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RESEARCH REPORT

Antioxidant stress response to fluctuations of dissolved oxygen and temperature in a semi-intensive aquaculture shrimp farm during high summer temperature

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Abstract

The enhancement of the antioxidant system in response to oxidative stress caused by environmental conditions has been amply reported in several animals. In many cases, the studies were done in controlled laboratory conditions and few studies are available under natural or semi-natural settings. In a shrimp farm with semi-intensive culture exposed to natural high temperature and oxygen changes, we evaluated selected antioxidant enzymes gene expression and enzymatic activity, as biochemical indicators. Also, for cell stress, carbonylated proteins and caspase-3 were evaluated, and lactate content for the shift to anaerobic metabolism in *Litopenaeus vannamei.* The shrimp were collected at four time-points: highest temperature (T1); an intermediate time-point (T2, control); lowest dissolved oxygen (T3) and reoxygenation (T4) conditions. In gills, antioxidant enzymes and caspase-3 expression were up-regulated during T3 and T4 compared to the control, carbonylated proteins were reduced, and the activity of antioxidant enzymes was unaffected. At T1, only the expression of *mMnSOD* was induced and lactate content was maintained in all sampling points. In hepatopancreas, only *cMnSOD* expression in T3 and T4 was induced. Carbonylated protein levels were unaffected in all sampling points, but at T1 and T4, caspase-3 expression increased, and antioxidant enzymes activity was reduced. Lactate content decreased in T3 and T4. Our results show a rapid response by the antioxidant enzymes of *L. vannamei* and endurance to cope with environmental changes in a semi-intensive aquaculture pond.

Key Words: hypoxia; high temperature; reoxygenation; oxidative stress; antioxidant; *Litopenaeus vannamei*

Introduction

The white shrimp *Litopenaeus vannamei* is native from the Pacific coast of Baja California in Mexico, to Northern Peru (Dugassa and Gaetan, 2018) and it is the most valuable commercial species of shrimp around the world. The global aquaculture production of *L. vannamei* was 4.9 million tons in 2018, representing 53% of the total crustacean worldwide production (FAO, 2020). Mexico was the sixth largest shrimp worldwide producer in 2019, only behind China, Vietnam, Ecuador, India, and Indonesia and with an expected growth of 180,000 tons for 2021 (Reyes, 2020). *L. vannamei* constitutes 35% of the wild shrimp caught in estuaries and bays in Mexico and 99% of the total production of farmed shrimp in aquaculture ponds (CONAPESCA, 2020). About 75% of the shrimp farms in Mexico are located in the coastlines of the Gulf of California, specifically in the States of Sonora and Sinaloa (Reyes, 2020).

In the coastal shrimp producing region of Sonora, Mexico, the zone of the Gulf of California is currently having an increasing trend in the average sea surface temperature (SST). The average values of the SST are ≥ 26 °C from June to October, with a maximum of 31.67 ± 0.51 °C in August and a low average of 18 ± 1.29 °C in November (García-Morales *et al*., 2017). Likewise, farmed shrimp in South America are produced in large quantities in extensive or semi-intensive systems in water temperatures between 26 – 32 °C (Bauer *et al*., 2021).

During the past 50 years, oxygen levels globally have been decreasing in the open ocean and coastal

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Fig. 1 Graphic representation of the coast of Hermosillo and the shrimp farm. Shrimp were sampled in a shrimp farm located in the coast of Hermosillo, Sonora, in northwestern Mexico, near Kino Bay. The image shows Mexico geographic area, Hermosillo coast outline location and satellite image of the shrimp farm. The exact site of sampling was 28° 38' 35.9" N 111° 47' 33.2" W

waters (Breitburg *et al*., 2018), and this event also commonly occurs in aquaculture ponds (Chang and Ouyang, 1988). The coastal zone of the Gulf of California suffers seasonal hypoxia (Diaz, 2001), where dissolved oxygen (DO) can be as low as 2 mg O_2/L (Diaz and Rosenberg, 2008), a threshold to establish hypoxia. Additionally, hypoxic conditions are exacerbated in higher water temperatures due to the lower oxygen solubility as temperature raises and also, the higher oxygen consumption by other organisms present (Breitburg *et al*., 2018). Both high temperature (Suzuki and Mittler, 2006) and hypoxia (Guzy and Schumacker, 2006) cause overproduction of reactive oxygen species (ROS) and these two stressors often occur simultaneously in aquatic environments (Levesque *et al*., 2019).

L. vannamei is the major shrimp species cultivated around the world due to the rapid growth compared to other shrimp species, making it the preferred shrimp species to be reared at high stocking densities. They have also high feed conversion rates, disease tolerance, and the ability to resist stressful environments (Jia *et al*., 2018; Fei *et al*., 2020; Sun *et al*., 2021). This shrimp species appears to have good physiological, metabolic, and molecular responses to survive concurrent stressors such as hypoxia and high temperature. One of the major defense responses is their antioxidant system to counteract oxidative stress and apoptosis caused by hypoxia and high temperature (González-Ruiz *et al*., 2021).

The first line of enzymes in the antioxidant system include superoxide dismutase (SOD) that converts superoxide radicals to hydrogen peroxide and oxygen, and catalase, that degrades hydrogen peroxide to oxygen and water (Pollitt *et al*., 1978). Previous studies in *L. vannamei* reported the nucleotide and amino acid sequences of catalase (Trasviña-Arenas *et al*., 2013) and both MnSODisoforms, the cytosolic (cMnSOD) (Gómez-Anduro *et al.*, 2006) and the mitochondrial (mMnSOD) (González-Ruiz *et al*., 2020). The expression of both MnSOD in *L. vannamei* are induced under hypoxia and reoxygenation at high temperature (González-Ruiz *et al*., 2020; Estrada-Cárdenas *et al*., 2021). Also, the enzymatic activities of SOD and catalase increased at high temperature in hypoxic and reoxygenation conditions (González-Ruiz *et al*., 2020; Estrada-Cárdenas *et al*., 2021). These studies indicate that

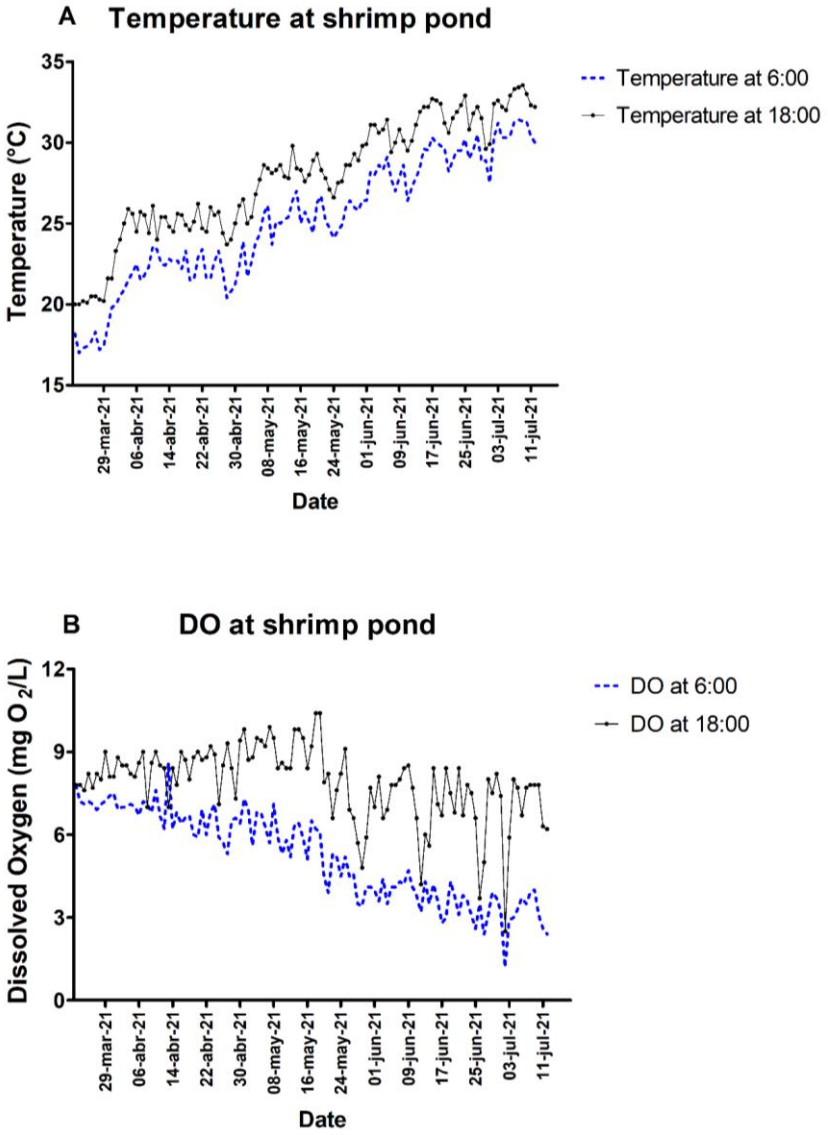

Fig. 2 Daily variation of temperature and oxygen in a semi-intensive commercial shrimp farming pond. Panel A: Temperature in the shrimp pond was recorded twice daily at 6:00 and at 18:00 (HH:MM). Panel B: Dissolved oxygen (DO) in the shrimp pond was recorded twice daily at 6:00 and at 18:00 (HH:MM). The data was collected from March 22nd 2021 – July 12th 2021

L. vannamei responds with the induction of their antioxidant system to counteract the effects of events that lead to redox imbalance such as hypoxia and their concomitant reoxygenation, as well as high temperature.

The early activation of the antioxidant system during oxidative stress has been reported in many organisms. It is known to occur in at least 83 animal species, including vertebrates and invertebrates (Giraud-Billoud *et al*., 2019), indicating an important and probably universal organismal response and has been called “Preparation for Oxidative Stress” (POS). Many of these types of studies have been done in “laboratory experiments” as mentioned by Diamond in 1983. In these cases, the investigator monitors one or more independent variables in controlled laboratory conditions to determine the effect over the dependent variables. However, laboratory experiments are only a proxy to natural settings, since ecosystems are constantly changing and some variables may not be considered Therefore, the “natural experiment” approach is used to test and confirm laboratory findings. In these types of studies, the experimenter only controls the site or time of sampling at which only the independent selected variables are changed and used to analyze their effect on the dependent variables, but all the other variables are “left alone”, without interference of the researcher. These experiments are more attached to the real conditions that occur in nature (Diamond, 1983). Some studies have been done to evaluate animal

responses in a natural setting to test the induction of POS response. These include the frogs *Proceratophrys cristiceps* (Moreira *et al*., 2020) and *Pleurodema diplolister* (Moreira *et al*., 2021a), the Asian common toad *Duttaphrynus melanostictus* (Patnaik and Sahoo, 2021), the characid *Astyanax elachylepis* (Ondei *et al*., 2020) and the mussel *Brachidontes solisianus* (Moreira *et al*., 2021) under a variety of natural environmental stressors.

We have previously evaluated in the white shrimp *L. vannamei*, several molecular and biochemical responses to environmental stressors such as hypoxia, reoxygenation, and high temperature, using laboratory-controlled bioassays. In this study, we used a natural experiment approach to investigate the changes in the antioxidant response and oxidative damage in shrimp *L. vannamei* in a semi-natural setting of temperature and dissolved oxygen DO in an aquaculture shrimp farm in Sonora, Mexico, during the high summer temperature prevalent in this region. We measured the relative expression of the genes for cMnSOD, mMnSOD, catalase, and caspase-3, the activities of SOD and catalase, and the oxidative stress shown by carbonylated proteins and also, lactate content as an indicator of anaerobic metabolism in the gills and hepatopancreas of *L. vannamei.*

Materials and Methods

Sampling and environmental data

Juvenile shrimp *L. vannamei* (19.57 ± 1.98 g) were sampled in a semi-intensive shrimp farm located in the coast of Hermosillo Sonora, in Northwestern Mexico near Kino Bay (28° 38' 35.9" N 111° 47' 33.2" W; Fig. 1). In this system, water exchange is used to improve water quality, but it was avoided during this sampling to maintain the conditions of the pond. The shrimp farm production is done in conventional earthen ponds with no temperature or DO level control. Before the sampling, the temperature and DO in the pond were monitored twice daily at 6:00 and 18:00 h (Fig. 2). The data helped us to design the sampling strategy with 4 time-points: at the highest temperature, during the lowest DO and two intermediate points. The sampling was done in July 2021. The first sampling time (T1) was at 17:45 h when the temperature in the pond was the highest. The second sampling time (T2) was at 22:00 h, an intermediate time-point between the highest temperature and the lowest DO at the pond. At this time, the sun had set and the temperature had dropped, while the oxygen was in adequate levels for the shrimp (Van Wyk and Scarpa, 1999). For these reasons, T2 was used as the control. The third sampling time (T3) was at 04:50 h, right before sunrise, when the DO in the pond was at the lowest level due to the lack of sunlight for photosynthesis of other organisms present in the farm and the consumption of oxygen. The last sampling time (T4) was at 07:50 h when the sun had already risen for at least 2:30 h; at this time photosynthesis was on course and a reoxygenation effect occurred in the farm (Table 1). During sampling, the seawater temperature and DO were recorded with an oximeter (YSI model 55, Yellow Spring, OH, USA) and the salinity conditions were measured with a refractometer. The salinity was ~38‰ at all sampling times.

Sampling and shrimp dissection were done in a portable table next to the shrimp pond, with the appropriate instrumentation and reagents to maintain the integrity of the samples. Animals were manually removed from the pond, quickly cleaned with paper towels, weighed, very quickly dissected, and the tissues of every individual shrimp were rapidly collected. For RNA isolation, parts of the gills and hepatopancreas were placed in a cold microcentrifuge tube containing approximately 10 volumes of TRI reagent (Sigma-Aldrich, St. Louis, MO, USA) (w/v) and the rest of the tissues were placed in another microcentrifuge tube for further analysis. All samples were immediately frozen after collection in dry ice, transported to the laboratory, and stored at -80 °C until analysis.

RNA extraction and cDNA synthesis

Total RNA was isolated from the hepatopancreas and gills of five individual shrimp separately (n=5 experimental units for each sampling time) using the TRI-Reagent and following the procedure provided by the manufacturer. RNA quantification and purity was done by measuring the A_{260} nm and the A_{260}/A_{280} nm ratio, respectively, in a

Table 1 Groups, collection times, temperature, DO and expected stress conditions

Group	Collection time (HH:MM)	T (°C)	DO (mg O_2/L)	Expected stress condition
T1	17:45	33.5	8.6	Highest temperature
T2	22:00	31.0	5.8	Recovery from high temperature
T3	04:50	30.3	3.7	Lowest DO
T4	07:50	30.0	4.8	Reoxygenation

DO = dissolved oxygen

Table 2 Primers and conditions used in RT-qPCR

Primer	Sequence (5'-3')	[Primers][a]	AT (°C)	GenBank accession number
cMnSODF	GCTGAGGCAAAGGAAGCTTAC	500 nM	58	DQ029053
cMnSODR	CTGAGCAACACCAGCCTGC			
mMnSODF	CAGACTTGCCCTACGATTAC	500 nM	58	MN037758
mMnSODR	AGATGGTGTGATTGATGTGAC			
FwCatRT	GTGAAGTTTTACACAGAAGAAGG	500 nM	58	AY518322
CatCBRTRv3	AGGGGTTCCTCTGTCAGAG			
Casp3-F	AGTTAGTACAAACAGATTGGAGCG	190 nM	55	KC660103
Casp3-R	TTGTGGACAGACAGTATGAGGC			
L8F2	TAGGCAATGTCATCCCCATT	800 nM	58	DQ316258
L8R2	TCCTGAAGGGAGCTTTACACG			

[a] = Primers concentration used in qPCR reactions
AT = annealing temperature of qPCR program used

NanoDrop One spectrophotometer (Thermo Scientific). The RNA integrity was verified by 1% agarose gel electrophoresis. RNA samples were treated with DNase I (Roche, Mannheim, Germany) to assure removal of any genomic DNA and was validated by quantitative PCR (qPCR). For each individual RNA sample, two complementary DNA (cDNA) syntheses were done with the Quantitect Reverse Transcription kit (Qiagen) using 500 ng of total RNA according to the manufacturer protocol. The cDNA samples were diluted to a final concentration of 25 ng/µL of the initial total RNA.

Quantification of relative gene expression of target genes by RT-qPCR

The relative expression of the genes was analyzed in five biological replicates or organisms (n = 5) with four technical replicates (two cDNAs for each RNA sample and two qPCR reactions for each cDNA) at each sampling time by RT-qPCR using specific primers for each gene (Table 2). The expression of the ribosomal protein L8 gene was used as a constitutive gene for normalization since it has been validated before as stable in shrimp during hypoxia (Cota-Ruiz *et al*., 2015). qPCR reactions (15 µL) contained: 7.5 µL of 2x SYBR green qPCR master mix (Bimake, Houston, TX, USA) (for cMnSOD, catalase and L8) or 7.5 µL of SsoAdvanced Universal SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) (for mMnSOD and caspase-3), 1 µL of cDNA (equivalent to 25 ng of total RNA), the corresponding primers specific for each gene at specific concentrations (Table 2) and nuclease-free water to complete the reaction. Non-template controls were included in every run to ensure no reagent contamination. The qPCR analyses were performed in a CFX96 Real-Time PCR Detection System (Bio-Rad) under the following cycling conditions: For cMnSOD, mMnSOD, catalase, and L8: 95 °C, 3 min (1 cycle); 95 °C, 30 s, 58 °C, 35 s, 72 °C, 55 s (40 cycles); with a melting curve analysis done at the end of each program by measuring the fluorescence from 65 to 95 °C with an increase of 0.3 °C each 5 s, to confirm the specificity of amplification and no primer dimer formation. For caspase-3: 95 °C, 3 min (1 cycle); 95 °C, 15 s, 55.5 °C, 20 s, 72 °C, 30 s (40 cycles); with the same program at the end to obtain the melting curve. The amplification efficiency for each primer set was calculated from standard quantification curves made with five-fold serial dilutions ranging from 25 to 0.2 ng/µL of cDNA (derived from total RNA) for cMnSOD, catalase, caspase-3 and L8 and 25 to 0.2 ng/µL of specific PCR product for mMnSOD. Relative expression of target genes was normalized to L8 expression and reported as relative expression to the second sampling time T2 (control), using the Pfaffl method (Pfaffl, 2001).

Biochemical assays

Samples of hepatopancreas and gills were homogenized with phosphate buffer (50 mM K_2HPO_4, 50 mM KH_2PO_4, 1 mM EDTA, 1 mM PMSF, pH 7) in proportions 1:3 and 1:2 (w/v), respectively. Hepatopancreas were homogenized with a Kontes™ Pellet Pestle™ Cordless Motor and gill with a sonifier Ultrasonic Branson 250 (Branson Ultrasonic Corporation, Danbury, CT, USA). The samples were kept on ice during homogenization and then, centrifuged at 1000×g for 20 min at 4 °C to remove debris, the supernatant was collected and centrifuged at 1200×g for 10 min at 4 °C. The homogenates were again centrifuged at 10,000×g for 15 min at 4 °C. The resulting supernatant was separated in two aliquots, one aliquot was used to measure antioxidant enzyme activity and the other aliquot for carbonylated proteins and lactate contents.

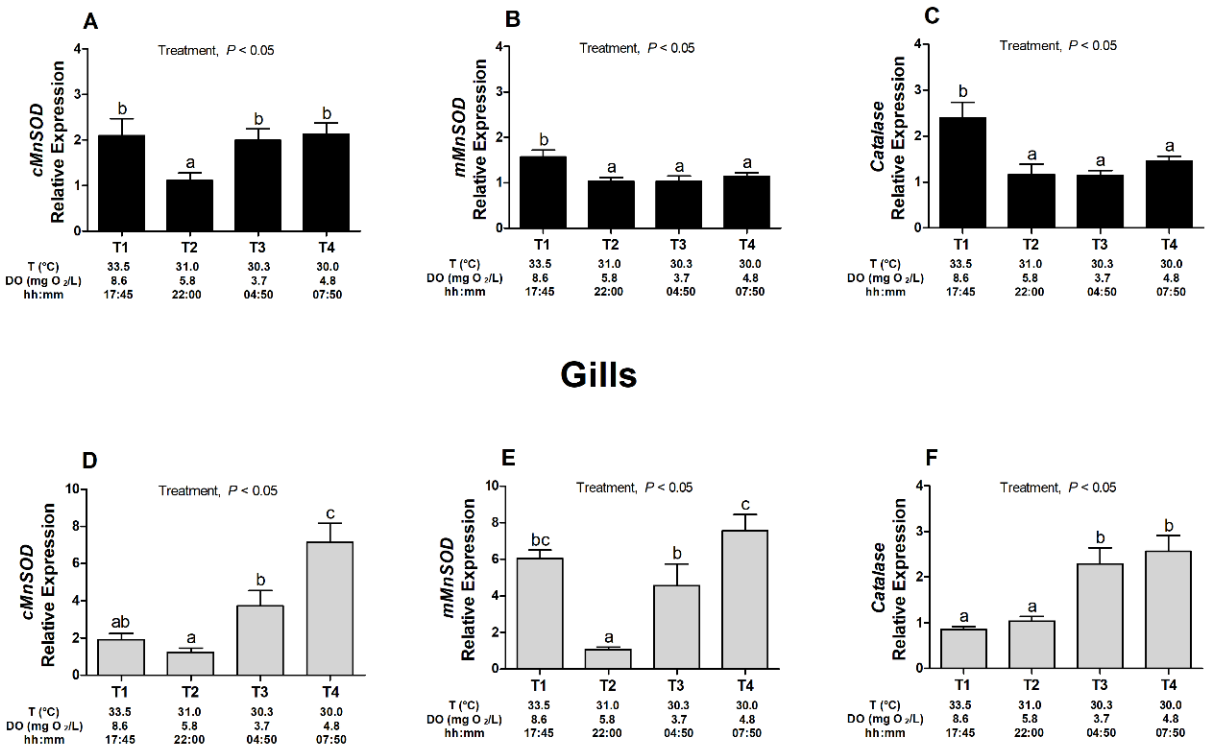

Fig. 3 Relative expression of *cMnSOD, mMnSOD and catalase* in hepatopancreas and gills under the natural temperature and DO of a semi-intensive commercial shrimp farming pond. Panel A: *cMnSOD* relative expression in hepatopancreas. Panel B: *mMnSOD* relative expression in hepatopancreas. Panel C: *catalase* relative expression in hepatopancreas. Panel D: *cMnSOD* relative expression in gills. Panel E: m*MnSOD* relative expression in gills. Panel F: *catalase* relative expression in gills. Data shown are means ± SEM (n = 5). Different lowercase letters denote significant differences ($p < 0.05$) between each sampling point

Antioxidant enzyme activity

Right before these analyses, the aliquot separated for this purpose was brought to 1:10 (w/v) ratio with phosphate buffer (50 mM K_2HPO_4, 50 mM KH_2PO_4, 1 mM EDTA, 1 mM PMSF, pH 7) considering the weight of the tissue used to make the original homogenate. Activities of SOD and catalase were measured spectrophotometrically at 25 °C. Total SOD activity was measured in three technical replicates from five individuals with a commercial kit (RANSOD, Randox) as reported previously (González-Ruiz *et al*., 2020). Total SOD specific activity (units) was calculated as the amount of enzyme that caused an absorbance change of 0.001/min/mg protein at 490 nm, while catalase activity was determined in two technical replicates from five individuals using the method described by Johansson and Håkan Borg (1988) and modified by Trasviña-Arenas *et al*., (2013). The catalase activity assay is based on catalase peroxidase activity, using methanol as an electron donor and measuring formaldehyde production spectrophotometrically at 550 nm with the chromogen Purpald (4-amino-3-hydrazino-5-mercapto-1,2,4-triazole) and thus, it is specific to measure catalase and excludes other peroxidases. Enzymatic activities were normalized by protein content in the homogenates and expressed as U/mg of protein. Protein concentration in the homogenates was quantified using the BCA Protein Assay Kit (Thermo Scientific).

Protein carbonylation quantification

Before the quantification of carbonylated proteins and lactate, the aliquot of the homogenate separated for this purpose was brought to 1:6 (w/v) ratio with phosphate buffer (50 mM K_2HPO_4, 50 mM KH_2PO_4, 1 mM EDTA, 1 mM PMSF, pH 7) considering the original weight of the tissue used for the homogenate. Quantification of carbonylated proteins content was measured in two technical replicates from five individuals using a protein carbonyl content assay kit (MAK094, Sigma-Aldrich, Castle Hill, Australia) as reported previously (González-Ruiz *et al*., 2021). The protein carbonylation assay determines stable dinitrophenyl (DNP) hydrazine adducts produced by the derivatization of protein carbonyl groups with 2,4-dinitrophenylhydrazine (DNPH) by spectrophotometric

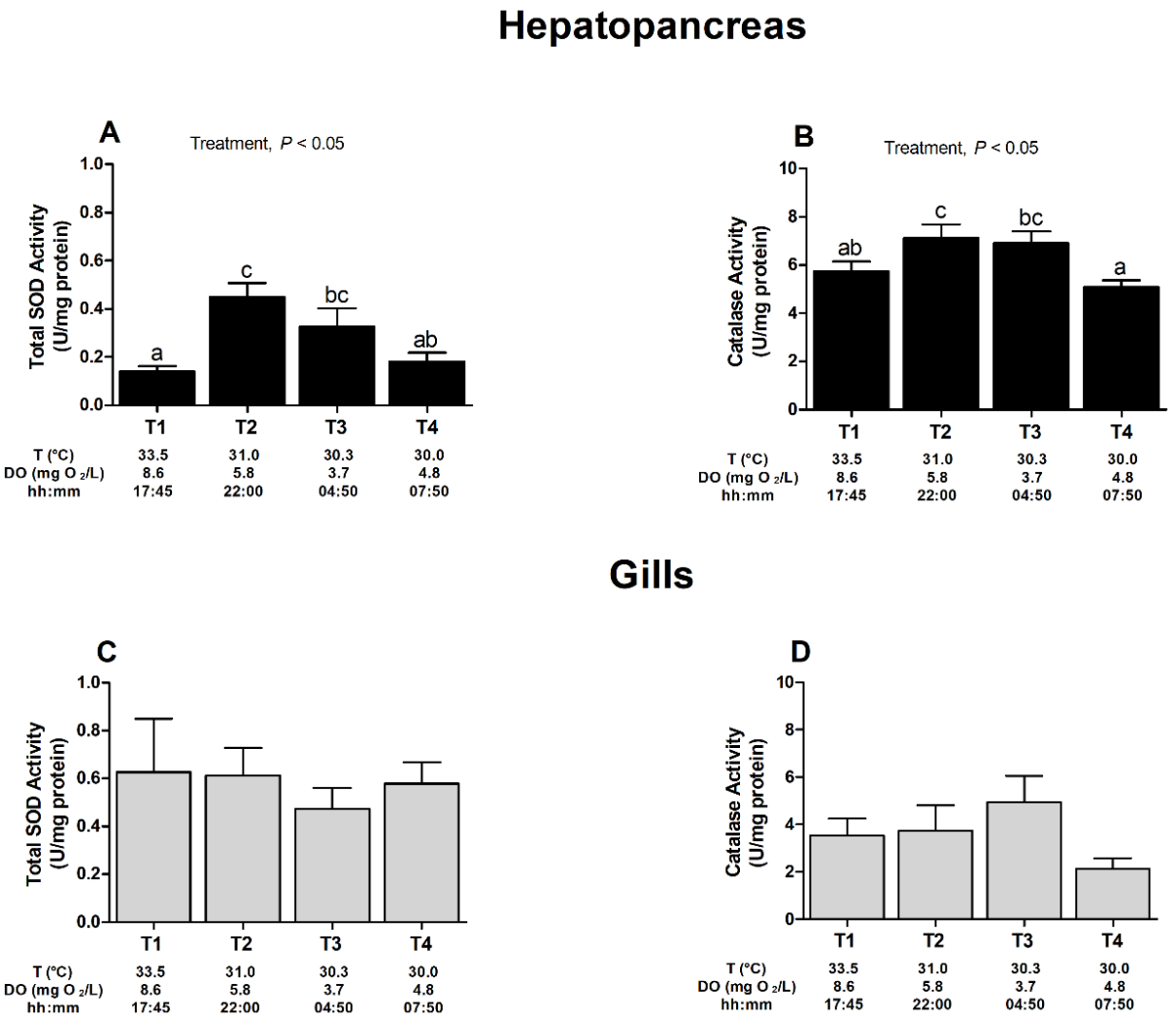

Fig. 4 Total SOD activity and catalase activity in hepatopancreas and gills under the natural temperature and DO of a semi-intensive commercial shrimp farming pond. Panel A: Total SOD activity in hepatopancreas. Panel B: Catalase activity in hepatopancreas. Panel C: Total SOD activity in gills. Panel D: Catalase activity in gills. Data shown are means ± SEM (n = 5). Different lowercase letters denote significant differences ($p < 0.05$) between each sampling point

detection at 375 nm. DNP hydrazine adducts are proportional to the carbonylated proteins present in the sample. The absorbance at 375 nm was measured using a Multiskan Sky microplate spectrophotometer (Thermo Scientific, Waltham, MA, USA) at 25 °C. Carbonylated protein content was expressed as the amount of protein carbonyls in nmol per mg protein. Protein concentration in the homogenates was quantified using the BCA Protein Assay Kit (Thermo Scientific).

Lactate quantification

Concentration of lactate was measured in the aliquot that was brought to 1:6 (w/v) ratio with phosphate buffer described above. Lactate content was quantified with the Lactate Assay kit (Randox) according to the manufacturer instructions. The method was adapted to microplate volume (200 µL final volume), the reactions were done at 37 °C and the absorbance at 550 nm was measured using a Multiskan Sky microplate spectrophotometer. Lactate content was determined in two technical replicates from five individuals and normalized to the protein concentration in each sample. Mean lactate concentrations in each sample were expressed in mg per mg of protein. Protein concentration was quantified using the BCA Protein Assay Kit (Thermo Scientific).

Statistical analysis

Kolmogorov-Smirnov and Levene's tests for normality and homogeneity of variance, respectively, were done in the IBM SPSS Statistics 26.0 software. After that, we used one-way ANOVA to compare the groups of sampling times. When significant differences were found, a *post-hoc*

Table 3 Protein carbonylation and lactate content in hepatopancreas and gills

	Sampling time					*p*-value
Hepatopancreas	T1 n = 5	T2 n = 5	T3 n = 5	T4 n = 5	SEM	Treatment
Protein carbonyl (nmol/mg protein)	13.0	10.5	12.5	12.8	0.90	$p > 0.05$
Lactate (mg/mg protein)	$1.6E^{-02}$	$1.3E^{-02}$	$0.6E^{-02}$*	$0.4E^{-02}$*	0.2	$p < 0.05$
Gills						
Protein carbonyl (nmol/mg protein)	18.6	18.7	10.4*	9.4*	1.20	$p < 0.05$
Lactate (mg/mg protein)	$4.6E^{-03}$	$3.8E^{-03}$	$4.4E^{-03}$	$4.2E^{-03}$	0.94	$p > 0.05$

comparison test was used with Fisher's Least significant difference (LSD) test for multiple comparison. This was done for all the variables. One-way ANOVA and LSD *post hoc* tests were done using the NCSS and PASS, 2007 software. Differences between the means of each group in the analyses were considered significant when $p < 0.05$. Data were plotted as mean ± standard error of the mean (SEM) using the software GraphPad Prism version 5.

Results

Expression of antioxidant enzymes

The relative expression of the antioxidant enzymes genes cMnSOD, mMnSOD, and catalase by RT-qPCR was evaluated in hepatopancreas and gills of *L. vannamei* exposed to natural temperature and DO in a semi-intensive aquaculture shrimp pond (Fig. 3). In hepatopancreas, cMnSOD expression in the sampling times at the highest temperature (T1), at the lowest DO level (T3) and reoxygenation (T4) was 0.9, 0.8 and 0.9-fold more ($p < 0.05$), respectively, compared to T2 (control) (Fig. 3A). For mMnSOD, expression at T1 was 0.5-fold more ($p < 0.05$) compared to T2 (Fig. 3B). Catalase expression at T1 was 1-fold ($p < 0.05$) more compared to T2 (Fig. 3C). In gills, cMnSOD expression at T3 and T4 was 2.0 and 5.2-fold more ($p < 0.05$), respectively, compared to T2 (Fig. 3D). For mMnSOD, expression at T1, T3, and T4 was 4.6, 3.2, and 6.0-fold more ($p < 0.05$) compared to T2 (Fig. 3E). Catalase expression at T3 and T4 was 1.2 and 1.5-fold more ($p < 0.05$) compared to T2 (Fig. 3F).

Antioxidant enzymes activity

The enzymatic activity of SOD and catalase in hepatopancreas and gills changed in a tissue-specific manner (Fig. 4). In hepatopancreas, total SOD activity in T1 and T4 was 220 and 150 % lower ($p < 0.05$) compared to T2 (Fig. 4A), whereas catalase activity in T1 and T4 was 25 and 40 % lower ($p < 0.05$) compared to T2 (Fig. 4B). In gills, total SOD and catalase activities did not significantly change in any of the sampling times.

Indicators of cell stress

In addition to the measurements of the expression and activity of antioxidant enzymes, we evaluated indicators of cell stress to determine if the shrimp had a shift to anaerobic metabolism, oxidative stress and apoptosis. In hepatopancreas, carbonylated protein content remained similar in all four sampling times, indicating that the different conditions of the semi-natural setting did not influence this indicator (Table 3). Lactate content in T3 and T4 was 1.1 and 2.3-fold lower ($p < 0.05$) compared to T2 (Table 3). Caspase-3 expression in T1 and T4 was 1.5 and 1.4-fold more ($p < 0.05$) compared to T2 (Fig. 5A). In gills, carbonylated protein in T3 and T4 was 0.8 and 1.0-fold less ($p < 0.05$) compared to T2 (Table 3). Lactate content did not change in all four sampling times; thus, the different conditions of the semi-natural setting did not influence this indicator (Table 3). Caspase-3 expression in T3 and T4 was 1.5 and 2.7-fold more ($p < 0.05$) compared to T2 (Fig. 5B).

Discussion

Oxygen depletion in aquatic environments has been a common trend during the past 50 years, with oxygen decreasing worldwide in the open ocean and coastal waters in benthic and intertidal zones (Breitburg *et al*., 2018) and in aquaculture ponds, especially in extensive and semi-intensive shrimp farms (Chang and Ouyang, 1988). In addition, hypoxic conditions are aggravated by water temperature increases in the environment due to global warming (Breitburg *et al*., 2018). Shrimp is widely distributed across marine environments and because of their tolerance to hypoxic and reoxygenation events (Giraud-Billoud *et al*., 2019)

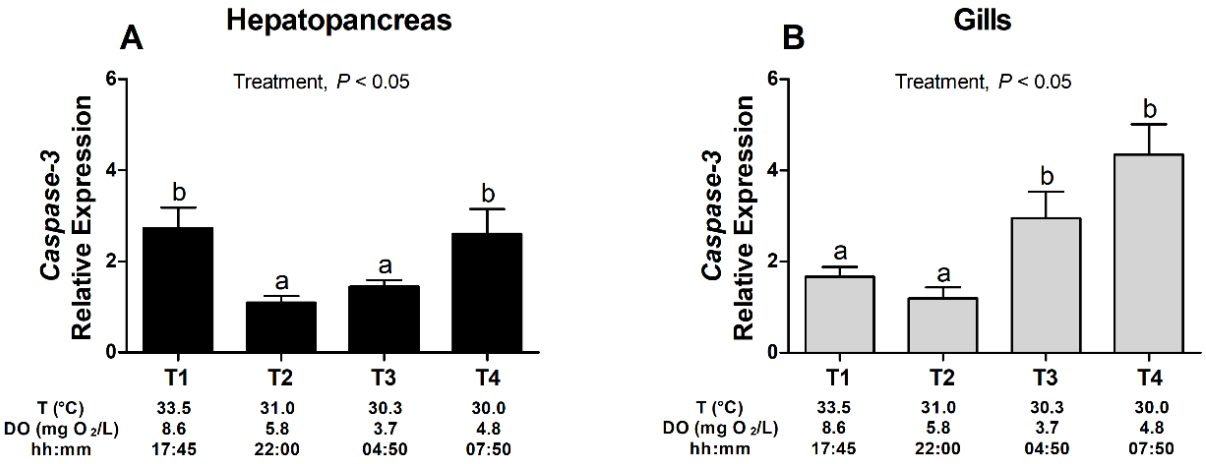

Fig. 5 Relative expression of *caspase-3* in hepatopancreas and gills under the natural temperature and DO of a semi-intensive commercial shrimp farming pond. Panel A: *Caspase-3* relative expression in hepatopancreas. Panel B: *Caspase-3* relative expression in gills. Data shown are means ± SEM (n = 5). Different lowercase letters denote significant differences ($p < 0.05$) between each sampling point

and warming temperatures (Zhou *et al*., 2010; González-Ruiz *et al*., 2020), several studies using laboratory controlled conditions have been done on the redox metabolism of these animals. In the present study, we used a natural experimental approach to analyze the effects of natural variations on temperature and DO in a semi-intensive pond that is "left alone" in an aquaculture shrimp farm, i.e. that was not controlled by us, only the sampling times. Generally, we found an up-regulation of the expression of antioxidants enzymes as the climate conditions became harsher in a tissue- and condition-specific manner. As expected, the highest temperature time did not coincide with the lowest DO time and the major changes occurred in the harsher climate conditions.

The response in gills is more sensitive to oxidative stress conditions compared to hepatopancreas

Tissue-specific responses were found between hepatopancreas and gills. More changes were detected in expression patterns compared to enzyme activities. For gills, higher differences in expression in all the cases were found in the sampling times compared to hepatopancreas. For gills, the highest expression was T4 (reoxygenation) in the 3 cases, followed by T3 (hypoxia) and then T1 (highest temperature) for cMnSOD and catalase, but T3 was similar to T1 in mMnSOD. This contrast with hepatopancreas, taking T2 as the best condition and sampling control, the highest temperature in T1 or the low DO in T3 and T4 resulted in similar expression for mMnSOD, but for cMnSOD and catalase, the highest expression is in the highest temperature of T1, with no differences in the other 3 conditions. In contrast to the changes in expression, no differences in total SOD and catalase were detected in gills in all the sampling times, but in hepatopancreas, the highest activity is in T2 for total SOD, while for catalase, the overall changes were smaller, but the highest were T3 and T2. It should be considered that at the sampling points, the shrimp are exposed to gradually changing conditions. Therefore, the production of ROS and the response by changes in expression and enzyme activity were also occurring gradually with differences between the 2 tissues. There are very likely differences between gills and hepatopancreas in mRNA translation efficiencies and posttranslational modifications needed for the fully active enzymes and those were reflected in the data obtained, but this could be further studied in the future.

The conditions at the sampling points should be noticed. At T3, the DO was 3.7 mg O_2/L, a low DO but still above the recognized level of hypoxia that is below 2 mg O_2/L in marine environments (Vaquer-Sunyer and Duarte, 2008). For shrimp, a good oxygen concentration for growth is around 5.0 mg O_2/L and only from 3.0 mg O_2/L or lower, corrections are recommended in the farms (Wyk and Scarpa, 1999). Events of hypoxia and reoxygenation occur in culture ponds, particularly in intensive or semi-intensive culture ponds with high density stocks, since the oxygen levels decrease during the night due to the respiration of plants and animals (Cheng *et al*., 2002) and also, from bacteria using oxygen, thus intensifying the level of hypoxic events (Vinatea *et al*., 2009). However, despite the drop of DO at night by hypoxia, during daylight, oxygen is produced by photosynthetic aquatic grass and reoxygenation occurs (Xu *et al*., 2022). At the time of sampling of T4, reoxygenation was occurring *in situ*. The sunrise on July 14th (2021) was at 5:40 am, thus reoxygenation had started at about 2 h before the sampling for T4 (Table 1).

ROS content increases in mitochondria during low oxygen (hypoxia or anoxia) and even more during reoxygenation, and this can result in oxidative stress and damage to biomolecules.

However, according to the Preparation for Oxidative Stress (POS) theory, the early activation of the antioxidant system helps to mitigate and minimize cellular harm via ROS signaling molecules (Hermes-Lima *et al*., 2015). On the other hand, while the expression of antioxidant enzymes was induced in gills, in hepatopancreas, this response had just begun.

In addition to the more sensitive molecular response in gills, carbonylated proteins decreased in T3 (lowest DO) and T4 (reoxygenation), while in hepatopancreas there was no statistically significant change. Carbonylated proteins results from ROS attack to the amino acid side chains (Requena *et al*., 2003) and free radicals can also damage nucleic acids, carbohydrates and lipids (Grune, 2000). These carbonyl derivatives can change proteins conformation. Carbonylated proteins can be cleaved by proteases in mild oxidative environments, but they may persist or increase in severe environmental oxidative conditions, suggesting a disruption of normal protein metabolism and accumulation of damaged molecules (Almroth *et al*., 2005). The decrease in carbonylated proteins in gills at T3 and T4 (hypoxia and reoxygenation, respectively), correlates with higher expression of the SODs and CAT. However, the enzyme activities that ultimately protect the cellular components were not higher in those time points. Cell protection can also derive from other enzymes as glutathione peroxidases, glutathione s-transferases or GSH. Interestingly, in *L. vannamei* gills have higher levels of GSH compared to hepatopancreas (Estrada-Cárdenas *et al*., 2021) and theferore, this could lead to a milder oxidizing environment compared to hepatopancreas and less carbonylated proteins content. However, it seems that the conditions of natural temperature and DO in the shrimp farm were not stressful enough to cause meaningful damage to proteins, probably thanks to the expression induction of the genes. These contrast to previous reports for gills of *L. vannamei* where hypoxia, reoxygenation, and high temperature or combinations of these stressors in a controlled environment in the laboratory caused higher carbonylated proteins levels (González-Ruiz *et al*., 2021). However, that study tested continuous harsher conditions, lasted 24 h in hypoxia (1.5 mg/ L) and high temperature (35 ^{o}C) or their combination, and 24 h of hypoxia followed by 1 h of reoxygenation (25 h) at control or high temperature. In contrast, in this study the environmental conditions changed gradually, occurred for shorter times and were less extremes, and thus, the natural environmental conditions were milder.

Previous studies under controlled laboratory conditions have highlighted the induction of the expression of antioxidant enzymes in *L. vannamei* at high temperature, hypoxia, reoxygenation and the combination of this stressors (González-Ruiz *et al*., 2020; Estrada-Cárdenas *et al*., 2021) and the importance of one of these enzymes (mMnSOD) to avoid high carbonylated protein levels and caspase-3 activity (González-Ruiz *et al*., 2021). Moreover, in these studies it was clear that the enzyme activity was less induced than the expression of their genes. Therefore, both in the natural DO and temperature setting and in controlled studies in the laboratory, the POS response in *L. vannamei* is more evident in gene expression rather than in induced activity of these enzymes.

The higher response of gills is reasonable due to their key function for respiration since oxygen is diffused from the surrounding environment to enter the body. Thus, a high ROS production is expected during hypoxia and reoxygenation, while in high temperature, an increased metabolic rate causes ROS overproduction. Moreover, most crustaceans present bradycardia (a reduction in the amount of energy spent by the cardiovascular system) under hypoxic conditions, and also, they can redirect blood to other tissues requiring higher levels of oxygen (i.e., more energy) (Paschke *et al*., 2010; Guadagnoli *et al*., 2011). Hence, the results from studies of natural DO and temperature setting, and also, from laboratory conditions, indicate more changes in gills in response to oxidative stress compared to other tissues, and in addition to other physiological responses, allows this shrimp species to thrive in harsh environments.

Changes in caspase-3 expression and lactate substantiate stress in natural temperature and DO

As mentioned before, accumulation of ROS damages membrane lipids, proteins and nucleic acids, and that could lead ultimately, to cell death (Welker *et al*., 2013). Cell death by apoptosis prevents the propagation of genomically compromised cells produced by irreparable DNA damage (Delia and Mizutani, 2017) and can be caused by many harsh factors, including high temperature, hypoxia and reoxygenation. Apoptosis is mediated by caspases that ultimately cleave hundreds of vital proteins to ensure programmed cell death and culminate with the activation of effector caspases, especially caspase-3 (Matsuura *et al*., 2016). Thus, caspase-3 induction could be used as an indicator of the activation of apoptosis. We analyzed the expression of caspase-3 and found induction in a tissue-specific manner and occurring in all the conditions, but with more changes in gills compared to hepatopancreas.

In gills, caspase-3 expression followed the same pattern of expression of the antioxidant enzymes genes expression with induction in T3 (lowest) and T4 (reoxygenation) compared to T2 (control), while in T1 (high temperature) the expression was unaffected. This is probably because at T3 and T4, the hypoxia and reoxygenation conditions were beginning with a likely concomitant increase in ROS production that in turn, could have induced the initiation of the intrinsic apoptosis pathway. Acute hypoxia causes DNA damage in the gills and hepatopancreas of *L. vannamei* (Li *et al*., 2016), with DNA damage activating death signals to lead to intrinsic apoptosis. Also, apoptosis occurs after reoxygenation with the activation of caspase-3 (Kang *et al*., 2000). However, during T1 only the mitochondrial MnSOD was induced, probably because mitochondria is a major source of ROS. In gills from *L. vannamei,* high temperature induces the expression and activity of the antioxidant enzymes mMnSOD (González-Ruiz *et al*., 2020)

and cMnSOD (Estrada-Cárdenas *et al*., 2021), and the induction of both MnSODs expression could help the shrimp to prevent the activation of caspase-3 and avoid apoptosis driven by ROS overproduction (González-Ruiz *et al*., 2021).

In hepatopancreas caspase-3 expression was induced in T1 and T4 compared to T2 (control). This pattern correlates with the decrease in antioxidant enzyme expression in the same sampling times, suggesting a less sensitive antioxidant response of hepatopancreas to environmental stress compared to gills. For example, even though the shrimp had been gradually exposed to high temperatures for at least 3 h at the time of sampling of T1 and the antioxidant enzymes expression was up-regulated, the activity of these enzymes decreased. The increase in caspase-3 agrees with more apoptotic cells in hepatopancreas of *L. vannamei* collected in the warm season at high water temperatures compared to cold season in shrimp farms from Iran (Basir and Peyghan, 2021). Therefore, high temperature was producing intracellular stress, likely by more ROS, thereby activating apoptosis when the antioxidant response was just beginning to be induced in the hepatopancreas, suggesting a lower sensitive antioxidant response.

In T4 the reoxygenation effect had started for about 2 h at the time of sampling and in gills, the expression of antioxidant enzymes was induced, responding quickly to the overproduction of ROS. In contrast, in hepatopancreas, the expression of most of the antioxidant enzymes was not induced, except for cMnSOD, the activity of these enzymes decreased, while the expression of caspase-3 increased. This shows that the hepatopancreas response to reoxygenation was different to gills. On the other hand, caspase-3 expression was not induced in T3 (lowest DO) in the hepatopancreas. We suggest that although the DO in the aquaculture pond at this sampling time was low, the critical level of hypoxia nor the time of exposure needed for this condition were reached to activate apoptosis. A previous study reported caspase-3 induction in hepatopancreas of *L. vannamei* only after 48 h of hypoxia (1.54 ± 0.28 mg O_2/L), while the same level of hypoxia but at a shorter exposure time (24 h) did not affect caspase-3 expression (Nuñez-Hernandez *et al*., 2018).

Interestingly, it appears that the DO level at T3 was low enough to induce the POS mechanism and caspase-3 expression in gills, corroborating the differences in responsiveness to oxidative environmental stress between gills and hepatopancreas in shrimp. Altogether, these results fit with the premise that there are two main effects of ROS overproduction, the induction of antioxidant defenses, at least in organisms that have the POS strategy, and an oxidative damage to biomolecules in the cell (Moreira *et al*., 2021b); with gills tissue of *L. vannamei* responding quickly and in a sensitive manner at the onset of the oxidative insults in the natural temperature and DO setting.

Shrimp cells resist fluctuations in DO by adjusting their energy metabolism. Exposure to hypoxia induces lactate dehydrogenase (LDH) (Soñanez-Organis *et al*., 2012), resulting in the production of more lactate and corroborating the acceleration of anaerobic metabolism rate in low oxygen conditions (Racotta *et al*., 2002; Pérez-Rostro *et al*., 2004; Soñanez-Organis *et al*., 2010; Camacho-Jiménez *et al*., 2019).

On the other hand, exposure of marine invertebrates to their critical temperatures causes the onset of anaerobic metabolism despite an adequate oxygen availability in the environment. This produces a subsequent accumulation of anaerobic end products such as lactate (Frederich *et al*., 2009). Lactate content is different in gills and hepatopancreas of shrimp exposed to the natural high temperature and DO of the aquaculture pond. This metabolite did not change in all four sampling times in the gills, while in hepatopancreas there was a decrease in T3 (lowest DO) and T4 (reoxygenation). In addition, lactate content was higher in hepatopancreas compared to gills in all sampling times. These results suggest that hepatopancreas is the major tissue involved in carbohydrate metabolism (Yepiz-Plascencia *et al*., 2000), however, since lactate levels are expected to increase under hypoxia, this might seem contradictory.

Still, in addition to being an anaerobic metabolite in low oxygen conditions, lactate is also an aerobic metabolite with adequate cellular oxygen concentration when the utilization of glucose or glycogen as a fuel is sustained (Gladden, 2004). Brooks introduced what is now known as the cell-to-cell lactate shuttle (Brooks, 1985). This hypothesis postulates that there is a successive distribution of lactate throughout the body after its formation. Therefore, lactate formed as an energy intermediate in tissues undergoing accelerated glycolysis may latter be used as a precursor for gluconeogenesis or glycogenesis (Ferguson *et al*., 2018).

Conclusions

In summary, this study shows that *L. vannamei* responds to oxidative stress inducing its endogenous antioxidants when exposed to high temperature, low DO, and concomitant reoxygenation conditions caused by the natural fluctuation of DO and temperature in a semi-intensive aquaculture pond. The enhancement of the antioxidant system was in a tissue-specific manner, with the gills being more sensitive than hepatopancreas, and in gene expression rather than in enzymatic activity. In addition, the biochemical stress indicators of caspase-3 expression and lactate content, corroborated that the natural fluctuation of DO and temperature in the semi-intensive aquaculture pond caused some stress. The results reported here for natural setting are in line with the proposal of the occurrence of the POS strategy in shrimp exposed to high temperature, low DO and reoxygenation under controlled laboratory conditions that have been reported before (González-Ruiz *et al*., 2020; 2021, 2022; Estrada-Cárdenas *et al*., 2021). Therefore, the rapid responses of *L. vannamei* are crucial for its widely reported tolerance to oxidative stress conditions that help this species to adapt and survive to the current harsh conditions of marine environments.

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