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Research Article

Eyestalk ablation and acclimation temperatures affect juveniles *Penaeus vannamei* thermo-tolerance: molecular biomarkers of cell protection oxidative stress, and compensatory mechanisms

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ABSTRACT. This study investigated the effect of acclimation temperatures and eyestalk ablation (EA) on the thermal tolerance of juvenile *Penaeus vannamei.* In each case, the relative expression of genes involved in cell protection (*hsp70* and *hsp90*), oxidative stress (*cMnSOD* and *GPx*), and anaerobic metabolism (*hif1a*) was assessed. For this purpose, shrimp were acclimated to 20, 26, and 32°C for 21 days. After acclimation, the critical thermal maxima (CTmax) was determined in non-eyestalk ablated, unilaterally, and bilaterally eyestalk ablated organisms. An effect of acclimation temperatures on CTmax values was observed, with shrimp acclimated at 32°C having the highest rates. Likewise, EA resulted in lower thermal tolerance to CTmax in organisms acclimated at 20 and 26°C. The shrimp's protective and cellular repair responses were evidenced by increased *hsp70* and *hsp90* gene expression after CTmax and were intensified by the EA. In contrast, the results showed that *cMnSOD* was very sensitive to CTmax, and its expression was intensified with EA, while for *GPx*, there was an increase in the relative gene expression, mainly in shrimp acclimated at 20°C. In the case of *hif1a*, overexpression was observed at the acclimation temperature of 26°C, showing the activation of compensatory mechanisms such as anaerobic metabolism. EA caused a significant molecular response during CTmax of molecular biomarkers involved in heat stress response, oxidative stress, and compensatory mechanisms.

Keywords: *Penaeus vannamei*; thermal acclimation; critical thermal maxima; heat shock proteins; oxidative stress response; hypoxia

INTRODUCTION

Temperature is one of the environmental factors influencing the physiology of most poikilothermic organisms. The responses of aquatic ectotherms can develop at behavioral, metabolic, energetic, or even molecular levels, depending on the intensity and exposure times (Pörtner 2010, Sokolova 2013, Schulte 2015). These responses allow poikilothermic aquatic organisms to maintain or recover homeostasis. For example, under acute or chronic heat stress, poikilotherms need different mechanisms to maintain cellular integrity, cope with cellular oxidation and regulate compensatory responses that allow them to survive (Pörtner 2012, Madeira et al. 2017). Although the scientific literature on the effects of temperature on the population, behavioral, or metabolic level in aquatic organisms is extensive, with the help of molecular tools, it is possible to contribute new approaches to understanding the responses of poikilotherms to abnormal temperature events.

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For example, at the molecular level, heat shock proteins (HSP70 and HSP90) are one of the first intracellular defense systems in the minimal stress proteome and act as key markers in response to heat stress in different aquatic organisms (Clark et al. 2008, Logan & Somero 2011, Yabu et al. 2011, Yebra-Pimentel et al. 2019, Larios-Soriano et al. 2020). While the expression of *hsp70* is the first line of defense, the expression of the *hsp90* gene is considered the ultimate response under heat stress (Qian et al. 2012), *hsp70* prevents the degradation of intracellular proteins, and cytoplasmic *hsp90* activates hundreds of target proteins in cellular regulatory pathways, including kinases, transcription factors, and steroid receptors (Li et al. 2009b).

In addition to the damage to cell structure, activation of antioxidant responses due to increased reactive oxygen species (ROS) has been observed when heat shocks occur (De Souza et al. 2014, Xu et al. 2018). ROS can damage DNA, proteins, or lipids, and their increase can disrupt normal physiological pathways leading to apoptosis (Wang et al. 2009, Zhou et al. 2010a). In this case, ROS are rapidly removed by antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione transferase (GST) that directly detoxify harmful ROS and other compounds involved in ROS generation (Wang et al. 2009, Zhou et al. 2010a).

Finally, the thermal tolerance of poikilotherms is directly regulated by the oxygen transport capacity from the blood to the cells (Pörtner 2010, Sokolova et al. 2012). Thus, anaerobic compensatory processes to generate energy may play a key role in response to the thermal increase. In this case, the hypoxia inducible factor protein complex is one of the transcription factors regulating different mechanisms involved in activating anaerobic pathways. Although these molecular markers (*hsp70*, *hsp90*, *GPx*, *cMnSOD*, and *hif1α*) have been previously studied in different organisms, their interaction could be fundamental to understanding the effect of temperature on the physiological responses of poikilotherms.

Temperature is a regulatory factor of different physiological and metabolic processes in poikilotherms (Somero 2005, Angilletta 2009). The effects of temperature increase are closely related, and the integration of different molecular markers is essential to know the level of affectation. In this case, it is proposed to link the mechanisms to avoid denaturation of cellular protein complexes, the mechanisms to regulate cell damage by oxidative stress, and the compensatory mechanism to generate energy in an environment where lower oxygen solubility and limited oxygen transport capacity into cells is expected (Pörtner 2010). All this simultaneously occurs when the temperature and the respiratory rate of poikilotherms (mechanism to recover energy homeostasis) increase, which could generate a higher proportion of ROS. If the temperature increase intensifies, it will harm cellular integrity.

Increasing temperature due to global warming is one of the main factors impacting aquaculture development worldwide (Doney et al. 2012, Madeira et al. 2017). Successful aquaculture development could be found in studying species that tolerate a wide range of temperatures. In this regard, the Pacific white shrimp *Penaeus vannamei* is one of them, as it has a wide range of thermal tolerance (15-33°C) that positions it as one of the most important aquaculture resources (Kumlu et al. 2011, Re et al. 2012). However, this organism is exposed to events that can harm its physiological responses to temperature variation. For example, in global aquaculture, eyestalk ablation (EA) has been used to induce gonadal maturation to accelerate reproductive rates channeling energy toward reproduction. However, it induces several consequences that affect the overall biological response. The best-known effects on spawners after spawning are increased bioenergy demand, which affects their survival (Palacios et al. 1999, Sainz-Hernández et al. 2008, Kumlu et al. 2011, Re et al. 2012).

There is no doubt that shrimp EA is one of the most widely used techniques in shrimp farming. It implies the extirpation of the X-organ sinus gland complex located in the eyestalk, the major neuroendocrine organs in crustaceans where important hormones are synthesized, stored, and secreted into hemolymph to regulate several metabolic processes (Sainz-Hernández et al. 2008). This homeostatic imbalance induces several consequences affecting the overall biological responses and changes its temperature tolerance and thermal preference (Palacios et al. 1999, Sainz-Hernández et al. 2008, Kumlu et al. 2011, Re et al. 2012). However, with new challenges emerging due to climate change and the availability of molecular-genomic tools, it is essential to analyze the consequences of these practices from new perspectives. In this sense, the present work aims to evaluate gene expression of molecular biomarkers (*hsp70*, *hsp90*, *GPx*, *cMnSOD*, and *hif1α*) under different acclimation temperatures (20, 26, and 32°C) and after determining the critical thermal maxima (CTmax) as well as comparing the expression levels of those genes with organisms subjected to unilateral and bilateral EA. This

study will provide a basis for establishing the effect of EA on the thermal tolerance of organisms at the genomic level, which will help to develop future research into the mechanisms controlling gene expression, such as DNA methylation in *P. vannamei*.

MATERIALS AND METHODS

Biological material

The animals were obtained from Acuícola Pacar, a limited liability shrimp farming company in Mexicali, Baja California, Mexico. Pacific white shrimp *P. vannamei* (PL12) postlarvae were placed in oval tanks (2000 L) with continuous seawater flow (35 of salinity) and maintained at 28 ± 1°C with 1000 W heaters connected to a digital temperature control system (Finnex; HC-0810M, Chicago, IL, USA). They were fed twice daily with 35% protein Aquaprofile pellet (Purina Co., Sonora, Mexico) at 8% of their biomass throughout the experiment until they reached 8-10 g wet weight. In this work, the shrimps were sexed according to the characteristics proposed by Perez-Farfante & Kinsley (1997), obtaining a 1:1 ratio of males and females. The intermolt stage of each animal was determined as half the interval between two successive molts assessed by the observation of exuviae deposited in each container. Since feeding is known to affect glycemia, at the intermolt stage, the shrimp fasted for 24 h before the experiments (Camacho-Jiménez et al. 2015).

Acclimation

Three oval tanks (500 L) were set up at 20, 26, and 32 ± 1°C, respectively, and 50 shrimps (of indistinct sex) were placed in each tank. The warm temperatures (26 and 32°C) were controlled with 1000 W titanium heaters (Finnex, Chicago, IL, USA) and connected to a digital temperature control system (Finnex, Chicago, IL, USA). At the same time, the cold temperature (20°C) was maintained with a water chiller connected to a digital temperature control system and monitored for 21 days. Room temperature was kept with an air conditioner system to ensure experimental temperatures. All tanks were constantly aerated, and seawater was replaced daily with an adjusted open flux of filtered seawater at a rate of 348 mL min^{-1} at 20 ± 1°C. On day 21, five shrimp were removed from each temperature and dried with absorbent paper. These five non-eyestalk ablated organisms were used as controls (C) because they were only kept at acclimation temperatures for 21 days and tested in real-time polymerase chain reaction (PCR) to determine the expression levels of selected transcripts under each acclimation temperature. Gill tissues were dissected and preserved in cold nucleic acid preservation buffer (NAP) (Camacho-Sánchez et al. 2013) and stored at -80°C until use.

Eyestalk ablation (EA)

The remaining 45 shrimp were used to determine the effect of EA on thermal tolerance during the CTmax experiment. Fifteen shrimps were unilaterally eyestalk ablated (UA), and 15 were bilaterally eyestalk ablated (BA) from each acclimation temperature. All shrimp (UA and BA) were kept separately in individual containers within the same tank (500 L). The remaining (n = 15) shrimp of each acclimated temperature were non-eyestalk ablated and used as a control for the CTmax experiment (CU). The EA procedure was performed with sterile scissors; the eyestalk was cut at the base and cauterized. Animals were fed two days after each EA procedure.

Critical thermal maxima (CTmax)

The CTmax of CU, UA, and BA shrimps acclimated to 20, 26, and 32°C were determined. For this purpose, in each case, five shrimps were placed in a 60 L experimental aquarium equipped with a 1000 W heater and provided with permanent aeration to control the temperature and maintain dissolved oxygen levels close to saturation levels. Organisms were maintained for 30 min at the acclimation temperature to reduce handling stress (Díaz et al. 2013); after that, CU, UA, and BA shrimps were exposed to temperature increments at a rate of 1°C min^{-1} (Lutterschmidt & Hutchison 1997a,b). The stress event recorded was the loss of verticality, which was the precise moment when shrimp were on their backs and unable to regain their upright posture or remained reclined at a 90° angle for at least 3 s (Díaz et al. 2013). Once shrimp reached CTmax, they were immediately removed from the aquarium and dried with absorbent paper; whole gills were dissected out, preserved in cold NAP buffer (Camacho-Sánchez et al. 2013), and stored at -80°C.

RNA extraction, DNase treatment, and cDNA synthesis

Total RNA was extracted from 100 mg of gills from each organism (C, CU, UA, and BA) using a Tri Reagent solution (SigmaAldrich, St. Louis, USA).

Briefly, the tissue was homogenized with 2 mm zirconia/silica beads (Biospec Products, Bartlesville, OK, USA) in a Fastprep-24 Instrument tissue homogenizer (MP Biomedicals, Solon, OH, USA) twice for

30 s at 5.0 m s^{-1} speed. RNA pellets were eluted in 50-100 µL of water treated with 0.1% diethylpyrocarbonate (DEPC) (Ambion, Austin, TX, USA). RNA integrity was assessed by electrophoresis on a 1.0% agarose gel stained with 10,000x Gel Red dye (Biotium Inc., Hayward, CA). RNA concentration and purity were measured with a NanoDrop Spectrophotometer (Thermo Scientific, Wilmington, DE, USA). Then, 6 µg of total RNA was treated with DNase RQ1 (Promega, Madison, WI, USA) to remove genomic DNA contamination following the manufacturer's protocol with additional purification and precipitation steps (López-Galindo et al. 2019). Treated RNA samples were further confirmed for the absence of DNA contamination by 1.2% agarose gel electrophoresis and PCR using the *LvEf1α* gene as an amplification control. The cDNA was synthesized with the ImProm-II™ and Oligo-dT Reverse Transcription System (Promega, Madison, WI, USA) with 1.0 µg of purified total RNA in a total reaction volume of 20 µL (50 ng μL^{-1}) following the manufacturer's protocol. The obtained cDNA was stored at -20°C until used for PCR reactions.

Relative gene expression

Transcript levels of *hsp70*, *hsp90*, *cMnSOD*, *GPx*, and *hif1α* genes were measured in the gills of Pacific white shrimp *P. vannamei*. Acclimated and exposed to CTmax by quantitative real-time PCR (qPCR) on a CFX-96 Touch™ real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA, USA).

The specific primers for the different genes were designed with PerlPrimer software (Marshall 2004) based on the mRNA sequences available in the GenBank database of the National Center for Biotechnology Information (NCBI, Table 1). In addition to the target genes, five potential reference genes were included: elongation factor 1-alpha (*Ef1α*), ribosomal protein L8 (*rpL8*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), and ribosomal protein subunit 18 (*18S* rRNA). The thermal cycling conditions were 95°C for 3 min, followed by 40 cycles of 95°C for 10 s and 60°C for 30 s. After stability analyses using geNorm, Normfinder, and Bestkeeper software, *rpL*8 and *Ef1α* were used as housekeeping genes. Efficiency values for each gene standard curve (E = $(10^{1/slope} - 1) \times 100$) were calculated from PCR products with serial dilutions (1:5). The qPCR reactions were performed in triplicate in 10 µL final reaction volume with 1x IQ™ SYBR® Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA), 0.2 µM forward and reverse primers, 3 µL cDNA template (diluted 1:5 and equivalent to 30 ng total RNA) and 1.6 µL sterile nuclease-free water. For reference genes, the primer concentration per reaction was 0.3 µM. A melting curve analysis (95°C for 10 s, 65 to 95°C for 5 s, with 0.5°C increments) was included to corroborate the specificity of the PCR product, as shown in a single peak.

For the *hif1α* gene, the thermal cycling conditions were 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 35 s, and extension at 72°C for 55 s. A melting curve was included with the same program as the other genes.

Data analysis

CTmax data were analyzed using a two-way analysis of variance (ANOVA) for acclimation temperature and EA variables. Normality was tested through the Shapiro-Wilk W test, and homogeneity of variances through Levene's test. A *post-hoc* analysis of means was performed using a Tukey honestly-significant-difference (HSD) test ($P < 0.05$).

Relative expression data were evaluated with the qBase+ software (Biogazelle, BE). This software uses a modified algorithm based on the classical ΔΔCt method, which considers the amplification efficiency for each target or reference gene. The model estimated normalized relative expression quantities (NRQs) from the quantification cycle using normalized target genes based on stable reference genes (Hellemans et al. 2007). All NRQs were log-transformed (log10) for statistical analysis and back-transformed for a graphical representation (Zar 1999), and differences were analyzed using a two-way analysis of variance (ANOVA) for acclimation temperature and EA variables. Normality was tested with a Shapiro-Wilk W test. A *post-hoc* analysis of means was performed using Fisher's least significant difference (LSD) test ($P < 0.05$). All statistical analyses were performed with Statistica 7.1 (StatSoft, Tulsa, OK, USA).

The authors confirm that the journal's ethical policies, as stated on the journal's author guidelines page, have been followed and that approval from the appropriate review committee, including ethical aspects, of the National Science and Technology Foundation of Mexico, has been received before the conduct of this study. Shrimp stress management does not require ethical approval.

RESULTS

Effect of acclimation temperatures and EA on the CTmax

CTmax increased significantly with increasing acclimation temperature from 20 to 32°C. The mean

Table 1. Accession numbers and primers sequences for qPCR. F: forward primer; R: reverse primer; bp: base pairs; *e*: primer efficiency. †Primers designed in PerlPrimer software for this study.

Gene/Primer	GenBank accession no.	Primer sequence (5'→3')	Amplicon length (bp)	*e*	Reference
LvEF1α	GU136229	(F) GAAATCCGACAACATGGGCT (R) CCAATCTTGTACACGTCCTG	161	1.803	†
LvrpL8	DQ316258	(F) ATGAACCCTGTAGAGCATCCTC (R) CTTTGTACCACGGATGAGACCA	141	1.929	†
Hsp70	AY645906	(F) CTCCTGCGTGGGTGTGTT (R) GCGGCGTCACCAATCAGA	120	1.973	Qian et al. (2012)
LvHsp90	HQ008268	(F) CGAAGGAGAACCAGAAGCAC (R) TGCTGAACGCAGTATTCGTC	145	1.908	†
Hif1α	FJ807918	(F) GGAGTCTTTGAGAGAGAG (R) GCCTCCTTCCGTGATCTTC	160	1.873	Soñanez-Organis et al. (2009)
cMnSOD	DQ005531	(F) CGTAGAGGGTATTGTCGT (R) TTGAAATCATACTTGAGGG	153	2.002	Wang et al. (2009), Zhou et al. (2010a)
LvGPx	AY973252	(F) GGACTTCCACCAGATGAACC (R) CTCGAAGTTGTTCCCAGGAC	154	1.970	†

CTmax ranged from 36.4 ± 0.9 to 41.0 ± 0.5°C in CU; between 33.9 ± 0.8 and 41.4 ± 0.5°C in UA; and between 33.3 ± 0.5 and 40.5 ± 0.9°C in BA. Overall, our results showed differences between acclimation temperatures and EA conditions ($P < 0.05$) and suggested a substantial reduction in CTmax in the UA and BA animals compared to the CU from acclimations at 20 and 26°C. Shrimps acclimated at 32°C showed a significant increase in all CTmax values according to the values determined at 20 and 26°C, significantly higher in UA shrimp ($P < 0.05$).

Effect of temperature and EA on gene expression cell maintenance and repair

Acclimated shrimps (20, 26, and 32°C) were tested to obtain the gene expression patterns of *hsp70* and *hsp90* at the end of the acclimation period. The expression values of *hsp70* (1.5, 0.6, 0.5-fold, respectively) and *hsp90* (0.8,0.5, and 0.2-fold, respectively) decreased inversely proportional to the acclimation temperature (Suppl. Fig. 1a-b).

The CTmax showed a significant effect ($P < 0.05$) on the expression levels of transcripts related to cell maintenance and repair response (*hsp70*, *hsp90*). CTmax significantly increased *hsp70* expression in UA and BA shrimp at different acclimation temperatures. The highest *hsp70* gene expression value was observed in UA and BA at 20°C and in BA at 32°C (Fig. 1a). The expression level of *hsp90* also increases with CTmax, denoting a significant increment in UA and BA shrimps compared to CU shrimps. The *hsp90* showed the highest expression values in UA and BA shrimps at 32°C (Fig. 1b).

Antioxidant response

Acclimated shrimps at 20°C showed the highest *GPx* expression value compared to those acclimated at 26 and 32°C. No significant differences were observed in *cMnSOD* gene expression among acclimation temperatures ($P > 0.05$; Suppl. Fig. 1c-d).

The CTmax induced the highest *GPx* gene expression in UA and BA organisms at 20°C ($P < 0.05$; Fig. 2a).

On the other hand, the CTmax and EA significantly affected the *cMnSOD* expression among different acclimation temperatures ($P < 0.05$, Fig. 2b). The *cMnSOD* expression did not follow the same pattern between acclimation temperatures and EA condition. BA organisms exposed to CTmax showed increased *cMnSOD* expression to increasing acclimation temperature (Fig. 2b). The highest *cMnSOD* expression values were detected in UA shrimps at 20°C, UA and BA at 26°C, and BA at 32°C.

Hypoxia-associated response according to the expression of *hif1α*

Acclimated shrimps at 26°C showed the highest *hif1α* relative expression value ($P < 0.05$) compared to those at 20 and 32°C (Suppl. Fig. 1e). CTmax significantly induced the *hif1α* gene in UA and BA at 26°C and BA at 32°C compared to CU (Fig. 3).

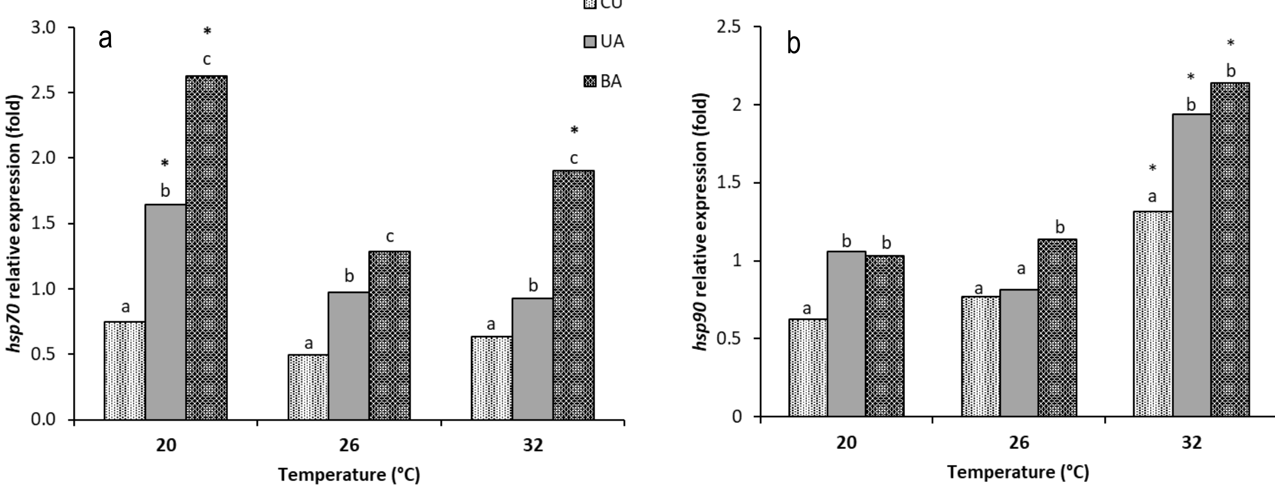

Figure 1. Relative expression (back-transformed log-means) of a) *hsp70* and b) *hsp90* in gills of *P. vannamei* juveniles exposed to critical thermal maxima (CTmax) acclimated at different temperatures under different eyestalk ablation (EA) conditions. CU: non-eyestalk ablated, UA: unilaterally eyestalk ablated, BA: bilaterally eyestalk ablated shrimps. Mean values are shown in bars; different letters indicate statistically significant differences among EA conditions ($P < 0.05$) and an asterisk for statistical differences among acclimation temperatures.

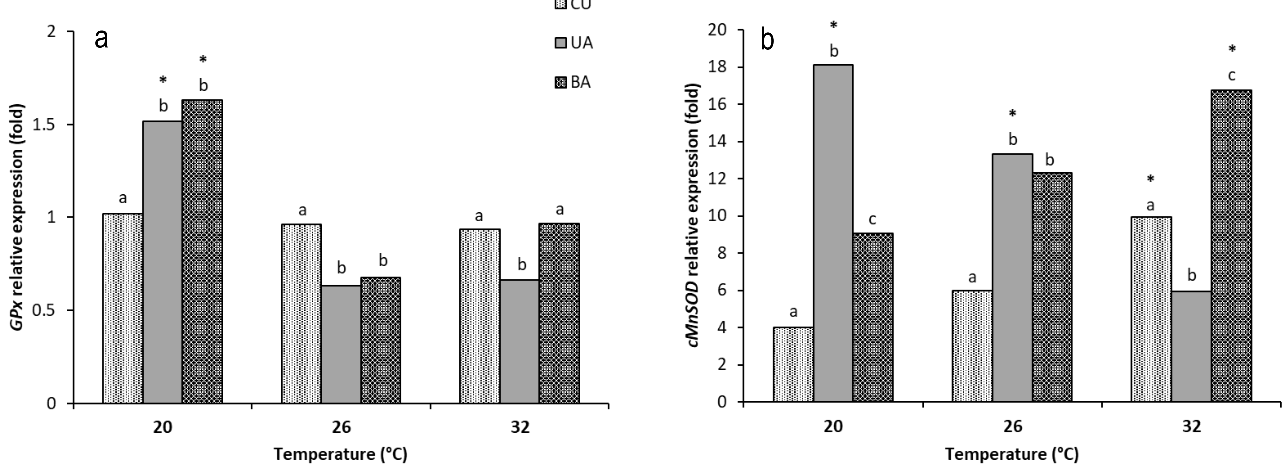

Figure 2. Relative expression (back-transformed log-means) of a) *Gpx* and b), *cMnSOD* in the gills of *P. vannamei* juveniles exposed to critical thermal maxima (CTmax) acclimated at different temperatures under different eyestalk ablation (EA) conditions. CU: non-eyestalk ablated, UA: unilaterally eyestalk ablated, BA: bilaterally eyestalk ablated shrimps. Mean values are shown in bars; different letters indicate statistically significant differences among EA conditions ($P < 0.05$) and an asterisk for statistical differences among acclimation temperatures.

DISCUSSION

The CTmax of *P. vannamei* was taken as loss of verticality (White 1983), described according to Beitinger et al. (2000) and Díaz et al. (2013) as the thermal point before death at which locomotor responses become disorganized due to neuromuscular blockade and presynaptic failure at which the shrimp loses the ability to escape conditions that may ultimately lead to death. This CTmax is considered a measure of the thermal tolerance of aquatic organisms and allows for determining the temperature at which the first signs of stress occur (Paladino et al. 1980, Re et al. 2012, Díaz et al. 2013). A direct relationship between CTmax and the temperature to which the organism acclimates has been previously reported in several crustacean species, such as *Palaemonetes varians*, *Macrobrachium acanthurus*, *Cherax quadricarinatus*, *Mirocaris fortunate*, *Litopenaeus stylirostris* and *P. vannamei* (Ravaux et al. 2003, 2012, Díaz et al. 2004a,b,

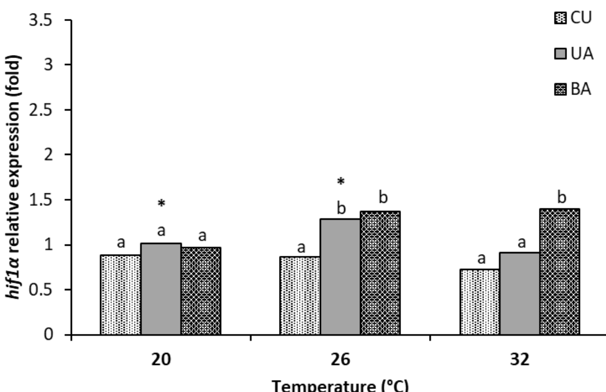

Figure 3. Relative expression (back-transformed log-means) of *hif1α* in the gills of *P. vannamei* juveniles exposed to critical thermal maxima (CTmax) acclimated at different temperatures under different eyestalk ablation (EA) conditions. CU: non-eyestalk ablated, UA: unilaterally eyestalk ablated, BA: bilaterally eyestalk ablated shrimps. Mean values are shown in bars; different letters indicate statistically significant differences among EA conditions ($P < 0.05$) and an asterisk for statistical differences among acclimation temperatures.

Re et al. 2005, 2006, 2012, Shillito et al. 2006, Kumlu Türkmen & Kumlu 2010). In this study, acclimation temperature and EA in Pacific white shrimp significantly affected CTmax. Therefore, the physiological response was assessed by analyzing the gene expression of some transcripts involved in response to heat stress, oxidative stress, and hypoxia. To our knowledge, this is the first report evaluating the effect of EA on the acute heat stress response in juvenile *P. vannamei*.

Expression patterns of genes involved in the heat stress response

Vertebrate and aquatic invertebrate organisms have evolved protective mechanisms in response to heat stress conditions, primarily based on the induction of chaperone proteins (HSPs) (Zhenyu et al. 2004, Luan et al. 2010, Qian et al. 2012). These chaperones serve as regulators of cellular functions, preventing the denaturation of other proteins, assisting the refolding of denatured proteins, minimizing the aggregation of non-native proteins, preventing cytotoxic aggregations, and degrading and removing labeled denatured proteins out of the cell (Feder & Hofmann 1999, Guo et al. 2010, Madeira et al. 2012). The role of HSP genes in response to heat shock has been well documented in crustacean species such as *Penaeus monodon* (Lo et al. 2004), *Fenneropenaeus chinensis* (Zhenyu et al. 2004, Luan et al. 2009, 2010), *Crangon crangon*, *Palaemon elegans*, *Palaemon longirostris* (Madeira et al. 2012) and *Macrobrachium rosenbergii* (Liu et al. 2004). For example, Rungrassamee et al. (2010) showed that *hsp70* expression in the hepatopancreas of *P. monodon* was significantly induced within the first hour of heat stress. On the other hand, it was observed in *F. chinensis* that *hsp70* expression was drastically induced under heat shock (Li et al. 2009a,b, Luan et al. 2009). In addition, Qian et al. (2012) showed that the expression of *hsp60*, *hsp70*, *hsc70*, and *hsp90* was induced under acute heat stress and the transcript level of *hsp70* was the most sensitive to temperature fluctuations among the four genes evaluated. All these studies were assessed under time-lapse heat shock stress. However, since in this study, the *hsp70* expression values were lower in shrimp acclimated at 26 and 32°C for 21 days, we can argue that the shrimp had the opportunity to acclimatize to these thermal conditions in this period. Interestingly, this was not the case for shrimps acclimated at 20°C, where a constant activation of the cellular repair and maintenance system was evident by observing the positive regulation of the *hsp70* gene.

The expression of the *hsp90* gene exhibited the same trends as *hsp70*. After the acclimation period, *hsp90* gene expression decreased proportionally with temperature. In contrast, at CTmax, its expression increased proportionally, showing an inverse pattern, suggesting that the physiological mechanisms due to the previous thermal acclimation were disrupted by the lack of the X-organ sinus gland complex, reducing the shrimp's capability to support homeostasis and making them susceptible to thermal changes. CTmax elicited a response of the *hsp70* gene only after EA, where its expression was enhanced. The low expression of *hsp70* and *hsp90* genes in organisms acclimated at 26 and 32°C for 21 days seems sufficient for these organisms to acclimatize and maintain their gill cell processes. It also appears that EA does not limit the maintenance and repair mechanisms mediated by the *hsp's* analyzed but rather intensifies these responses.

According to Qian et al. (2012), *hsp90* gene expression was maintained at low levels in the hepatopancreas of *P. vannamei* acclimated at 27 ± 1°C. This observation was similar to that of Rungrassamee et al. (2010), who reported low levels of expression in *P. monodon* gills acclimated at 27 ± 2°C in non-heat-stressed organisms, whereas, in 35°C acclimated organisms subjected to heat shock, *hsp90* expression was induced after 1 h of exposure and remained stable up to 2.5 h. The *hsp90* gene showed the same pattern under EA stress in this study. We hypothesize that at CTmax, there is a homeostatic imbalance of the cell due

to the number of denatured proteins and damage to processes involving *hsp90*, such as signal transduction systems, transcription factors, cell cycle regulation and steroid hormone receptors (Rungrassamee et al. 2010). It is possible to point out that high acclimation temperatures induced significantly higher *hsp90* expression in response to CTmax even when shrimp were eyestalk ablated. In our study, the response to 20°C temperature was modulated mainly by *hsp70*, while for the 32°C temperature, it was *hsp90*. Finally, EA intensified the expression of both genes (*hsp70* and *hsp90*).

Expression patterns of genes involved in the oxidative stress response

All aerobic organisms produce ROS under physiological conditions that result in oxidative stress. SOD is the first and most important line of antioxidant enzymes that scavenge superoxide radicals by converting them into H_2O_2 and oxygen (Gómez-Anduro et al. 2006, 2007, 2012). Therefore, characterization, gene expression, and gene structure of *cMnSOD* have been reported in several decapod crustaceans, such as the tiger shrimp *P. monodon* (AY726542), the Chinese shrimp *F. chinensis* (GQ168792), the kuruma shrimp *Marsupenaeus japonicus* (GQ181123), and the blue crab *Callinectes sapidus* (AF26403030), among others (Brouwer et al. 2003, Chai et al. 2010, Sookruksawong et al. 2013). In particular, in *P. vannamei,* studies have focused on the level of mRNA expression in gill, hepatopancreas, and hemocytes directly related to the immune system (Sookruksawong et al. 2013).

Previous studies have shown that temperature affects ROS production in crustacean species such as *Macrobrachium nipponense* (Wang et al. 2006). Sookruksawong et al. (2013) suggested that *P. vannamei* increased *cMnSOD* production to reduce oxidative stress induced by high temperatures in their environment. Zhou et al. (2010a) evaluated the differential expression of seven genes encoding antioxidant enzymes in hemocytes, hepatopancreas, and gills following acute heat stress and observed temperature-induced *cMnSOD* gene expression in hemocytes and hepatopancreas mainly. In contrast, in gill tissue, they reported low mRNA expression. In the present study, we found that *cMnSOD* expression levels in shrimp gill were induced by CTmax being stronger in organisms acclimated at 20 and 26°C after UA and in organisms acclimated at 32°C after bilateral EA. Non-ablated organisms had the lowest *cMnSOD* expression levels in all cases. Although heat stress induced through CTmax generated a significant *cMnSOD* response, ablation of the eye peduncles (unilaterally and bilaterally) was the main factor modulating this *cMnSOD* mediated antioxidant response to scavenge superoxide radicals in *P. vannamei* gills.

On the other hand, GPx is a ubiquitous antioxidant enzyme that protects cells against oxidative damage by reducing hydrogen peroxide and a wide range of organic peroxides using glutathione as a reducing substrate during phagocytosis or physiological metabolism (Wang et al. 2012). This enzyme is part of the main enzymatic line of defense against oxidative membrane damage with other enzymes, such as SOD, GST, and CAT, mainly (Wang et al. 2012). Some authors, such as Zhou et al. (2010 a,b), observed that acute heat stress induced the expression of *CAT*, *GST*, ferritin, and *hsp60* genes in *P. vannamei* gills exposed to 22 and 28°C for 6 h. However, they did not observe any differences in the expression of *CAT*, *GST*, ferritin, and *hsp60* genes in *P. vannamei* gills exposed to 22 and 28°C for 6 h. However, they observed no difference in *GPx* gene expression in gill and hepatopancreas tissues relative to the control temperature (15°C).

Interestingly, in the present study, *GPx* expression in shrimp gills seems to be mainly induced by the acclimation temperature of 20°C, where the highest levels of this transcript were observed regardless of the EA state or CTmax. In this case, the two genes were chosen to analyze the temperature, and EA's effect on shrimp's oxidative stress seems to respond as molecular stress markers. While *cMnSOD* was induced by ablation of the eye peduncles to scavenge superoxide radicals.

Hypoxia-associated response to *hif1α* expression

Pörtner (2010), with the general principle of thermal tolerance limited by oxygen-carrying capacity, explained how the aerobic range was limited on both sides in the thermal window and established the performance window in the organisms, with an optimum close to the upper temperature of the *pejus*. Thermal limitation results from insufficient oxygen-carrying capacity, which is reflected in a decrease in cellular oxygen levels and, eventually, the transition to anaerobic metabolism (Pörtner 2010). This statement establishes the width and positioning of the organisms' thermal windows on the temperature scale. Thus, the temporal limitation of thermal tolerance starts beyond peaking temperatures. Pörtner (2010) also mentions that, during protective acclimation, critical temperature triggers anaerobic capacity through HIF-1 and metabolic depression. In this case, compensatory mechanisms, such as anaerobic metabolism, heat shock

response, and oxidative stress, and their capacity, can be modified by acclimation (Pörtner 2010).

All existing reports on the relative expression of *hif1α* have been evaluated under hypoxic and normoxic conditions. Soñanez-Organis et al. (2009) observed that under normoxic conditions in *P. vannamei* gill tissue maintained at 28°C, *hif1α* gene expression was higher than in shrimp exposed to hypoxia. These results are consistent with those of Hardy et al. (2012), who showed that *hif1α* gene expression levels in gill tissue were significantly higher under normoxic than under hypoxic conditions in both female and male *C. sapidus* (blue crab) acclimated to 25°C. The gills play an essential role in the HIF response to hypoxia as *hif1α* transcript levels are higher in this tissue under normoxic and hypoxic conditions (Soñanez-Organis et al. 2009, Piontkivska et al. 2011, Hardy et al. 2012).

In this study, a pattern of increased *hif1α* gene expression was observed in organisms during acclimation to different temperatures for 21 days, as reported in other studies under normoxic conditions. When the organisms reached CTmax, the relative expression of *hif1α* decreased and showed no significant difference between the different acclimation temperatures. The decreased *hif1α* gene expression pattern has been reported under hypoxic conditions (Soñanez-Organis et al. 2009, Piontkivska et al. 2011, Hardy et al. 2012). However, significant differences in the relative expression of *hif1α* were observed between acclimation temperatures and CTmax. At the 26°C acclimation temperature, a higher relative expression of *hif1α* was observed. The acclimation temperature of 26°C, included in the optimal thermal range, could be considered a normal condition for these organisms. This result coincides with that reported by Soñanez-Organis et al. (2009), who observed that under normoxic conditions, the expression of the *hif1α* gene was higher than under experimental conditions. This response can be explained by the mechanism used by these organisms to respond to hypoxia, the reversible metabolic depression characterized by a drastically reduced but balanced steady state between ATP supply and consumption (Gorr et al. 2006). The *hif1α* gene was overexpressed under thermal acclimation; therefore, we hypothesized that this gene was essential in the thermal acclimation processes of the organisms (Kawabe & Yokoyama 2012).

In conclusion, CTmax induced a significant expression of *hsp70* and *hsp90* by intensifying their expression with EA. Although *cMnSOD* and *GPx* genes were susceptible to EA, *GPx* was the main gene in antioxidant response at the acclimation temperature of 20°C, whereas *cMnSOD* was significantly induced by EA. On the other hand, *P. vannamei* showed an energy conservation strategy to temperature changes; therefore, EA could be causing an energy imbalance, making them highly susceptible to a lack of the X-organ sinus gland complex and atypical temperatures. Thus, EA has consequences at the molecular level on the expression of these genes involved in cellular maintenance and compensatory processes not only when temperature increases but also at the metabolic, immunological, and reproductive levels. These results indicated that the processes maintained by the eyestalk were strongly affected by its removal, reducing the shrimp's ability to maintain homeostasis. This response has also been observed at the physiological level with increased bioenergetic demands. It was affecting survival, increasing molting, metabolic, and feeding rates and modifying thermal preference and tolerance, and reproductively by reducing spawning viability, abortive spawning, low fertilization, and hatching rates, among others (Palacios et al. 1999, Sainz-Hernández et al. 2008, Kumlu et al. 2011, Re et al. 2012). This work demonstrates that removing the X-organ sinus gland complex and the hormone changes has a negative effect on breeding organisms and juvenile *P. vannamei* organisms, impairing physiological-molecular responses and thermal tolerance. Therefore, we strongly recommend further studies on the effects of the implications of EA on progeny through genomic and epigenetic perspectives.

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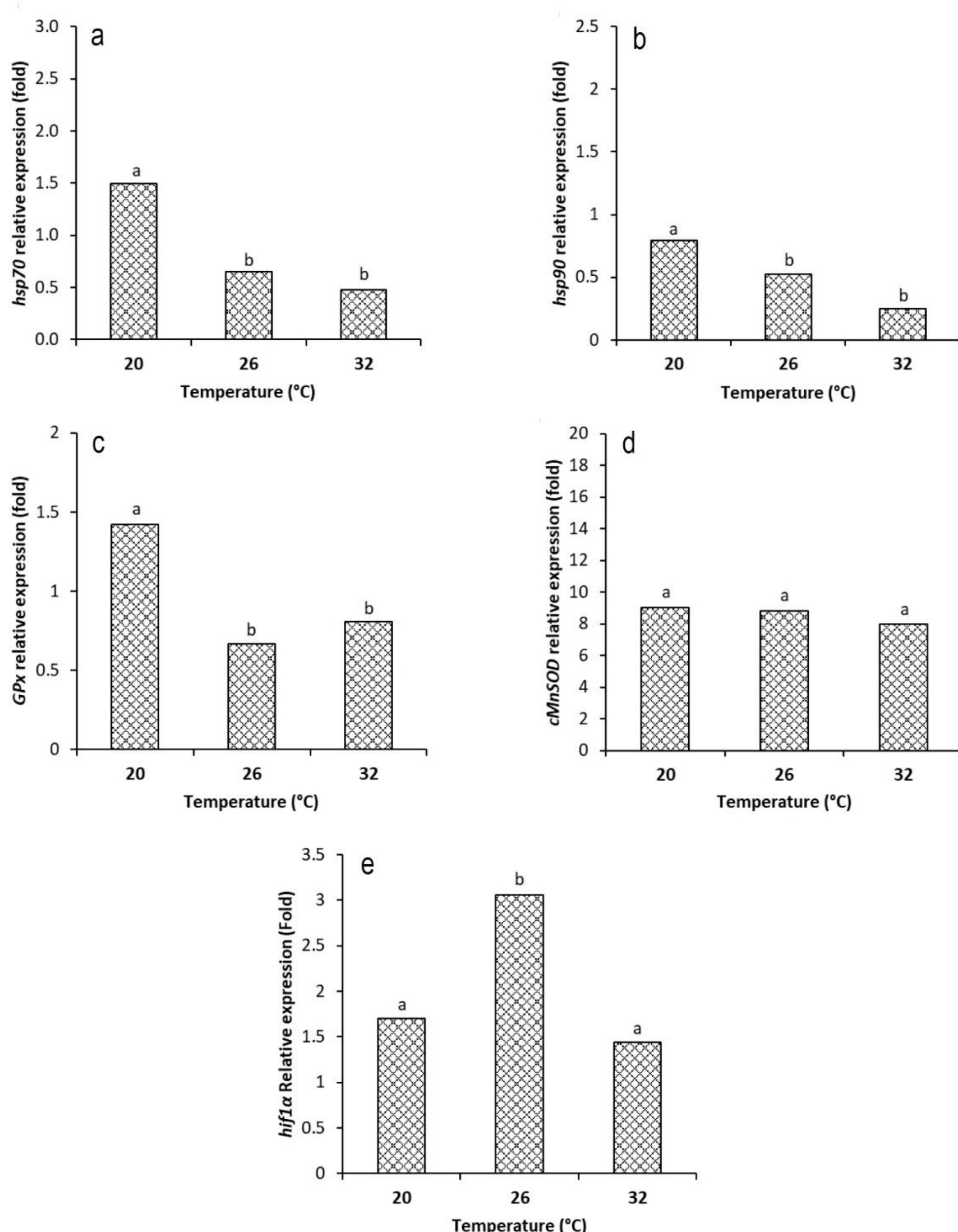

Supplementary Figure 1. Relative expression (back-transformed log-means) of a) *hsp70*, b) *hsp90*, c) *Gpx*, d) *cMnSOD*, and e) *hif1α* in the gills of *P. vannamei* juveniles non-eyestalk ablated acclimated at different temperatures. Mean values are shown in bars, and different letters indicate statistically significant differences among acclimation temperatures ($P < 0.05$).