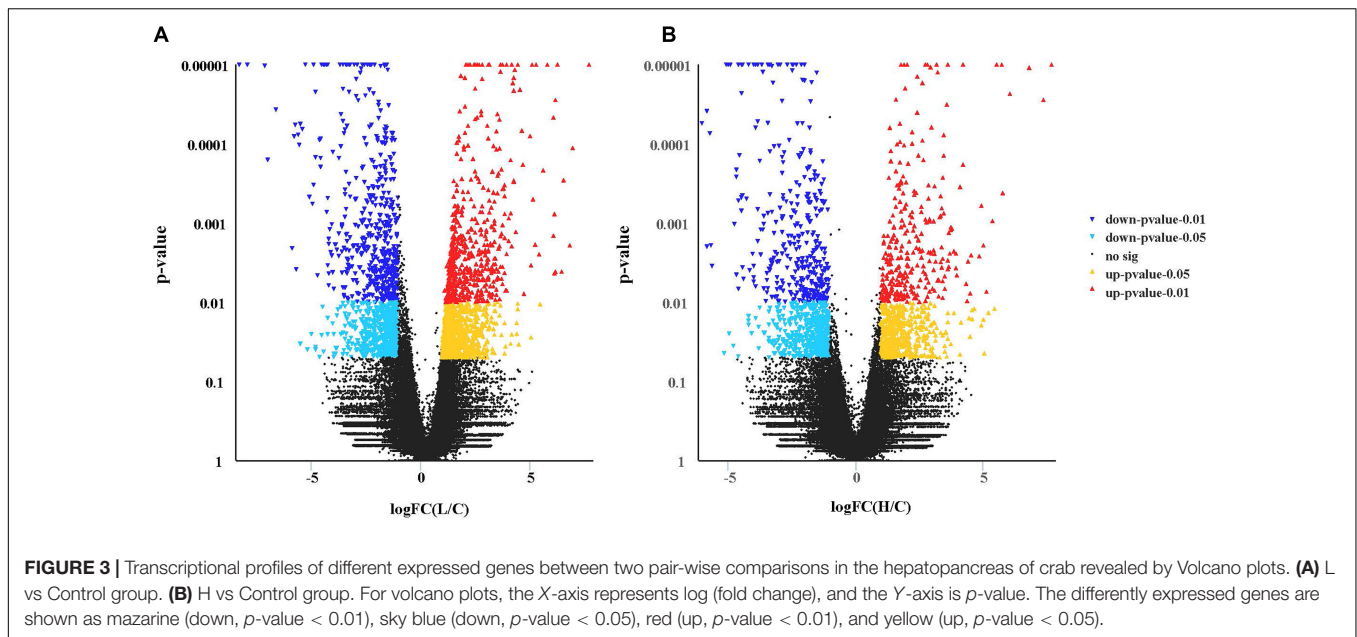


FIGURE 2 | Effects of acidic and alkaline stress on the content of malondialdehyde (A), the activities of superoxide dismutase (B), total hemocyte counts (C), acid phosphatase (D), and alkaline phosphatase (E) of *E. sinensis*. The results were presented as mean $\pm$ SE and different lowercase letters above each column mean significant differences by Duncan's test ($P < 0.05$).

TABLE 1 | Basic information of the transcriptome analysis.

	Min length	Max length	Mean length	Percent GC	N50	Total nucleotides	Total numbers
Transcripts	201	20,188	1027	46.92	1624	85,306,988	83,025
Genes	201	20,188	904	46.67	1389	58,750,515	64,995



any pH groups after 8 weeks, indicating the Chinese mitten crab can survive in pH 6.5–9.5. But there was also a study showing that 40% healthy crabs (about 50 g) died after exposure to pH 9.5 for 2 weeks (Pan et al., 2017). That is because smaller crabs have greater tolerance to environmental stress than bigger ones (Tood and Dehnel, 1960). Weight gain was reduced by water pH stress, and the acidic pH had more negative effect, which is consistent with the result of pH effect on protein utilization. The negative effect of pH stress may be mediated by the disruption of metabolic regulation, extra energy expenditure, and reduction of nutrient absorption (Leal et al., 2011), which may explain why weight gains in the low pH and high pH groups are low in the current study. The other possible reason may be that acidification may alter the dynamics of muscle water content, which would lead to the changes of weight gain.

Environmental stress can trigger the over production of reactive oxygen species (ROS) and result in a severe damage to cells (Du et al., 2013). MDA is one of the most known products of lipid peroxidation and is a well-established marker of oxidative stress, which can be induced by a wide range of environmental stress (Wang et al., 2004). Therefore, the increase of MDA is usually associated with various environmental stress and the pathological state of animals (Zhang Y. et al., 2015). Aquatic crustaceans lack an adaptive immune response and mostly rely on innate immune responses, among which the antioxidant defense system is important to reduce ROS damage (Muta and Iwanaga, 1996; Zhang W. et al., 2015). SOD is a key antioxidant enzyme directly participating in the removal of ROS (Kobayashi et al., 2019). ACP and AKP are highly conserved enzymes and play an important role in the non-specific immunity of crustaceans (Yang et al., 2007; Gisbert et al., 2018). Thus, higher ACP and AKP activities have a positive effect on the defense against external microbial invasion (Wu et al., 2019). Total hemocyte numbers of crustaceans can vary greatly after exposure to environmental

stress and infections by bacteria, fungi, and viruses (Truscott and White, 1990; Vargas et al., 1997; Iwanaga and Lee, 2005; Vazquez et al., 2009). Any increase in THC could lead to an increase of immune defense ability (Min et al., 2015). The results of the present study reveal that all three immune parameters of the crabs were significantly affected after exposure to alkaline acid stress. Also, an increase of OH^- concentration seems to have more stimulative effects on immune function of the crabs than those at a high H^+ concentration, which may be the reason why the MDA content in the high pH group was lower than that in the low pH group.

When suffering in environmental stress, crustaceans require additional energy to maintain homeostasis (Wang et al., 2016). Carbohydrates are often included in artificial diets to serve as an energy source, and they can also meet the high energy demand of aquatic animals under stress (Tseng and Hwang, 2008; Wang et al., 2017). Mitochondrial oxidative phosphorylation provides over 90% of the energy produced by aerobic organisms; therefore, the regulation of oxidative phosphorylation is a major issue for coping with the environment changes to meet more energy need (Bermejo-Nogales et al., 2015). However, animals would use glycolysis to produce energy in the absence of adequate oxygen. In the presence of adequate oxygen levels, the intracellular pH might be a factor that determines which way to obtain energy. For instance, oxidative phosphorylation could be driven in an acidic condition and aerobic glycolysis is driven in an alkaline condition (Khalifat et al., 2008; Calderon-Montano et al., 2011). Similar to these findings, the significant changes in oxidative phosphorylation, TCA cycle, and pyruvate metabolism pathways at low pH may be driven by the acidic medium, and the glycolysis/gluconeogenesis pathways can be driven by high pH. However, the primary response in aquatic animals is usually accompanied with high levels of plasma glucose and anaerobic

TABLE 2 | Significantly changed genes and KEGG pathways involved in metabolism response in the hepatopancreas of Chinese mitten crab under pH 6.5 vs in the control group for 8 weeks.

Pathway term	Involved genes	P-value
Oxidative phosphorylation	CYC1, CYT1, petC SDHA, SDH1 NDUFA12 COX5B ATPeV1H NDUFS7ATPF1D, atpH ATPeF1B, ATP5B, ATP2 QCR2, UQCRC2 ATPeV1F, ATP6S14 UQCRCFS1, RIP1, petA QCR8, UQCRCQ SDHC, SDH3 ATPeF0B, ATP5F1, ATP4 NDUFA4 NDUFAB1 COX5A NDUFS3 ATPeF0O, ATP5O, ATP5 ATPF0C, atpE NDUFB2 ATPF1B, atpD ATPeF1D, ATP5D, ATP16 ppa NDUFB7	3.30E-06
Glycolysis/gluconeogenesis	frmA, ADH5, adhC PDHX LDH, ldh E4.1.1.32, pckA, PEPCK PGK, pgk ENO, eno DLAT, aceF, pdhC PTS-Glc-EIIA, crr GPI, pgil ACSS, acs PDHA, pdhA GAPDH, gapA DLD, lpd, pdhD FBA, fbaA	6.07 E-04
Citrate cycle (TCA cycle)	PDHX IDH1, IDH2, icd E4.1.1.32, pckA, PEPCK SDHC, SDH3 DLAT, aceF, pdhC MDH2 SDHA, SDH1 PDHA, pdhA ACO, acnA DLD, lpd, pdhD	8.39 E-04
Pyruvate metabolism	PDHX LDH, ldh E4.1.1.32, pckA, PEPCK MDH2 DLAT, aceF, pdhC ACSS, acs PDHA, pdhA DLD, lpd, pdhD	0.011
Starch and sucrose metabolism	UGT UGDH, ugd E3.2.1.28, treA, treF E3.2.1.4 MGAM SI PTS-Glc-EIIA, crr UGP2, galU, galF GPI, pgil TPS	0.011
Taurine and hypotaurine metabolism	CSAD GADL1, CSADC, ADC	0.034
Glutathione metabolism	IDH1, IDH2, icd GST, gst E1.11.1.9 GCLC E4.1.1.17, ODC1, speC, speF	0.042
Amino sugar and nucleotide sugar metabolism	E3.2.1.14 PTS-Nag-EIIC, nagE HEXA_B PTS-Glc-EIIA, crr UGP2, galU, galF GPI, pgi	0.045
Steroid biosynthesis	E1.14.13.72, SC4MOL, ERG25 DHCR24	0.046
Pentose and glucuronate interconversions	UGT UGDH, ugd DHDH UGP2, galU, galF DCXR	0.050

TABLE 3 | Significantly changed genes and KEGG pathways involved in metabolism response in the hepatopancreas of Chinese mitten crab under pH 9.5 vs in the control group for 8 weeks.

Pathway term	Associated genes	P-value
Starch and sucrose metabolism	MGAM E3.2.1.1, amyA, malS E3.2.1.28, treA, treF SI PTS-Glc-EIIA, crr GPI, pgil TPS	1.75 E-04
Glycolysis/gluconeogenesis	ENO, eno E4.1.1.32, pckA, PEPCK GAPDH, gapA ADPGK FBA, fbaA LDH, ldh PTS-Glc-EIIA, crr PGK, pgk GPI, pgil GAPDH, gapA gpmI	8.07 E-04
One carbon pool by folate	FTCD MTHFS metF, MTHFR	0.010
Amino sugar and nucleotide sugar metabolism	nagA, AMDHD2 PTS-Nag-EIIC, nagE PTS-Glc-EIIA, crr GPI, pgi manA, MPI	0.010
Linoleic acid metabolism	PLA2G, SPLA2 CYP2J CYP3A	0.015
Galactose metabolism	MGAM SI	0.025
Glycosaminoglycan degradation	NAGLU SGSH	0.043
Taurine and hypotaurine metabolism	CSAD	0.048

glycolysis (Arends et al., 1999; Acerete et al., 2004; Fazio et al., 2015). This may explain why both oxidative phosphorylation and glycolysis/glycogenesis pathways were upregulated in the low pH group. Due to the enhanced glucose activities, the digestion of carbohydrates was increased in terms of the upregulated starch and sucrose metabolism pathways in both acidic and alkaline groups.

Except for conventional carbohydrates, the presence of non-glycogenic carbohydrates also contributes to the concentration of total carbohydrates in the hepatopancreas of most crustaceans (Chang and O'Connor, 1983). The amino sugar glucosamine (GlcN) and *N*-acetylglucosamine (GlcNAc) are prevalent in the biosphere. For instance, amino sugars are the major component of the exoskeleton of crustaceans (Zeng et al., 2016). The changes in the amino sugar pathways in the current study indicate that acidic and alkaline stress might affect the molt activity of Chinese mitten crab. Similarly, some other studies also found that environmental stress would change molting frequency in ammonia-exposed *Penaeus monodon*, saponin-exposed *Penaeus*

japonicus, copper sulfate- exposed *P. monodon*, nitrite-exposed *P. monodon*, and acid-exposed *Macrobrachium rosenbergii* (Chen and Chen, 1992, 2003; Chen and Lin, 1992). In the current study, the finding is further verified by the changes of pentose and glucuronate interconversions, galactose metabolism pathways, and steroid biosynthesis pathways in low pH and high pH groups.

Glycosaminoglycans are heteropolysaccharides composed by a repeat disaccharide unit in which one of the two monosaccharides always contains an amino sugar (*N*-acetylglucosamine or GlcNAc) (Antonio et al., 2013). A previous study reported that the crayfish *Orconectes virilis* might depend on the pentose pathway during intermolt and glycolysis during premolt in carbohydrate metabolism (Mcwhinnie, 1962). The glycolytic, pentose phosphate, and glucuronate pathways are operated as catabolic pathways for glucose utilization in intermolt crayfish, *Pacifastacus leniusculus* (Puyear et al., 1965). Galactose also appears frequently during the premolt of period to satisfy the carbohydrate need of molting (Vernberg and Vernberg, 1972).

As a member of gonad stimulatory hormones, steroid plays a pivotal role in molting and reproduction in crustaceans (Subramoniam, 2017). Steroid hormones also have antioxidant and free radical scavenging activities and the physiological metabolisms involved in steroid in the hepatopancreas of crustacean would promote SOD activity (Cai et al., 2002; Wu et al., 2016). The SOD activity of the crab in low pH group was enhanced in the current study. Enzymes such as SOD, glutathione peroxidases (GPx), and some non-enzymatic defense such as glutathione (GSH), tocopherols, taurine, and urate constitute the first line of defense to inactive ROS and scavenge free radicals (Sharma et al., 2004; Wang et al., 2019). The thiol group in the cysteine moiety of GSH is a reducing agent and can be reversibly oxidized and reduced (Meister and Anderson, 1983). However, GSH is an acidic molecule characterized by a γ -linked amino acid, and the cysteine residue can reduce its stability in an alkaline environment (Anik et al., 2016; Giustarini et al., 2016). That is why the GSH metabolism pathways were significantly changed in the low pH group but not in high pH group. Taurine, hypotaurine, and their metabolic precursors (cysteamine, cystic acid, and cysteinesulfinic acid) may protect the organisms against a variety of oxidants (Konukoglu et al., 1999). As antioxidants could protect the crustaceans from oxidative stress, the taurine and hypotaurine metabolism pathways were significantly changed in both low and high pH groups.

Some crustaceans could also convert the linolenic acid to the highly polyunsaturated fatty acids such as eicosapentaenoic acid in the similar way to what has been reported in fish (Kabeya et al., 2018). Long chain polyunsaturated fatty acids (LC-PUFA, involved in eicosapentaenoic acid) have an important role in physiological processes such as immune and stress responses (Tocher, 2010; Norambuena et al., 2015). The changes of the linoleic acid metabolism in the high pH group indicate that alkaline stress may stimulate *de novo* synthesis of EPA, and improve the ability of stress resistance in crab.

In general, the crab growth was suppressed by both acidic and alkaline stress, especially at pH 6.5. The increase of OH⁻ concentration seems to have a more stimulative effect on antioxidant ability and immunity and stronger ability of stress resistance than the crabs under acidic stress. Environmental stress would increase the metabolic activity of animals to compensate the change and maintain homeostasis (Koehn and Bayne, 1989). In the current study, data of transcriptome reveal that the

main metabolic changes were the pathways related to glucose metabolism as carbohydrates can rapidly provide energy to meet the high energy demand of aquatic animals in a stress condition. Oxidative phosphorylation might be the main source of energy under acidic stress, while the aerobic glycolysis supplies most energy during alkaline stress. The pathway analysis indicates that pH stress may affect the molting process, but this claim needs a longer term field study to confirm.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the PRJNA554226.

ETHICS STATEMENT

The animal study was reviewed and approved by the Committee on the Ethics of Animal Experiments of East China Normal University.

AUTHOR CONTRIBUTIONS

XW, ZH, EL, and LC designed the research. XW, ZH, CW, and CQ conducted the research and contributed to the data acquisition and analysis. XW and LC contributed to the draft and the final writing of the manuscript. ZG and JQ revised the manuscript. All authors agreed to be accountable for all aspects of the work and approved the final manuscript.

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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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