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

Laura López-Galindo1,2, Ernesto Larios-Soriano1,3, Claudia Ventura-López1, Fernando Díaz1, Denise Re1, & Clara E. Galindo-Sánchez1

1Departamento de Biotecnología Marina, Centro de Investigación Científica y de Educación Superior de Ensenada, Ensenada, Baja California, México
2Instituto de Investigaciones Oceanológicas, Universidad Autónoma de Baja California, Ensenada, Baja California, México
3Facultad de Ciencias Marinas, Universidad Autónoma de Baja California, Ensenada, Baja California, México
Corresponding author: Clara E. Galindo-Sánchez (cgalindo@cicese.mx)

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 (Porțner 2010, Sokolova 2013, Schulte 2015). These responses allow poikilothermic aquatic organisms to maintain or recover homeostasis. For example, under acute or chronic heat stress, poikilo-
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% diethylpyrocarboxylate (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 LxEF1α 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 for 30 s. After stability analyses using geNorm, Normfinder, and Bestkeeper software, *rpL8* and *Ef1α* were used as housekeeping genes. Efficiency values for each gene standard curve (E = (10^(1/slope) - 1) x 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.

<table>
<thead>
<tr>
<th>Gene/Primer</th>
<th>GenBank accession no.</th>
<th>Primer sequence (5′→3′)</th>
<th>Amplicon length (bp)</th>
<th>e</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>LvEF1α</td>
<td>GU136229</td>
<td>(F) GAAATCCGACACATGGGCT (R) CCAATCTTGTACACGTCTCTG</td>
<td>161</td>
<td>1.803†</td>
<td></td>
</tr>
<tr>
<td>LvrpL8</td>
<td>DQ316258</td>
<td>(F) ATGAAACCCTGTAAGACATCCTC (R) CTTGTACCACGCAGATAAGCA</td>
<td>141</td>
<td>1.929†</td>
<td></td>
</tr>
<tr>
<td>Hsp70</td>
<td>AY645906</td>
<td>(F) CTCCTGCTGGGTGTTGTTT (R) GCGGCGTCACCAAATCAGA</td>
<td>120</td>
<td>1.973†</td>
<td></td>
</tr>
<tr>
<td>Lvhsp90</td>
<td>HQ008268</td>
<td>(F) CGAAGGAGAACCAGAGACAC (R) TGCTGAACGCAGATATTCGTC</td>
<td>145</td>
<td>1.908†</td>
<td></td>
</tr>
<tr>
<td>Hif1α</td>
<td>FJ807918</td>
<td>(F) GGAGTCTTTGAGAGAG (R) GCCTCCTTCCGTTGATCTTC</td>
<td>160</td>
<td>1.873†</td>
<td></td>
</tr>
<tr>
<td>cMnSOD</td>
<td>DQ005531</td>
<td>(F) CGTAGAGGGATATTGTCGT (R) TTGAATACATCTTGGAGG</td>
<td>153</td>
<td>2.002†</td>
<td></td>
</tr>
<tr>
<td>LvGPx</td>
<td>AY973252</td>
<td>(F) GGACTTCCACCAGAACC (R) CTCGAAGTTGTTCCAGGAC</td>
<td>154</td>
<td>1.970†</td>
<td></td>
</tr>
</tbody>
</table>

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).
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, 2013).
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.

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 H2O2 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 hif1a 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, hif1a gene expression was higher than in shrimp exposed to hypoxia. These results are consistent with those of Hardy et al. (2012), who showed that hif1a 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 hif1a 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 hif1a 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 hif1a decreased and showed no significant difference between the different acclimation temperatures. The decreased hif1a 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 hif1a were observed between acclimation temperatures and CTmax. At the 26°C acclimation temperature, a higher relative expression of hif1a 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 hif1a 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 hif1a 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 reproducitively 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.

ACKNOWLEDGMENTS

This research was supported by the CICESE project (682123). The authors thank CONACYT for the master's scholarship (280194) awarded to Laura Liliana Lopez Galindo as part of her master of science thesis at CICESE. We thank all members of the functional genomics laboratory for technical advice and D. Fischer for editorial services.

REFERENCES


zinc superoxide dismutase by a novel cytosolic manganese superoxide dismutase has replaced cytosolic copper, zinc superoxide dismutase in crustaceans that use copper (hemocyanin) for oxygen transport. Biochemical Journal, 374: 219-228. doi: 10.1042/ bj20030272


Palacios, E., Carreño, D., Rodríguez-Jaramillo, M.C. & Racotta, I.S. 1999. Effect of eyestalk ablation on maturation, larval performance, and biochemistry of white Pacific shrimp, Penaeus vannamei, broodstock.


Somero, G.N. 2005. Linking biogeography to physiology: evolutionary and acclimatory adjustments of thermal


Received: May 23, 2022; Accepted: January 17, 2023


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 ($P < 0.05$).