DISSERTATION

ROLE OF STRESS-RESPONSIVE GENES IN THE MOLT CYCLE OF DECAPOD CRUSTACEANS

Submitted by

Diego Alejandro López-Cerón

Department of Biology

In partial fulfillment of the requirements

For the Degree of Doctor of Philosophy

Colorado State University

Fort Collins, Colorado

Fall 2019

Doctoral Committee:

Advisor: Donald Mykles

Deborah Garrity
Shane Kanatous
Santiago Di Pietro

ABSTRACT

ROLE OF STRESS-RESPONSIVE GENES IN THE MOLT CYCLE OF DECAPOD CRUSTACEANS

The main objective of this study was to understand the molecular mechanisms underlying the inhibition of molting by environmental stress in decapods. The general hypothesis describes that environmental stressors inhibit synthesis and secretion of molting hormones (ecdysteroids) by the molting gland, or Y-organ (YO), through stress response signaling genes in the mTOR signaling pathway. It is predicted that premolt animals of *Gecarcinus lateralis* will be more sensitive to thermal stress compared to those in intermolt stages, and that *G. lateralis* will be more sensitive to environmental stresses such as temperature or desiccation than *Carcinus maenas*. The aims of this study were to review of state of the art on molting in decapods, regulation of molt cycle by environmental stress, design of model of stress response in decapods (Chapter 1), Isolate, identify and characterize cDNAs encoding stress response genes in *G. lateralis* and *C. maenas* (Chapter 2), Quantify the effects of acute thermal stress on the Y-organ of Multiple Leg Autotomy (MLA) and Eyestalk Ablated (ESA) intermolt, premolt, and postmolt *G. lateralis* (Chapter 3), and Quantify the effects of desiccation on gene expression and mTOR activity in intermolt animals of *C. maenas* (Chapter 4).

TABLE OF CONTENTS

CHAPTER ONE: STRESS IN DECAPOD CRUSTACEANS

INTRODUCTION

Regulation of molting in decapods

Molting or ecdysis is defined as the act of molting the integument (McLaughlin, 1980), a natural behavior all crustaceans exhibit. In decapods, molting involves biochemical, morphological, physiological and behavioral changes to prepare the organisms for recovery and recovery from ecdysis (Burggren and McMahon, 1988). Most of the crustaceans pass part of their lives preparing for, or recovering from, ecdysis (Table 1-1).

At the premolt, or proecdysis, stage decapods regenerate lost limbs, cuticular components are resorbed after partially breaking the old exoskeleton, a new cuticle is deposited, water and ions are absorbed, calcium is resorbed and stored in the tissues, and claw muscles atrophy (Factor, 1995; Mykles, 2001). At ecdysis, total shedding of the old exoskeleton occurs, and the size of the animals increases by a high hydrostatic pressure, in consequence of absorption of water without osmotic gradient between haemolymph and sea water (Burggren and McMahon, 1988; Mykles, 1980). After ecdysis (postmolt or metecdysis), synthesis of the new integument is completed and hardened using stored calcium and claw muscles are restored; additional tissue is secreted and replaces water previously absorbed (Skinner, 1962, 1965). During the intermolt stage, tissue grows and fills the available space until the next molt (Burggren and McMahon, 1988; Mykles, 1980).

The molecular mechanisms that control ecdysis in crustaceans are associated with growth and developmental processes (Devaraj and Natarajan, 2006). In crustaceans, neuropeptides formed by neurosecretory cells in the X-organ are accumulated and released from the sinus gland

in the eyestalk (Hopkins, 2001; Hopkins, 2012). The neuroendocrine system in crustaceans' eyestalks (Figure 1-1) is formed by the brain, medullas terminalis, and the X-organ (XO) -Sinus Gland (SG) system (XO-SG), which transports and releases neuropeptides into the hemolymph by exocytosis via axonic flow to the SG (Huberman et al., 1995). Molting in crustacean decapods responds to signals of two major neuropeptides with a common amino acid composition originally identified in crustaceans: the eyestalk CHH/MIH/GIH gene family (Chan et al., 2003). These neuropeptides, Crustacean Hyperglycemic Hormone (CHH) and Molt-Inhibiting Hormone (MIH), have their source in the X-organ-sinus gland complex and regulate growth, reproduction and some aspects of metabolism (Webster, 1997). Premolt and preparation for the ecdysis phase are controlled by endocrine systems that respond to internal and external signals from XO-SG over Y-organ (YO) (Factor, 1995). YO controls and regulates development through production of growth hormone or ecdysteroids. YO or ecdysial glands are paired cephalic endocrine organs that are located in the anterior cephalothorax of crustaceans and control molting, reproduction and other physiological activities (Quackenbush, 1986).

According to Chang et al. (1990), CHH and MIH share a high degree of amino acid identity and conserved cysteine residues at the same relative positions. Within the CHH/MIH gene family, Lee et al. (2007) described two groups or subfamilies of neuropeptides. A CHH subfamily or group I, composed by all the CHH peptides and MIHs of several crustacean species such as *Homarus americanus*, *Procambarus bouvieri*, *Penaeus vannamei*, and the mandibular organ-inhibiting hormone (MOIH) of *Libinia emarginata*. CHH peptides are hormones of 72–74 amino acids residues, which contain a crustacean hyperglycemic hormone precursor related peptide (CPRP), a signal peptide and mature peptide sequences (Jeon et al., 2012). The group II or GIH subfamily (gonad- or vitellogenesis-inhibiting hormone, GIH or VIH) includes MOIH of

Cancer pagurus, GIH of *H. americanus* and *Armadillium vulgare*, and all other MIHs. These peptides are 74–81 amino acids length and contain only a signal sequence and a mature peptide. In contrast to CHH subfamily, GIH subfamily has a glycine at the position 12 in the mature hormone.

CHH, when stimulated by environmental signals, is fundamental in various processes like glucose regulation, molting and water uptake (Chung and Webster, 2003; Webster, 1997; Webster et al., 2012). CHH is most abundant in the XO-SG, but it has been also characterized in other tissues such as the second root of thoracic ganglia, subesophageal ganglia, pericardial organs, gut, heart, antennal glands, and gills (Chung et al., 2010; Zheng et al., 2010). After eyestalk ablation, CHH influences physiological processes such as food intake, digestion and nutrient transport, molting, and reproduction (Christie et al., 2010). CHH interacts with guanylyl cyclase membrane receptors on the YO membrane, leading to an increase in cGMP and inhibiting the ecdysteroid biosynthesis (Figure 1-2) (Lee and Mykles, 2006). CHH characterization and expression profile has been determined on several crustacean species (Jeon et al., 2012; Li et al., 2010; Ventura-Lopez et al., 2016; Xie et al., 2014; Zheng et al., 2010).

MIH is released from XO neurosecretory cells of the eyestalk and inhibits molting by suppressing secretory activity of the YO (Soumoff and O'Connor, 1982). During intermolt of *Gecarcinus lateralis*, ecdysteroids circulate in hemolymph at low levels by MIH inhibition, but once eyestalk ablation is performed, the intermolt period is shortened and the number of molt cycles increase (Figure 1-2) (Lee et al., 2007). Mattson and Spaziani (1986) determined through *in vitro* assays on *Cancer antennarius* that ecdysteroid secretion by YO is controlled by MIH, with a significantly fluctuation of hemolymph ecdysteroid concentration during various molting stages and inducing advanced shedding of exoskeleton. MIH is thought to interact with specific

membrane receptors over YO, following to an increase in cAMP and a sustained augment in cGMP as second messengers (Mykles, 2011). MIH characterization and expression profile has been determined on several crustacean species (Huang et al., 2015a; Huang et al., 2014; Luo et al., 2015; Robert et al., 2016; Techa and Chung, 2015).

Effect of stress on molting in decapods

In their review of land crabs, Burggren and McMahon (1988) described that the activation or inhibition of molting is caused by intrinsic and extrinsic factors: intrinsic such as sexual maturity of the individual and the loss and regeneration of limbs, and extrinsic corresponding to environmental variables such as light, temperature, and rainfall. Many studies have correlated growth and development in decapods, based on the intrinsic and extrinsic factors, by the manipulation of molting or characterization of behavior and physiological response to stressors. *G. lateralis* was studied by Bliss and Boyer (1964) to determine that some environmental factors are regulators of growth. After exposure to two different temperatures, blackback land crabs delayed the proecdysial limb growth at high temperatures but were able to restart limb growth at moderate and lower temperatures. In the same way, whereas animals staying on dry sand with light and other crabs repressed or delayed proecdysial limb growth, those in darkness on moist sand tend to resume limb growth. Surprisingly, some of these factors delay molting but do not prevent it, meaning an adaptive value when the exact conditions are appropriate. Diet and nutrient deprivation are another key factor regulating growth and development of decapods. *Ocypode quadrata*, a species of sandy beaches, spends most of the winter season underground with no food resource. After 15 days of food deprivation demonstrated to be adapted to fasting or starvation with no significant changes in expression levels of CHH (Vinagre and Chung, 2016).

Few studies combine different environmental stressors with molting and neuropeptides expression. *Callinectes sapidus* is a commercially important species in Chesapeake Bay that migrates and experiences changes in environmental conditions such as temperature and dissolved oxygen (Brown-Peterson et al., 2005). To determine profiles of CHH in response to stress, individuals of *C. sapidus* were exposed to gradients of oxygen and temperature (Chung and Zmora, 2008). Additionally, levels of expression of eyestalk- (ES-CHH) and pericardial-CHH (PO-CHH) were measured under normoxic and hypoxic conditions, to determine their physiological role. Hyperglycemia and hyperlactemia were observed on crabs exposed to hypoxia and emersion, with a great reduction of ES-CHH and an increase of PO-CHH after hypoxia and emersion. Temperature substantially influenced an increase on both ES-CHH and PO-CHH and lactate levels. These results have been found by Webster (1996) on *C. pagurus* as well. Emersion of *C. pagurus* individuals resulted in hypoxia and hyperglycemia with CHH levels increased in the first hour. Webster's conclusions indicate that emersion and posterior hypoxia promote massive and rapid release of CHH from sinus gland. Molting is represented as a straight forward system involving two antagonistic hormones: a molt-inhibiting neuropeptide and a molt-promoting steroid (Factor, 1995).

Molecular mechanisms of stress response in decapods

Under stress conditions, decapods species exhibit molecular mechanisms and gene expression patterns to survive and maintain a physiological stability. Although many molecules and proteins are involved directly or indirectly with perturbations at cellular level, some specific genes have critical functions in stress response.

AMP-activated protein kinase (or Adenosine Monophosphate-activated protein kinase, AMPK) is a master metabolic regulator and a heterotrimer formed by an α-catalytic subunit and two non-catalytic β- and γ-subunits (Kim et al., 2016). AMPK has many important functions for instance cell protection in physiological and pathological stress, including hypoxia and heat shock by reacting to reductions in the ATP:AMP/ADP ratio, shifting to generation of ATP on metabolism by blocking energy consumption (Sanli et al., 2014). In order to conserve energy through metabolic stress and a broad array of disturbing situations such as energy depletion, AMPK inhibits protein translation and cellular growth by stopping the mammalian mechanistic Target Of Rapamycin (mTOR) (Inoki et al., 2012). A high intracellular AMP/ATP ratio activates AMPK to respond to the reduction of cellular energy, after two AMP molecules bind to four tandem cystathionine β-synthase domains (CBS) into the γ-subunit, allowing the kinase complex to sense levels of adenine nucleotide (Faubert et al., 2015). That AMPK activation requires phosphorylation at Thr172, which lies in the activation segment of the amino-terminal kinase domain of the α-subunit, leading to a various hundred-fold increase in activity of AMPK (Cetrullo et al., 2015). In view of this information, AMPK as a biomarker of various stressors during environmental tension, has been found and activated during anaerobiosis due to O_2 depletion and temperature stress in the rock crab *Cancer irroratus* in a tissue-specific manner (Pinz et al., 2005). On air-exposed individuals of *Portunus trituberculatus* at 8 °C, AMP/ATP ratio increased and AMPKα expression was significantly upregulated by hypothermia (Lu et al., 2016). From individuals of *C. irroratus* exposed to sublethal temperatures, Frederich et al. (2009) determined that AMPK is a better temperature stress indicator that Heat Shock Proteins (HSP) in early stress response when animals reach a thermal threshold or pejus limit. In a broader observation, AMPK is a very effective feature to explain the ability to stay alive at

different temperatures like some crustacean species living in different habitats that demonstrate different behavior as they approach to a critical environmental temperature. The green crab *Carcinus maenas* (living in the intertidal zone) is especially considered to have a broader pejus range than *C. irroratus* (subtidal zone) and *H. americanus* (permanently in the subtidal zone), as a result of less variations of water temperature in the subtidal ambient (Jost et al., 2012).

Sirtuin stands for Silent Information Regulator 2 proteIN (Sirt2). Sirtuin is a family of seven highly conserved proteins included in four classes, which deacetylase target substrates on nicotinamide adenine dinucleotide (NAD^+) availability as a cofactor, by removing acetyl groups from lysine residues (Carafa et al., 2012). Each of these proteins comprises a catalytic domain conserved in all Sirtuins with different N- and C- terminal regions, where characteristic residues give characteristic biological properties to these proteins (Costantini et al., 2013). They exhibit three subcellular localizations: SIRT1, SIRT2, SIRT6 and SIRT7 are present in the nucleus and cytoplasm, and SIRT3, SIRT4 and SIRT5 are found in mitochondria (Frye, 2000). Their function has been associated with caloric restriction, energy metabolism, and stress resistance (Gasser and Cockell, 2001). In stress occurrence, Sirtuins promote resistance and cell survival through suppression of genes and pathways associated to specific transcription factors identified in mammals (Bosch-Presegue and Vaquero, 2014). Since Sirtuins proteins act as energy sensor and metabolic regulator, they are relevant biomarkers on stress response by hypoxia and temperature. To date, only one research paper examines effects of Sirtuin on non-decapods crustaceans response to heat shock stress (Schumpert et al., 2016). After a severe heat shock exposure, SIRT2 showed overexpression on *Daphnia pulicaria* individuals in a similar level to the mammalian SIRT1, providing protection and reducing cell death by proteotoxic stress. However, it has been used as energy sensor in other invertebrates to determine response at proteomic level

after acute heat stress (Tomanek and Zuzow, 2010). SIRT5 was lower after exposure of *Mytilus trossulus* to 24-28 °C for 1 hour possibly regulating energy and metabolic costs derived from heat shock. High temperature effects were similarly studied on *Cellana toreuma* to determine how metabolic regulation by SIRT1 occur (Han et al., 2013). SIRT1 increased transcriptionally after limpets were exposed to temperatures from 22 to 40 °C, with initial expression at 30 °C, maximum at 32 °C and inactivation at 36 °C. At cellular level, SIRT1 has showed increased activity during caloric restriction whereas HIF signaling lowered, indicating these proteins may act in stress-dependent manner (Dioum et al., 2009). Westerheide et al. (2009) have also showed a high relation in recruitment of heat shock factors (HSF) after a long-term heat shock, reducing one-fourth on its expression and supporting the SIRT1 role as an *in vivo* regulator of HSF and HSP expression.

The Heat Shock Proteins (HSP) are molecules involved in protection of cells and maintenance of proteins homeostasis from the effects of heat and some other stress forms (Lindquist and Craig, 1988). HSPs are classified based on the nature of their function and molecular mass, with the most important role being protein folding and disaggregation after proteotoxic stress (Schlesinger, 1990). HSPs are classified generally into six major families: small heat shock proteins (sHSPs), HSP40 (J-proteins), HSP60 (chaperonins), HSP70, HSP90, and HSP100 (De Maio, 1999). Many studies make use of HSP to determine how fast they are produced after temperature elevation and the relationship between temperatures of induction and environmental with reproduction and development. Some factors induce HSPs expression such as exposure to ethanol, heavy metals, and hypoxia, but they also trigger thermotolerance in most of the model decapods. Temperature and thermal tolerance are two major factors controlling distribution and activities of many aquatic and terrestrial decapod species. They have been two

of the most studied stress factors in decapods as described in the next review. Thermal limits and HSP70 production were studied on *Pachygrapsus marmoratus* to determine intraspecific differences and how temperature affects some population factors such as reproduction and mortality (Madeira et al., 2012). HSP70 expression not only increased significantly with high temperatures but also in females, juveniles and coastal individuals, compared with males, adults and estuarine crabs. Since several stress factors trigger HSPs up- or down-regulation during general cellular stress, HSP70 might not be an adequate and better biomarker than AMPk for temperature stress on *C. irroratus* (Frederich et al., 2006). Thus, AMPk differential expression in time and type of tissue may be correlated to energy relocation after a critical point of the thermal stress. One of the most extremes marine habitats are the deep-sea vents with some highly resilient decapod fauna associated (Mann and Lazier, 2006). Specimens of *Rimicaris exoculata* reached a peak of thermal resistance at 33 °C and died after 8 h of exposure at 45 °C (Ravaux et al., 2003). HSP70 expression was weaker in non-heat-shocked animals with a maximum survival and induction threshold at temperatures of 15-20 °C. *R. exoculata* might not tolerate high temperatures in the 33-37 °C range and die above this limit if exposed during long periods. HSPs expression levels can be correlated with other environmental factors for example hypoxia and CO_2 presence. HSP70 and HSP90 transcripts of the arctic crab *Hyas araneus* were upregulated at intermediate and high temperature and CO_2 pressure with no changes at 10 °C (Harms et al., 2014). Along with an increase of mRNA transcripts of proteins of antioxidant defense, HSPs transcripts showed being a chaperone on cellular damage.

Hypoxia Inducible Factor (HIF) are proteins continuously made and accumulated in hypoxic cells, but rapidly degraded and almost absent in normoxic cells (Carroll and Ashcroft, 2005). HIF, a heterodimer consisting of a HIFα and a HIFβ subunits, are proteins that contain one basic

helix-loop-helix (bHLH) domain and two PER-ARNT (Aryl hydrocarbon Receptor Nuclear Translocator)-SIM (PAS) domains in their N-terminal regions (Sharp and Bernaudin, 2004). HIFα is usually regulated by posttranslational acetylation and phosphorylation, although other mechanisms of regulation occur (Yoon et al., 2014). Between their N- and C-terminal domains, there are two oxygen-dependent degradation domain (ODD) (Sharp and Bernaudin, 2004). In normoxia, after the hydroxylation of two proline residues (Pro402 and Pro564) within the ODD domain of the α-subunit happens, the von Hippel–Lindau tumor suppressor gene (pVHL) tags the α-subunit with polyubiquitin, allowing recognizing and degradation by the proteasome (Zagorska and Dulak, 2004). In hypoxia, hydroxylation of Proline residues is inhibited because of oxygen limitation, preventing pVHL from recognizing α-subunit, and leading to its accumulation and dimerization with the β-subunit (Ke and Costa, 2006). Responses of decapods to occurrence of typical stressors such as hypoxia, may lead to either a typical tolerance reaction or negative consequences for survival after oxygen reduction. For instance, expression of HIF in the shrimp *Palaemonetes pugio* had not significant changes after individuals of grass shrimps were exposed continuously to moderate and severe hypoxia from 7 to 14 days (Li and Brouwer, 2007). The unchangeable expression of HIF may support the fact that HIF is regulated at the translational level as in vertebrates and being dependent of grade and duration of hypoxia stress. These results could be observed in other species. Expression of both HIF subunits was unchanged after exposure of *C. sapidus* to hypoxia and hypercapnia for 1 h (Hardy et al., 2012). Differences in HIF subunits expression between types of muscle fibers were detectable but not significant compared to normoxic animals. As in *P. pugio*, levels of response of HIF to short-term hypoxia had not important variations but higher levels in gill and light muscle fibers, suggesting a tissue-specific response and glycolytic processes. The unusual response of HIF to

hypoxia in some decapods species may suggest a different model in HIF regulation and expression than that in typical mammalian systems. A transcriptomic study on *Macrobrachium nipponense* described tissue-specific differences in mechanisms and pathways after response to chronic hypoxia stress, with zero variations of HIF levels with hypoxia or anoxia (Sun et al., 2015). At first point, hypoxia would have a similar behavior to thermal tolerance exhibited by many crustacean species as a regular adaptation associated with habitat and lifestyle. However, response may occur in either additive or synergistic manner (Mann and Lazier, 2006).

Molecular biomarkers may be more accurate and sensitive to physiological responses to stress such as hypoxia, thermal stress, energy depletion and caloric restriction (Chang, 2005).

Stress response in decapod crustaceans

Multiple adaptations help crustaceans to respond to metabolic and environmental stress through diverse pathways, including metabolic pathways and molting cycle control. Stress events trigger reactions of decapods to external overstimulation and may disturb the performance of native and non-native species. It has an important consequence since global changes in habitats and climate affect especially aquatic systems in comparison with terrestrial ecosystems: aquatic and semi-aquatic species should be more resistant than populations in terrestrial zones (Perez et al., 2016; Sorte et al., 2013). Decapod crustaceans are few of the animal groups which have conquered both aquatic and terrestrial environments (Johnson and Uglow, 1985).

The resistance of decapod species with respect to stressors factors such as temperature, emersion, desiccation and gradual hypoxia anticipate their potential distribution in both aquatic and terrestrial environments (Cuculescu et al., 1998; Quijón et al., 2001). During stress response, decapods deal with major physiological arrangements which include heart and breathing rates

increased, release of stored energy, and oxygen and nutrients redistribution to location of the stress in the body (Hill et al., 2012). The extension of the adaptations and response to stressors is partially dependent of the typical habitat of the decapods, and describes that organisms use specific adjustments. Species can inhabit a range of environments from subtidal such as *H. americanus* (Factor, 1995; Hines et al., 2013; Spees et al., 2002), intertidal where *C. maenas* inhabits, (Grosholz and Ruiz, 1996; Klassen and Locke, 2007; Taylor et al., 1973; Tepolt and Somero, 2014), to terrestrial with *G. lateralis* as one of the most representative terrestrial species (Bliss, 1968, 1979; Burggren and McMahon, 1988; Capistran-Barradas et al., 2003; Factor, 1995). Various studies have shown the physiological effects of hypoxia and thermal stress on decapod crustaceans and focused on metabolic and molecular adjustments.

Generally, temperature determines the distribution of decapods in aquatic and continental environments. Distribution of decapod species has been correlated with maximum, minimum and mean ambient temperature that determines the thermal range within a particular population inhabit and prevail (Stillman and Somero, 1996; Wilkens and Milton, 1965). Thermal variation additionally has been studied by influencing essential biological functions such as reproduction and growth in *Chasmagnathus granulate*, a species existing in salt marshes (D'Incao et al., 1992). The extent of thermal resistance adaptation has also been determined on *C. maenas*, *C. irroratus* and *H. americanus*, by measuring their temperature tolerance to a range of temperatures representative of the ambient temperature range experienced in their natural habitats (Jost et al., 2012). During low tide, strong sunlight can produce high temperatures on exposed shore that may raise above the normal limits of sea organisms. Certain species that occupy temperate regions tend to be eurythermic as they are exposed to a wide range of temperatures when are compared to tropical and/or subtropical populations. However, some

genus such as *Petrolisthes spp.* are distributed globally and their species depict varied distribution patterns along an extensive latitudinal and vertical zonation (Gunderson and Stillman, 2015; Jensen and Armstrong, 1991). Contrarily, some stenothermal species (e.g. *Uca spp.*) are characterized by poorer tolerance to drastic alterations in thermal limits (Wilkens and Milton, 1965). Moreover, Vernberg and Tashian (1959) used the response to acclimation to low temperature and shifting to thermal death limits, to report *Uca rapax* as a tropical species more resistant to extreme temperatures than *Uca pugnax*, a temperature dweller. In fact, their findings are counterintuitive to the climate variability hypothesis of Janzen (1967), which states that temperate species in climates with high climate variability should exhibit broader thermal tolerances, compared to that tropical species evolve to have narrower thermal limits in low variability environments (Figure 1-3).

Emersion and subsequent desiccation are common stressors affecting intertidal species. In contrast to subtidal and supratidal organisms that inhabit environments with low variation of the abiotic factors, intertidal individuals may lose water by evaporation and die from desiccation. For instance, red color morphs of *C. maenas* are supposed to have a lower ability to deal desiccation, because of the lesser time they spend on the shore respect to green morphs (Reid and Aldrich, 1989). Even though red morphs underwent desiccation and death faster than those green morphs, Reid and Aldrich (1989) did not determine a significant correlation regarding deadly conditions for both colors. Although metabolic and functional differences are not established in morph types of some species regarding emersion, there are typical biochemical and physiological responses decapods show to face hypoxic conditions. In early life stages of decapods, Alter et al. (2015) describe reduced mobility and hear rate, anaerobic metabolism and reduction of metabolic activity. In some cases, emersion affects species (e.g. *Cancer productus*) that

maintained a position on the shore and were emersed because of their location (Defur et al., 1983). By keeping a position into hypoxic seawater, *C. productus* is forced to keep CO_2 excretion and maintain branchial functions. In a rare adaptation, the crayfish *Orconectes rusticus* raises the anterior portion of the carapace over the hypoxic water and reverses the direction of the normal ventilator current, by pumping air into the branchial cavity (McMahon and Wilkes, 1983). During emersion periods, species also set up new habits to avoid an acid-base unbalance. *Necora puber* has reduced intertidal assemblages because of elevated pCO_2 and temperature, that leads to slower recovery after emersion and struggle to remove extracellular CO_2 and lactate (Rastrick et al., 2014). Some species, such as *Chionoecetes bairdi*, suffer of exposure to air, subfreezing temperatures, desiccation, and limb loss (Carls and Oclair, 1995) after capture. On this species, Haukenes et al. (2009) ran experiments of stress gradient with changing time and temperature with exposure to a second stressor. Based on oxygen consumption, these authors determined that physiological and behavioral responses might be a combined result after collective stressors. Anthropogenic manipulation of organisms is an important source of stress to species captured from commercial fisheries. Most of the extremes conditions decapods face in their natural environment can be modelled as they possibly occur in commercial fishery.

Both aquatic and terrestrial decapod species cope with variations on oxygen supply and demand. In the littoral realm, crustaceans struggle with reductions of oxygen tension and are exposed to hypoxic and anoxic conditions because of tidal cycle (Taylor and Spicer, 1987). Decapods that permanently transitioned from water to land have developed more specific adaptations to the severities of the terrestrial environment (Vermeer, 1987). During hypoxia periods, decapods must utilize anaerobic pathways and morphological modifications to survive or switch permanently to conditions of low oxygen levels. Some gecarcinids, *G. lateralis* and

Cardisoma guanhumi, have high airflow rates through the gills with low extraction efficiency; a characteristic comparable to other crustacean at similar temperatures and weight (Cameron, 1975). Although *O. quadrata* is considered one of the most land-adapted crabs, it spends little time in the ocean filling its branchial chambers with water and air; it cannot survive long periods in immersion (Gray, 1957). As other terrestrial decapods (e.g. *Cardisoma sp.* and *Gecarcinus sp.*), *O. quadrata* has fewer gills and a reduced surface area on them to diminish the possibility of suffering desiccation through transpiration, even as long as 20 hours after air exposure (Bliss, 1968). Other species occupy terrestrial environments with merely partial adaptations to its natural conditions: *Neohelice granulata* chooses to spend more time on land to avoid the hypoxic water conditions on salt marshes and estuarine areas of the Atlantic coast of South America (Geihs et al., 2013). However, to tolerate short periods of exposure to air because of a large gill area, *N. granulata* carries water into the branchial chamber when exposed to air and recirculate it over the carapace to re-oxygenate (de Lima et al., 2015). At reduced oxygen pressure gradients, affinity between haemocyanin and oxygen must rise to increase and/or sustain oxygen levels in the gills. It can be achieved by a rearrangement of protein structure to obtain a molecule with a higher affinity for oxygen (Bernasconi and Uglow, 2008). Decapod species of intertidal and terrestrial habitats go through emersion naturally with tidal cycle or a seasonal dry period. Moreover, burrowing and benthic species may experience periodic or random periods of hypoxia.

This project uses a comparative approach to understand the molecular mechanisms underlying the inhibition of molting by environmental stress. The central hypothesis is that environmental stressors inhibit synthesis and secretion of molting hormones (ecdysteroids) by the YO, through stress response signaling genes in the mTOR signaling pathway. I predict that

G. lateralis will be more sensitive to environmental stresses, such as temperature and desiccation, than *C. maenas*. Secondly, we hypothesize that premolt animals will be more sensitive to thermal stress and hypoxia, compared to those in intermolt stages, as previous studies indicate that stress response genes are downregulated at the committed and repressed stages of molt cycle (Brown-Peterson et al., 2005; Das and Mykles, 2016; de la Vega et al., 2007; Harms et al., 2014). The differences in sensitivity will be reflected by differential regulation and activities of stress response genes and YO ecdysteroidogenesis. Heat shock and desiccation may affect mTOR activity by acting on stress proteins linked to the mTOR-signaling network, inhibiting ecdysteroid secretion and altering the molt cycle (Figure 1-4). Inhibition of YO ecdysteroidogenesis under stressful conditions could be beneficial, as it would allow the animal to delay molting until favorable conditions return.

Table 1-1. Premolt and postmolt events of the *G. lateralis* molt cycle (Skinner, 1962, 1965).

	Stage	Time	Events
Premolt	D_0		Limb regeneration, atrophy of claw muscle
	D_1		Separation of layers from old exoskeleton and absorption of cuticular components
	D_2	2½ weeks	Formation of epicuticle and exocuticle
	D_3		Decrease in size of epidermal cells
	D_4		High concentrations astaxanthin in blood (pink blood)
Ecdysis		1-2 h	Shedding of the old exoskeleton
Postmolt	A	0-2 days	Resorption of gastroliths
	B	2 weeks	Endocuticle synthesis
	C_1-C_3		Endocuticle synthesis, membranous layer
Intermolt	C_4		

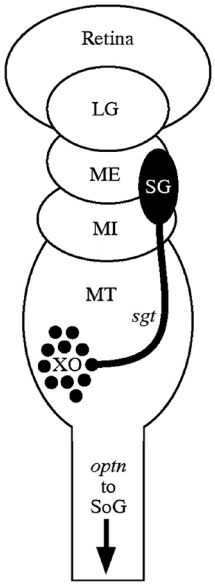


Figure 1-1. Schematic diagram of the location and organization of the X-organ-Sinus Gland (XO-SG) system. The neuroendocrine system within the eyestalk consists of medulla terminalis (MT), medulla interna (MI), medulla externa (ME), lamina ganglionaris (LG), and retina. This system of ganglia is connected to the supraesophageal ganglion (SoG) via the optic nerve (optn). In the MT, a loosely associated collection of neurosecretory somata termed the X-organ (XO) is located. The release site of hormones produced by XO is the sinus gland (SG), which is located at the junction of the MI and ME. The sinus gland tract (sgt) associates the XO and SG (Hsu et al., 2006).

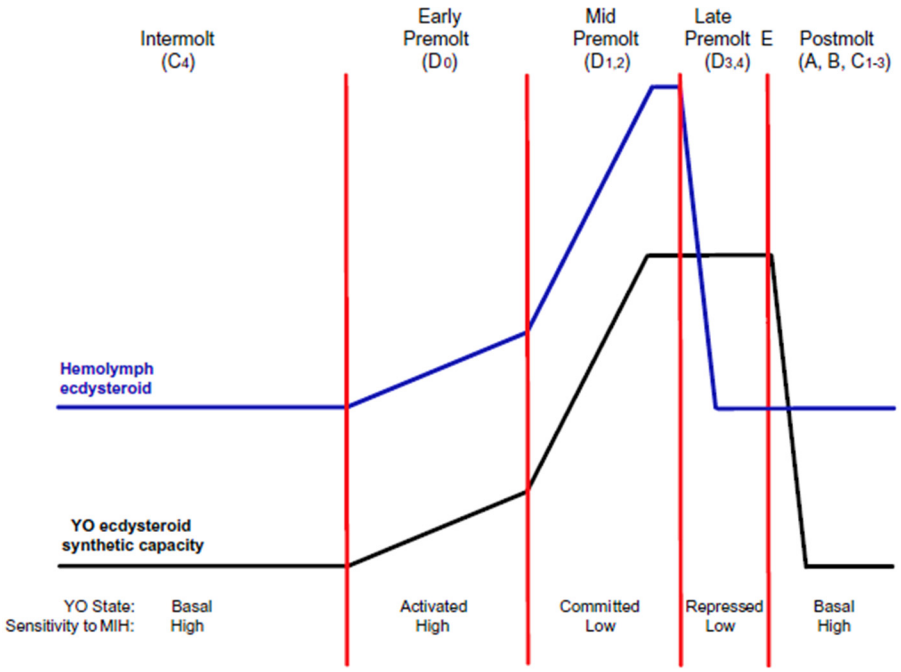


Figure 1-2. Hormonal regulation of molting in the blackback land crab, *G. lateralis*. Diagram shows the relationship between molt stage, YO state, YO sensitivity to MIH, YO ecdysteroid synthetic capacity, and hemolymph ecdysteroid titer. During postmolt (A, B, $C_{1–3}$), intermolt (C_4), early premolt (D_0), and mid premolt (D_{1-2}), hemolymph ecdysteroid titers are correlated with YO synthetic capacity; during late premolt (D_{3-4}), high ecdysteroid represses YO ecdysteroidgenesis and ecdysteroid titer falls. The YO transitions through four physiological states during the molt cycle: basal (B), activated (A), committed (C), and repressed (R). At the A to C transition, the animal becomes committed to molt, as the YO is less sensitive to MIH At the C to R transition, YO ecdysteroid synthetic capacity remains high, but high hemolymph ecdysteroid titer inhibits ecdysteroid secretion. Molting, or ecdysis (E), marks the R to B transition, during which the YO atrophies and becomes sensitive to MIH (Chang and Mykles, 2011).

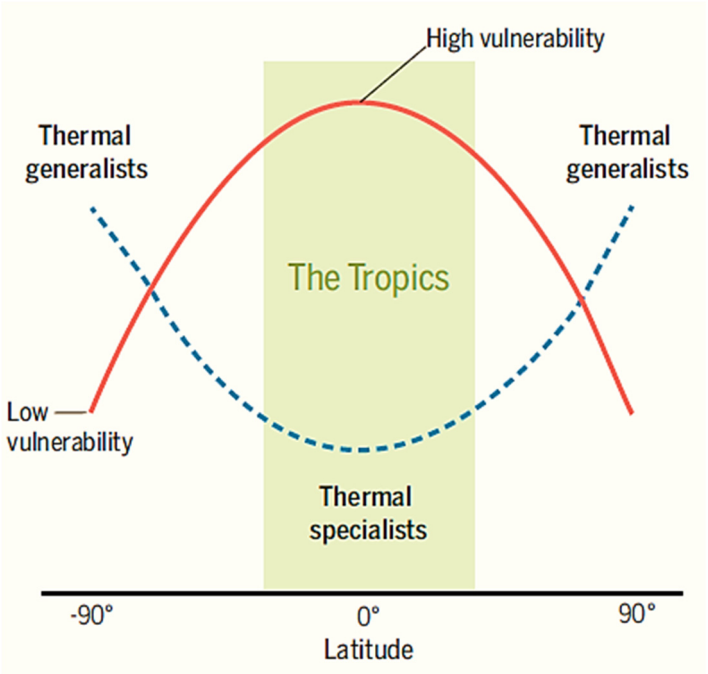


Figure 1-3. Tropical species, on average, have narrower climatic niches and greater sensitivity to climate change compared to their temperate counterparts. In general, tropical populations exhibit narrower climatic limits and greater sensitivity to climate change compared to temperate species (Janzen, 1967; Perez et al., 2016).

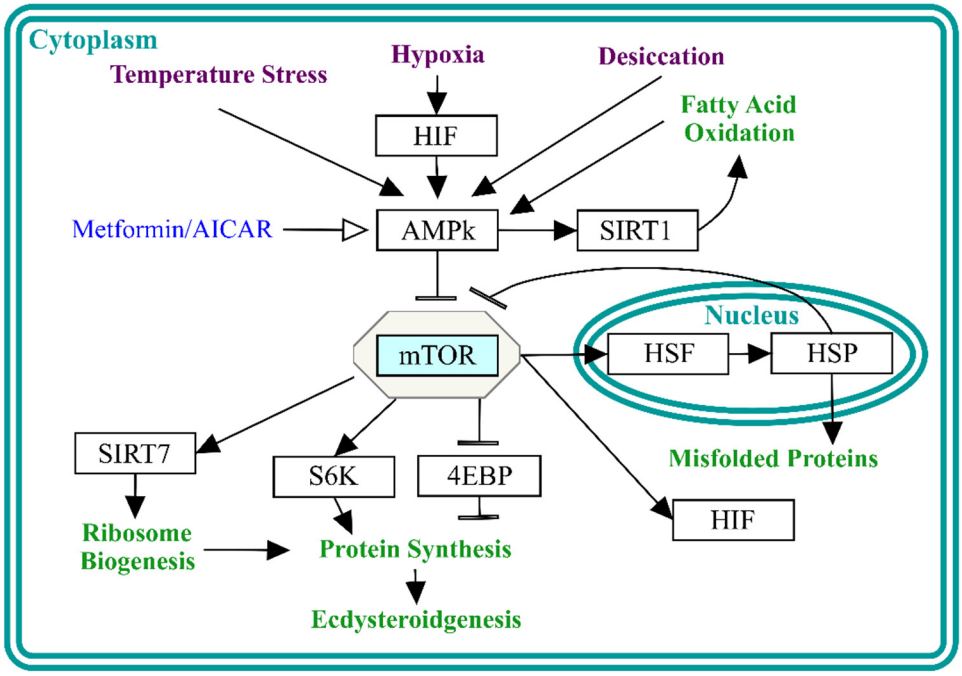


Figure 1-4. Model for stress response signaling in the crustacean YO. mTOR-dependent protein synthesis, which is regulated by environmental stresses via AMPk, is required for ecdysteroid biosynthesis. Downstream effectors are Silent Information Regulators (SIRTs), heat shock protein (HSPs), and hypoxia inducible factor (HIF). AICAR and Metformin activate AMPk.

REFERENCES

Alter, K., Paschke, K., Gebauer, P., Cumillaf, J.-P. and Pörtner, H.-O. (2015). Differential physiological responses to oxygen availability in early life stages of decapods developing in distinct environments. *Marine Biology* **162**, 1111-1124.

Bernasconi, C. J. and Uglow, R. F. (2008). Effects of emersion-induced hypoxia on some haemolymph constituents of *Nephrops norvegicus*. *Dis Aquat Organ* **82**, 135-43.

Bliss, D. E. (1968). Transition from water to land in decapod crustaceans. *American Zoologist* **8**, 355-392.

Bliss, D. E. (1979). From sea to tree - saga of a land crab. *American Zoologist* **19**, 385-410.

Bliss, D. E. and Boyer, J. R. (1964). Environmental regulation of growth in the decapod crustacean *Gecarcinus lateralis*. *Gen Comp Endocrinol* **4**, 15-41.

Bosch-Presegue, L. and Vaquero, A. (2014). Sirtuins in stress response: guardians of the genome. *Oncogene* **33**, 3764-75.

Brown-Peterson, N. J., Larkin, P., Denslow, N., King, C., Manning, S. and Brouwer, M. (2005). Molecular indicators of hypoxia in the blue crab *Callinectes sapidus*. *Marine Ecology Progress Series* **286**, 203-215.

Burggren, W. W. and McMahon, B. R. (1988). Biology of the land crabs. New York, US: Cambridge University Press.

Cameron, J. N. (1975). Aerial gas exchange in the terrestrial Brachyura *Gecarcinus lateralis* and *Cardisoma guanhumi*. *Comp Biochem Physiol A Comp Physiol* **52**, 129-34.

Capistran-Barradas, A., Defeo, O. and Moreno-Casasola, P. (2003). Density and

population structure of the red land crab *Gecarcinus lateralis* in a tropical semi-deciduous forest in Veracruz, Mexico. *Interciencia* **28**, 323-+.

Carafa, V., Nebbioso, A. and Altucci, L. (2012). Sirtuins and disease: the road ahead. *Front Pharmacol* **3**, 4.

Carls, M. G. and Oclair, C. E. (1995). Responses of tanner crabs, *Chionoecetes-Bairdi*, exposed to cold-air. *Fishery Bulletin* **93**, 44-56.

Carroll, V. A. and Ashcroft, M. (2005). Targeting the molecular basis for tumour hypoxia. *Expert Rev Mol Med* **7**, 1-16.

Cetrullo, S., D'Adamo, S., Tantini, B., Borzi, R. M. and Flamigni, F. (2015). mTOR, AMPK, and Sirt1: key players in metabolic stress management. *Crit Rev Eukaryot Gene Expr* **25**, 59-75.

Chan, S. M., Gu, P. L., Chu, K. H. and Tobe, S. S. (2003). Crustacean neuropeptide genes of the CHH/MIH/GIH family: implications from molecular studies. *Gen Comp Endocrinol* **134**, 214-9.

Chang, E. S. (2005). Stressed-out lobsters: crustacean hyperglycemic hormone and stress proteins. *Integr Comp Biol* **45**, 43-50.

Chang, E. S. and Mykles, D. L. (2011). Regulation of crustacean molting: a review and our perspectives. *Gen Comp Endocrinol* **172**, 323-30.

Chang, E. S., Prestwich, G. D. and Bruce, M. J. (1990). Amino acid sequence of a peptide with both molt-inhibiting and hyperglycemic activities in the lobster, *Homarus americanus*. *Biochemical and Biophysical Research Communications* **171**, 818-826.

Christie, A. E., Stemmler, E. A. and Dickinson, P. S. (2010). Crustacean neuropeptides. *Cell Mol Life Sci* **67**, 4135-69.

Chung, J. S. and Webster, S. G. (2003). Moult cycle-related changes in biological activity of moult-inhibiting hormone (MIH) and crustacean hyperglycaemic hormone (CHH) in the crab, *Carcinus maenas*. From target to transcript. *Eur J Biochem* **270**, 3280-8.

Chung, J. S. and Zmora, N. (2008). Functional studies of crustacean hyperglycemic hormones (CHHs) of the blue crab, *Callinectes sapidus* - the expression and release of CHH in eyestalk and pericardial organ in response to environmental stress. *FEBS J* **275**, 693-704.

Chung, J. S., Zmora, N., Katayama, H. and Tsutsui, N. (2010). Crustacean hyperglycemic hormone (CHH) neuropeptidesfamily: Functions, titer, and binding to target tissues. *Gen Comp Endocrinol* **166**, 447-54.

Costantini, S., Sharma, A., Raucci, R., Costantini, M., Autiero, I. and Colonna, G. (2013). Genealogy of an ancient protein family: the Sirtuins, a family of disordered members. *BMC Evol Biol* **13**, 60.

Cuculescu, M., Hyde, D. and Bowler, K. (1998). Thermal tolerance of two species of marine crab, *Cancer pagurus* and *Carcinus maenas*. *Journal of Thermal Biology* **23**, 107-110.

D'Incao, F., Ruffino, M. L., da Silva, K. G. and da Costa Braga, A. (1992). Responses of *Chasmagnathus granulata* Dana (Decapoda: Grapsidae) to salt-marsh environmental variations. *Journal of Experimental Marine Biology and Ecology* **161**, 179-188.

Das, S. and Mykles, D. L. (2016). A comparison of resources for the annotation of a de novo assembled transcriptome in the molting gland (Y-organ) of the blackback land crab, *Gecarcinus lateralis*. *Integr Comp Biol* **56**, 1103-1112.

de la Vega, E., Hall, M. R., Wilson, K. J., Reverter, A., Woods, R. G. and Degnan, B. M. (2007). Stress-induced gene expression profiling in the black tiger shrimp *Penaeus monodon*. *Physiol Genomics* **31**, 126-38.

de Lima, T. M., Geihs, M. A., Nery, L. E. and Maciel, F. E. (2015). Air exposure behavior of the semiterrestrial crab *Neohelice granulata* allows tolerance to severe hypoxia but not prevent oxidative damage due to hypoxia-reoxygenation cycle. *Physiol Behav* **151**, 97-101.

De Maio, A. (1999). Heat shock proteins: facts, thoughts, and dreams. *Shock* **11**, 1-12.

Defur, P. L., McMahon, B. R. and Booth, C. E. (1983). Analysis of hemolymph oxygen levels and acid-base status during emersion 'in situ' in the red rock crab, *Cancer productus*. *Biol Bull* **165**, 582-590.

Devaraj, H. and Natarajan, A. (2006). Molecular mechanisms regulating molting in a crustacean. *FEBS J* **273**, 839-46.

Dioum, E. M., Chen, R., Alexander, M. S., Zhang, Q., Hogg, R. T., Gerard, R. D. and Garcia, J. A. (2009). Regulation of hypoxia-inducible factor 2alpha signaling by the stress-responsive deacetylase sirtuin 1. *Science* **324**, 1289-93.

Factor, J. R. (1995). Biology of the lobster *Homarus americanus*, vol. 1, pp. 528: Academic Press.

Faubert, B., Vincent, E. E., Poffenberger, M. C. and Jones, R. G. (2015). The AMP-activated protein kinase (AMPK) and cancer: many faces of a metabolic regulator. *Cancer Lett* **356**, 165-70.

Frederich, M., O'Rourke, M. R., Furey, N. B. and Jost, J. A. (2009). AMP-activated protein kinase (AMPK) in the rock crab, *Cancer irroratus*: an early indicator of temperature stress. *J Exp Biol* **212**, 722-30.

Frederich, M., O'Rourke, M. and Towle, D. (2006). Is AMP activated protein kinase expression in *Cancer irroratus* a better signal for temperature stress then HSP70? *The MDIBL Bulletin* **45**, 37-39.

Frye, R. A. (2000). Phylogenetic classification of prokaryotic and eukaryotic Sir2-like proteins. *Biochem Biophys Res Commun* **273**, 793-8.

Gasser, S. M. and Cockell, M. M. (2001). The molecular biology of the SIR proteins. *Gene* **279**, 1-16.

Geihs, M. A., Maciel, F. E., Vargas, M. A., Cruz, B. P. and Nery, L. E. M. (2013). Effects of hypoxia and reoxygenation on the energetic metabolism of the crab *Neohelice granulata* (Decapoda, Varunidae). *Journal of Experimental Marine Biology and Ecology* **445**, 69-78.

Gray, I. E. (1957). A comparative study of the gill area of crabs. *The Biological Bulletin* **112**, 34-42.

Grosholz, E. D. and Ruiz, G. M. (1996). Predicting the impact of introduced marine species: lessons from the multiple invasions of the European green crab *Carcinus maenas*. *Biological Conservation* **78**, 59-66.

Gunderson, A. R. and Stillman, J. H. (2015). Plasticity in thermal tolerance has limited potential to buffer ectotherms from global warming. *Proc Biol Sci* **282**, 20150401.

Han, G. D., Zhang, S., Marshall, D. J., Ke, C. H. and Dong, Y. W. (2013). Metabolic energy sensors (AMPK and SIRT1), protein carbonylation and cardiac failure as biomarkers of thermal stress in an intertidal limpet: linking energetic allocation with environmental temperature during aerial emersion. *J Exp Biol* **216**, 3273-82.

Hardy, K. M., Follett, C. R., Burnett, L. E. and Lema, S. C. (2012). Gene transcripts encoding hypoxia-inducible factor (HIF) exhibit tissue- and muscle fiber type-dependent responses to hypoxia and hypercapnic hypoxia in the Atlantic blue crab, *Callinectes sapidus*. *Comp Biochem Physiol A Mol Integr Physiol* **163**, 137-46.

Harms, L., Frickenhaus, S., Schiffer, M., Mark, F. C., Storch, D., Held, C., Portner, H. O. and Lucassen, M. (2014). Gene expression profiling in gills of the great spider crab *Hyas araneus* in response to ocean acidification and warming. *BMC Genomics* **15**, 789.

Haukenes, A. H., Buck, C. L. and El Mejjati, S. Y. (2009). Effects of emersion temperature on the oxygen consumption rates of male tanner crabs, *Chionoecetes Bairdi*. *Journal of Crustacean Biology* **29**, 91-95.

Hill, R., Wyse, G. and Anderson, M. (2012). Animal Physiology. Sunderland, Massachusetts: Sinauer Associates.

Hines, D. J., Clark, K. F. and Greenwood, S. J. (2013). Global gene expression profiling of *Homarus americanus* (Crustacea) larval stages during development and metamorphosis. *Invertebrate Reproduction & Development* **58**, 97-107.

Hopkins, P. M. (2001). Limb regeneration in the fiddler crab, *Uca pugilator*: hormonal and growth factor control. *American Zoologist* **41**, 389-398.

Hopkins, P. M. (2012). The eyes have it: a brief history of crustacean neuroendocrinology. *Gen Comp Endocrinol* **175**, 357-66.

Hsu, Y. W., Messinger, D. I., Chung, J. S., Webster, S. G., de la Iglesia, H. O. and Christie, A. E. (2006). Members of the crustacean hyperglycemic hormone (CHH) peptide family are differentially distributed both between and within the neuroendocrine organs of *Cancer crabs*: implications for differential release and pleiotropic function. *J Exp Biol* **209**, 3241-56.

Huang, H., Fu, C., Chen, X., Gong, J., Huang, X. and Ye, H. (2015). Molt-inhibiting hormone (MIH) gene from the green mud crab *Scylla paramamosain* and its expression during the molting and ovarian cycle. *Aquaculture Research* **46**, 2665-2675.

Huang, X., Ye, H., Huang, H., Yang, Y. and Gong, J. (2014). An insulin-like androgenic gland hormone gene in the mud crab, *Scylla paramamosain*, extensively expressed and involved in the processes of growth and female reproduction. *Gen Comp Endocrinol* **204**, 229-38.

Huberman, A., Aguilar, M. B. and Quackenbush, L. S. (1995). A neuropeptide family from the sinus gland of the Mexican crayfish, *Procambarus bouvieri* (Ortmann). *Aquaculture* **135**, 149-160.

Inoki, K., Kim, J. and Guan, K. L. (2012). AMPK and mTOR in cellular energy homeostasis and drug targets. *Annu Rev Pharmacol Toxicol* **52**, 381-400.

Janzen, D. H. (1967). Why mountain passes are higher in the tropics. *The American Naturalist* **101**, 233-249.

Jensen, G. C. and Armstrong, D. A. (1991). Intertidal zonation among congeners: factors regulating distribution of porcelain crabs *Petrolisthes spp.* (Anomura: Porcellanidae). *Marine Ecology Progress Series* **73**, 47-60.

Jeon, J. M., Kim, B. K., Lee, J. H., Kim, H. J., Kang, C. K., Mykles, D. L. and Kim, H. W. (2012). Two type I crustacean hyperglycemic hormone (CHH) genes in morotoge shrimp (*Pandalopsis japonica*): cloning and expression of eyestalk and pericardial organ isoforms produced by alternative splicing and a novel type I CHH with predicted structure shared with type II CHH peptides. *Comp Biochem Physiol B Biochem Mol Biol* **162**, 88-99.

Johnson, I. and Uglow, R. F. (1985). Some effects of aerial exposure on the respiratory physiology and blood chemistry of *Carcinus maenas* (L.) and *Liocarcinus puber* (L.). *Journal of Experimental Marine Biology and Ecology* **94**, 151-165.

Jost, J. A., Podolski, S. M. and Frederich, M. (2012). Enhancing thermal tolerance by

eliminating the pejus range: a comparative study with three decapod crustaceans. *Marine Ecology Progress Series* **444**, 263-274.

Ke, Q. and Costa, M. (2006). Hypoxia-inducible factor-1 (HIF-1). *Mol Pharmacol* **70**, 1469-80.

Kim, J., Yang, G., Kim, Y., Kim, J. and Ha, J. (2016). AMPK activators: mechanisms of action and physiological activities. *Exp Mol Med* **48**, e224.

Klassen, G. and Locke, A. (2007). A Biological Synopsis of the European Green Crab, *Carcinus maenas*. *Canadian Manuscript Report of Fisheries and Aquatic Sciences 2818*, 75.

Lee, K. J., Kim, H. W., Gomez, A. M., Chang, E. S., Covi, J. A. and Mykles, D. L. (2007). Molt-inhibiting hormone from the tropical land crab, *Gecarcinus lateralis*: cloning, tissue expression, and expression of biologically active recombinant peptide in yeast. *Gen Comp Endocrinol* **150**, 505-13.

Lee, S. G. and Mykles, D. L. (2006). Proteomics and signal transduction in the crustacean molting gland. *Integr Comp Biol* **46**, 965-77.

Li, S., Li, F., Wang, B., Xie, Y., Wen, R. and Xiang, J. (2010). Cloning and expression profiles of two isoforms of a CHH-like gene specifically expressed in male Chinese shrimp, *Fenneropenaeus chinensis*. *Gen Comp Endocrinol* **167**, 308-16.

Li, T. and Brouwer, M. (2007). Hypoxia-inducible factor, gsHIF, of the grass shrimp *Palaemonetes pugio*: molecular characterization and response to hypoxia. *Comp Biochem Physiol B Biochem Mol Biol* **147**, 11-9.

Lindquist, S. and Craig, E. A. (1988). The heat-shock proteins. *Annu Rev Genet* **22**, 631-77.

Lu, Y., Zhang, D., Wang, F. and Dong, S. (2016). Hypothermal effects on survival,

energy homeostasis and expression of energy-related genes of swimming crabs *Portunus trituberculatus* during air exposure. *J Therm Biol* **60**, 33-40.

Luo, X., Chen, T., Zhong, M., Jiang, X., Zhang, L., Ren, C. and Hu, C. (2015). Differential regulation of hepatopancreatic vitellogenin (VTG) gene expression by two putative molt-inhibiting hormones (MIH1/2) in Pacific white shrimp (*Litopenaeus vannamei*). *Peptides* **68**, 58-63.

Madeira, D., Narciso, L., Cabral, H. N., Diniz, M. S. and Vinagre, C. (2012). Thermal tolerance of the crab *Pachygrapsus marmoratus*: intraspecific differences at a physiological (CTMax) and molecular level (Hsp70). *Cell Stress Chaperones* **17**, 707-16.

Mann, K. H. and Lazier, J. R. N. (2006). Dynamics of marine ecosystems: biological-physical interactions in the oceans. Malden, MA, USA/Oxford, UK/Victoria, Australia: Wiley-Blackwell.

Mattson, M. P. and Spaziani, E. (1986). Evidence for ecdysteroid feedback on release of molt-inhibiting hormone from crab eyestalk ganglia. *The Biological Bulletin* **171**, 264-273.

McLaughlin, P. A. (1980). Comparative morphology of recent crustacea: W. H. Freeman.

McMahon, B. R. and Wilkes, P. R. H. (1983). Emergence responses and aerial ventilation in normoxic and hypoxic crayfish *Orconectes rusticus*. *Physiological Zoology* **56**, 133-141.

Mykles, D. L. (1980). Mechanism of Fluid Absorption at Ecdysis in the American Lobster, Homarus-Americanus. *Journal of Experimental Biology* **84**, 89-&.

Mykles, D. L. (2001). Interactions between limb regeneration and molting in decapod crustaceans. *American Zoologist* **41**, 399-406.

Mykles, D. L. (2011). Ecdysteroid metabolism in crustaceans. *J Steroid Biochem Mol Biol* **127**, 196-203.

Perez, T. M., Stroud, J. T. and Feeley, K. J. (2016). Thermal trouble in the tropics. *Science* **351**, 1392-3.

Pinz, I., Perry, D. and Frederich, M. (2005). Activation of 5'-AMP activated protein kinase during anaerobiosis in the rock crab, *Cancer irroratus*. *The MDIBL Bulletin* **44**, 31-32.

Quackenbush, L. S. (1986). Crustacean endocrine toxicology: a review. *Canadian Journal of Fisheries and Aquatic Sciences* **43**, 2271-2282.

Quijón, P., Jaramillo, E. and Contreras, H. (2001). Distribution and habitat structure of *Ocypode gaudichaudii* H. Milne Edwards & Lucas, 1843, in sandy beaches of Northern Chile. *Crustaceana* **74**, 91-103.

Rastrick, S. P., Calosi, P., Calder-Potts, R., Foggo, A., Nightingale, G., Widdicombe, S. and Spicer, J. I. (2014). Living in warmer, more acidic oceans retards physiological recovery from tidal emersion in the velvet swimming crab, *Necora puber*. *J Exp Biol* **217**, 2499-508.

Ravaux, J., Gaill, F., Le Bris, N., Sarradin, P. M., Jollivet, D. and Shillito, B. (2003). Heat-shock response and temperature resistance in the deep-sea vent shrimp *Rimicaris exoculata*. *J Exp Biol* **206**, 2345-54.

Reid, D. G. and Aldrich, J. C. (1989). Variations in response to environmental hypoxia of different colour forms of the shore crab, *Carcinus maenas*. *Comparative Biochemistry and Physiology Part A: Physiology* **92**, 535-539.

Robert, A., Monsinjon, T., Delbecque, J. P., Olivier, S., Poret, A., Foll, F. L., Durand, F. and Knigge, T. (2016). Neuroendocrine disruption in the shore crab *Carcinus maenas*: Effects of serotonin and fluoxetine on chh and mih-gene expression, glycaemia and

ecdysteroid levels. *Aquat Toxicol* **175**, 192-204.

Sanli, T., Steinberg, G. R., Singh, G. and Tsakiridis, T. (2014). AMP-activated protein kinase (AMPK) beyond metabolism: a novel genomic stress sensor participating in the DNA damage response pathway. *Cancer Biol Ther* **15**, 156-69.

Schlesinger, M. J. (1990). Heat shock proteins. *J Biol Chem* **265**, 12111-4.

Schumpert, C. A., Anderson, C., Dudycha, J. L. and Patel, R. C. (2016). Involvement of *Daphnia pulicaria* Sir2 in regulating stress response and lifespan. *Aging (Albany NY)* **8**, 402-17.

Sharp, F. R. and Bernaudin, M. (2004). HIF1 and oxygen sensing in the brain. *Nat Rev Neurosci* **5**, 437-48.

Skinner, D. M. (1962). The structure and metabolism of a crustacean integumentary tissue during a molt cycle. *The Biological Bulletin* **123**, 635-647.

Skinner, D. M. (1965). Amino acid incorporation into protein during the molt cycle of the land crab, *Gecarcinus lteralis*. *J Exp Zool* **160**, 225-33.

Sorte, C. J., Ibanez, I., Blumenthal, D. M., Molinari, N. A., Miller, L. P., Grosholz, E. D., Diez, J. M., D'Antonio, C. M., Olden, J. D., Jones, S. J. et al. (2013). Poised to prosper? A cross-system comparison of climate change effects on native and non-native species performance. *Ecol Lett* **16**, 261-70.

Soumoff, C. and O'Connor, J. D. (1982). Repression of Y-organ secretory activity by molt inhibiting hormone in the crab *Pachygrapsus crassipes*. *Gen Comp Endocrinol* **48**, 432-9.

Spees, J. L., Chang, S. A., Snyder, M. J. and Chang, E. S. (2002). Thermal acclimation and stress in the American lobster, *Homarus americanus*: equivalent temperature shifts elicit unique gene expression patterns for molecular chaperones and polyubiquitin. *Cell*

Stress Chaperones **7**, 97-106.

Stillman, J. and Somero, G. (1996). Adaptation to temperature stress and aerial exposure in congeneric species of intertidal porcelain crabs (genus *Petrolisthes*): correlation of physiology, biochemistry and morphology with vertical distribution. *J Exp Biol* **199**, 1845-55.

Sun, S., Xuan, F., Fu, H., Zhu, J., Ge, X. and Gu, Z. (2015). Transciptomic and histological analysis of hepatopancreas, muscle and gill tissues of oriental river prawn (*Macrobrachium nipponense*) in response to chronic hypoxia. *BMC Genomics* **16**, 491.

Taylor, A. C. and Spicer, J. I. (1987). Metabolic responses of the prawns *Palaemon elegans* and *P. serratus* (Crustacea: Decapoda) to acute hypoxia and anoxia. *Marine Biology* **95**, 521-530.

Taylor, E. W., Butler, P. J. and Sherlock, P. J. (1973). The respiratory and cardiovascular changes associated with the emersion response of *Carcinus maenas* (L.) during environmental hypoxia, at three different temperatures. *Journal of Comparative Physiology* **86**, 95-115.

Techa, S. and Chung, J. S. (2015). Ecdysteroids regulate the levels of molt-inhibiting hormone (MIH) expression in the blue crab, *Callinectes sapidus*. *PLoS One* **10**, e0117278.

Tepolt, C. K. and Somero, G. N. (2014). Master of all trades: thermal acclimation and adaptation of cardiac function in a broadly distributed marine invasive species, the European green crab, *Carcinus maenas*. *J Exp Biol* **217**, 1129-38.

Tomanek, L. and Zuzow, M. J. (2010). The proteomic response of the mussel congeners *Mytilus galloprovincialis* and *M. trossulus* to acute heat stress: implications for thermal tolerance limits and metabolic costs of thermal stress. *J Exp Biol* **213**, 3559-74.

Ventura-Lopez, C., Gomez-Anduro, G., Arcos, F. G., Llera-Herrera, R., Racotta, I.

S. and Ibarra, A. M. (2016). A novel CHH gene from the Pacific white shrimp *Litopenaeus vannamei* was characterized and found highly expressed in gut and less in eyestalk and other extra-eyestalk tissues. *Gene* **582**, 148-60.

Vermeer, G. K. (1987). Effects of air exposure on desiccation rate, hemolymph chemistry, and escape behavior of the spiny lobster, *Panulirus-Argus*. *Fishery Bulletin* **85**, 45-51.

Vernberg, F. J. and Tashian, R. E. (1959). Studies on the physiological variation between tropical and temperate zone fiddler crabs of the genus *Uca* I. Thermal death limits. *Ecology* **40**, 589-593.

Vinagre, A. S. and Chung, J. S. (2016). Effects of starvation on energy metabolism and crustacean hyperglycemic hormone (CHH) of the Atlantic ghost crab *Ocypode quadrata* (Fabricius, 1787). *Marine Biology* **163**.

Webster, S. (1996). Measurement of crustacean hyperglycaemic hormone levels in the edible crab *Cancer pagurus* during emersion stress. *J Exp Biol* **199**, 1579-85.

Webster, S. G. (1997). High-affinity binding of putative moult-inhibiting hormone (MIH) and crustacean hyperglycaemic hormone (CHH) to membrane-bound receptors on the Y-organ of the shore crab *Carcinus maenus*. *Proceedings of the Royal Society of London. Series B: Biological Sciences* **251**, 53-59.

Webster, S. G., Keller, R. and Dircksen, H. (2012). The CHH-superfamily of multifunctional peptide hormones controlling crustacean metabolism, osmoregulation, moulting, and reproduction. *Gen Comp Endocrinol* **175**, 217-33.

Westerheide, S. D., Anckar, J., Stevens, S. M., Jr., Sistonen, L. and Morimoto, R. I. (2009). Stress-inducible regulation of heat shock factor 1 by the deacetylase SIRT1. *Science* **323**, 1063-6.

Wilkens, J. L. and Milton, F. (1965). Heat tolerance and temperature relationships of the fiddler crab, *Uca pugilator*, with reference to body coloration. *Biological Bulletin* **128**, 133-141.

Xie, X., Zhu, D., Yang, J., Qiu, X., Cui, X. and Tang, J. (2014). Molecular cloning of two structure variants of crustacean hyperglycemic hormone (CHH) from the swimming crab (*Portunus trituberculatus*), and their gene expression during molting and ovarian development. *Zoolog Sci* **31**, 802-9.

Yoon, H., Shin, S. H., Shin, D. H., Chun, Y. S. and Park, J. W. (2014). Differential roles of Sirt1 in HIF-1alpha and HIF-2alpha mediated hypoxic responses. *Biochem Biophys Res Commun* **444**, 36-43.

Zagorska, A. and Dulak, J. (2004). HIF-1: the knowns and unknowns of hypoxia sensing. *Acta Biochim Pol* **51**, 563-85.

Zheng, J., Chen, H. Y., Choi, C. Y., Roer, R. D. and Watson, R. D. (2010). Molecular cloning of a putative crustacean hyperglycemic hormone (CHH) isoform from extra-eyestalk tissue of the blue crab (*Callinectes sapidus*), and determination of temporal and spatial patterns of CHH gene expression. *Gen Comp Endocrinol* **169**, 174-81.

CHAPTER TWO: *IN SILICO* AND *IN VIVO* CHARACTERIZATION OF GENES OF STRESS RESPONSE IN THE BLACKBACK LAND CRAB, *GECARCINUS LATERALIS* AND THE GREEN SHORE CRAB, *CARCINUS MAENAS*

SUMMARY

Stress response in crustaceans can be induced by changes in environmental temperature, oxygen and energy stability. Physiological and behavioral adaptations are implemented by decapods to cope with environmental and internal alterations. Adaptive molecular mechanisms that involve changes in gene expression patterns are also directed to maintain homeostasis under stress. The purpose of this study was to identify and characterize the genes of stress response in the blackback land crab, *Gecarcinus lateralis*, and the green shore crab *Carcinus maenas* using an *in silico* and *in vivo* approach. Orthologs of *AMPkα*-, *β*-, and *γ*-subunits; *SIRT1*; *SIRT7*; *HSP60*; *HSP70*; and *HIFα*- and *β*-subunits, in intermolt, early premolt, mid premolt, late premolt, and postmolt stages of the molt cycle of *G. lateralis* were isolated, identified and characterized. Stress responsive genes in *G. lateralis* and *C. maenas* shared structural properties among arthropod species and exhibited unique profiles of expression in Y-organs, claw muscle, gill, heart, and thoracic ganglion. Expression of genes was mostly higher in Y-organ and heart and lower in claw muscles in both species, with non-specific tissue distribution. Collectively, these data expand the knowledge of genes of stress response in a tropical species and a domestic temperate invader and provide a database for experiments directed to understand the role of stress in crustacean decapods.

INTRODUCTION

Previous studies have shown that stress response has an impact in the physiology of crustacean species at the molecular level and on the cellular energy allocation (Sun et al., 2015; Zheng et al., 2019). Growth and metabolism in crustaceans are controlled by the metabolic function of the mammalian **m**echanistic **T**arget **O**f **R**apamycin (mTOR) (Cetrullo et al., 2015). Its activity regulates synthesis of proteins necessary for cell growth, cell division, and response to cellular stress (Saxton and Sabatini, 2017). Nevertheless, mTOR activation relies on both availability of energy and the control from other signaling pathways, as well as cellular stress conditions such as DNA damage, heat stress, and oxidative stress for its activation and function (Kaur and Sharma, 2017). The blackback land crab *Gecarcinus lateral*is is a decapod that inhabits the dry zone of sandy beaches and low hills in Bahamas, Florida Keys, Texas, Yucatan, Colombia and Venezuela (Bliss et al., 1978; von Prahl and Manjarrés, 1984). As a permanent terrestrial crustacean, they must return to breed and moisten their gills in the ocean, although its blood has a higher oxygen carrying capacity (Taylor and Davies, 1982). Inhabiting widely sheltered habitats and with high humidity of coastal areas, *G. lateral*is has developed adaptations to ensure moderate temperatures and constant moisture (Taylor and Davies, 1981). The green crab *Carcinus maenas* is an intertidal decapod in estuarine habitats and semi-exposed rocky coasts, native from Europe and Africa, and highly adaptable to survive air exposure and extreme low and high temperatures (Chaves et al., 2010; Roman and Palumbi, 2004). *C. maenas* has invaded the Atlantic and Pacific coasts of North America, South Africa, Australia, South America, and Asia (Behrens Yamada et al., 2015; Burden et al., 2014; Cosham et al., 2016; Tepolt and Somero, 2014; Vinuesa, 2007). With the adaptations to two adverse environments and their capability to cope with extreme temperatures, desiccation, and possibly temporal

hypoxia, *G. lateralis* and *C. maenas* represent excellent models for the study of stress response and its effect on molting and growth (Abuhagr et al., 2016; Chang and Mykles, 2011; Chung and Webster, 2003, 2005; Das et al., 2016; Oliphant et al., 2018). Adenosine Monophosphate-activated Protein kinase (AMPk) is a heterotrimer regulator of energy composed of an α-catalytic subunit and two non-catalytic β- and γ-subunits (Stapleton et al., 1997). By shifting to generation of ATP on metabolism and by blocking energy consumption, AMPk protects cells under physiological stress, such as heat shock and possibly hypoxia (Han et al., 2013). A reduction in the ATP:AMP/ADP ratio will activate AMPk by binding two AMP molecules to four tandem cystathionine β-synthase domains (CBS) into the γ-subunit, and with the phosphorylation of Thr172 in the amino-terminal kinase domain of the α-subunit, increasing AMPk activity various hundred-fold (Cetrullo et al., 2015). Under metabolic stress and energy depletion, AMPk inhibits protein translation and therefore cellular growth by downregulating the expression of mTOR as a result of autophagy (Kaur and Sharma, 2017). Silent Information Regulator 2 protein (SIRT2) is a family of seven proteins included in four classes, and with a highly conserved catalytic domain and diverse N- and C- terminal residues (Carafa et al., 2012). Class I contains SIRT1, SIRT2 (nucleus and cytoplasm), and SIRT3 (mitochondria). Class II comprises SIRT4 and Class III comprises SIRT5 (both in mitochondria). Class IV includes SIRT6 and SIRT7 (nucleus and cytoplasm) (Frye, 2000). On availability of nicotinamide adenine dinucleotide (NAD^+), Sirtuins remove acetyl groups from lysine residues on target substrates (Gillum et al., 2011). Sirtuins are involved in regulation of energy in metabolism, stress resistance, and caloric restriction (Greiss and Gartner, 2009), which are functions of an energy sensor and metabolic regulator and closely related to AMPk activity. For this connection, Gillum et al. (2011) also describe that Sirtuins

promote survival and recovery of cells under stress, by activating pathways associated to specific transcription factors such as gluconeogenesis and fatty acid oxidation.

As a taxonomic group which are mostly aquatic, crustaceans cope with variations in environmental temperature and have acquired mechanisms of thermal response at physiological and molecular level (Zheng et al., 2018). In addition, crustaceans experiencing stress by environmental factors exhibit induced expression of molecular chaperones that promote folding of proteins and repair of those unfolded or denatured (Chang et al., 1999). Heat shock proteins (HSPs) act as molecular chaperones to prevent protein denaturation, repair and refold denatured or misfolded proteins (Yi et al., 2018). They can also operate in regular cellular processes such as protein transport, DNA replication, and protein synthesis, and are widely used as biochemical markers of exposure to environmental stresses, such as temperature change and pollution (Yang et al., 2016). Based on their molecular weight and homology, HSPs are classified into small HSPs (sHSPs, with molecular masses ranging from 12 to 40 kDa), HSP40, HSP60, HSP70, HSP90, and HSP100 (Zheng et al., 2018). As thermal stress in crustaceans, hypoxia plays a role on reproduction and survival in function of the spatially, daily, and seasonally variation of environmental oxygen (Li and Brouwer, 2007). Environmental hypoxia obstructs the optimal development of crustaceans through physiological stress, causing less frequency of molts, metabolic changes, slow growth and high mortality (Sun et al., 2015). In this way, aquatic crustaceans have developed physiological and biochemical adaptations to survive hypoxia, presenting a possibility to study genes of stress response from the evolutionary, functional and regulatory point of view and their role in the environmental acclimatization (Klumpen et al., 2017; Li and Brouwer, 2007). The transcription factor Hypoxia Inducible Factor (HIF) consists of two subunits (HIF-α and HIF-β) (Semenza, 2001b). Under normoxia conditions, the newly

synthesized HIF-α subunit will undergo degradation by ubiquitin-mediated proteasomal degradation (Semenza, 2001a). During hypoxia, absence of oxygen represses proteasomal degradation, accumulating and allowing HIF-α subunit to translocate, bind to the HIF-β and trigger the expression of HIF-controlled genes by binding to elements to responsive hypoxia (Semenza, 2001a). From this it is even possible to use species susceptible to hypoxic conditions compared to other crustaceans as models of response to hypoxia (Sun et al., 2015).

The purpose of this study was to isolate, identify, and characterize cDNAs encoding genes of stress response (AMPk, SIRT, HSP and HIF) in intermolt (IM), early premolt (EP), mid premolt (MP), late premolt (LP), and postmolt (PM) stages of the molt cycle of the blackback land crab, *Gecarcinus lateralis*, and the green shore crab *Carcinus maenas*. The hypothesis describes that these genes share structural properties among arthropod species and that each molt stage exhibits a distinct gene expression profile. Contigs encoding cDNAs and relative expression of nine genes were obtained from the *G. lateralis* YO *de novo* transcriptome (Das et al., 2018) and from two *C. maenas de novo* transcriptomes (Tepolt and Palumbi, 2015; Verbruggen et al., 2015). Profiles of tissue distribution of genes of stress response in *G. lateralis* and *C. maenas* were described as well.

MATERIALS AND METHODS

Isolation and characterization of sequences encoding genes of stress response in *G. lateralis* and *C. maenas*

Nine mRNA sequences encoding six proteins were obtained from the *Gecarcinus lateralis* Y-organ (YO) *de novo* transcriptome (Das et al., 2018), and from two *Carcinus maenas de novo* transcriptomes (Tepolt and Palumbi, 2015; Verbruggen et al., 2015). Sequences

encoding for *AMPkα*-, *β*-, and *γ*-subunits, *SIRT1*, *SIRT7*, *HSP60*, *HSP70*, and *HIFα*-, and *β*-subunits, were extracted using the stand-alone software prfectBLAST 2.0 (Santiago-Sotelo and Ramirez-Prado, 2012). To verify the correct identification of the genes, both nucleotide sequences and predicted protein sequences were blasted against NCBI databases and statistical significance of the matches was obtained. To confirm the isolation of full encoding contigs, open reading frames (ORF) of the nucleotide sequences were translated to six reading frames. Basic protein structure and conserved domains were obtained from the Conserved Domain Database (CDD) on National Center for Biotechnology Information (NCBI, http://ncbi.nlm.nih.gov) and InterPro on the European Bioinformatics Institute (EBI, https://www.ebi.ac.uk). ClustalX 2.0 (Larkin et al., 2007) and GeneDoc (Nicholas and Nicholas, 1997) were used to create multiple alignments with orthologs from other crustacean species on the NCBI database to reveal sequence identity. The identified nucleotide and deduced amino acid sequences from *G. lateralis* reported in this chapter, were deposited in the GenBank nucleotide sequence database.

RNA isolation and cDNA synthesis of genes of stress response in *G. lateralis* and *C. maenas*

YO, claw muscle (CM), gill (G), heart (H), and thoracic ganglion (TG) tissues from IM *G. lateralis* and *C. maenas* animals were harvested to assess the tissue distribution of genes of stress response. In 1 ml TRIzol (Life Technologies, Grand Island, NY), YOs were homogenized with a pestle mixer (VWR, Denver, CO) for 5 min. CM, G, H, and TG tissues were homogenized using a TissueLyser II (QIAGEN, Germantown, MD). Samples were run at 180 oscillations min^{-1} for 2 min and 1 ml TRIzol (Life Technologies, Grand Island, NY) was added to resuspend the tissue. Homogenized tissues were centrifuged at 16,000 g for 15 min to extract 1 ml of supernatant. Total RNA was treated with chloroform/isopropanol/ethanol (70%) and pellets were

resuspended in 20 µl of RNase/DNase free water. Samples were treated with 3 µl 10x DNase buffer, 0.75 µl Ribolock, 3.25 µl RNase/DNase free water and 3 µl DNase I (Life Technologies, Grand Island, NY) to remove genomic DNA. A second RNA extraction was done by using acidic phenol/chloroform isoamyl alcohol 24:1, and RNA was precipitated with isopropanol/sodium acetate (pH 5.2). Pellets were rehydrated in 22 µl RNase/DNase free water and RNA purity and concentration was determined by measuring absorbance at 260 and 280 nm using a Nanodrop ND1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE). Total RNA was reverse transcribed to cDNA using SuperScript IV Reverse Transcriptase (Thermo Fisher Scientific, Wilmington, DE), according to the manufacturer's instructions.

Tissue expression of genes of stress response in *G. lateralis* and *C. maenas*

Quantitative PCR (qPCR) was used to quantify the mRNA levels of genes of stress response in tissues from intermolt (IM) animals, early premolt (EP), mid premolt (MP), late premolt (LP), and postmolt (PM) stages. Eukaryote Elongation Factor (EF-2) was used as a positive control of cDNA quality. QPCR was developed using a LightCycler 480 thermal cycler (Roche Applied Science, Indianapolis, IN). QPCR reactions consisted of 0.5 µl each of 10 µmol l^{-1} forward and reverse primer (Table 2-2), 3 µl PCR-grade water, 5 µl SYBR Green I Master Mix, and 1 µl first strand cDNA. PCR conditions were set in an initial denaturation at 95 °C for 5 min, 45 cycles at 95 °C for 5 s, annealing at 60 °C for 5 s, and extension at 72 °C for 20 s. Absolute concentration of transcripts was calculated using a standard curve generated by serial dilutions ranging 10^{-9} to 10^{-18} ng μl^{-1} of purified cDNA product of each gene. Final qPCR transcript concentration was standardized to copy number per µg of total RNA used in the cDNA synthesis, and $\log_{10}$ transformed prior to analysis.

Relative expression of genes of stress response in the *G. lateralis* YO transcriptome

Relative expression of genes of stress response were obtained from the *de novo* YO transcriptome (Das et al., 2018) and was expressed in FPKM (Fragments per Kilobase Per Million Reads). Mean FPKM values were clustered with an average linkage using the Euclidean clustering distance and clusters were represented as a heatmap with the use of the R statistical environment.

Statistical analysis

Statistical analysis was performed on Statgraphics 18 (The Plains, VA, USA) and PAST (Hammer et al., 2001). Graphs were created on SigmaPlot 12 (San Jose, CA, USA). Gene expression of five molt cycle stages and tissue expression patterns in *G. lateralis* and *C. maenas* were analyzed using one-way ANOVA and Tukey-Kramer HSD *post-hoc* multiple comparisons test. Characterized sequences were presented as a consensus sequence of the *G. lateralis* and *C. maenas* isolated contigs. Data were plotted as mean ±1 S.E.M. and all statistical analysis were set at $\alpha>0.05$ level of significance.

RESULTS

Molecular characteristics of genes of stress response in *G. lateralis* and *C. maenas*

Nucleotide sequences of arthropods and crustacean species available on gene databases were used to isolate contigs encoding genes of stress response in *Gecarcinus lateralis* and *Carcinus maenas*. All contig sequences encoded the entire open reading frame (Table 2-1). Contigs encoding *Gl-AMPkα* (GenBank accession MK046865), *Gl-AMPkβ* (GenBank accession

MK046866), *Gl-AMPKγ* (GenBank accession MK059957), *Gl-SIRT1* (GenBank accession MK372514), *Gl-SIRT7* (GenBank accession MK372515), *Gl-HSP60* (GenBank accession MK372512), *Gl-HSP70* (GenBank accession MK372513), *Gl-HIFα* (GenBank accession MK372510), and *Gl-HIFβ* (GenBank accession MK372511) were obtained.

Sequence analysis revealed that *Gl-AMPkα*-, *β*-, and *γ*-subunits have a full-length coding sequence (CDS) of 1614, 882, and 1713 bp, encoding polypeptides with 537, 293, and 570 amino acid residues and a molecular weight of 60.59, 32.83, and 64.25 kDa, respectively. *Cm-AMPkα*-, *β*-, and *γ*-subunits have a full-length CDS of 1566, 879, and 1392 bp, encoding polypeptides with 522, 293, and 464 amino acid residues and a molecular weight of 58.86, 32.76, and 61.16 kDa, correspondingly. The mRNA and deduced amino acid sequences for *Gl-AMPk* and *Cm-AMPk* subunits are presented in Figure 2-1, Figure 2-3 and Figure 2-5. The *AMPkα* sequence encoded the catalytic domain, UBA-like autoinhibitory domain, and the C-terminal regulatory domain (Figure 2-1). QPCR primers targeted to the catalytic domain of *Gl-AMPkα* produced a 201-bp product and to the C-terminal regulatory of *Cm-AMPkα* domain produced a 219-bp product. The *AMPkβ* sequence encoded the N-terminal Early Set domain or glycogen binding domain, and an interaction domain (Figure 2-3). QPCR primers targeted to the N-terminal Early Set domain of both species produced a 204-bp product in *G. lateralis* and a 225-bp product in *C. maenas*. A product of 235-bp in *Gl-AMPkγ* and 247-bp in *Cm-AMPkγ* was amplified from the first of the two pairs of CBS domains encoded by the isolated sequences (Figure 2-5). The two CBS-pair superfamily domains were located at the 178-251 (CBS domain pair 1), 262-316 (CBS domain pair 2),333-398 (CBS domain pair 3),409-452 (CBS domain pair 4) positions.

Isolated CDS of *Gl-SIRT1* and *SIRT7* had 1230 and 1161 bp, encoding polypeptides with 409 and 386 amino acid residues and a theorical molecular weight of 45.46 and 43.40 kDa, respectively. *Cm-SIRT1* and *SIRT7* are sequences of 1230 and 1596 bp, encoding proteins with 410 and 532 amino acid residues and molecular weight of 45.54 and 59.39 kDa, appropriately. The mRNA and deduced amino acid sequences for *SIRT1* and *SIRT7* from both species are presented in Figure 2-7 and Figure 2-9. A NAD^+-dependent deacetylation domain, a region between the 550 and 800 nucleotides, was amplified to obtain products with 216-bp from *Gl-SIRT1* and 240-bp from *Cm-SIRT1* (Figure 2-7). Similarly, a region between 390 and 770 nucleotides associated to the NAD^+-dependent deacetylation domain, was amplified to obtain products with 215-bp from *Gl-SIRT7* and 249-bp from *Cm-SIRT7* (Figure 2-9).

Contigs of *Gl-HSP60* and *Gl-HSP70* have full-length CDS with 1737 and 1953 bp, encoding polypeptides with 578 and 650 amino acid residues and a molecular weight of 61.35 and 71.20 kDa, respectively. In *C. maenas*, *Cm-HSP60* and *Cm-HSP70* are sequences with 1734 and 1965 bp, encoding polypeptides with 578 and 655 amino acid residues and a molecular weight of 61.26 and 71.49 kDa, respectively. The mRNA and deduced amino acid sequences for *HSP60* and *HSP70* from both species are presented in Figure 2-11 and Figure 2-13. An amplified region of *HSP60* from both species corresponded to the GroEL-like type I chaperonin domain (eukaryotic homolog), with products of 204- bp in *G. lateralis* and 227- bp in *C. maenas* (Figure 2-11). In *HSP70*, products of 215-bp in *Gl-HSP70* and 160-bp in *Cm-HSP70* were obtained from the C-terminal end of the Nucleotide-Binding Domain (NBD, Figure 2-13).

Finally, full-length CDS for *Gl-HIFα* and *-β* have 3606 and 1983 bp, encoding polypeptides with 1201 and 660 amino acid residues and a molecular weight of 131.28 and 72.71 kDa, respectively. In *C. maenas*, *HIFα* and *-β* subunits are sequences of 3255 and 1986 bp,

encoding polypeptides with 1085 and 662 amino acid residues and a molecular weight of 107.91 and 72.48 kDa, respectively. The mRNA and deduced amino acid sequences for *HIFα* and *HIFβ* from both species are presented in Figure 2-15 and Figure 2-17. The *HIFα* sequence encoded the basic Helix-Loop-Helix domain, a pair of PAS domains, and the C-terminal transactivation domain. QPCR primers targeted to the one of the two PAS domains in *HIFα* of both species produced a 235-bp product in *G. lateralis* and a 233-bp product in *C. maenas*. The *HIFβ* sequence was formed by a basic Helix-Loop-Helix domain and a pair of PAS domains. The first PAS domain was amplified in both species and produced a 235-bp product in *G. lateralis* and a 224-bp product in *C. maenas*.

Multiple sequence alignments of crustacean orthologs of stress responsive genes in *G. lateralis* and *C. maenas*

Sequence alignments of stress responsive genes revealed high degree of homology to proteins from other crustacean and arthropod species, with most amino acids conserved especially in the conserved domains. Amino acid sequences of *AMPkα* subunits show significant similarity in the catalytic core region, an N-terminal Ser/Thr kinase domain followed by an ubiquitin-associated (UBA)-like autoinhibitory domain (AID) but differ in the C-terminal regulatory tails (Figure 2-2). *AMPkα* showed a 93.7% similarity with *Scylla paramamosain* (GenBank accession KP161206.1) and 91.7% with *Cancer irroratus* (GenBank accession FJ496868.1). *AMPkβ* subunit sequences revealed the E or "early" Set domain associated with the catalytic region at the N-terminal end, the interaction domain at the C-terminal region, and made possible to identify the glycogen-binding sites as well (Figure 2-4). Sequences of this subunit had a 74.0% similarity with *Litopenaeus vannamei* (GenBank accession KP272117.1) and 52.2%

with the beetle *Dastarcus helophoroides* (GenBank accession KX121427.1). Finally, aligned sequences of *AMPkγ* localized two tandem repeats of the CBS motifs or Bateman domains (Figure 2-6), and showed a 71.46% similarity with *L. vannamei* (GenBank accession KP272118.1) and 62.8% with the fruit fly *Drosophila melanogaster* (GenBank accession AF094764.1).

Two members of the family of proteins SIRT2 were also identified. A conserved portion of 257 amino acids corresponded to the core region of *SIRT1* with multiple NAD^+ and Zinc binding sites (Figure 2-8). Although it had a similarity of 81.6% with *Penaeus vannamei* (GenBank accession MG321242.1) and 64.7% with the moth *Helicoverpa armigera* (GenBank accession KY363351.1), about 10 to 30 amino acids at the terminal ends were not highly aligned to other species' sequences. *SIRT7* had a well conserved N-terminal region and a core region of 239 amino acids, with high variation in about 180 residues at the C-terminal end (Figure 2-10). This may explain its lower conservative similarity of 76.5% with *P. vannamei* (GenBank accession MG491309.1) and 54.5% with the parasitic copepod *Lepeophtheirus salmonis* (GenBank accession BT077928.1).

Orthologs of the HSP family included HSP60 and HSP70 contigs. Aligned HSP60 sequences revealed a remarkable high similarity determined by the well-conserved GroEL-like type I chaperonin domain (eukaryotic homolog) and several ATP/Mg^{2+}-binding sites (Figure 2-12). It also reveals a high similitude of 88.7% with Chinese mitten crab *Eriocheir sinensis* (GenBank accession KP642083.1) and 85.2% with *Portunus trituberculatus* (GenBank accession JN628037.1). HSP70 isolated contigs had a ~93% of similarity with *E. sinensis* (GenBank accession EU857483.1) and *Pachygrapsus marmoratus* (GenBank accession DQ173922.1). Alignments showed a highly conserved N- terminal and middle region of the sequence, and

defined multiple nucleotide binding sites, BAG/HSP70 interaction sites, and NEF/HSP70 interaction sites (Figure 2-14).

Alignments of HIF sequences confirmed the complex structure of the α and β subunits. *HIFα* is a large protein that contains a typical helix-loop-helix at the N- terminal region but about ~160 amino acids in this portion barely matched with ~20 residues between *G. lateralis* and the other crustacean species (Figure 2-16). DNA binding sites, an E-box/N-box specificity site, and putative active sites were also identified and located inside of the conserved domains,. This subunit had a similarity of 85-90.0% with *E. sinensis* (GenBank accession KF825558.1) and *Cancer magister* (GenBank accession DQ535030.1). *HIFβ* alignments among arthropod species showed an E-box/N-box specificity site, putative active sites, and more significantly, a dimerization interface in addition to a helix-loop-helix and two PAS domains (Figure 2-18). *HIFβ* isolated sequences had a ~85% similarity with *P. trituberculatus* (GenBank accession KU681174.1) and *Macrobrachium nipponense* (GenBank accession KP050353.1).

Transcriptional analysis of stress responsive genes in *G. lateralis* and *C. maenas*

Ecdysteroid titers in hemolymph depicted a typical pattern of the *G. lateralis* molt cycle: low titers at IM, increasing values from EP to LP, and the lowest levels at PM stage (Figure 2-19). The relative expression levels of stress responsive genes were obtained from the YO transcriptome and represented in a heatmap (Figure 2-19). Relative expression of *Gl-EF2*, *Gl-SIRT1*, *Gl-HSP70*, *Gl-HIFα* and *Gl-HIFβ* was mostly highest in IM and significantly downregulated at premolt and PM stages ($P < 0.05$). Other genes such as *Gl-AMPkα*, *Gl-AMPkβ*, *Gl-AMPKγ*, *Gl-SIRT7*, and *Gl-HSP60* were also downregulated from IM to PM with no significant changes among molt stages.

Tissue expression patterns of stress responsive genes in *G. lateralis* and *C. maenas*

All stress responsive genes were widely distributed in all examined tissues of *G. lateralis* and *C. maenas* (Figure 2-20) and their level of expression varied among genes and tissues.

In *G. lateralis*, the highest mRNA levels of all genes were detected in the heart, while the claw muscle had the lowest mRNA levels ($P < 0.05$, Table 2-3). *Gl-AMPK* subunits expression was highest in heart ($P < 0.05$), followed by gill and YO. For *Gl-SIRT1* and *Gl-SIRT7*, expression levels were ordered with heart ($P < 0.05$) > YO > gill > thoracic ganglion > claw muscle. *Gl-HSP70* mRNA expression was significantly higher than *Gl-HSP60* expression in heart ($P < 0.05$) and generally in YO, gill, thoracic ganglion, and claw muscle. *Gl-HIFα* and *Gl-HIFβ* displayed similar and significant expression patterns among the tested tissues, with higher expression in *Gl-HIFα*.

In *C. maenas*, the highest mRNA levels of all genes were mostly detected in the YO and heart, while the gill usually had the lowest mRNA levels ($P < 0.05$, Table 2-4). *Cm-AMPK* subunits expression was highest in heart ($P < 0.05$), followed by YO and claw muscle. For *Cm-SIRT1* and *Cm-SIRT7*, expression levels were higher in the YO ($P < 0.05$) > heart > thoracic ganglion > claw muscle. *Cm-HSP70* mRNA expression was slightly higher than *Cm-HSP60* expression in YO and heart ($P < 0.05$) but no significant differences were observed among tissues. *Cm-HIFα* had higher expression levels in heart, thoracic ganglion, and claw muscle than *Cm-HIFβ* but no significant differences were observed among the tested tissues, although expression levels in most tissues were generally higher than those of the *Cm-HIFβ* subunit.

DISCUSSION

Homologs of genes of stress response play an important role of biomarkers in the physiology of crustacean species (Lv et al., 2014; Semmouri et al., 2019; Zheng et al., 2019). Most of them are part of complex cell and metabolic processes and are synthesized and accumulated during stress events. Highly conserved and abundant across invertebrate species, most of these genes are expressed strictly in response to environmental and physiological stress (Han et al., 2013). AMPK is a metabolic stress protein that senses AMP/ATP ratio and acts as a glycogen sensor as well (Stapleton et al., 1997). The protein functions as an alpha-beta-gamma heterotrimer. High sequence homology of the AMPkα subunit with proteins from other crustacean species, especially in the conserved domains, suggest they may interact with different proteins within these regions (Jeon, 2016). The Ser/Thr kinase domain found in vertebrates contains a conserved Thr at the position 172 that must be phosphorylated for activity in the activation loop (A-loop) (Willows et al., 2017). This residue lies at position 180 of the A-loop (ADFGLSNIMVDGEFLR^{180}TSCGSPN, Figure 2-2) which may be a variation in sequencing and assembly of the sequence in AMPkα. The C-terminal region corresponded to the interaction domain, a region that is necessary for interaction of the AMPkα subunit with the kinase complex (β and γ subunits), but is not only responsible for the interaction (Xiao et al., 2013). Characterization of AMPkβ revealed a carbohydrate-binding pocket (Figure 2-4), necessary for activation of this subunit by binding to glycogen (Oligschlaeger et al., 2015), which also suggests the regulatory role of AMPkβ in modulating AMPk activity. It's also been shown that the presence of a C-terminal interaction domain is required to bind to the α- and γ-subunits as scaffolds with no direct interaction between β- and γ-subunits (Wong and Lodish, 2006). Identification of the two CBS domains pairs in AMPkγ allow for multiple ligand binding sites,

known as amino acids binding AMP or ATP during events of metabolic regulation (Figure 2-6). The N-terminal region of AMPkγ exhibited a moderately low similarity to other proteins between the residues 15-60. Willows et al. (2017) describe it as a usual condition of these amino acids to be highly probable to variations and therefore possibly regulating AMPk activity.

Main differences in non-conserved regions of SIRT1 and SIRT7 isolated sequences may explain the typical highly variable N- and C- terminal domains (Figure 2-8 and Figure 2-10) across species of arthropods, and the differences in activity and function (Carafa et al., 2012). Except by few examples of other aquatic species and arthropods (Dong et al., 2016; Dong et al., 2014; Vasquez et al., 2017; Yuan et al., 2019), Sirtuins have been scarcely characterized from the evolutionary and molecular point of view in crustaceans (Schumpert et al., 2016). A basic structural and bioinformatic approximation was used to characterize the open reading frame of SIRT1 and SIRT7 in two decapod species. Bioinformatics tools can characterize structural and functional properties of Sirtuin species in essential growth and metabolic processes in crustaceans. Most of the information about subcellular localization and function in invertebrate species has been described from mammalian Sirtuins (Gillum et al., 2011; Greiss and Gartner, 2009). Sirtuins share a highly conserved core region enclosing an NAD^+ binding site and a catalytic domain formed by two subdomains: a large Rossmann fold subdomain and a small domain composed of two associated modules of zinc-binding sites (Carafa et al., 2012). In the core domain, Frye (2000) has described several short motifs including GAGISXXXGIPXXR, PXXXH, TQNID, and HG that are well conserved in prokaryotes and eukaryotes. These motifs were also found for the core domain of SIRT1 (^{139}GAGISTSAGIPDFR^{152}, ^{200}PTPSH^{204}, ^{221}TQNID^{225}, ^{222}HG^{223}) and SIRT7 (^{139}GAGISTAAGIPDFR^{152}, ^{91}PTLTH^{95}, ^{112}TQNID^{116}, ^{133}HG^{134}). Additionally, a ^{69}GVWTL^{73} motif present four residues C-terminal to the

^{139}GAGISTSAGIPDFR^{152} motif of SIRT7 was characterized as a signature of class IV sequences (Frye, 2000). Inside the catalytic domain, the zinc-binding motifs can be identified by the consensus sequence Cys-$X_{2–4}$-Cys-$X_{15–40}$-Cys-$X_{2–4}$-Cys (Moniot et al., 2012). These modules were identified for both SIRT1 (^{50}CIACHKEYSQEWMKEEIFKDNIPSCTEC^{277}) and SIRT7 (^{141}CNLCQRQFVRSSAATTVGQKSEGKACPARRGNGRRC^{176}) (Figure 2-8 and Figure 2-10).

Sirtuins activity may be related to the interaction with other stress responsive genes. In cases of activation of energy-producing pathways, AMPk activates PGC1a through direct phosphorylation, regulating mitochondrial biogenesis (Cetrullo et al., 2015), thus promoting a NAD^+-dependent activation of PGC1a by SIRT1 (Canto et al., 2009). *In vitro* studies of HeLa cells found that inhibition of SIRT1 by nicotinamide reduces expression of HSP27, HSP40, HSP70, and HSP90 (Westerheide et al., 2009). Hypoxia leads to the transcription of HIFα subunit, which is essential to synthesize a heterodimeric DNA-binding complex with the β-subunit, targeting downstream genes for oxygen regulation (Sun et al., 2016). It has been shown in *in vitro* and *in vivo* hypoxia models that inhibition of SIRT1 accumulation impairs the transcription of HIFa (Laemmle et al., 2012; Yoon et al., 2014). This is an important linkage with the energy homeostasis pathway, as crustaceans undergo changes in cellular energy status by environmental factors, such as salinity fluctuation (Zhang et al., 2016), thermal stress (Mohamad et al., 2018) or starvation (Sugumar et al., 2013) during the molt cycle.

The structure and function of heat shock proteins as a chaperone in protein folding in the cell have been mostly curated in arthropods other than crustaceans (King and MacRae, 2015). Their characterization is an essential objective to explain the physiology and development of crustaceans under extreme conditions such as thermal and hypoxic stress (Chang et al., 1999). Their architecture includes better hydrogen bonds, better hydrophobic internal packing, and an

enhanced secondary structure (Feder and Hofmann, 1999), which explains their multifunctional activity in cellular stress and how these proteins are unaffected by denaturation. A HSP60 chaperonins signature (^{429}AAIEEGIVPGGG^{440}), many ATP/ADP binding sites (54-56, 110, 114, 173, 422, 439, 478), and several Gly-Gly-Met repeat motifs at the C-terminus were typical motifs identified in this study (Figure 2-12), and also found in *Marsupenaeus japonicus* (Zheng et al., 2018), and *Scylla paramamosain* (Yang et al., 2013). The presence of these highly conserved motifs among HSP60 sequences (Figure 2-12) ultimately determines a mechanism of coupling to the substrate based on the ATP hydrolysis during the refolding process (Yi et al., 2018). Indeed, HSP60 or chaperonins are one of the cooperative chaperones folding newly synthesized or stress-denatured proteins. For substrate encapsulation, HSP60 (or the bacterial model GroEL-like type I chaperonin identified in this study) binds to a detachable "lid" structure (GroES/HSP10) that binds in an ATP-dependent fashion (Xu et al., 2014), forming a barrel-like complex. A HSP70 sequence with two conserved functional domains was isolated as well: the N-terminal Nucleotide Binding Domain (NBD) and a Substrate Binding Domain (SBD, BAG/NEF interaction site) at the C-terminus connected through a highly conserved region (Figure 2-14). HSP70 sequence also included the three typical conserved signatures of this protein (^{8}GIDLGTTY^{15}, ^{199}DLGGGTFD^{206}, ^{335}VLVGGSTRIPKIQ^{347}) and found in other invertebrates such as *Sterechinus neumayeri* (Gonzalez-Aravena et al., 2018) and many crustacean species (Baringou et al., 2016). The specificity and activity of HSP70 has been determined by their interaction with J-proteins (HSP40) as a chaperone complex (Mayer and Bukau, 2005) similarly as the HSP60-HSP10 interaction. HSP70 will form an extensive network of chaperones as a result of that J-protein family members (DnaJ, DnaK) are found in large numbers and exceed to HSP70 (Mayer et al., 2001). In this way, it may be possible to approach indirectly to the level of

expression of HSP40 while only the response of HSP70 is measured under stress conditions (Chen et al., 2018; Yang et al., 2016).

Identification of HIFα and -β subunits relied on the detection of the two major conserved domains: basic Helix-Loop-Helix (bHLH) and PER-ARNT-SIM (PAS) (Figure 2-16 and Figure 2-18). The bHLH domains were identified by many DNA binding sites and one E-box/N-box specificity residue along a ~60 residue motif (Sonanez-Organis et al., 2009), which validates the activity in oligomerization and DNA binding of the bHLH domains (Semenza, 2001a; Semenza, 2001c). Both bHLH domains in HIFα (^{167}KRKEQSRNAARNRR^{180}-^{203}QLDK^{206}) and HIFβ (^{67}ERRRRNKMTAYIAEL^{81}-^{98}LTILRMAVAHMKALR^{112}) contained multiple DNA binding residues and one E-box/N-box specificity residue but, in general, differed in non-described residues. The PAS domain may be considered the most important conserved amino acids in HIF subunits, as PAS motifs appear in archaea, eubacteria and eukarya (Taylor and Zhulin, 1999), and have been found to give the target gene specificity, and to act as sensor for oxygen in signal transduction (Semenza, 1998). Sun et al. (2016) found that *Macrobrachium nipponense* PAS domains contain two 50–100 conserved residue sequences (PAS A/B), HIFα and -β subunits of *G. lateralis* and *C. maenas* contained similar number of amino acids. Under normoxic conditions, HIF-1α is degraded by post-translational hydroxylation of conserved proline residues within the oxygen-dependent degradation domain (ODD) (Semenza, 2001a; Semenza, 2001b). Li and Brouwer (2007) found in *Palaemonetes pugio* that the proline residues subjected to hydroxylation are conserved and reside at the N-terminal end of the ODD within a LXXLAP sequence motif (^{459}LTHLAP^{464}), and in the C-terminal ODD (Pro- 564, ^{634}MRAPFIP^{640}). These two sequence motifs are also conserved in the HIFα subunit of *G. lateralis* and *C. maenas* (^{615}LTHLAP^{620}, ^{808}MRAPYIP^{814}).

As the structure and function of these genes were described here, they have been functionally categorized into the cellular and metabolic processes and described on other crustacean transcriptome studies as well (Tagmount et al., 2010; Teranishi and Stillman, 2007; Zheng et al., 2019). This indicates that genes of stress response are typically highly conserved throughout evolution and essential for multicellular survival in crustaceans. Relative expression of the isolated genes in *G. lateralis* may be categorized within the profile 1 described by Das et al. (2018), as the highest relative values were in IM, a lower gene expression through premolt stages, and the lowest values in PM. Downregulated expression in the YO transcriptome (Figure 2-19) may be a consequence of crustacean transcripts in the molt cycle that are relatively poorly identified and characterized in databases. This factor may influence reads mapping, sequence clustering, and number of blast hits during the *de novo* transcriptome assembly (Suwansa-Ard et al., 2015).

The expression of stress responsive genes in the YOs, claw muscle, gill, heart, and thoracic ganglion was detected, and the results showed that the studied orthologs were generally expressed in all tested tissues, indicating they are multifunctional in *G. lateralis* and *C. maenas*. Although the comparison of tissues indicated that there is not tissue-specific expression, it exclusively relates to non-molting crabs in both species as only IM animals were analyzed. Moreover, stress responsive genes belong to multiple metabolic and growth processes across arthropod species (Gu et al., 2015; Klumpen et al., 2017; Schumpert et al., 2016), which determines their expression in some tissues is related to local cell and metabolic activity and regulation of molecular pathways.

CONCLUSIONS

Contigs encoding *AMPkα*-, *β*-, and *γ*-subunits, *SIRT1*, *SIRT7*, *HSP60*, *HSP70*, *HIFα*-, and *β*-subunits were isolated and characterized in the blackback land crab, *Gecarcinus lateralis*, and green shore crab, *Carcinus maenas*. The results showed that the deduced amino acid sequences of *AMPkα*-, *β*-, and *γ*-subunits, *SIRT1*, *SIRT7*, *HSP60*, *HSP70*, *HIFα*-, and *β*-subunits were homologous to the known sequences in other arthropod species and contained several highly conserved typical domains present in their orthologs. The genes were expressed in five tissues studied with non-tissue specific expression of the genes, supporting the multifunctional activity of stress responsive genes in cell growth, metabolism, and protein synthesis. The present study provided the basic data for the identification and characterization of genes of stress response in *G. lateralis* and *C. maenas* and generates a database to study these genes in decapods in different molt stages and/or under environmental stress.

Table 2-1. Contig length and calculated molecular weight of ortholog sequences for *G. lateralis* (*Gl*) and *C. maenas* (*Cm*). Bp: base pairs; aa: amino acids; kDa: kilodalton; *AMPkαβγ*: AMP-activated protein kinase alpha, beta, gamma subunit; *SIRT*: Silent Information Regulator 2; *HSP*: Heat Shock Protein; *HIFαβ*: Hypoxia Inducible Factor alpha, beta subunit.

Gene	Contig Length (bp)	ORF Length (aa)	Molecular Weight (kDa)
Gl-AMPkα	1614	537	60.59
Gl-AMPkβ	882	293	32.83
Gl-AMPKγ	1713	570	64.25
Gl-SIRT1	1230	409	45.46
Gl-SIRT7	1161	386	43.40
Gl-HSP60	1737	578	61.35
Gl-HSP70	1953	650	71.20
Gl-HIFα	3606	1201	131.28
Gl-HIFβ	1983	660	72.71
Cm-AMPkα	1566	522	58.86
Cm-AMPkβ	879	293	32.76
Cm-AMPKγ	1392	464	64.16
Cm-SIRT1	1230	410	45.54
Cm-SIRT7	1596	532	59.39
Cm-HSP60	1734	578	61.26
Cm-HSP70	1965	655	71.49
Cm-HIFα	3255	1085	107.91
Cm-HIFβ	1986	662	72.48

Table 2-2. Oligonucleotide primer sequences used for expression analysis of *G. lateralis* (*Gl*) and *C. maenas* (*Cm*) genes. F, forward; R, reverse; Tm, melting temperature; bp, base pair; *EF2*, elongation factor 2; *AMPkαβγ*: AMP-activated protein kinase alpha, beta, gamma subunit; *SIRT*: Silent Information Regulator 2; *HSP*: Heat Shock Protein; *HIFαβ*: Hypoxia Inducible Factor alpha, beta subunit.

Primer	Sequence (5' – 3')	Tm (°C)	Product (bp)
Gl-EF2-F1	TTCTATGCCTTTGGCCGTGTC	60.7	227
Gl-EF2-R1	ATGGTGCCCGTCTTAACCA	60.2	227
Gl-AMPkα-F1	CTCAAGCTGTTTCGTCATCC	60.8	201
Gl-AMPkα-R1	CATATGTCTGTGGCAGTAGTCC	62.0	201
Gl-AMPkβ-F1	TTCGTCATCAAGTGGGCTG	62.1	204
Gl-AMPkβ-R1	GTTGCTGCTGTTGTTGTTGG	62.6	204
Gl-AMPkγ-F1	CTCACCATCACCGACTTC	59.2	235
Gl-AMPkγ-R1	ATAGCACATTCCCTGTAGCC	61.5	235
Gl-SIRT1-F1	TATCCAGGTGCTTTCAACCC	62.0	216
Gl-SIRT1-R1	TTCCTCTTTCATCCACTCTTGG	61.9	216
Gl-SIRT7-F1	TTGGGCAGAAGAGTGAAGG	61.6	215
Gl-SIRT7-R1	AGTGTCTACAGGGATGTTGC	61.6	215
Gl-HSP60-F1	GGATGACACACTGCTACTGAA	61.8	204
Gl-HSP60-R1	GGTAAACGAGAAGAAGGACCG	62.1	204
Gl-HSP70-F1	ACCCGTATTCCTAAGATTCAGC	61.8	215
Gl-HSP70-R1	ATCAGCTTGGTCATCACTCC	61.7	215
Gl-HIFα-F1	CCTTCGTCAGCAGACACTC	61.8	235
Gl-HIFα-R1	GTCACCAGCCACACATAGC	62.6	235
Gl-HIFβ-F1	CAGTCACACCAGTCCTTAACC	62.1	233
Gl-HIFβ-R1	GACCTTCATGCGGCAGAT	62.0	233
Cm-EF2-F1	CCATCAAGAGCTCCGACAATG	61.6	278
Cm-EF2-R1	CATTTCGGCACGGTACTTCTG	61.8	278
Cm-AMPkα-F1	TTGCACCACTGAGAGAACG	61.9	219
Cm-AMPkα-R1	AACCTCCTGTTATTGGGTTCC	62.0	219
Cm-AMPkβ-F1	CTCCCACCAGTACAAGTTCC	61.7	225
Cm-AMPkβ-R1	TCATCTTCCTCTTGCATACCG	61.7	225
Cm-AMPkγ-F1	TGGAGGACCATCGCTTGGAGACT	61.9	247
Cm-AMPkγ-R1	GGCTTGTGTAGGATTGACGGCT	59.5	247
Cm-SIRT1-F1	CTTTCTTTGTGCTGGCTAAGG	61.4	240
Cm-SIRT1-R1	CCTCCTTCATCCAGTCTTGG	61.5	240
Cm-SIRT7-F1	GGTTATGTCCAGTACGTGGT	61.3	249
Cm-SIRT7-R1	CCAGTCCAGGATGTTGTCAT	61.7	249
Cm-HSP60-F1	ATTGCCGAGGATGTTGATGG	62.6	227
Cm-HSP60-R1	TGAATCTCGCCAACCATTCC	62.5	227
Cm-HSP70-F1	GACAACAGAATGGTGAATCACTTCGCTC	59.6	160
Cm-HSP70-R1	GAGAGTCGATCTCTATGCTGGCCTG	60.3	160
Cm-HIFα-F1	CCTTCGTGAGCAGACACTC	61.8	235
Cm-HIFα-R1	GTCACCAGCCACACGTAAC	62.6	235

Cm-HIFβ-F1	TATTCTTGAGGCTGCTGATGG	62.0	224
Cm-HIFβ-R1	CGGTCTTGAGGTCCAATATACG	62.0	224

Table 2-3. ANOVA results for tissue distribution in *G. lateralis* (*Gl*). df, degrees of freedom; *EF2*, elongation factor 2; *AMPkαβγ*: AMP-activated protein kinase alpha, beta, gamma subunit; *SIRT*: Silent Information Regulator 2; *HSP*: Heat Shock Protein; *HIFαβ*: Hypoxia Inducible Factor alpha, beta subunit.

	df1	Mean square	df2	Mean square	F	P = value
Gl-EF2	4	0.962	10	0.124	7.792	0.004
Gl-AMPkα	4	1.037	10	0.160	6.478	0.008
Gl-AMPkβ	4	0.723	10	0.081	8.889	0.002
Gl-AMPkγ	4	0.737	10	0.101	7.281	0.005
Gl-SIRT1	4	0.641	10	0.131	4.906	0.019
Gl-SIRT7	4	0.960	10	0.169	5.671	0.012
Gl-HSP60	4	0.824	10	0.094	8.788	0.003
Gl-HSP70	4	0.612	10	0.110	5.569	0.013
Gl-HIFα	4	1.546	10	0.141	11.000	0.001
Gl-HIFβ	4	1.245	10	0.127	9.812	0.002

Table 2-4. ANOVA results for tissue distribution in *C. maenas* (*Cm*). df, degrees of freedom; *EF2*, elongation factor 2; *AMPkαβγ*: AMP-activated protein kinase alpha, beta, gamma subunit; *SIRT*: Silent Information Regulator 2; *HSP*: Heat Shock Protein; *HIFαβ*: Hypoxia Inducible Factor alpha, beta subunit.

	df1	Mean square	df2	Mean square	F	P = value
Cm-EF2	4	1.861	10	2.228	0.835	0.533
Cm-AMPkα	4	1.767	10	2.617	0.675	0.624
Cm-AMPkβ	4	2.382	10	0.422	5.648	0.012
Cm-AMPkγ	4	2.192	10	0.241	9.086	0.002
Cm-SIRT1	4	1.007	10	0.286	3.519	0.048
Cm-SIRT7	4	1.195	10	0.292	4.094	0.032
Cm-HSP60	4	1.460	10	0.207	7.056	0.006
Cm-HSP70	4	2.150	10	0.691	3.112	0.066
Cm-HIFα	4	1.126	10	1.843	0.611	0.664
Cm-HIFβ	4	0.709	10	0.218	3.253	0.059

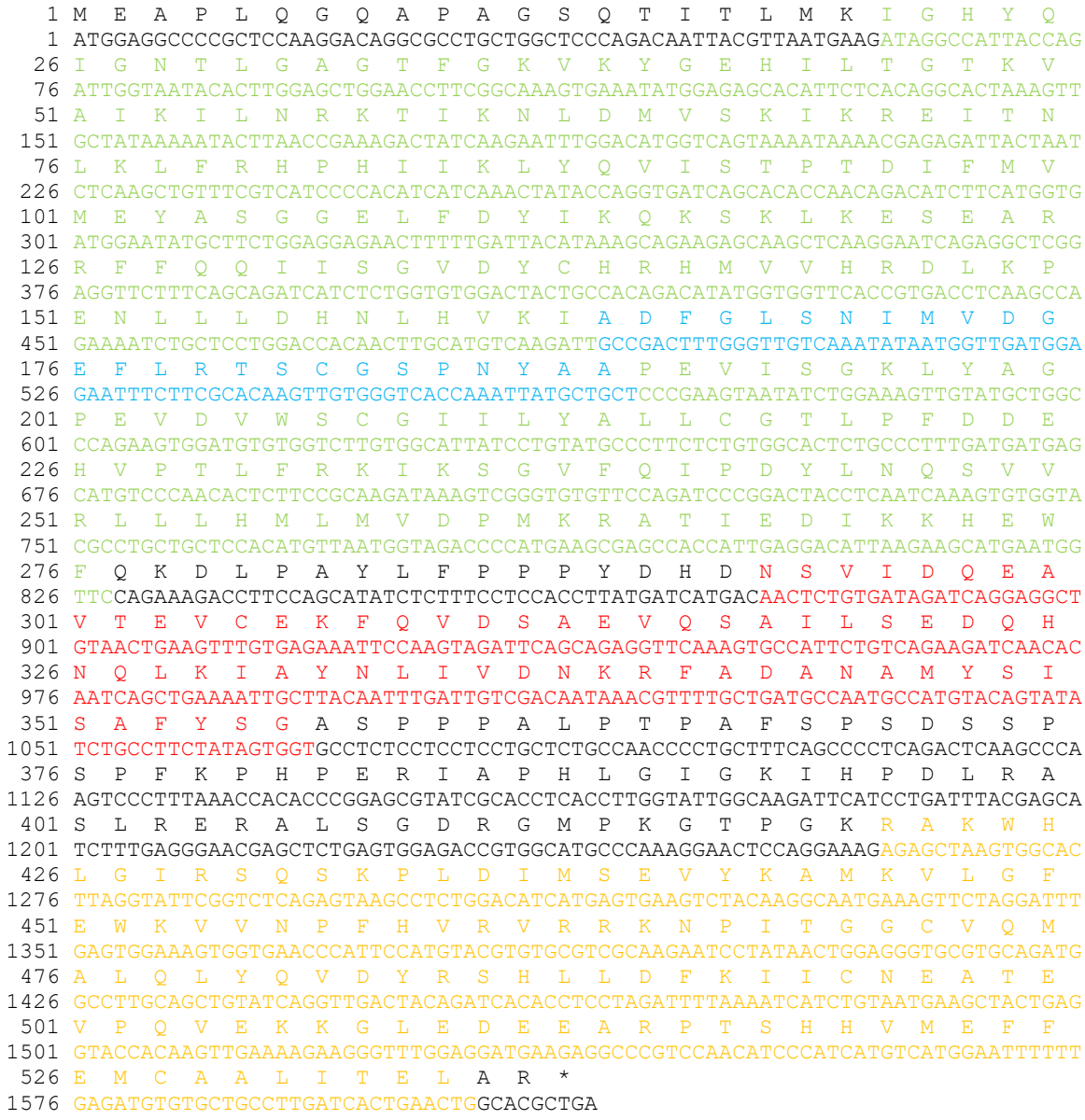


Figure 2-1. Consensus sequence of the isolated nucleotide and deduced amino acid sequences encoding *AMPKα* in *G. lateralis* and *C. maenas*. Green: Catalytic domain; **blue**: activation loop (A-loop); **red**: UBA-like autoinhibitory domain; **orange**: C-terminal regulatory domain. The amino acid position is marked below the figure, the position of the nucleic acid is marked above the figure.

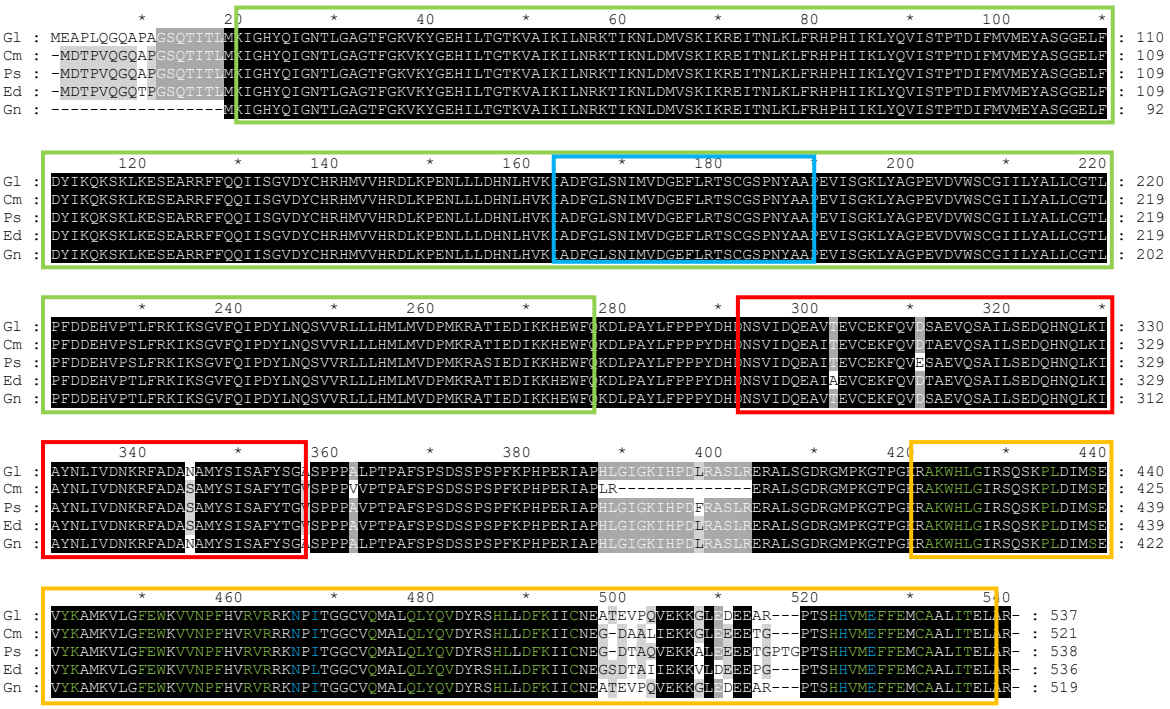


Figure 2-2. Multiple alignment of deduced amino acid sequences of the *AMPKα* in *G. lateralis* and *C. maenas* and orthologs from other crustacean species. **Green box**: Catalytic domain; **blue box**: activation loop (A-loop); **red box**: UBA-like autoinhibitory domain; **orange box**: C-terminal regulatory domain; **green letter**: beta subunit interface; **blue letter**: gamma subunit interface. Gl: *Gecarcinus lateralis* contig c252370_g1_i1, Cm: *Carcinus maenas* comp87977_c1_seq7 (Verbruggen et al., 2015) accession GBXE01117363.1, Ps: *Portunus sanguinolentus* CL1125 Contig6 accession GFZC01005444.1, Ed: *Eurypanopeus depressus* TR282636-c0_g1_i5 accession GFJG01075064.1, Gn: *Gecarcoidea natalis* contig4202 accession GFXJ01004202.1. Amino acid residues shaded in black are 100% identical in all sequences, shaded in dark gray are 80% similar in all sequences, and shaded in light gray are 60% similar in all sequences.

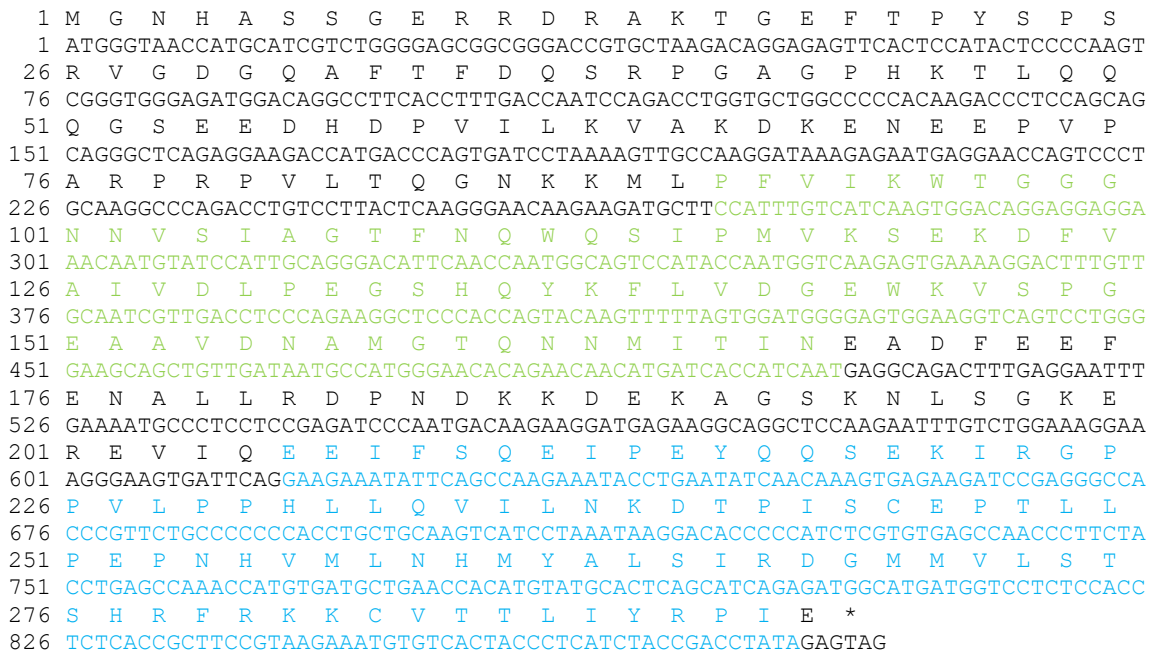


Figure 2-3. Consensus sequence of the isolated nucleotide and deduced amino acid sequences encoding *AMPkβ* in *G. lateralis* and *C. maenas*. Green: N-terminal Early set domain; **blue**: Interaction domain. The amino acid position is marked below the figure, the position of the nucleic acid is marked above the figure.

Figure 2-4. Multiple alignment of deduced amino acid sequences of the *AMPkβ* in *G. lateralis* and *C. maenas* and orthologs from other crustacean species. **Green box**: N-terminal Early set domain; **blue box**: Interaction domain; **green letter**: glycogen binding site. Gl: *Gecarcinus lateralis* c174533_g1_i1, Cm: *Carcinus maenas* comp87324_c3_seq1 (Verbruggen et al., 2015) accession GBXE01111349.1, Cf: *Charybdis feriata* CL5672.Contig2 accession GGFD01017064.1, Cj: *Charybdis japonica* comp30484_c0_seq2 accession GFPO01022754.1, Ps: *Portunus sanguinolentus* Unigene25737 accession GFZC01056119.1, Cs: *Callinectes sapidus* comp59499_c0_seq10 accession GEID01097740.1, So: *Scylla olivacea* Ref_Crab_Transcript_95652_2787 accession GDRN01095460.1. Amino acid residues shaded in black are 100% identical in all sequences, shaded in dark gray are 80% similar in all sequences, and shaded in light gray are 60% similar in all sequences.

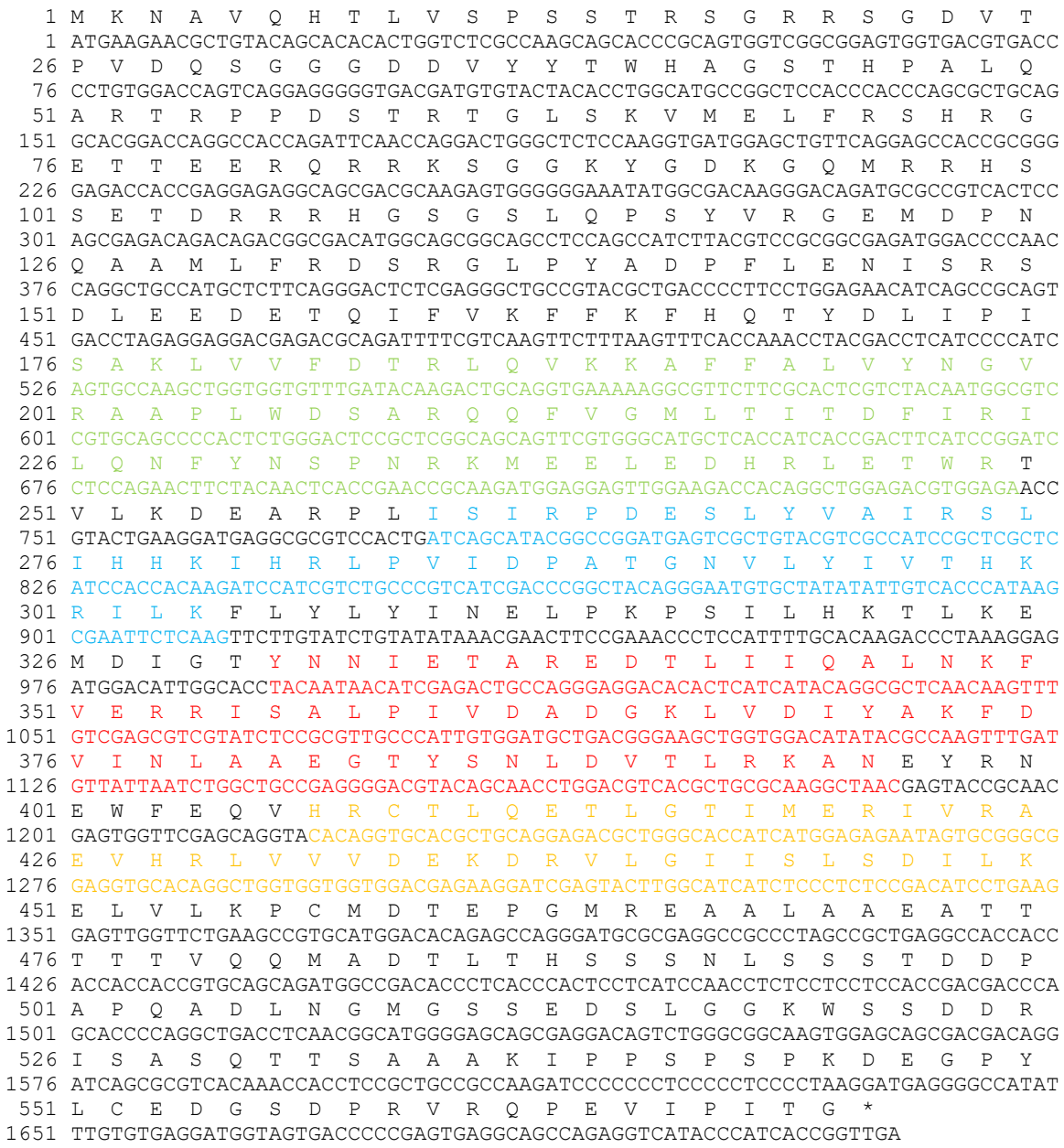


Figure 2-5. Consensus sequence of the isolated nucleotide and deduced amino acid sequences encoding *AMPkγ* in *G. lateralis* and *C. maenas*. Green: CBS domain pair 1; **blue**: CBS domain 2; **red**: CBS domain 3; **orange**: CBS domain 4. CBS domains occur in tandem repeats associated called Bateman domain or a CBS pair based. The amino acid position is marked below the figure, the position of the nucleic acid is marked above the figure.

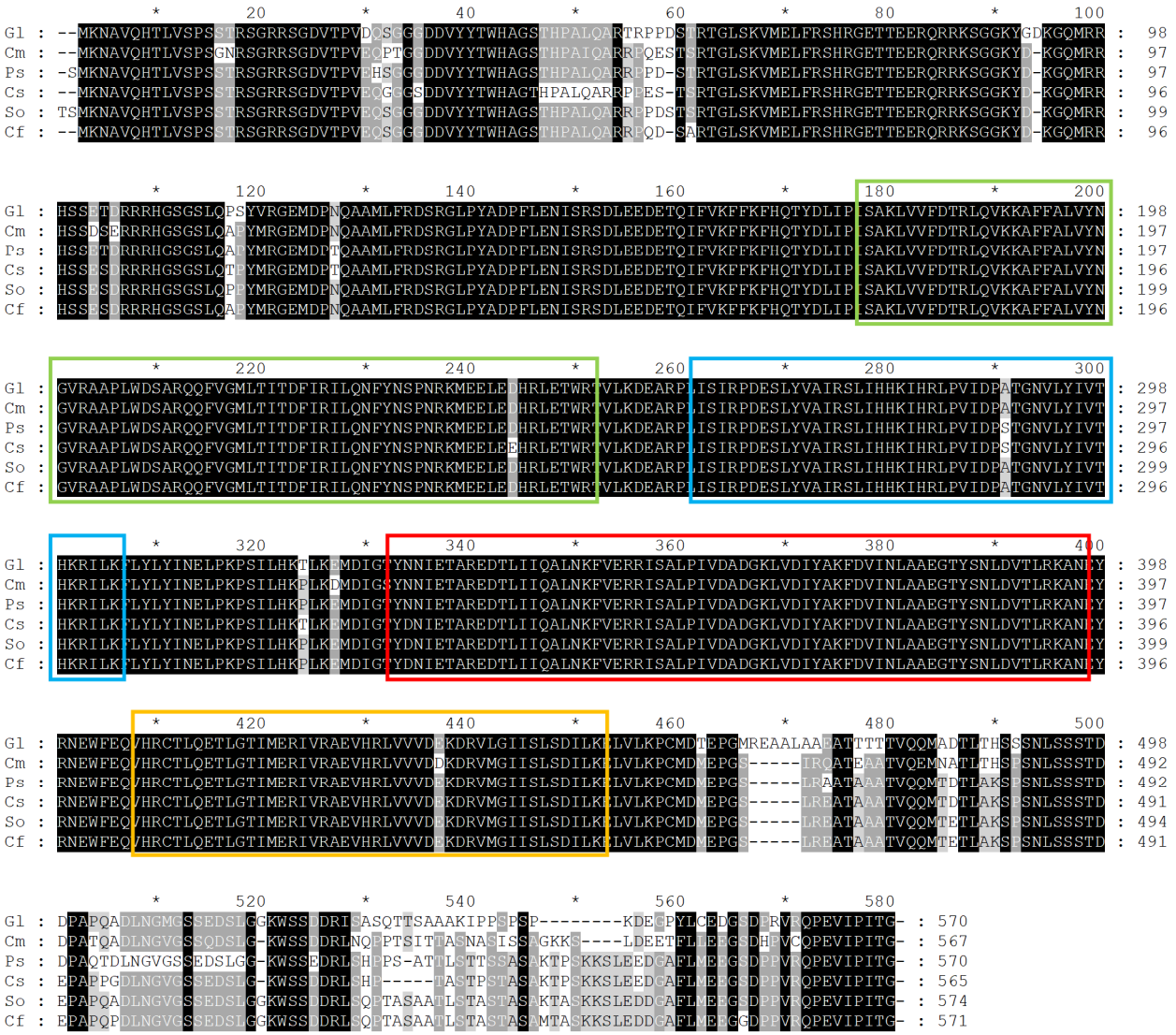


Figure 2-6. Multiple alignment of deduced amino acid sequences of the *AMPKγ* in *G. lateralis* and *C. maenas* and orthologs from other crustacean species. **Green box**: CBS domain pair 1; **blue box**: CBS domain 2; **red box**: CBS domain 3; **orange box**: CBS domain 4. CBS domains occur in tandem repeats associated called Bateman domain or a CBS pair based. Gl: *Gecarcinus lateralis* c268592_g2_i2, Cm: *Carcinus maenas* comp85552_c0_seq3 (Verbruggen et al., 2015) accession GBXE01097595.1, Ps: *Portunus sanguinolentus* CL790.Contig1 accession GFZC01003955.1, Cs: *Callinectes sapidus* comp60648_c1_seq1 accession GEID01111247.1, So: *Scylla olivacea* Ref_Crab_Transcript_106234_2423 accession GDRN01106031.1, Cf: *Charybdis feriata* CL1870.Contig5 accession GGFD01007250.1. Amino acid residues shaded in black are 100% identical in all sequences, shaded in dark gray are 80% similar in all sequences, and shaded in light gray are 60% similar in all sequences.

```
   1 M  A  D  S  K  E  V  A  S  R  V  L  T  G  E  A  E  A  P  K  E  T  C  K  G
   1 ATGGCAGACTCCAAGGAAGTAGCAAGCAGAGTGTTGACAGGCGAGGCAGAGGCACCCAAAGAGACATGTAAAGGC
  26 S  T  S  T  P  A  E  G  G  T  D  S  K  E  F  E  G  A  I  G  G  M  R  D  P
  76 AGCACATCAACACCAGCTGAAGGAGGAACAGACTCAAAGGAGTTTGAGGGAGCCATTGGGGGAATGAGAGACCCC
  51 S  K  I  N  E  E  E  D  S  D  E  E  E  D  D  Y  E  D  D  L  D  D  D  D  I
 151 AGCAAGATTAATGAGGAGGAAGATAGTGATGAAGAAGAAGATGATTATGAAGATGACTTGGATGATGATGACATT
  76 E  D  E  D  D  G  E  D  H  E  G  F  M  S  M  L  Q  N  F  I  M  S  K  L  D
 226 GAAGATGAGGATGATGGGGAGGACCATGAAGGATTCATGAGTATGTTACAGAACTTTATAATGAGCAAACTGGAC
 101 L  S  G  H  G  E  V  E  K  V  L  E  E  V  S  V  E  G  V  V  K  Y  I  K  S
 301 CTAAGTGGCCATGGAGAGGTTGAAAAGGTCTTGGAAGAGGTTAGTGTCGAGGGAGTGGTGAAGTACATCAAGAGT
 126 G  Q  C  K  N  I  I  T  M  A  G  A  G  I  S  T  S  A  G  I  P  D  F  R  S
 376 GGACAGTGCAAGAACATCATCACCATGGCTGGTGCCGGCATCTCCACTTCTGCAGGTATCCCAGACTTTCGTTCA
 151 P  G  T  G  L  Y  N  N  L  E  K  Y  N  L  P  F  P  E  A  I  F  D  I  D  F
 451 CCAGGTACAGGTTTATACAACAACCTTGAGAAATATAACCTGCCCTTCCCAGAGGCCATATTTGACATTGATTTC
 176 F  K  S  N  P  K  P  F  F  V  L  A  R  D  L  Y  P  G  A  F  N  P  T  P  S
 526 TTTAAAAGCAACCCCAAGCCTTTCTTTGTCCTTGCAAGAGACTTATATCCAGGTGCTTTCAACCCCACTCCCTCC
 201 H  W  F  I  R  L  L  H  E  K  G  L  L  L  R  H  Y  T  Q  N  I  D  T  L  E
 601 CACTGGTTCATTCGACTCCTCCACGAGAAGGGACTCCTTTTACGGCATTATACACAGAACATTGACACACTAGAG
 226 H  V  A  G  L  P  A  E  K  V  V  E  A  H  G  T  F  R  T  S  H  C  I  A  C
 676 CATGTTGCTGGACTACCTGCTGAGAAGGTGGTGGAGGCACATGGTACCTTCCGCACCTCTCACTGCATCGCCTGT
 251 H  K  E  Y  S  Q  E  W  M  K  E  E  I  F  K  D  N  I  P  S  C  T  E  C  S
 751 CACAAGGAGTACAGCCAAGAGTGGATGAAAGAGGAAATCTTCAAGGATAACATACCCTCATGTACAGAGTGCAGC
 276 G  L  V  K  P  D  I  V  F  F  R  E  N  L  P  T  R  F  F  H  L  M  Q  S  D
 826 GGCCTTGTCAAGCCTGATATTGTGTTCTTCCGTGAAAACCTTCCAACTAGATTCTTTCATTTGATGCAGTCAGAC
 301 F  P  K  C  D  L  L  L  I  M  G  T  S  L  T  V  Q  P  F  A  M  L  I  N  N
 901 TTCCCTAAGTGTGACCTGCTACTTATCATGGGCACCTCCCTCACTGTGCAACCTTTTGCTATGCTGATCAACAAT
 326 V  P  S  S  C  P  R  L  L  I  N  R  E  K  A  G  T  V  D  P  M  M  A  M  L
 976 GTGCCATCCTCCTGTCCACGGCTTCTTATAAACCGAGAGAAAGCAGGAACGGTGGACCCAATGATGGCCATGCTA
 351 Q  G  M  L  S  S  S  G  S  G  L  A  L  D  S  P  R  N  T  R  D  V  A  L  L
1051 CAGGGAATGCTGAGTAGCAGTGGCAGTGGCTTGGCCCTTGACTCTCCCAGGAACACTCGAGATGTTGCTTTACTG
 376 G  D  C  D  D  G  C  V  K  L  V  D  M  L  D  W  K  E  D  F  E  K  L  L  T
1126 GGAGACTGTGATGATGGCTGTGTCAAGCTAGTGGATATGCTGGACTGGAAGGAGGACTTTGAGAAGCTCCTCACC
 401 S  S  K  K  D  D  K  A  E  *
1201 TCTTCCAAGAAGGATGACAAGGCTGAATAG
```

Figure 2-7. Consensus sequence of the isolated nucleotide and deduced amino acid sequences encoding *SIRT1* in *G. lateralis* and *C. maenas*. Green: SIRT1, catalyze NAD+-dependent protein/histone deacetylation. The amino acid position is marked below the figure, the position of the nucleic acid is marked above the figure. The amino acid position is marked below the figure, the position of the nucleic acid is marked above the figure.

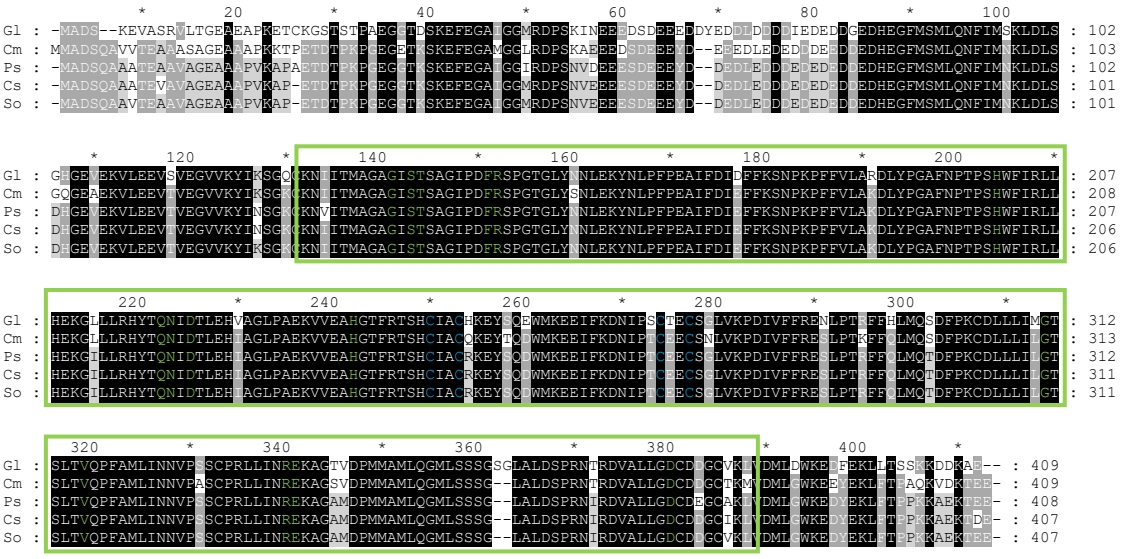


Figure 2-8. Multiple alignment of deduced amino acid sequences of the *SIRT1* in *G. lateralis* and *C. maenas* and orthologs from other crustacean species. **Green box**: SIRT1, catalyze NAD+-dependent protein/histone deacetylation; **green letter**: NAD^+ binding site; **blue letter**: Zn binding site. Gl: *Gecarcinus lateralis* c248059_g2_i1, Cm: *Carcinus maenas* comp82582_c4_seq1 (Verbruggen et al., 2015) accession GBXE01084825.1, Ps: *Portunus sanguinolentus* Unigene46301 accession GFZC01076660.1, Cs: *Callinectes sapidus* comp53729_c0_seq1 accession GEID01061618.1, So: *Scylla olivacea* Ref_Crab_Transcript_30385_2658 accession GDRN01030275.1. Amino acid residues shaded in black are 100% identical in all sequences, shaded in dark gray are 80% similar in all sequences, and shaded in light gray are 60% similar in all sequences.

```
   1 M  S  C  S  Y  A  E  G  L  S  K  Y  E  N  K  G  K  L  G  L  P  E  R  F  D
   1 ATGTCTTGCAGCTACGCCGAGGGGTTGAGCAAGTACGAGAACAAGGGGAAATTAGGCCTGCCAGAGAGGTTTGAC
  26 P  S  D  E  V  E  R  K  V  Q  I  L  A  Q  W  M  R  E  S  R  H  T  V  I  H
  76 CCATCAGATGAGGTGGAGCGGAAGGTACAGATCCTGGCCCAGTGGATGAGGGAGTCCAGACACACCGTCATCCAC
  51 T  G  A  G  I  S  T  A  A  G  I  P  D  F  R  G  P  N  G  V  W  T  L  E  K
 151 ACAGGCGCTGGGATTAGCACGGCAGCTGGAATCCCCGACTTTAGAGGTCCCAATGGAGTCTGGACACTGGAAAAG
  76 E  G  L  K  P  E  V  N  I  S  W  D  D  A  R  P  T  L  T  H  M  A  I  V  S
 226 GAGGGTCTCAAGCCAGAGGTTAATATATCATGGGACGATGCACGGCCCACACTCACACACATGGCCATCGTCAGT
 101 L  E  Q  R  G  Y  V  Q  Y  V  V  T  Q  N  I  D  G  L  H  L  R  S  G  L  Q
 301 CTGGAGCAGCGTGGGTATGTGCAGTATGTGGTGACGCAGAACATTGACGGGCTCCACCTACGCTCGGGTCTGCAG
 126 R  K  K  L  A  E  L  H  G  D  M  F  V  D  K  C  N  L  C  Q  R  Q  F  V  R
 376 CGTAAGAAGTTAGCGGAACTCCACGGGGACATGTTTGTGGACAAGTGCAACCTGTGTCAGAGGCAGTTCGTCAGG
 151 S  S  A  A  T  T  V  G  Q  K  S  E  G  K  A  C  P  A  R  R  G  N  G  R  R
 451 AGCTCAGCCGCCACAACTGTTGGGCAGAAGAGTGAAGGCAAGGCGTGTCCAGCAAGGCGCGGCAACGGCAGGCGG
 176 C  R  G  K  L  H  D  N  I  L  D  W  E  D  G  L  P  D  A  D  L  D  L  A  I
 526 TGTCGTGGGAAGCTGCACGACAACATTCTGGACTGGGAAGATGGCCTGCCAGATGCTGACCTGGACCTGGCTATC
 201 S  H  A  C  V  A  D  L  S  L  C  L  G  T  T  L  Q  I  V  P  S  G  N  I  P
 601 TCCCATGCCTGTGTGGCCGATCTCAGCCTGTGTCTAGGAACAACACTGCAGATTGTACCAAGTGGCAACATCCCT
 226 V  D  T  K  K  R  G  G  R  L  V  I  C  N  L  Q  P  T  K  Q  D  R  H  A  D
 676 GTAGACACTAAAAAGCGTGGTGGCAGGCTGGTGATATGCAACCTGCAGCCCACCAAACAGGACCGCCACGCTGAC
 251 L  I  I  N  T  Y  V  D  D  L  M  K  R  L  L  E  L  L  G  V  P  L  M  A  Y
 751 CTCATCATAAACACATACGTCGATGATCTCATGAAGCGTCTCCTGGAGCTGCTGGGTGTCCCCCTCATGGCTTAC
 276 T  P  E  D  D  P  L  K  I  T  Q  L  L  L  Q  G  S  K  V  I  G  R  S  D  E
 826 ACCCCTGAGGATGACCCCCTCAAGATCACACAGCTCCTTCTCCAGGGGTCGAAGGTCATCGGGAGGTCAGATGAA
 301 D  A  F  E  P  I  E  W  T  I  P  E  E  W  V  K  D  R  D  L  A  E  R  V  K
 901 GATGCATTTGAGCCAATAGAGTGGACGATCCCCGAAGAGTGGGTCAAGGATAGGGACTTAGCGGAGAGGGTCAAG
 326 S  R  K  V  I  R  K  R  P  G  V  K  E  E  V  I  E  R  K  K  V  K  N  S  Q
 976 TCAAGAAAGGTCATACGAAAGAGGCCTGGGGTCAAGGAAGAAGTGATTGAGAGGAAGAAGGTAAAAAATAGTCAG
 351 I  P  V  A  E  E  G  M  E  D  G  K  M  E  E  Q  V  G  L  Y  H  R  N  G  E
1051 ATTCCTGTGGCTGAGGAGGGAATGGAGGATGGGAAGATGGAAGAACAGGTGGGGTTATATCATAGGAATGGGGAG
 376 V  V  T  N  E  E  E  E  E  E  D  *
1126 GTGGTCACGAACGAGGAAGAGGAAGAGGAGGACTGA
```

Figure 2-9. Consensus sequence of the isolated nucleotide and deduced amino acid sequences encoding *SIRT7* in *G. lateralis* and *C. maenas*. Green: SIRT7, catalyze NAD+-dependent protein/histone deacetylation. The amino acid position is marked below the figure, the position of the nucleic acid is marked above the figure.

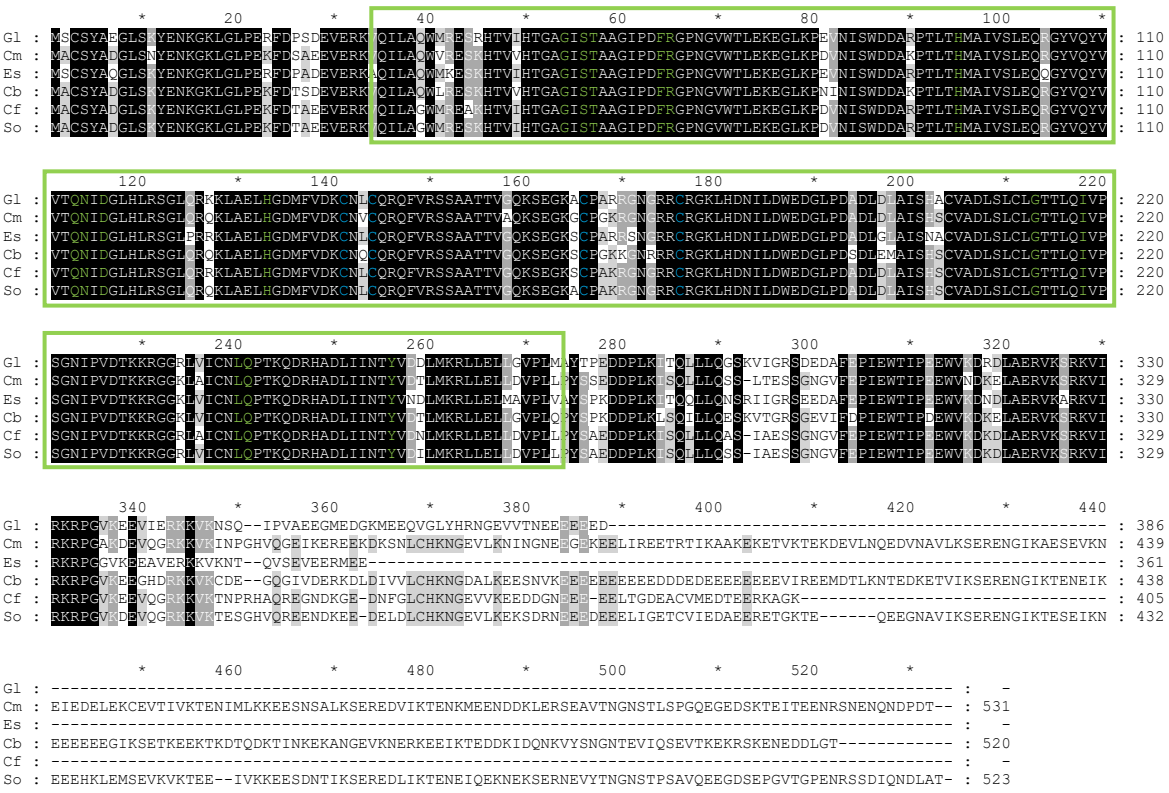


Figure 2-10. Multiple alignment of deduced amino acid sequences of the *SIRT7* in *G. lateralis* and *C. maenas* and orthologs from other crustacean species. **Green box**: SIRT7, catalyze NAD+-dependent protein/histone deacetylation; **green letter**: NAD^+ binding site; **blue letter**: Zn binding site. Gl: *Gecarcinus lateralis* c259766_g2_i1, Cm: *Carcinus maenas* comp87935_c0_seq1 (Verbruggen et al., 2015) accession GBXE01116841.1, Es: *Eriocheir sinensis* comp28640_c0_seq1 accession GBUF01002997.1, Cb: *Cancer borealis* contig_2654 accession GEFB01002691.1, Cf: *Charybdis feriata* Unigene48777 accession GGFD01072295.1, So: *Scylla olivacea* Ref_Crab_Transcript_ 81321_2623 accession GDRN01081144.1. Amino acid residues shaded in black are 100% identical in all sequences, shaded in dark gray are 80% similar in all sequences, and shaded in light gray are 60% similar in all sequences.

```
   1 M  Y  R  A  A  S  L  L  R  L  P  V  N  R  R  A  V  Q  Q  L  A  A  R  S  Y
   1 ATGTACCGTGCCGCCTCTCTCCTTCGGCTGCCCGTGAACCGCCGGGCAGTCCAGCAGCTGGCGGCGAGAAGCTAC
  26 A  K  D  V  K  F  G  S  E  V  R  A  M  M  L  Q  G  V  D  V  L  T  D  A  V
  76 GCTAAGGACGTTAAGTTCGGCTCAGAGGTGCGGGCCATGATGCTGCAGGGCGTCGACGTGCTGACAGACGCCGTG
  51 A  V  T  M  G  P  K  G  R  N  V  I  I  E  Q  S  W  G  S  P  K  I  T  K  D
 151 GCCGTCACCATGGGCCCTAAGGGACGCAATGTGATCATCGAACAGAGCTGGGGCAGCCCTAAGATCACCAAAGAT
  76 G  V  T  V  A  K  A  V  E  L  K  D  K  F  Q  N  I  G  A  K  L  V  Q  D  V
 226 GGAGTGACAGTGGCAAAGGCTGTGGAGCTGAAGGACAAGTTCCAAAACATTGGTGCCAAGCTAGTGCAGGATGTG
 101 A  N  N  T  N  E  E  A  G  D  G  T  T  T  A  T  V  L  A  R  T  I  A  K  E
 301 GCCAACAACACCAACGAGGAGGCTGGTGATGGCACCACCACCGCCACTGTGTTGGCCCGCACCATTGCCAAGGAA
 126 G  F  D  R  I  S  K  G  A  N  P  I  E  I  R  R  G  V  M  L  A  V  D  A  V
 376 GGCTTTGACAGGATCAGCAAGGGAGCAAACCCCATTGAAATCAGGCGAGGTGTGATGCTAGCAGTTGATGCCGTC
 151 I  E  H  L  R  T  L  S  R  Q  V  T  T  P  E  E  I  A  Q  V  A  T  I  S  A
 451 ATTGAACACCTGCGTACTCTCTCCCGCCAGGTGACTACTCCTGAAGAGATTGCTCAGGTGGCCACAATCTCTGCC
 176 N  G  D  S  E  V  G  Q  L  I  S  A  A  M  E  K  V  G  R  N  G  V  I  T  V
 526 AATGGTGACAGTGAAGTGGGCCAACTCATTTCTGCAGCCATGGAGAAGGTTGGTCGTAATGGAGTCATCACTGTC
 201 K  D  G  K  T  L  K  D  E  L  E  V  I  E  G  M  K  F  D  R  G  Y  I  S  P
 601 AAGGATGGCAAGACACTGAAGGATGAGCTAGAGGTCATTGAGGGCATGAAGTTTGACAGGGGTTACATTTCTCCA
 226 Y  F  I  N  T  T  K  G  A  K  V  E  Y  Q  D  A  L  V  L  L  S  E  K  K  I
 676 TACTTCATTAACACCACGAAAGGTGCCAAGGTGGAGTATCAGGATGCCTTGGTCCTTCTGTCTGAGAAGAAAATC
 251 S  S  I  Q  S  I  I  P  A  L  E  I  A  N  Q  Q  R  K  P  L  L  I  I  A  E
 751 TCCTCCATTCAGTCAATCATCCCAGCACTGGAGATTGCCAACCAGCAGAGGAAGCCTCTGTTGATCATTGCTGAA
 276 D  V  D  G  E  A  L  S  T  L  V  V  N  R  L  K  I  G  L  Q  I  A  A  V  K
 826 GATGTGGATGGTGAGGCACTCAGCACCTTGGTTGTGAATCGCCTCAAGATTGGCCTCCAAATTGCTGCTGTCAAG
 301 A  P  G  F  G  D  N  R  K  N  T  I  Q  D  I  A  I  A  T  G  A  L  V  F  N
 901 GCCCCAGGCTTCGGGGACAATCGCAAGAACACCATTCAGGACATTGCCATTGCCACAGGTGCTCTTGTCTTTAAT
 326 D  E  A  S  M  V  K  M  E  D  V  Q  V  H  D  L  G  M  V  G  E  V  Q  I  T
 976 GATGAGGCCAGCATGGTCAAGATGGAGGATGTGCAGGTGCATGACCTTGGCATGGTGGGGGAGGTGCAGATCACC
 351 K  D  D  T  L  L  L  K  G  K  G  K  P  S  D  I  E  R  R  V  K  Q  I  R  D
1051 AAGGATGACACACTGCTACTGAAGGGCAAGGGCAAGCCATCAGACATTGAGAGGCGAGTCAAGCAAATCCGTGAC
 376 Q  M  E  D  S  N  S  E  Y  E  K  E  K  M  Q  E  R  V  A  R  L  S  N  G  V
1126 CAGATGGAAGACAGCAACTCAGAGTACGAGAAGGAGAAGATGCAGGAGCGCGTGGCAAGGCTATCTAACGGTGTA
 401 A  V  V  K  V  G  G  S  S  E  V  E  V  N  E  K  K  D  R  V  N  D  A  L  C
1201 GCTGTTGTCAAGGTCGGAGGCTCATCGGAAGTGGAGGTGAATGAGAAGAAGGACCGAGTGAATGATGCTCTCTGT
 426 A  T  R  A  A  I  E  E  G  I  V  P  G  G  G  V  A  L  L  R  C  L  P  A  L
1276 GCCACACGAGCTGCTATCGAGGAAGGAATCGTGCCAGGCGGCGGTGTGGCACTCCTCCGCTGCCTCCCTGCCCTG
 451 D  A  L  T  P  A  N  D  D  Q  K  M  G  I  D  I  I  R  K  A  I  R  T  P  C
1351 GATGCCCTCACCCCTGCTAATGATGACCAGAAGATGGGCATTGATATCATCCGCAAGGCCATCCGGACCCCATGC
 476 H  T  I  V  S  N  A  G  I  D  A  A  V  I  V  N  K  V  E  E  A  T  G  D  H
1426 CACACCATTGTTAGTAACGCCGGCATCGACGCGGCTGTCATCGTGAATAAGGTGGAGGAGGCGACAGGTGACCAC
 501 G  Y  D  A  A  T  G  T  F  V  N  L  V  E  A  G  I  I  D  P  T  K  V  V  R
1501 GGTTATGATGCTGCCACCGGGACCTTCGTTAACCTGGTGGAAGCCGGAATCATTGACCCAACCAAGGTTGTACGC
 526 T  A  L  T  D  A  S  G  V  A  S  L  L  T  T  A  E  A  V  I  T  E  V  P  K
1576 ACAGCCCTCACCGATGCTTCAGGAGTGGCCTCTCTGCTCACCACAGCAGAGGCAGTCATCACTGAGGTCCCCAAG
 551 E  E  P  A  G  G  M  G  G  G  M  G  G  M  G  G  M  G  G  M  G  G  M  G  G
1651 GAGGAGCCAGCAGGCGGCATGGGTGGTGGAATGGGGGGCATGGGAGGCATGGGCGGTATGGGAGGAATGGGTGGT
 576 M  G  M  *
1726 ATGGGAATGTAA
```

Figure 2-11. Consensus sequence of the isolated nucleotide and deduced amino acid sequences encoding *HSP60* in *G. lateralis* and *C. maenas*. Green: GroEL-like type I chaperonin. The amino acid position is marked below the figure, the position of the nucleic acid is marked above the figure.

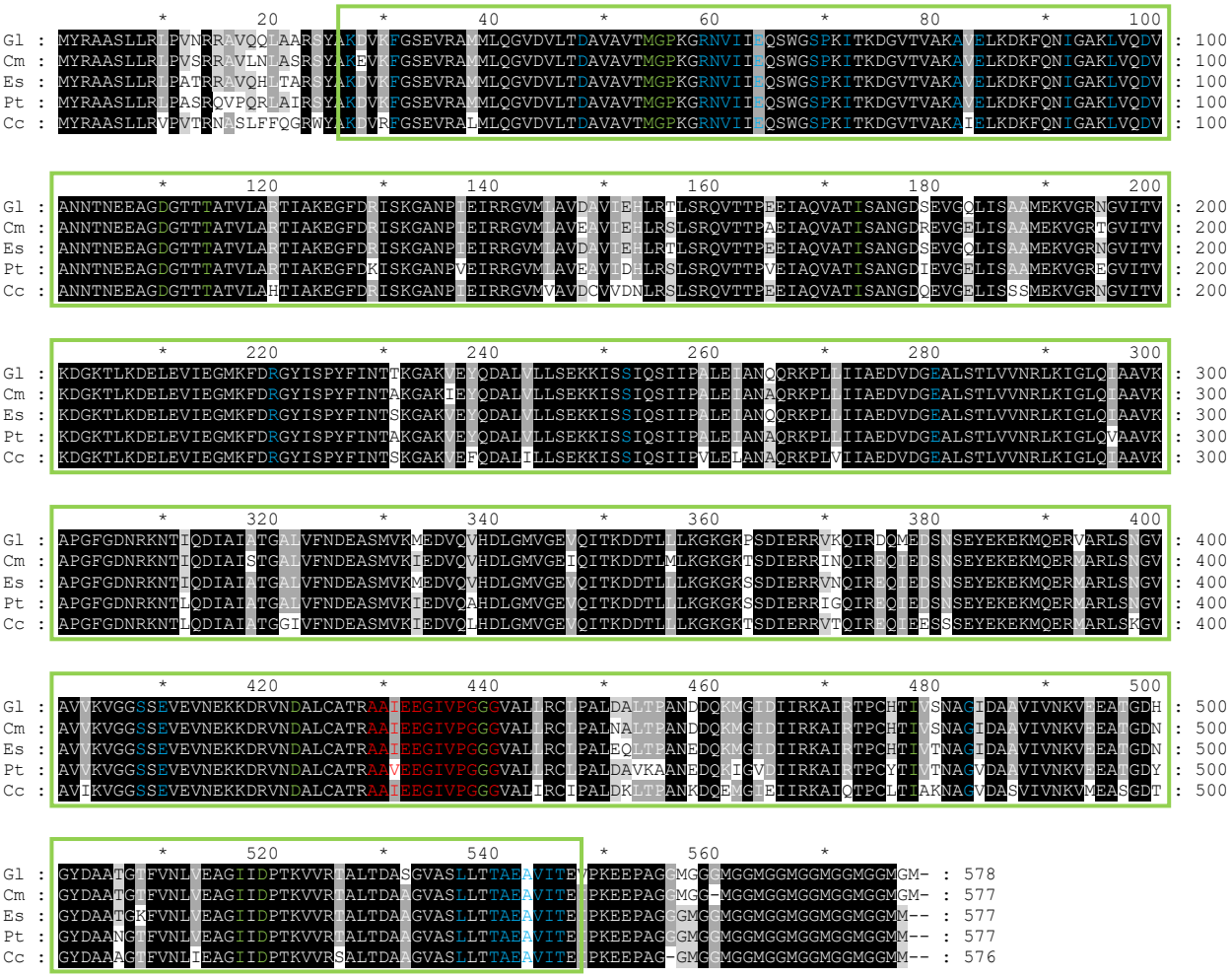


Figure 2-12. Multiple alignment of deduced amino acid sequences of the *HSP60* in *G. lateralis* and *C. maenas* and orthologs from other crustacean species. **Green box**: GroEL-like type I chaperonin; **green letter**: ATP/Mg binding site, **blue letter**: ring oligomerization interface, **green letter**: chaperonins cpn60 signature. Gl: *Gecarcinus lateralis* c252415_g1_i2, Cm: *Carcinus maenas* contig_1518 (Tepolt and Palumbi, 2015) accession https://doi.org/10.5061/dryad.g8b96, Es: Eriocheir sinensis accession KP642083.1, Pt: *Portunus trituberculatus* accession JN628037.1, Cc: *Cherax cainii* accession KR058803.1. Amino acid residues shaded in black are 100% identical in all sequences, shaded in dark gray are 80% similar in all sequences, and shaded in light gray are 60% similar in all sequences.

```
   1 M  S  K  G  S  A  V  G  I  D  L  G  T  T  Y  S  C  V  G  V  F  Q  H  G  K
   1 ATGTCTAAGGGATCAGCAGTGGGTATCGACCTGGGGACCACCTACTCCTGCGTGGGTGTGTTCCAGCATGGCAAG
  26 V  E  I  I  A  N  D  Q  G  N  R  T  T  P  S  Y  V  A  F  T  D  T  E  R  L
  76 GTCGAGATTATTGCCAACGACCAGGGCAACAGAACCACGCCCTCCTATGTCGCCTTCACCGACACCGAGCGTCTT
  51 I  G  D  A  A  K  N  Q  V  A  M  N  P  N  N  T  V  F  D  A  K  R  L  I  G
 151 ATCGGCGACGCCGCCAAGAACCAGGTGGCGATGAACCCCAACAACACAGTATTCGATGCCAAGAGACTCATCGGC
  76 R  K  F  N  D  H  N  V  Q  S  D  M  K  H  W  P  F  D  V  I  D  D  N  T  K
 226 AGGAAGTTCAACGATCACAATGTGCAGAGCGACATGAAACACTGGCCGTTCGACGTGATCGATGATAACACAAAG
 101 P  K  I  K  V  E  Y  K  G  E  S  K  S  F  Y  P  E  E  I  S  S  M  V  L  I
 301 CCAAAGATCAAAGTAGAGTACAAGGGCGAGTCCAAGTCGTTCTACCCCGAGGAGATCTCATCCATGGTGCTGATC
 126 K  M  K  E  T  A  E  A  Y  L  G  T  T  V  K  D  A  V  I  T  V  P  A  Y  F
 376 AAGATGAAGGAGACGGCCGAAGCATATCTTGGCACCACCGTAAAGGACGCTGTCATCACCGTCCCGGCCTACTTC
 151 N  D  S  Q  R  Q  A  T  K  D  A  G  T  I  S  G  L  N  V  L  R  I  I  N  E
 451 AACGACTCCCAGCGTCAGGCCACCAAGGACGCTGGAACCATCTCTGGTCTCAATGTGCTGCGTATCATCAACGAA
 176 P  T  A  A  A  I  A  Y  G  L  D  K  K  V  G  G  E  R  N  V  L  I  F  D  L
 526 CCCACCGCCGCCGCCATCGCCTACGGTCTCGACAAGAAGGTAGGCGGTGAGCGCAACGTGCTCATCTTCGATCTG
 201 G  G  G  T  F  D  V  S  I  L  T  I  E  D  G  I  F  E  V  K  S  T  A  G  D
 601 GGCGGTGGGACCTTCGATGTATCCATCCTGACCATCGAGGATGGCATCTTCGAAGTAAAGTCCACCGCTGGAGAC
 226 T  H  L  G  G  E  D  F  D  N  R  M  V  N  H  F  L  Q  E  F  K  R  K  Y  K
 676 ACTCACTTGGGCGGGGAGGACTTTGACAACAGGATGGTGAACCACTTCCTCCAGGAGTTCAAGAGGAAATACAAG
 251 K  D  P  S  E  S  K  R  A  L  R  R  L  R  T  A  C  E  R  A  K  R  T  L  S
 751 AAGGATCCTTCTGAGAGTAAGCGTGCTCTCCGACGCCTCCGCACCGCCTGTGAACGTGCGAAGCGTACCCTCTCC
 276 S  S  A  Q  A  S  V  E  I  D  S  L  F  E  G  I  D  F  Y  T  S  I  T  R  A
 826 TCCTCCGCCCAGGCCAGCGTGGAGATCGACTCTCTCTTCGAGGGCATCGACTTCTACACCTCCATCACCCGTGCC
 301 R  F  E  E  L  C  A  D  L  F  R  G  T  L  E  P  V  E  K  A  L  R  D  A  K
 901 CGCTTCGAGGAGCTCTGCGCTGACCTGTTCCGCGGCACCCTGGAGCCCGTGGAGAAAGCCCTGCGTGATGCGAAG
 326 M  D  K  A  Q  I  H  D  I  V  L  V  G  G  S  T  R  I  P  K  I  Q  K  L  L
 976 ATGGACAAGGCCCAGATCCATGACATCGTGCTTGTGGGCGGTTCCACCCGCATTCCTAAGATCCAGAAGCTTCTG
 351 Q  D  F  F  N  G  K  E  L  N  K  S  I  N  P  D  E  A  V  A  Y  G  A  A  V
1051 CAGGACTTCTTCAACGGCAAAGAGCTCAACAAGTCCATCAACCCCGACGAGGCTGTGGCCTACGGCGCGGCGGTG
 376 Q  A  A  A  I  L  C  G  D  K  S  E  A  V  Q  D  L  L  L  L  D  V  T  P  L  S
1126 CAGGCCGCTATTCTGTGCGGCGACAAGTCCGAGGCCGTGCAGGACCTGCTGCTGCTCGACGTGACGCCTCTGTCC
 401 L  G  I  E  T  A  G  G  V  M  T  A  L  I  K  R  N  T  T  I  P  T  K  Q  T
1201 CTGGGTATCGAAACTGCCGGTGGCGTGATGACTGCTCTCATCAAGCGTAACACCACCATCCCCACTAAGCAGACT
 426 Q  T  F  T  T  Y  S  D  N  Q  P  G  V  L  I  Q  V  Y  E  G  E  R  A  M  T
1276 CAGACTTTCACCACCTACTCCGACAACCAGCCAGGTGTGCTCATCCAGGTATACGAGGGAGAGCGAGCCATGACA
 451 K  D  N  N  L  L  G  K  F  E  L  T  G  I  P  P  A  P  R  G  V  P  Q  I  E
1351 AAGGACAACAACCTTCTTGGCAAGTTCGAATTGACTGGCATTCCCCCAGCGCCCCGAGGCGTGCCTCAGATCGAG
 476 V  T  F  D  I  D  A  N  G  I  L  N  V  S  A  V  D  K  S  T  G  K  E  N  K
1426 GTGACCTTTGACATCGACGCTAACGGCATCTTGAATGTGTCTGCCGTGGACAAGTCGACTGGCAAAGAGAACAAG
 501 I  T  I  T  N  D  K  G  R  L  S  K  E  E  I  E  R  M  V  Q  D  A  E  K  Y
1501 ATCACTATCACCAACGACAAGGGCCGCCTCAGCAAGGAGGAGATCGAACGCATGGTGCAGGACGCCGAAAAGTAC
 526 K  A  E  D  E  K  Q  R  D  R  I  G  A  K  N  A  L  E  S  Y  C  F  N  M  K
1576 AAGGCCGAGGACGAGAAGCAGAGGGACCGTATCGGTGCCAAGAATGCCCTCGAGTCCTATTGCTTCAACATGAAG
 551 S  T  V  E  E  E  K  F  K  D  K  V  S  E  E  D  R  N  K  I  L  E  A  C  N
1651 TCCACCGTGGAGGAGGAGAAGTTCAAGGACAAGGTGAGCGAGGAAGACCGTAACAAGATCCTAGAGGCCTGCAAC
 576 D  A  I  K  W  L  D  A  N  Q  L  G  E  K  E  E  Y  E  H  K  Q  K  D  L  E
1726 GACGCCATCAAGTGGCTTGATGCCAACCAGCTGGGTGAGAAGGAAGAGTACGAACACAAGCAGAAGGATCTCGAA
 601 Q  I  C  N  P  I  I  T  K  M  Y  Q  A  A  G  G  A  P  P  G  G  M  P  G  G
1801 CAGATCTGCAACCCCATTATCACCAAGATGTACCAGGCCGCAGGCGGTGCTCCTCCAGGTGGCATGCCTGGTGGC
 626 F  P  G  A  G  G  A  P  G  A  A  P  G  G  G  S  S  G  P  T  I  E  E  V  D
1876 TTCCCTGGTGCTGGCGGCGCCCCAGGTGCTGCCCCCGGCGGTGGTTCCTCTGGACCCACCATCGAGGAGGTCGAC
 651 *
1951 TAA
```

Figure 2-13. Consensus sequence of the isolated nucleotide and deduced amino acid sequences encoding *HSP70* in *G. lateralis* and *C. maenas*. Green: heat shock protein 70 (HSP70). The amino acid position is marked below the figure, the position of the nucleic acid is marked above the figure.

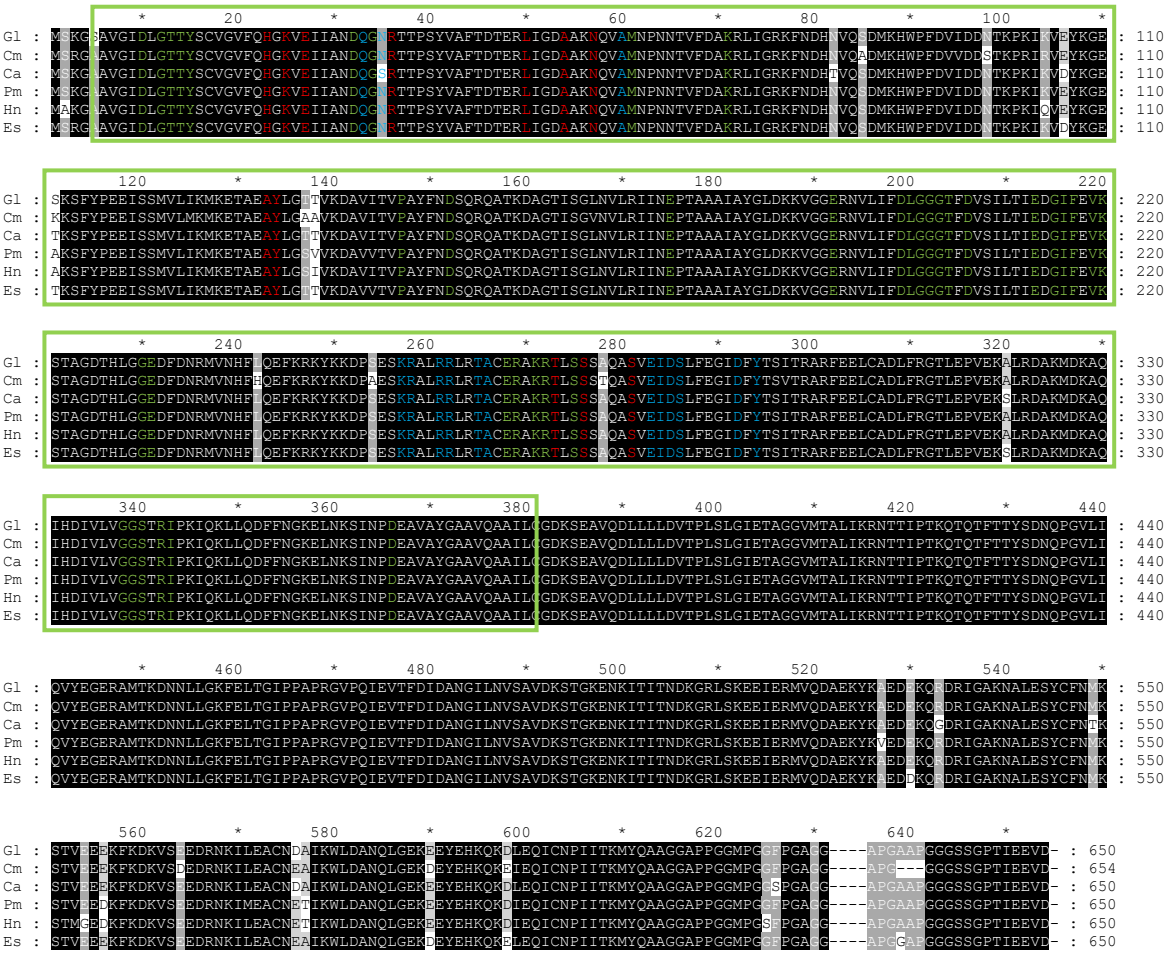


Figure 2-14. Multiple alignment of deduced amino acid sequences of the *HSP70* in *G. lateralis* and *C. maenas* and orthologs from other crustacean species. **Green box**: heat shock protein 70 (HSP70); **green letter**: nucleotide binding site, **blue letter**: BAG/HSP70 interaction site; **red letter**: NEF/HSP70 interaction site. Gl: *Gecarcinus lateralis* c237074_g1_i1, Cm: *Carcinus maenas* contig_345 (Tepolt and Palumbi, 2015) accession https://doi.org/10.5061/dryad.g8b96, Ca: *Cardisoma armatum* accession KU613077.1, Pm: *Pachygrapsus marmoratus* accession DQ173922.1, Hn: *Hemigrapsus nudus* accession KU613086.1, Es: *Eriocheir sinensis* accession EU857483.1. Amino acid residues shaded in black are 100% identical in all sequences, shaded in dark gray are 80% similar in all sequences, and shaded in light gray are 60% similar in all sequences.

```
   1 M  G  P  Q  M  S  P  Q  A  G  N  F  A  P  G  Y  H  G  G  A  T  I  P  G  T
   1 ATGGGGCCCCAGATGAGCCCTCAAGCAGGGAACTTTGCGCCAGGTTACCATGGCGGAGCGACCATTCCCGGGACT
  26 Q  C  M  S  S  P  M  A  P  P  D  C  R  S  S  G  Y  P  V  S  F  G  Q  V  A
  76 CAGTGCATGTCGTCGCCCATGGCCCCGCCCGACTGCAGGTCGTCAGGCTACCCGGTCAGCTTCGGGCAGGTGGCA
  51 P  M  E  A  S  R  T  P  Y  G  N  P  H  A  H  S  Y  A  G  Q  L  C  A  G  Y
 151 CCCATGGAGGCCAGCAGAACGCCCTACGGCAACCCTCACGCCCACAGCTACGCGGGGCAGCTCTGCGCCGGCTAC
  76 N  T  P  L  D  N  T  N  G  Y  D  R  Q  V  F  R  S  P  P  A  M  P  Q  G  Q
 226 AACACGCCCTTAGACAATACTAACGGATACGACCGTCAGGTGTTCCGCTCGCCGCCCGCCATGCCTCAGGGCCAA
 101 V  F  P  E  Y  R  A  A  H  Y  R  E  Y  P  P  P  L  E  E  H  R  P  P  G  C
 301 GTGTTCCCCGAGTACCGCGCGGCTCACTACAGGGAGTACCCGCCGCCCCTTGAGGAGCACCGCCCGCCAGGTTGT
 126 E  G  R  L  P  E  Q  G  H  L  I  P  K  T  E  P  E  A  R  P  D  A  E  L  Q
 376 GAGGGCCGCCTGCCGGAACAGGGGCACCTCATCCCCAAGACTGAGCCTGAGGCGCGGCCCGACGCAGAACTACAG
 151 D  A  Q  P  I  K  L  P  R  K  R  G  R  N  S  D  K  R  K  E  Q  S  R  N  A
 451 GACGCCCAACCCATCAAACTACCGCGGAAAAGAGGCAGAAACAGCGACAAGCGAAAGGAACAGTCCCGCAATGCC
 176 A  R  N  R  R  G  K  E  S  Q  I  F  N  E  L  A  S  V  L  P  L  P  P  Q  T
 526 GCCCGCAACCGCCGAGGGAAGGAGAGTCAGATATTCAACGAGCTGGCCTCGGTGCTTCCACTCCCCCCTCAGACC
 201 I  H  Q  L  D  K  A  S  I  M  R  L  T  I  T  K  L  E  V  R  A  L  L  E  T
 601 ATCCACCAGCTGGACAAAGCCTCCATCATGCGGCTCACCATCACCAAACTGGAGGTCCGCGCCCTGCTTGAAACT
 226 G  L  T  K  R  I  K  G  S  G  G  S  K  L  D  Q  E  M  D  E  H  H  M  K  A
 676 GGACTCACGAAGCGCATCAAGGGCAGCGGCGGGAGCAAGCTGGACCAGGAGATGGACGAGCACCACATGAAGGCG
 251 L  D  G  F  L  L  V  V  N  T  D  G  R  V  I  Y  T  S  E  N  I  A  T  F  L
 751 CTGGACGGCTTCCTGCTGGTGGTGAACACGGACGGCCGCGTCATCTACACCTCAGAGAACATCGCCACATTCCTG
 276 G  H  Q  Q  E  E  V  M  G  S  S  L  Y  H  Y  T  N  M  V  D  H  S  E  V  E
 826 GGACACCAACAGGAGGAGGTGATGGGCAGCTCCCTGTACCACTACACCAACATGGTGGACCACAGCGAGGTGGAG
 301 Q  L  T  S  F  K  N  P  H  Q  P  R  R  A  F  L  R  L  K  C  T  L  T  S  K
 901 CAGCTGACGTCCTTCAAGAATCCCCACCAGCCCCGCCGCGCCTTCCTTCGCCTCAAGTGCACCCTGACTTCCAAG
 326 G  R  S  V  N  F  K  N  A  T  D  K  V  V  Q  V  I  G  E  V  V  G  E  K  A
 976 GGCCGCTCCGTCAACTTCAAAAACGCCACCGACAAGGTGGTGCAGGTGATCGGTGAGGTGGTGGGCGAGAAGGCA
 351 E  K  A  W  L  V  A  L  A  I  P  V  P  H  P  S  N  I  E  F  P  L  D  K  Q
1051 GAGAAGGCCTGGCTGGTGGCCCTGGCCATCCCGGTGCCGCATCCCTCAAACATTGAATTTCCGCTCGACAAGCAA
 376 T  F  V  S  R  H  S  L  D  M  K  F  T  Y  V  D  T  N  V  K  E  F  C  G  Y
1126 ACCTTCGTCAGCAGACACTCCCTCGACATGAAGTTCACCTACGTCGACACCAACGTGAAGGAATTCTGCGGCTAC
 401 A  S  E  E  L  V  G  R  S  V  Y  E  L  H  H  A  L  D  T  M  L  I  Q  D  A
1201 GCGTCGGAGGAGCTCGTGGGTCGCTCGGTTTACGAACTGCACCACGCACTTGACACCATGCTCATCCAGGACGCC
 426 Y  K  N  L  L  N  K  G  Q  V  E  T  S  R  Y  R  F  L  A  R  G  G  G  Y  V
1276 TACAAGAACCTGCTGAACAAGGGACAAGTGGAGACAAGCCGTTACCGCTTCCTGGCTCGCGGCGGAGGCTATGTG
 451 W  L  V  T  Q  A  T  L  I  H  G  P  R  E  N  K  P  Q  Y  V  V  C  L  N  Y
1351 TGGCTGGTGACACAGGCTACCCTCATTCATGGACCCCGGGAGAACAAGCCCCAATACGTTGTCTGCCTCAACTAC
 476 V  V  S  E  V  E  S  K  D  E  I  L  S  E  F  Q  L  M  C  S  S  S  N  S  D
1426 GTGGTCAGTGAAGTTGAGTCCAAGGATGAGATTCTTTCCGAGTTCCAGTTGATGTGTAGCAGTAGCAACAGCGAC
 501 S  S  D  T  N  N  N  D  S  E  K  E  I  T  S  T  S  S  K  A  S  L  K  S  G
1501 AGTAGTGATACCAATAATAACGACAGCGAAAAAGAGATTACCAGCACCAGCAGCAAAGCAAGCCTCAAGTCTGGC
 526 P  V  N  V  P  V  P  V  A  S  P  V  V  Q  G  L  P  T  P  P  Y  S  P  A  P
1576 CCCGTGAATGTACCTGTGCCCGTGGCCTCCCCAGTTGTACAGGGCTTGCCTACCCCACCCTACTCACCTGCCCCC
 551 R  T  T  T  A  Q  K  V  S  E  S  S  N  P  R  S  T  T  A  P  L  P  P  P  P
1651 AGGACCACCACAGCCCAGAAGGTGAGTGAAAGCTCCAACCCACGCAGCACCACAGCTCCTCTGCCACCACCACCA
 576 P  V  A  S  T  F  K  I  F  V  P  R  T  K  D  M  N  K  G  F  L  T  F  N  N
1726 CCTGTTGCCAGCACCTTTAAGATCTTTGTGCCACGAACAAAGGATATGAACAAAGGGTTCCTGACCTTCAATAAT
 601 N  D  P  H  S  T  V  L  K  D  E  P  E  D  L  T  H  L  A  P  S  G  G  D  T
1801 AATGATCCACACTCCACAGTGCTGAAGGACGAACCAGAAGACCTGACTCACCTTGCCCCATCGGGTGGTGATACT
 626 C  V  P  L  P  T  L  I  P  D  L  E  E  D  S  C  M  P  Q  D  V  S  T  F  I
1876 TGTGTGCCACTGCCTACACTGATACCTGACCTGGAAGAGGATTCATGTATGCCCCAAGATGTTTCCACATTCATA
 651 P  S  L  D  D  M  L  T  L  E  Y  P  L  Q  V  V  N  S  D  A  V  I  R  S  S
1951 CCAAGCCTAGACGATATGTTAACTCTAGAATACCCACTGCAAGTTGTGAACTCTGATGCTGTCATACGAAGCTCA
 676 S  A  E  K  I  D  D  Q  D  L  S  P  K  Y  L  Y  E  E  N  Q  I  Q  E  G  V
2026 AGTGCAGAGAAAATTGACGACCAAGACTTGAGCCCCAAGTATTTGTATGAAGAAAACCAGATACAAGAAGGAGTC
 701 K  S  V  N  S  L  S  G  G  R  L  L  L  N  R  S  R  C  N  T  P  S  P  D  C
2101 AAGTCTGTAAACAGCTTAAGTGGTGGGAGACTGTTACTTAACAGGAGCAGGTGCAACACACCATCACCAGACTGT
 726 N  S  V  H  S  G  G  L  G  T  P  E  P  P  K  P  L  L  S  E  A  A  L  P  Q
2176 AACAGTGTACACTCCGGTGGATTGGGTACACCAGAGCCCCCAAAGCCTCTGTTGAGTGAGGCTGCCCTCCCACAG
 751 P  I  K  K  Q  S  P  I  P  F  G  S  S  H  P  R  T  T  T  E  S  L  F  M  P
2251 CCCATCAAAAAACAATCACCGATTCCATTCGGAAGCAGTCATCCCAGAACCACCACGGAGTCTCTCTTCATGCCG
 776 L  D  E  S  N  S  G  G  S  S  E  L  F  S  K  L  D  L  K  F  E  N  Q  N  M
```

```
2326 CTAGATGAAAGCAATTCTGGAGGCTCCTCAGAGCTGTTCAGTAAATTGGATCTCAAGTTTGAGAATCAGAACATG
 801 D  S  D  E  F  E  M  R  A  P  Y  I  P  P  S  N  E  L  L  L  F  L  D  H  E
2401 GACTCTGATGAATTTGAAATGAGAGCACCATACATTCCTCCAAGTAATGAACTTTTGTTATTTCTGGACCATGAG
 826 D  L  F  S  G  P  E  S  E  I  A  I  S  P  K  C  L  R  D  G  K  F  S  T  A
2476 GATCTCTTCTCAGGGCCAGAGTCAGAAATTGCCATCTCCCCTAAATGTCTCAGGGATGGAAAGTTTTCTACTGCT
 851 T  D  K  E  D  S  S  L  A  Q  L  L  R  D  T  D  P  S  I  A  G  N  S  P  G
2551 ACAGACAAAGAAGACTCCAGTCTGGCTCAGCTTTTGCGAGACACAGACCCCAGCATAGCTGGGAATAGTCCTGGC
 876 R  N  I  Q  L  D  H  S  P  G  V  Q  R  S  Q  Y  Q  Q  N  K  F  L  D  G  G
2626 AGAAATATCCAACTTGATCACTCTCCAGGGGTTCAGAGGAGCCAGTATCAACAGAACAAATTCTTGGATGGTGGA
 901 G  N  F  V  D  P  N  K  V  L  P  G  H  F  A  G  K  D  E  L  E  S  I  G  S
2701 GGAAATTTTGTAGATCCAAATAAAGTGCTGCCTGGACACTTCGCTGGCAAAGATGAGCTGGAAAGCATTGGAAGT
 926 D  S  Q  L  A  D  P  P  P  V  L  V  Q  E  P  V  E  P  P  P  P  L  L  G  T
2776 GACTCCCAACTTGCGGACCCACCCCCTGTACTTGTGCAGGAACCTGTGGAGCCACCGCCTCCCCTGCTGGGCACA
 951 D  S  Y  H  T  S  G  Q  V  K  R  G  H  S  P  L  S  S  P  T  L  H  H  K  K
2851 GATTCGTATCACACTTCAGGGCAGGTGAAGCGAGGTCACTCGCCACTCTCTTCACCCACACTCCACCACAAAAAG
 976 L  C  P  P  K  C  Q  Q  P  Q  Q  K  V  P  E  R  C  Q  D  G  G  N  N  H  Q
2926 CTGTGTCCACCTAAATGTCAACAGCCACAACAAAAGGTTCCTGAACGATGTCAAGATGGAGGGAATAACCATCAA
1001 Q  N  Q  Q  H  G  G  L  R  L  L  T  P  H  T  P  I  M  Q  Q  L  L  I  S  K
3001 CAGAATCAGCAACATGGAGGACTGCGCTTGCTCACTCCACACACCCCCATTATGCAACAGTTGTTAATCAGCAAG
1026 D  P  I  T  V  R  G  G  R  P  T  A  G  G  I  S  A  S  R  N  L  L  T  D  K
3076 GATCCGATCACTGTACGTGGAGGACGTCCCACTGCTGGAGGTATCTCAGCCTCCAGGAATTTACTTACTGACAAG
1051 S  H  S  V  L  R  N  L  L  N  V  N  E  D  N  S  I  V  L  K  D  A  H  G  G
3151 AGTCACTCAGTGCTTCGGAACCTACTGAATGTAAATGAAGACAACAGCATAGTACTAAAGGATGCTCATGGAGGT
1076 G  L  T  S  T  P  T  A  L  R  I  P  K  D  R  M  A  T  M  L  L  T  S  T  G
3226 GGTCTTACTTCCACTCCCACAGCCTTACGTATTCCCAAAGACAGAATGGCTACTATGTTGCTGACCAGCACTGGA
1101 G  K  S  G  T  S  L  S  Y  P  T  L  R  L  V  A  G  G  P  T  S  L  K  Q  A
3301 GGCAAGTCAGGAACCTCTCTGTCTTATCCTACACTGAGATTAGTAGCAGGAGGCCCCACCAGTTTGAAGCAAGCA
1126 F  K  L  T  T  N  S  S  S  H  S  E  G  C  G  S  R  F  G  Q  R  Q  D  P  F
3376 TTCAAACTGACTACAAACTCAAGCAGTCATTCTGAAGGATGTGGCAGCAGGTTTGGCCAGCGACAAGATCCCTTT
1151 L  L  M  S  S  D  T  I  P  T  L  L  E  L  T  Q  Q  D  Y  E  V  N  A  P  A
3451 TTGCTGATGAGCTCAGACACCATCCCCACTTTGCTAGAGCTGACCCAGCAGGACTATGAGGTCAATGCACCAGCC
1176 N  N  S  L  L  Q  G  A  E  L  L  M  A  L  D  Q  S  S  E  A  A  L  L  D  G
3526 AACAACAGTCTCCTTCAGGGAGCAGAACTCCTCATGGCACTGGACCAAAGCAGTGAAGCAGCTTTGCTTGATGGG
1201 K  *
3601 AAGTAA
```

Figure 2-15. Consensus sequence of the isolated nucleotide and deduced amino acid sequences encoding *HIFα* in *G. lateralis* and *C. maenas*. Green: basic Helix-Loop-Helix domain, 60-100 amino acids long; **blue**: PAS domain A/B, sensor for and oxygen in signal transduction; **red**: C-terminal transactivation domain. The amino acid position is marked below the figure, the position of the nucleic acid is marked above the figure.

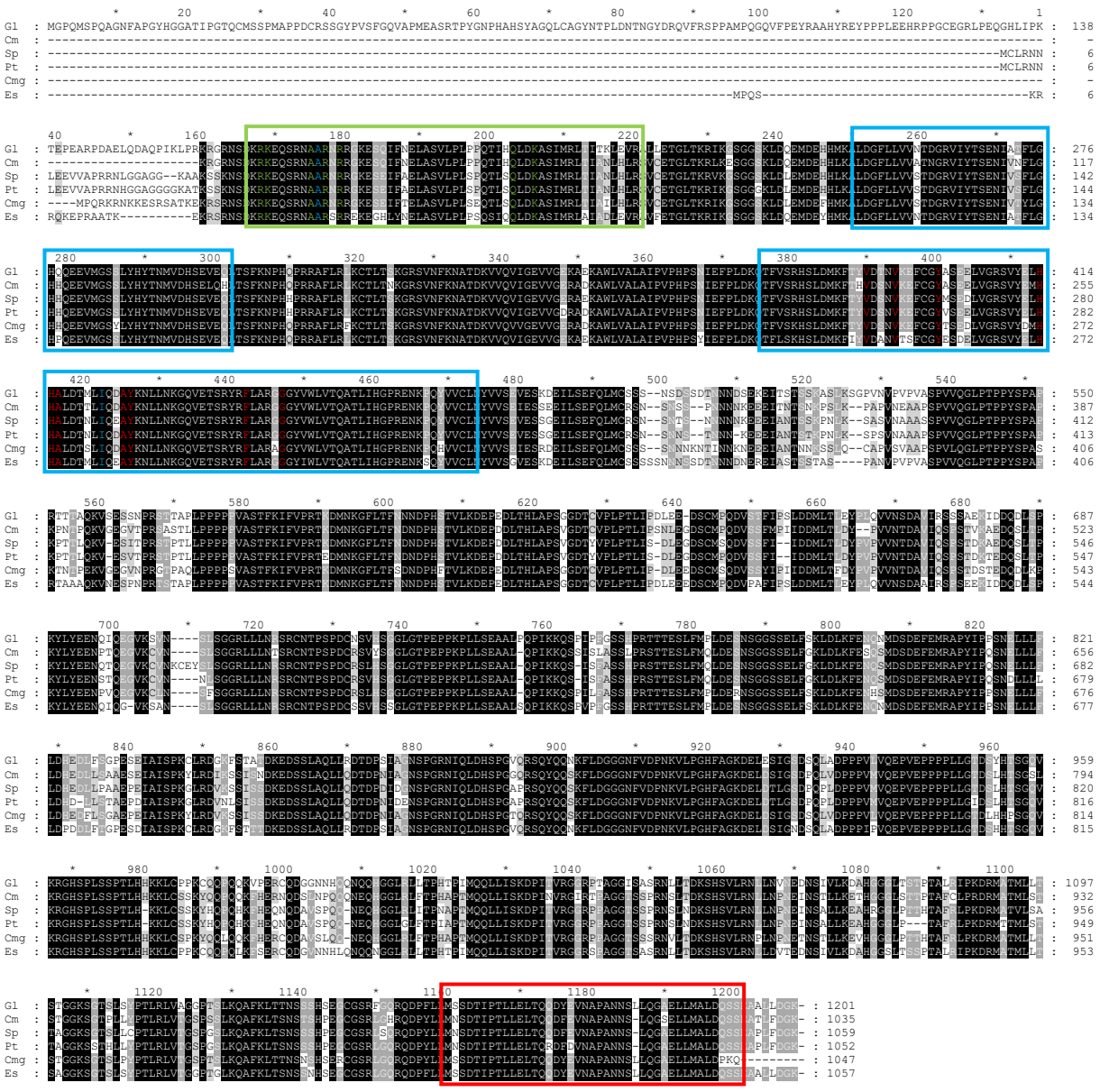


Figure 2-16. Multiple alignment of deduced amino acid sequences of the *HIFα* in *G. lateralis* and *C. maenas* and orthologs from other crustacean species. **Green box**: Helix-loop-helix domain, 60-100 amino acids long; **blue box**: PAS domain A/B, sensor for and oxygen in signal transduction; **red box**: C-terminal transactivation domain; **green letter**: DNA binding site, **blue letter**: E-box/N-box specificity site; **red letter**: putative active site. Gl: *Gecarcinus lateralis* c240194_g1_i2, Cm: *Carcinus maenas* comp88298_c2_seq1 (Verbruggen et al., 2015) accession GBXE01120594.1, Sp: *Scylla paramamosain* accession KU644140.1, Pt: *Portunus trituberculatus* accession KU681173.1, Cmg: *Cancer magister* accession DQ535030.1, Es: *Eriocheir sinensis* accession KF825558.1. Amino acid residues shaded in black are 100% identical in all sequences, shaded in dark gray are 80% similar in all sequences, and shaded in light gray are 60% similar in all sequences.

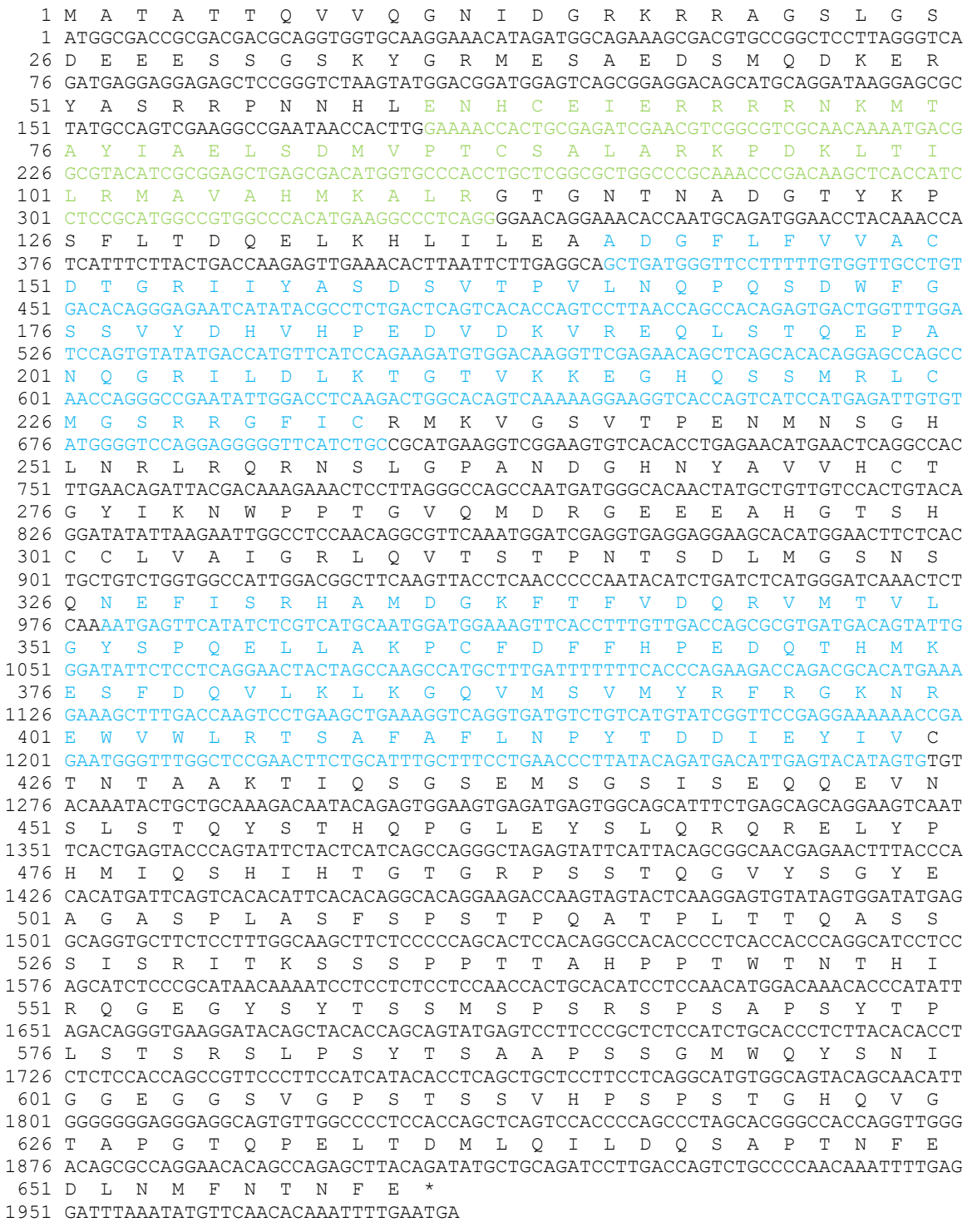


Figure 2-17. Consensus sequence of the isolated nucleotide and deduced amino acid sequences encoding *HIFβ* in *G. lateralis* and *C. maenas*. Green: basic Helix-Loop-Helix domain, 60-100 amino acids long; **blue**: PAS domain A/B, sensor for and oxygen in signal transduction. The amino acid position is marked below the figure, the position of the nucleic acid is marked above the figure.

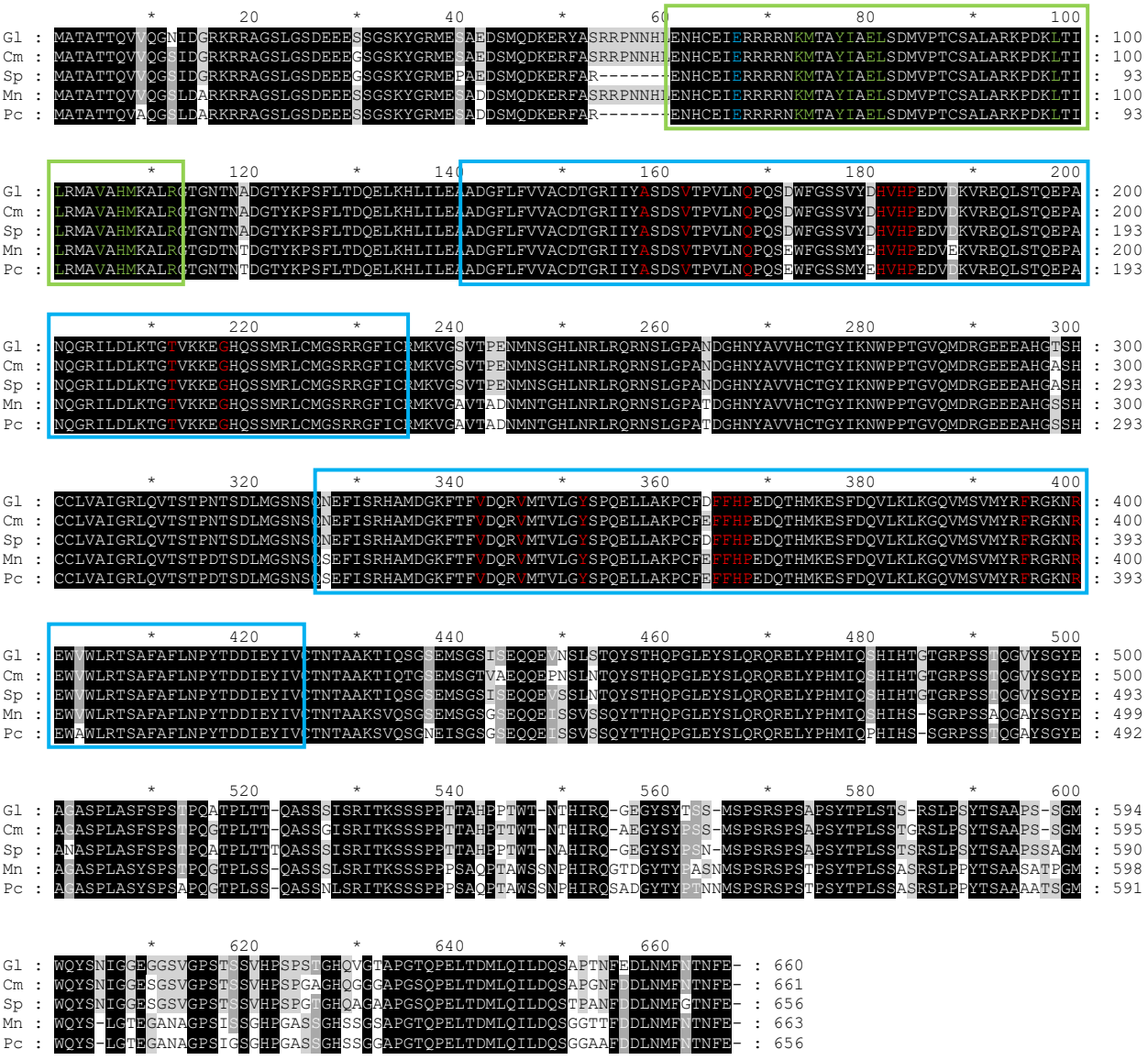


Figure 2-18. Multiple alignment of deduced amino acid sequences of the *HIFβ* in *G. lateralis* and *C. maenas* and orthologs from other crustacean species. **Green box**: basic Helix-Loop-Helix domain, 60-100 amino acids long; **blue box**: PAS domain A/B, sensor for and oxygen in signal transduction; **green letter**: E-box/N-box specificity site, **blue letter**: dimerization interface; **red letter**: putative active site. Gl: *Gecarcinus lateralis* c231200_g1_i2, Cm: *Carcinus maenas* comp86713_c2_seq1 (Verbruggen et al., 2015) accession GBXE01106236.1, Sp: *Scylla paramamosain* accession KU644141.1, Mn: *Macrobrachium nipponense* accession KP050353.1, Pc: *Palaemon carinicauda* accession KU647692.1. Amino acid residues shaded in black are 100% identical in all sequences, shaded in dark gray are 80% similar in all sequences, and shaded in light gray are 60% similar in all sequences.

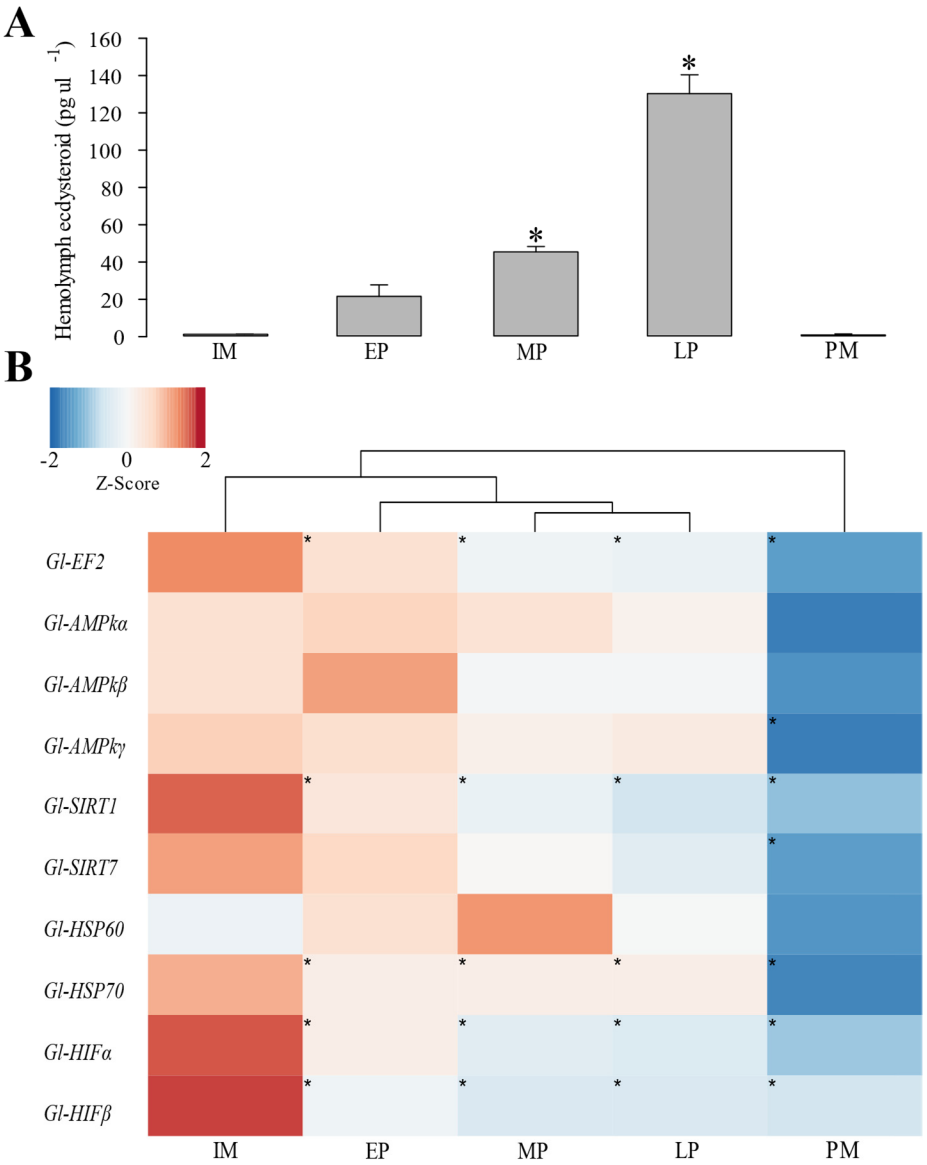
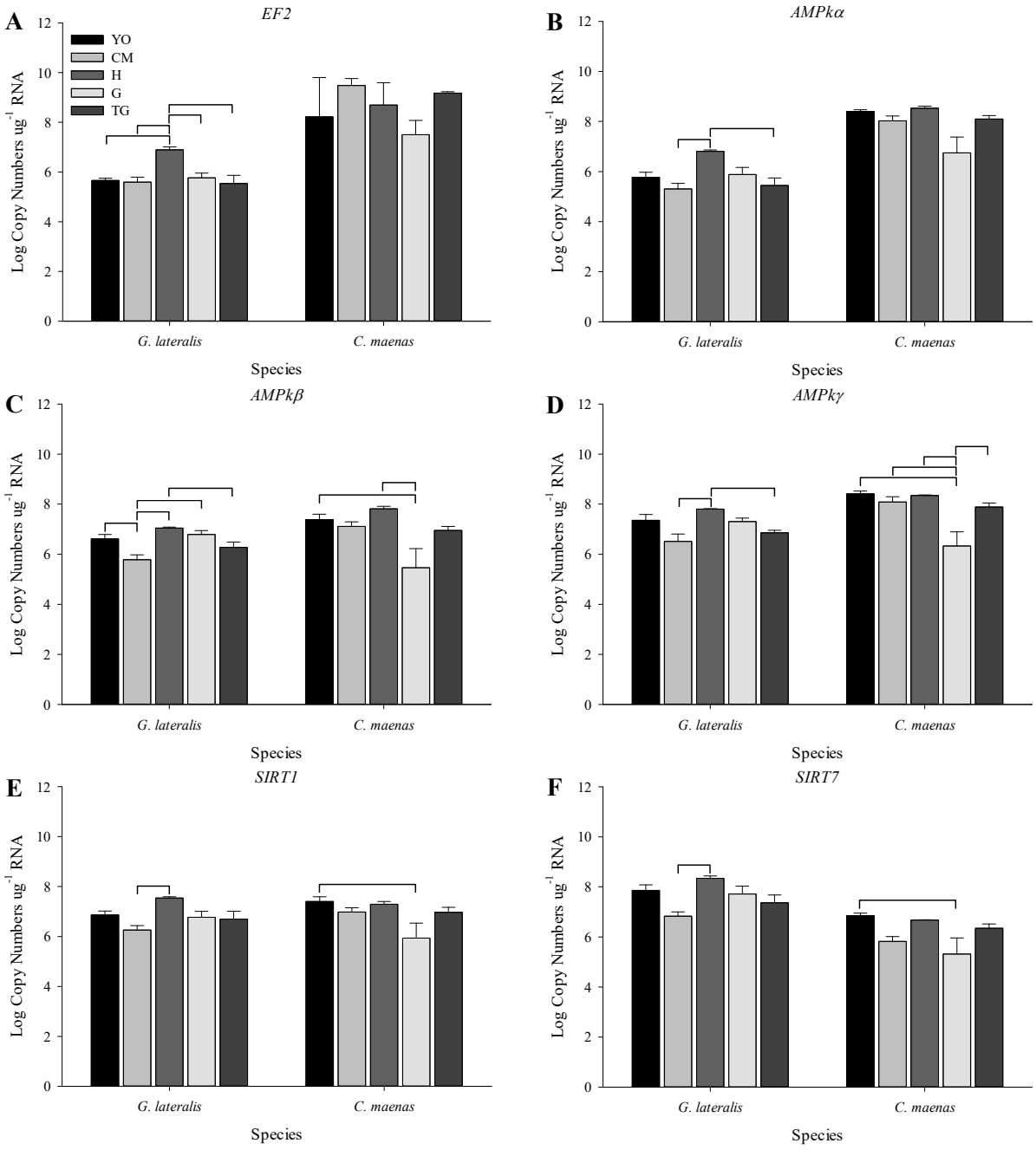


Figure 2-19. Ecdysteroid titers and heatmap of gene expression values depicting clustering of genes of stress-response in the molt cycle of *G. lateralis*. (A) Ecdysteroid titers obtained from Das et al. (2018), (B) Mean $\log_{10}$ FPKM values were used to depict the expression across five molt cycle stages obtained from Das et al. (2018). Molt cycle stages are represented in columns and genes are represented in rows. Genes are clustered together based on a Euclidean average similarity across five molt cycle stages. Low to high expression is represented by a change of color from blue to red, respectively. The color key scale bar at upper left shows Z-score values for the heatmap. Relative expression was mostly highest in IM and lowest in PM. Values are mean ± S.E. M. (n = 3). *: significant differences compared to IM stage ($P < 0.05$).


A
EF2
YO
CM
H
G
TG
Log Copy Numbers ug^{-1} RNA
G. lateralis
C. maenas
Species
B
AMPkα
C
AMPkβ
D
AMPkγ
E
SIRT1
F
SIRT7

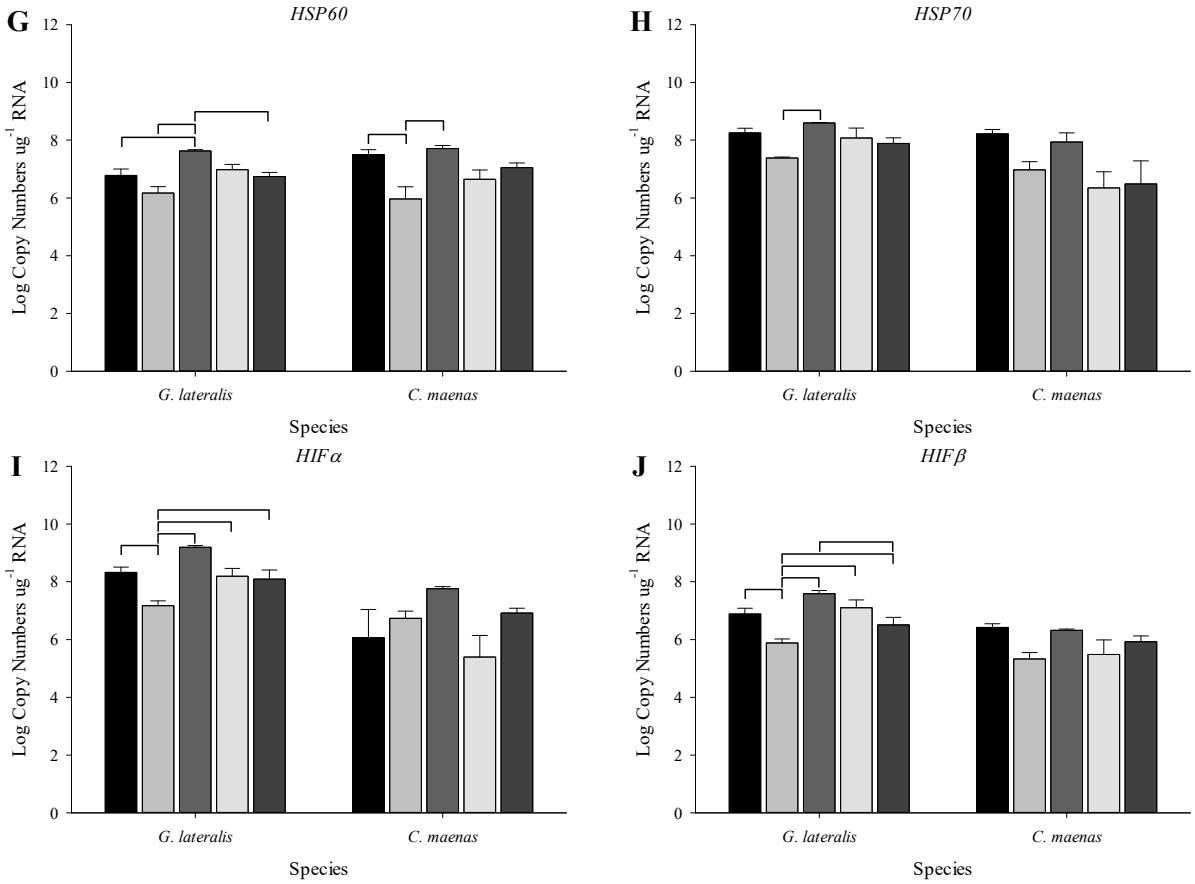


Figure 2-20. Tissue expression of *EF2*, *AMPkα*-, *β*-, and *γ*-, *SIRT1*, *SIRT7*, *HSP60*, *HSP70*, *HIFα*, and *β* in *G. lateralis* and *C. maenas*. (A) MLA-*EF2*, (B) MLA-*AMPKα*, (C) MLA-*AMPkβ*, (D) MLA-*AMPkγ*, (E) MLA-*SIRT1*, (F) MLA-*SIRT7*, (G) MLA-*HSP60*, (H) MLA-*HSP70*, (I) MLA-*HIFα*, (J) MLA-*HIFβ*. QPCR was used to qualitatively assess the tissue distribution of genes of stress response in Y-organ (YO), claw muscle (CM), heart (H), gill (G), and thoracic ganglion (TG). Values are means ± S.E. M. (n = 3). Bars that share a bracket indicate significant differences among different tissues ($P < 0.05$).

REFERENCES

Abuhagr, A. M., MacLea, K. S., Mudron, M. R., Chang, S. A., Chang, E. S. and Mykles, D. L. (2016). Roles of mechanistic target of rapamycin and transforming growth factor-beta signaling in the molting gland (Y-organ) of the blackback land crab, *Gecarcinus lateralis*. *Comp Biochem Physiol A Mol Integr Physiol* **198**, 15-21.

Baringou, S., Rouault, J. D., Koken, M., Hardivillier, Y., Hurtado, L. and Leignel, V. (2016). Diversity of cytosolic HSP70 Heat Shock Protein from decapods and their phylogenetic placement within Arthropoda. *Gene* **591**, 97-107.

Behrens Yamada, S., Peterson, W. T. and Kosro, P. M. (2015). Biological and physical ocean indicators predict the success of an invasive crab, *Carcinus maenas*, in the northern California Current. *Marine Ecology Progress Series* **537**, 175-189.

Bliss, D. E., van Montfrans, J., van Montfrans, M. and Boyer, J. R. (1978). Behavior and growth of the land crab *Gecarcinus lateralis* (Fréminville) in southern Florida. *Bull. Am. Mus. Nat. Hist* **160**, 111-152.

Burden, C. T., Stow, A. J., Hoggard, S. J., Coleman, M. A. and Bishop, M. J. (2014). Genetic structure of *Carcinus maenas* in southeast Australia. *Marine Ecology Progress Series* **500**, 139-147.

Canto, C., Gerhart-Hines, Z., Feige, J. N., Lagouge, M., Noriega, L., Milne, J. C., Elliott, P. J., Puigserver, P. and Auwerx, J. (2009). AMPK regulates energy expenditure by modulating NAD+ metabolism and SIRT1 activity. *Nature* **458**, 1056-60.

Carafa, V., Nebbioso, A. and Altucci, L. (2012). Sirtuins and disease: the road ahead. *Front Pharmacol* **3**, 4.

Cetrullo, S., D'Adamo, S., Tantini, B., Borzi, R. M. and Flamigni, F. (2015). mTOR, AMPK, and Sirt1: key players in metabolic stress management. *Crit Rev Eukaryot Gene Expr* **25**, 59-75.

Chang, E. S., Chang, S. A., Keller, R., Reddy, P. S., Snyder, M. J. and Spees, J. L. (1999). Quantification of stress in lobsters: crustacean hyperglycemic hormone, stress proteins, and gene expression. *American Zoologist* **39**, 487-495.

Chang, E. S. and Mykles, D. L. (2011). Regulation of crustacean molting: a review and our perspectives. *Gen Comp Endocrinol* **172**, 323-30.

Chaves, M. L., Horta, M. S., Chainho, P., Costa, M. J. and Costa, J. L. (2010). New additions to the feeding ecology of Carcinus maenas (L., 1758) in a South-western Europe estuary (Portugal). *Cahiers De Biologie Marine* **51**, 229-238.

Chen, T., Lin, T., Li, H., Lu, T., Li, J., Huang, W., Sun, H., Jiang, X., Zhang, J., Yan, A. et al. (2018). Heat shock protein 40 (HSP40) in Pacific white shrimp (*Litopenaeus vannamei*): molecular cloning, tissue distribution and ontogeny, response to temperature, acidity/alkalinity and salinity stresses, and potential role in ovarian development. *Front Physiol* **9**, 1784.

Chung, J. S. and Webster, S. G. (2003). Moult cycle-related changes in biological activity of moult-inhibiting hormone (MIH) and crustacean hyperglycaemic hormone (CHH) in the crab, *Carcinus maenas*. From target to transcript. *Eur J Biochem* **270**, 3280-8.

Chung, J. S. and Webster, S. G. (2005). Dynamics of in vivo release of molt-inhibiting hormone and crustacean hyperglycemic hormone in the shore crab, *Carcinus maenas*. *Endocrinology* **146**, 5545-51.

Cosham, J. A., Beazley, K. F. and McCarthy, C. (2016). Local knowledge of

distribution of European green crab (*Carcinus maenas*) in southern Nova Scotian coastal waters. *Human Ecology* **44**, 409-424.

Das, S., Pitts, N. L., Mudron, M. R., Durica, D. S. and Mykles, D. L. (2016). Transcriptome analysis of the molting gland (Y-organ) from the blackback land crab, *Gecarcinus lateralis*. *Comp Biochem Physiol Part D Genomics Proteomics* **17**, 26-40.

Das, S., Vraspir, L., Zhou, W., Durica, D. S. and Mykles, D. L. (2018). Transcriptomic analysis of differentially expressed genes in the molting gland (Y-organ) of the blackback land crab, *Gecarcinus lateralis*, during molt-cycle stage transitions. *Comp Biochem Physiol Part D Genomics Proteomics* **28**, 37-53.

Dong, Y.-w., Zhang, S. and Woods, A. (2016). Ecological relevance of energy metabolism: transcriptional responses in energy sensing and expenditure to thermal and osmotic stresses in an intertidal limpet. *Functional Ecology* **30**, 1539-1548.

Dong, Y. W., Han, G. D. and Huang, X. W. (2014). Stress modulation of cellular metabolic sensors: interaction of stress from temperature and rainfall on the intertidal limpet *Cellana toreuma*. *Mol Ecol* **23**, 4541-54.

Feder, M. E. and Hofmann, G. E. (1999). Heat-shock proteins, molecular chaperones, and the stress response: evolutionary and ecological physiology. *Annu Rev Physiol* **61**, 243-82.

Frye, R. A. (2000). Phylogenetic classification of prokaryotic and eukaryotic Sir2-like proteins. *Biochem Biophys Res Commun* **273**, 793-8.

Gillum, M. P., Erion, D. M. and Shulman, G. I. (2011). Sirtuin-1 regulation of mammalian metabolism. *Trends Mol Med* **17**, 8-13.

Gonzalez-Aravena, M., Calfio, C., Mercado, L., Morales-Lange, B., Bethke, J., De Lorgeril, J. and Cardenas, C. A. (2018). HSP70 from the Antarctic sea urchin Sterechinus

neumayeri: molecular characterization and expression in response to heat stress. *Biol Res* **51**, 8.

Greiss, S. and Gartner, A. (2009). Sirtuin/Sir2 phylogeny, evolutionary considerations and structural conservation. *Mol Cells* **28**, 407-15.

Gu, S. H., Chen, C. H., Hsieh, Y. C., Lin, P. L. and Young, S. C. (2015). Modulatory effects of bombyxin on ecdysteroidogenesis in Bombyx mori prothoracic glands. *J Insect Physiol* **72**, 61-69.

Hammer, Ø., Harper, D. A. T. and Ryan, P. D. (2001). PAST: paleontological statistics software package for education and data analysis. *Palaeontologia Electronica* **4**, 9.

Han, G. D., Zhang, S., Marshall, D. J., Ke, C. H. and Dong, Y. W. (2013). Metabolic energy sensors (AMPK and SIRT1), protein carbonylation and cardiac failure as biomarkers of thermal stress in an intertidal limpet: linking energetic allocation with environmental temperature during aerial emersion. *J Exp Biol* **216**, 3273-82.

Jeon, S. M. (2016). Regulation and function of AMPK in physiology and diseases. *Exp Mol Med* **48**, e245.

Kaur, A. and Sharma, S. (2017). Mammalian target of rapamycin (mTOR) as a potential therapeutic target in various diseases. *Inflammopharmacology* **25**, 293-312.

King, A. M. and MacRae, T. H. (2015). Insect Heat Shock Proteins During Stress and Diapause. In *Annual Review of Entomology, Vol 60*, vol. 60: *Annual Review of Entomology*. (ed. M. R. Berenbaum), pp. 59-75.

Klumpen, E., Hoffschroer, N., Zeis, B., Gigengack, U., Dohmen, E. and Paul, R. J. (2017). Reactive oxygen species (ROS) and the heat stress response of *Daphnia pulex*: ROS-mediated activation of hypoxia-inducible factor 1 (HIF-1) and heat shock factor 1 (HSF-1) and the clustered expression of stress genes. *Biol Cell* **109**, 39-64.

Laemmle, A., Lechleiter, A., Roh, V., Schwarz, C., Portmann, S., Furer, C., Keogh, A., Tschan, M. P., Candinas, D., Vorburger, S. A. et al. (2012). Inhibition of SIRT1 impairs the accumulation and transcriptional activity of HIF-1alpha protein under hypoxic conditions. *PLoS One* **7**, e33433.

Larkin, M. A., Blackshields, G., Brown, N. P., Chenna, R., McGettigan, P. A., McWilliam, H., Valentin, F., Wallace, I. M., Wilm, A., Lopez, R. et al. (2007). Clustal W and Clustal X version 2.0. *Bioinformatics* **23**, 2947-8.

Li, T. and Brouwer, M. (2007). Hypoxia-inducible factor, gsHIF, of the grass shrimp *Palaemonetes pugio*: molecular characterization and response to hypoxia. *Comp Biochem Physiol B Biochem Mol Biol* **147**, 11-9.

Lv, J., Liu, P., Gao, B., Wang, Y., Wang, Z., Chen, P. and Li, J. (2014). Transcriptome analysis of the Portunus trituberculatus: de novo assembly, growth-related gene identification and marker discovery. *PLoS One* **9**, e94055.

Mayer, M. P., Brehmer, D., Gassler, C. S. and Bukau, B. (2001). Hsp70 chaperone machines. *Adv Protein Chem* **59**, 1-44.

Mayer, M. P. and Bukau, B. (2005). Hsp70 chaperones: cellular functions and molecular mechanism. *Cell Mol Life Sci* **62**, 670-84.

Mohamad, A., Arshad, A., Sung, Y. Y. and Jasmani, S. (2018). Effect of thermal stress on Hsp70 gene expression and female reproductive performance of giant freshwater prawn, *Macrobrachium rosenbergii*. *Aquaculture Research* **49**, 135-150.

Moniot, S., Weyand, M. and Steegborn, C. (2012). Structures, substrates, and regulators of Mammalian sirtuins - opportunities and challenges for drug development. *Front Pharmacol* **3**, 16.

Nicholas, K. B. and Nicholas, H. B. J. (1997). GeneDoc: a tool for editing and annotating multiple sequence alignments. *Distributed by the author*.

Oligschlaeger, Y., Miglianico, M., Chanda, D., Scholz, R., Thali, R. F., Tuerk, R., Stapleton, D. I., Gooley, P. R. and Neumann, D. (2015). The recruitment of AMP-activated protein kinase to glycogen is regulated by autophosphorylation. *J Biol Chem* **290**, 11715-28.

Oliphant, A., Alexander, J. L., Swain, M. T., Webster, S. G. and Wilcockson, D. C. (2018). Transcriptomic analysis of crustacean neuropeptide signaling during the moult cycle in the green shore crab, *Carcinus maenas*. *BMC Genomics* **19**, 711.

Roman, J. and Palumbi, S. R. (2004). A global invader at home: population structure of the green crab, *Carcinus maenas*, in Europe. *Mol Ecol* **13**, 2891-8.

Santiago-Sotelo, P. and Ramirez-Prado, J. H. (2012). prfectBLAST: a platform-independent portable front end for the command terminal BLAST+ stand-alone suite. *Biotechniques* **53**, 299-300.

Saxton, R. A. and Sabatini, D. M. (2017). mTOR Signaling in growth, metabolism, and disease. *Cell* **168**, 960-976.

Schumpert, C. A., Anderson, C., Dudycha, J. L. and Patel, R. C. (2016). Involvement of *Daphnia pulicaria* Sir2 in regulating stress response and lifespan. *Aging (Albany NY)* **8**, 402-17.

Semenza, G. L. (1998). Hypoxia-inducible factor 1: master regulator of O2 homeostasis. *Curr Opin Genet Dev* **8**, 588-94.

Semenza, G. L. (2001a). HIF-1 and mechanisms of hypoxia sensing. *Curr Opin Cell Biol* **13**, 167-71.

Semenza, G. L. (2001b). HIF-1, O2, and the 3 PHDs. *Cell* **107**, 1-3.

Semenza, G. L. (2001c). HIF-1, O2, and the 3 PHDs: How Animal Cells Signal Hypoxia to the Nucleus. *Cell* **107**, 1-3.

Semmouri, I., Asselman, J., Van Nieuwerburgh, F., Deforce, D., Janssen, C. R. and De Schamphelaere, K. A. C. (2019). The transcriptome of the marine calanoid copepod Temora longicornis under heat stress and recovery. *Mar Environ Res* **143**, 10-23.

Sonanez-Organis, J. G., Peregrino-Uriarte, A. B., Gomez-Jimenez, S., Lopez-Zavala, A., Forman, H. J. and Yepiz-Plascencia, G. (2009). Molecular characterization of hypoxia inducible factor-1 (HIF-1) from the white shrimp *Litopenaeus vannamei* and tissue-specific expression under hypoxia. *Comp Biochem Physiol C Toxicol Pharmacol* **150**, 395-405.

Stapleton, D., Woollatt, E., Mitchelhill, K. I., Nicholl, J. K., Fernandez, C. S., Michell, B. J., Witters, L. A., Power, D. A., Sutherland, G. R. and Kemp, B. E. (1997). AMP-activated protein kinase isoenzyme family: subunit structure and chromosomal location. *FEBS Lett* **409**, 452-6.

Sugumar, V., Vijayalakshmi, G. and Saranya, K. (2013). Molt cycle related changes and effect of short term starvation on the biochemical constituents of the blue swimmer crab Portunus pelagicus. *Saudi J Biol Sci* **20**, 93-103.

Sun, S., Xuan, F., Fu, H., Ge, X., Zhu, J., Qiao, H., Jin, S. and Zhang, W. (2016). Molecular characterization and mRNA expression of hypoxia inducible factor-1 and cognate inhibiting factor in *Macrobrachium nipponense* in response to hypoxia. *Comp Biochem Physiol B Biochem Mol Biol* **196-197**, 48-56.

Sun, S., Xuan, F., Fu, H., Zhu, J., Ge, X. and Gu, Z. (2015). Transciptomic and histological analysis of hepatopancreas, muscle and gill tissues of oriental river prawn (*Macrobrachium nipponense*) in response to chronic hypoxia. *BMC Genomics* **16**, 491.

Suwansa-Ard, S., Thongbuakaew, T., Wang, T., Zhao, M., Elizur, A., Hanna, P. J., Sretarugsa, P., Cummins, S. F. and Sobhon, P. (2015). In silico Neuropeptidome of Female Macrobrachium rosenbergii Based on Transcriptome and Peptide Mining of Eyestalk, Central Nervous System and Ovary. *PLoS One* **10**, e0123848.

Tagmount, A., Wang, M., Lindquist, E., Tanaka, Y., Teranishi, K. S., Sunagawa, S., Wong, M. and Stillman, J. H. (2010). The porcelain crab transcriptome and PCAD, the porcelain crab microarray and sequence database. *PLoS One* **5**, e9327.

Taylor, A. C. and Davies, P. S. (1981). Respiration in the Land Crab, Gecarcinus-Lateralis. *Journal of Experimental Biology* **93**, 197-208.

Taylor, A. C. and Davies, P. S. (1982). Aquatic respiration in the land crab, Gecarcinus lateralis (Fréminville). *Comparative Biochemistry and Physiology Part A: Physiology* **72**, 683-688.

Taylor, B. L. and Zhulin, I. B. (1999). PAS domains: internal sensors of oxygen, redox potential, and light. *Microbiol Mol Biol Rev* **63**, 479-506.

Tepolt, C. K. and Palumbi, S. R. (2015). Transcriptome sequencing reveals both neutral and adaptive genome dynamics in a marine invader. *Mol Ecol* **24**, 4145-58.

Tepolt, C. K. and Somero, G. N. (2014). Master of all trades: thermal acclimation and adaptation of cardiac function in a broadly distributed marine invasive species, the European green crab, *Carcinus maenas*. *J Exp Biol* **217**, 1129-38.

Teranishi, K. S. and Stillman, J. H. (2007). A cDNA microarray analysis of the response to heat stress in hepatopancreas tissue of the porcelain crab *Petrolisthes cinctipes*. *Comp Biochem Physiol Part D Genomics Proteomics* **2**, 53-62.

Vasquez, M. C., Beam, M., Blackwell, S., Zuzow, M. J. and Tomanek, L. (2017).

Sirtuins regulate proteomic responses near thermal tolerance limits in the blue mussels *Mytilus galloprovincialis* and *Mytilus trossulus*. *J Exp Biol* **220**, 4515-4534.

Verbruggen, B., Bickley, L. K., Santos, E. M., Tyler, C. R., Stentiford, G. D., Bateman, K. S. and van Aerle, R. (2015). De novo assembly of the *Carcinus maenas* transcriptome and characterization of innate immune system pathways. *BMC Genomics* **16**, 458.

von Prahl, H. and Manjárres, G. (1984). Cangrejos Gecarcinidos (Crustacea; Gecarcinidae) de Colombia. *Caldasia* **XIV**, 149-168.

Westerheide, S. D., Anckar, J., Stevens, S. M., Jr., Sistonen, L. and Morimoto, R. I. (2009). Stress-inducible regulation of heat shock factor 1 by the deacetylase SIRT1. *Science* **323**, 1063-6.

Willows, R., Navaratnam, N., Lima, A., Read, J. and Carling, D. (2017). Effect of different gamma-subunit isoforms on the regulation of AMPK. *Biochem J* **474**, 1741-1754.

Wong, K. A. and Lodish, H. F. (2006). A revised model for AMP-activated protein kinase structure: The alpha-subunit binds to both the beta- and gamma-subunits although there is no direct binding between the beta- and gamma-subunits. *J Biol Chem* **281**, 36434-42.

Xiao, B., Sanders, M. J., Carmena, D., Bright, N. J., Haire, L. F., Underwood, E., Patel, B. R., Heath, R. B., Walker, P. A., Hallen, S. et al. (2013). Structural basis of AMPK regulation by small molecule activators. *Nat Commun* **4**, 3017.

Xu, D., Sun, L., Liu, S., Zhang, L., Ru, X., Zhao, Y. and Yang, H. (2014). Molecular cloning of heat shock protein 10 (Hsp10) and 60 (Hsp60) cDNAs and their expression analysis under thermal stress in the sea cucumber Apostichopus japonicus. *Comp Biochem Physiol B Biochem Mol Biol* **171**, 49-57.

Yang, X. Q., Zhang, Y. L., Wang, X. Q., Dong, H., Gao, P. and Jia, L. Y. (2016).

Characterization of Multiple Heat-Shock Protein Transcripts from Cydia pomonella: Their Response to Extreme Temperature and Insecticide Exposure. *J Agric Food Chem* **64**, 4288-98.

Yang, Y., Ye, H., Huang, H., Li, S., Zeng, X., Gong, J. and Huang, X. (2013). Characterization and expression of SpHsp60 in hemocytes after challenge to bacterial, osmotic and thermal stress from the mud crab Scylla paramamosain. *Fish Shellfish Immunol* **35**, 1185-91.

Yi, J., Wu, H., Liu, J., Lai, X., Guo, J., Li, D. and Zhang, G. (2018). Molecular characterization and expression of six heat shock protein genes in relation to development and temperature in *Trichogramma chilonis*. *PLoS One* **13**, e0203904.

Yoon, H., Shin, S. H., Shin, D. H., Chun, Y. S. and Park, J. W. (2014). Differential roles of Sirt1 in HIF-1alpha and HIF-2alpha mediated hypoxic responses. *Biochem Biophys Res Commun* **444**, 36-43.

Yuan, Y., Sun, P., Jin, M., Wang, X. and Zhou, Q. (2019). Regulation of Dietary Lipid Sources on Tissue Lipid Classes and Mitochondrial Energy Metabolism of Juvenile Swimming Crab, Portunus trituberculatus. *Front Physiol* **10**, 454.

Zhang, D., Guo, X., Wang, F. and Dong, S. (2016). Effects of periodical salinity fluctuation on the growth, molting, energy homeostasis and molting-related gene expression of *Litopenaeus vannamei*. *Journal of Ocean University of China* **15**, 911-917.

Zheng, J., Cao, J., Mao, Y., Su, Y. and Wang, J. (2019). Comparative transcriptome analysis provides comprehensive insights into the heat stress response of Marsupenaeus japonicus. *Aquaculture* **502**, 338-346.

Zheng, J., Li, L., Dong, H., Mao, Y., Su, Y. and Wang, J. (2018). Molecular cloning of heat shock protein 60 from *Marsupenaeus japonicus* and its expression profiles at early developmental stages and response to heat stress. *Aquaculture Research* **49**, 301-312.

CHAPTER THREE: RESPONSE OF THE Y-ORGAN TO THERMAL STRESS IN INTERMOLT AND PREMOLT STAGES OF THE BLACKBACK LAND CRAB, *GECARCINUS LATERALIS*

SUMMARY

Molting in crustaceans requires the shedding of the old exoskeleton in order to develop and grow. Through intermolt, premolt, and postmolt stages regulated by the secretion of ecdysteroids from the Y-organ (YO), crustaceans undergo physiological, morphological, and behavioral changes. Molting can be induced by multiple leg autotomy (MLA) or eyestalk ablation (ESA). The research purpose was to determine the response to acute thermal stress on the blackback land crab *Gecarcinus lateralis*, and its impact on YO ecdysteroidogenesis and mRNA levels of the stress response genes *AMPk*, *SIRT1*, *SIRT7*, *HSP60*, *HSP70*, and *HIF*. I hypothesize that acute thermal stress inhibits ecdysteroidogenesis in the YO by upregulation of stress responsive genes, and that premolt animals are more thermal sensitive than intermolt animals. MLA crabs at five molt stages were exposed to 27, 32, or 35°C for 1 hour. Crabs at 0, 1, 3, 5, 7, 14 and 21 days post-ESA were exposed to 27 or 35°C for 1 hour. Ecdysteroid levels in MLA and ESA crabs were affected by molt stage and temperature and exhibited the typical pattern of other crustaceans. In MLA animals, *AMPk*, *SIRT1*, *SIRT7*, *HSP60* and *HIF* mRNA levels were mostly affected by temperature. Gene expression mostly varied in late premolt and postmolt stages with lower values at 27°C, and lower concentration at 35°C when compared with the same molt stage at lowest and mid temperature treatment. In ESA animals, *SIRT1*, *HSP60*, *HSP70* and *HIF* were moderately affected by ESA but not by temperature. Acute heat stress can be established as a system of protection and recovery at the molecular and physiological level.

INTRODUCTION

Molting is the faculty of all crustaceans to shed the exoskeleton periodically in order to develop and grow (Zhang et al., 2016). The molt cycle involves the intermolt, premolt, ecdysis and postmolt stages (Abuhagr et al., 2014b; Chang and Mykles, 2011; Mykles, 2011). This natural cycle strictly regulates all the biochemical, physiological and morphological changes previously and subsequently to ecdysis (Chang, 1995; Skinner, 1985). Crustaceans are held in intermolt stage through the secretion of the neuropeptides Molt Inhibiting Hormone (MIH) and Crustacean Hyperglycemic Hormone (CHH) from the X-organ-sinus gland complex (XOSG) in the eyestalks (Chang, 1995; Chung and Webster, 2005; Fu et al., 2016; Hopkins, 2012; Wittmann et al., 2018). The Y-organ (YO), or molting gland, synthesizes and secretes ecdysteroids that are released into hemolymph and regulate molting (Abuhagr et al., 2016; Pitts et al., 2017). While elevated concentration of MIH inhibits ecdysteroidgenesis in intermolt, reduced MIH titers halts inhibition of the Y-organ and triggers ecdysteroid synthesis in premolt with subsequent molting (Chung and Webster, 2005; Skinner, 1985). During the molt cycle, ecdysteroids exhibit a typical pattern of low concentration levels in intermolt (C_4) and postmolt (A, B, C_{1-3}), with peaks in premolt (D_0 to D_3) stages (Chang and Mykles, 2011; Skinner, 1985). Ecdysteroids may have a positive feedback mechanism to promote MIH activity in premolt stage, with the subsequent increased of MIH titers and inhibition of synthesis and release of ecdysteroids by YO in postmolt (Techa and Chung, 2015).

The intricate regulatory mechanism of molting can be activated or inhibited by 1) intrinsic factors such as loss and regeneration of limbs and, 2) extrinsic factors, such as photoperiod, temperature, and humidity (Hartnoll, 1988, 2001). Multiple Leg Autotomy (MLA)

triggers reduction of MIH (Chang and Mykles, 2011; Mykles, 2001) and induces molting in the way that crabs experience in response to limb loss from predation or injury (Hopkins, 2001; Skinner and Graham, 1972; Yu et al., 2002). Eyestalk Ablation (ESA) is used to activate ecdysteroidgenesis in the YO by eliminating the primary source of MIH (Chang and Bruce, 1980; Lee and Mykles, 2006). A typical pattern of ecdysteroid titers in hemolymph and whole-body ecdysteroid content of intact animals (comparable to those in MLA) is found in larvae, juvenile and adult crustaceans (Abuhagr et al., 2016; Chang and Bruce, 1981; Das et al., 2018; Durliat et al., 1988; Hopkins, 1983, 1986; Johnson, 2003; Lee et al., 1998; Mykles, 2011; Okumura et al., 1989; Soumoff and Skinner, 1983; Spindler and Anger, 1986). After eyestalk removal, increasing circulating ecdysteroids in the hemolymph shows the same pattern of other eyestalk-ablated crustaceans (Abuhagr et al., 2016; Chang et al., 1976; Hopkins, 1986; Lee and Mykles, 2006; McDonald et al., 2011; Medler et al., 2005; Okumura and Aida, 2001; Okumura et al., 1989; Pitts et al., 2017; Tamone et al., 2005).

Thermal stress triggers cellular and physiological responses such as energy relocation from gene and protein synthesis and changes in growth and metabolism (Verberk et al., 2018; Zheng et al., 2018). Cellular growth and metabolism integrate energy use and supply and molecular signaling, which are controlled and regulated by the mechanistic target of rapamycin (mTOR) eukaryotes (Cetrullo et al., 2015; Haissaguerre et al., 2014). Through an immediate response to intracellular energy levels, hormones and growth factors, mTOR controls gene transcription, protein synthesis, and many other intracellular activities (Haissaguerre et al., 2014; Sheng et al., 2012). Upregulation of the mTOR signaling pathway mediates the transition of *G. lateralis* from intermolt to premolt throughout the activation of the YO and the synthesis of ecdysteroids and proteins (Chang and Mykles, 2011; Das et al., 2018). With the phosphorylation

of the ribosomal protein S6 kinase (P70S6K) by mTOR, translation of specific mRNAs regulates ecdysteroidgenesis and protein synthesis (Abuhagr et al., 2016; Santiago and Sturgill, 2001). P70S6K is one of the mTOR signaling components crucial for the YO activation and sustained ecdysteroid synthesis (Das et al., 2018). AMP dependent protein kinase (AMPk), one of the upstream genes to the mTOR pathway, activates pathways for ATP production and blocks energy-consuming processes during metabolic stress conditions (Cetrullo et al., 2015; Giovannini and Bianchi, 2017). Under energetic stress conditions, inhibition of protein synthesis therefore mTOR activity is essential to conserve energy for cell survival and growth. AMPk can inhibit mTOR activity directly by 1) regulation of mTOR involving phosphorylation of Raptor in Ser722 and Ser792 and indirectly 2) by phosphorylation and activation the Tuberous sclerosis complex (TSC) (Inoki et al., 2012). Another metabolic sensor with no characterization in crustaceans is the members of the silent information regulator 2 family (SIRT2). Sirtuin activity is NAD^+-dependent, which explains the role of these proteins in metabolism and energetic state and the high levels of NAD^+ during exercise, fasting and energy restrictions (Cetrullo et al., 2015). Although Sirtuin activity coincides with AMPk functions, mTOR inhibition by suppression of energy source not always implicates an upregulated expression of Sirtuins (Giovannini and Bianchi, 2017). In some specific stimuli, such as low energy, low nutrients or hypoxia, Sirtuins promote a stress response by deacetylation of the TSC complex, with the inhibition of mTOR and thereby a reducing protein synthesis and cell growth (Ghosh et al., 2010). To this date, this is the only characterization of SIRT family members in crustaceans other than the Cladoceran *Daphnia pulicaria* (Schumpert et al., 2016). Low ATP/ADP or ATP/AMP ratios can also be a consequence of hypoxia. The hypoxia inducible factor (HIF), a dimeric protein, is responsible for upregulation or downregulation of numerous genes upon

hypoxic stress (Sheng et al., 2012; Thomas and Ashcroft, 2019). During hypoxia, low oxygen levels block production of ATP in mitochondria leading to lesser ATP cellular levels and activation of AMPk, with the subsequent inhibition of mTOR (Tan and Hagen, 2013). During hypo- and hyper-thermic stress, some proteins are translated as a primary and early response system. Heat shock proteins (HSP) are molecular chaperones that regulate protein homeostasis, prevent protein denaturation and misfolding, and support repair and transport or denatured and growing proteins (Tomanek, 2002; Zheng et al., 2018). During acute heat shock, mTOR may regulate HSP mRNA and protein expression through the phosphorylation of the heat shock factor (HSF), thus mediating the intensity and duration of response to thermal stress (Chou et al., 2012).

Among many stress factors, temperature can cause a significant delay or suspension of molting and growth (Aiken and Waddy, 1995). The Dungeness crab *Metacarcinus magister* accelerates molting within the optimal range of 5 to 20°C, and reaches high mortality levels above this temperature (Wittmann et al., 2018). The shrimp *Rhynchocinetes durbanensis* and the hermit crab *Calcinus laevimanus* showed an upregulated response of biomarkers to heat waves with prevalence of molecular chaperons (Madeira et al., 2018). Survival and *de novo* lipogenesis genes were differentially affected by temperature shifts in the two planktonic crustaceans *Tigriopus japonicus* and *T. kingsejongensis*, with a significant transcription of heat shock proteins in response to shifts (Han et al., 2018). Protein levels in general decreased at lower temperatures in the stenothermal species *Austropotamobius pallipes* and *Glyptonotus antarcticus* when compared with the temperate isopod *Idotea rescata* (Whiteley et al., 1997). In *Homarus americanus*, there was a deadly relationship between ecdysteroid levels and high temperatures in an accelerated and abnormal premolt stage (Aiken and Waddy, 1975). A combination of light

conditions and high temperature delayed *Gecarcinus lateralis* to enter premolt and display limb growth (Bliss and Boyer, 1964).

The purpose of this study was to determine the effect of acute thermal stress on ecdysteroidogenesis in the YO of intermolt (IM), early premolt (EP), mid premolt (MP), late premolt (LP), and postmolt (PM) individuals of the blackback land crab, *G. lateralis*. It has been hypothesized that acute thermal stress inhibits ecdysteroidogenesis in the YO of *G. lateralis* by upregulation of stress responsive genes, and that premolt animals will be more sensitive to thermal stress compared with those in intermolt stages, as previous studies indicate that stress response genes are downregulated at the committed and repressed stages of molt cycle (Brown-Peterson et al., 2005; de la Vega et al., 2007; Harms et al., 2014; Das and Mykles, 2016). The differences in sensitivity will be revealed by differential regulation of stress response genes and YO ecdysteroidogenesis.

MATERIALS AND METHODS

Animals and experimental design

Male *Gecarcinus lateralis* were collected from Dominican Republic. Animals were kept at ~27°C in 80% relative humidity on a 12 h:12 h dark:light cycle. Crabs were fed raisins, iceberg lettuce, and carrots twice a week. Experimental individuals were induced to molt by multiple leg autotomy (MLA) or eyestalk ablation (ESA). In MLA, where 5 or more walking legs are autotomized, triggers a reduction in MIH (Chang and Mykles, 2011; Mykles, 2001) and constitutes a molt induction method that the crabs experience in response to limb loss from predation or injury (Hopkins, 2001; Skinner and Graham, 1972; Yu et al., 2002). ESA is used to activate ecdysteroidgenesis in the YO by eliminating the primary source of MIH (Lee and

Mykles, 2006). Experimental animals were individually isolated into sand cages maintained in total darkness. To determine the molt stage, limb regeneration is used as a measure of growth known as regeneration index (or R-value) which ranges from 0 to 23. R-value is defined as the proportion of length of limb and carapace width multiplied by 100 (Bliss and Boyer, 1964; Skinner and Graham, 1972).

YOs of MLA animals at 27°C were harvested at intermolt (intermolt), early premolt (early premolt), mid premolt (mid premolt), late premolt (late premolt), and postmolt (postmolt) stages. Using a Gyromax benchtop incubator shaker (Amerex Instruments, San Francisco, CA), two groups of MLA individuals at the five molt stages were exposed to acute thermal stress at either 32 or 35°C for 1 hour individually in sand cages. Crabs where kept at 80% humidity. Cages were equilibrated at the experimental temperature before treatment. YOs were harvested after experiment, one was processed for qPCR and the other YO was processed for Western Blotting. YOs of ESA animals exposed to 27 or 35°C for 1 hour were harvested at 0, 1, 3, 5, 7, 14, and 21 days post-ESA. Thoracic ganglion (TG) and heart (H) were harvested only from 27°C group. After exposure, 100 µl of hemolymph was combined with 300 µl MeOH and ecdysteroids titers in the hemolymph were quantified by an competitive enzyme linked immunosorbent assay (ELISA) (Abuhagr et al., 2014a; Yorde et al., 1976).

RNA isolation and cDNA synthesis

In 1 ml TRIzol (Life Technologies, Grand Island, NY), YOs were homogenized with a pestle mixer (VWR, Denver, CO) for 5 min. TG and H tissues were homogenized using a TissueLyser II (QIAGEN, Germantown, MD). Samples were run at 180 oscillations min^{-1} for 2 min and 1 ml TRIzol (Life Technologies, Grand Island, NY) was added to resuspend the tissue.

Homogenized tissues were centrifuged at 16,000 g for 15 min to extract 1 ml of supernatant. Total RNA was treated with chloroform/isopropanol/ethanol (70%) and pellets were resuspended in 20 µl of RNase/DNase free water. Samples were treated with 3 µl 10x DNase buffer, 0.75 µl Ribolock, 3.25 µl RNase/DNase free water and 3 µl DNase I (Life Technologies, Grand Island, NY) to remove genomic DNA. A second RNA extraction was done by using acidic phenol/chloroform isoamyl alcohol 24:1, and RNA was precipitated with isopropanol/sodium acetate (pH 5.2). Pellets were rehydrated in 22 µl RNase/DNase free water and RNA purity and concentration was determined by measuring absorbance at 260 and 280 nm using a Nanodrop ND1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE). Total RNA was reverse transcribed to cDNA using SuperScript IV Reverse Transcriptase (Thermo Fisher Scientific, Wilmington, DE), according to the manufacturer's instructions.

Gene isolation and quantitative PCR

Complete cDNAs encoding *Gl-AMPkα* (GenBank accession MK046865), *Gl-AMPkγ* (GenBank accession MK059957), *Gl-SIRT1* (GenBank accession MK372514), *Gl-SIRT7* (GenBank accession MK372515), *Gl-HSP60* (GenBank accession MK372512), *Gl-HSP70* (GenBank accession MK372513), *Gl-HIFα* (GenBank accession MK372510), and *Gl-HIFβ* (GenBank accession MK372511) were isolated and characterized from the YO transcriptome (Das et al., 2018) (see Chapter 2). Highly conserved domains and motifs were identified using the Conserved Domain Database (CDD) on National Center for Biotechnology Information (NCBI, http://ncbi.nlm.nih.gov) and InterPro on the European Bioinformatics Institute (EBI, https://www.ebi.ac.uk). ClustalX 2.0 (Larkin et al., 2007) and GeneDoc (Nicholas and Nicholas, 1997) were used to create multiple alignments with orthologs from other crustacean species on

the NCBI database to reveal sequence identity. Conserved regions were amplified using specific primers (Table 2-2, Integrated DNA Technologies, Coralville, IA, USA).

Eukaryote Elongation Factor (EF-2) was used as a positive control of cDNA quality. Quantitative analysis (qPCR) was developed using a LightCycler 480 thermal cycler (Roche Applied Science, Indianapolis, IN). QPCR reactions consisted of 0.5 µl each of 10 µmol l^{-1} forward and reverse primer (Table 2-2), 3 µl PCR-grade water, 5 µl SYBR Green I Master Mix, and 1 µl first strand cDNA. PCR conditions were set in an initial denaturation at 95°C for 5 min, 45 cycles at 95°C for 5 s, annealing at 60°C for 5 s, and extension at 72°C for 20 s. Absolute concentration of transcripts was calculated using a standard curve generated by serial dilutions ranging 10^{-9} to 10^{-18} ng μl^{-1} of purified cDNA product of each gene. Final qPCR transcript concentration was standardized to copy number per µg of total RNA used in the cDNA synthesis, and $\log_{10}$ transformed prior to analysis.

Preparation of lysate from HeLa cell culture

HeLa cells culture was provided by Dr. O'Neil Wiggan from the department of Biochemistry & Molecular Biology at Colorado State University. HeLa cells were cultured at 37°C in Dulbecco's Modified Eagle's Medium (DMEM; Thermo Fisher Scientific, Wilmington, DE) until confluence. Prior to homogenization, cells were washed with ice-cold Phosphate-Buffered Saline 1X (PBS) and centrifuged at 8,000 g for 10 min. Cells were homogenized by sonication in 5 ml of homogenizing buffer containing 20 mmol l^{-1} Tris, 1 mmol l^{-1} EDTA, 20 mmol l^{-1} KCl, 50 µl Halt Protease Inhibitor Cocktail EDTA-Free containing AEBSF 1 mmol l^{-1}, Aprotinin 800 nmol l^{-1}, Bestatin 50 µmol l^{-1}, E64 15 µmol l^{-1}, Leupeptin 20 µmol l^{-1}, Pepstatin A 10 µmol l^{-1} (Thermo Fisher Scientific, Wilmington, DE), and 50 µl of Sodium Orthovanadate

200 mmol l^{-1} (Acros Organics, Belgium) for 3x1 min. The lysate in solution was centrifuged at 12,000 g for 20 min and supernatant was carefully retrieved. Protein concentration in the supernatant was determined by BCA protein assay (Thermo Fisher Scientific, Wilmington, DE) as described in Smith et al. (1985). Lysate was aliquoted and stored at -80°C.

Western Blotting

Tissues were frozen in liquid nitrogen before storage at -80°C until use. Tissues were homogenized in buffer containing 20 mmol l^{-1} Tris, 1 mmol l^{-1} EDTA, 20 mmol l^{-1} KCl, Halt Protease Inhibitor Cocktail EDTA-Free containing AEBSF 1 mmol l^{-1}, Aprotinin 800 nmol l^{-1}, Bestatin 50 µmol l^{-1}, E64 15 µmol l^{-1}, Leupeptin 20 µmol l^{-1}, Pepstatin A 10 µmol l^{-1} (Thermo Fisher Scientific, Wilmington, DE), and Sodium Orthovanadate 2 µmol l^{-1} (Acros Organics, Belgium) for 5 min. Homogenized samples were centrifuged at 16,000 g for 15 min. Sample supernatant and Laemmli sample buffer (Laemmli, 1970) were combined in a 1:1 ratio and incubated at 95°C for 10 min. 20 ug of samples and Precision Plus Protein standard (Bio-Rad Laboratories, Hercules, CA) were separated at 150-200 V for 30-45 min, on a Mini-Protean TGX 4–15% tris-glycine gel (Bio-Rad Laboratories, Hercules, CA) using a tris-glycine buffer system. Protein samples were transferred to a 0.45 µm PVDF membrane (100 V, 1 h, Amersham Hybond-P, GE Healthcare Life Sciences, Pittsburgh, PA) and stained with 0.01% Colloidal Gold to validate blotting. After blocking the membrane at room temperature for 1 h with VECTASTAIN Universal Elite ABC 2% horse serum (Vector Laboratories, Burlingame, CA) in 5% BSA-TTBS, the blot was probed overnight in a 1:500 dilution of primary rabbit anti-AMPKα (Thr172), rabbit anti-phospho-AMPKα (Thr172), (Millipore Sigma, Temecula, CA), mouse anti-P70 S6 Kinase (S6K), and mouse anti-phospho-P70 S6 Kinase (S6K, Ser434),

antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). Because AMPK has multiple phosphorylation sites, downstream targets can determine its activity. Nevertheless, an increase in phosphorylation of Thr172 is representative of several cellular effects on AMPK activation (Frederich and Balschi, 2002; Frederich et al., 2009). Membrane was incubated in VECTASTAIN Universal Elite ABC biotinylated secondary antibody 1:50 or 1:100 for 30 min (Vector Laboratories, Burlingame, CA) and developed with WesternBright Sirius chemiluminescent HRP substrate (Advansta, Menlo Park, CA) or SuperSignal West Pico PLUS chemiluminescent substrate (Thermo Fisher Scientific, Wilmington, DE). Images were obtained using a ChemiDoc XRS+ molecular imager (Bio-Rad Laboratories, Hercules, CA). For each lane and band in the Western blots, the associated Colloidal Gold staining of the whole lane (all proteins) served as type of normalization by sum. Protein concentration was calculated by densitometry with ImageJ (NIH, Bethesda, MD) and $\log_{10}$ transformed prior to analysis.

Statistical analysis

Statistical analysis was performed on Statgraphics 18 (The Plains, VA, USA) and PAST (Hammer et al., 2001). Graphs were created on SigmaPlot 12 (San Jose, CA, USA). Outliers were detected using the Grubbs' Test, and removed. Kruskal-Wallis and Tukey-Kramer HSD *post-hoc* multiple comparisons tests were used to test significant effects of molt stage or day post-ESA and temperature on ecdysteroid titers. Two-way ANOVA and Tukey-Kramer HSD *post-hoc* multiple comparisons tests were used to test significant effects of molt stage and temperature on transcript concentration and protein abundance and activity of MLA animals. Linear regressions and correlation coefficients of Pearson (r) were implemented to determine a relationship between log10-transformed protein abundance and log10-transformed ecdysteroid

titers. One-way (TG-H) or two-way (YO) ANOVA and Tukey-Kramer HSD *post-hoc* multiple comparisons tests were used to test significant effects of day post-ESA and temperature on transcript concentration of ESA animals. qPCR data of TG samples at day 3 post-ESA were excluded from the analysis, as errors in RNA isolation and cDNA synthesis were evident. Data were plotted as mean ±1 S.E.M. and all statistical analysis were set at $\alpha>0.05$ level of significance.

RESULTS

Effects of molt stage and acute heat stress on ecdysteroid titers and mRNA levels of MLA crabs

Haemolymph ecdysteroid levels of *G. lateralis* MLA individuals exposed to 27, 32 or 35°C varied by molt stage and temperature ($\chi^2(14) = 265.6$, $P<0.0001$, Figure 3-1A). In general, ecdysteroid titers in the hemolymph depicted a typical pattern of the *G. lateralis* molt cycle: low titers at intermolt, increasing values from early premolt to late premolt, and the lowest levels at postmolt stage. Levels at 32°C were higher in all molt stages than levels observed at 27°C. Hemolymph titers at 35°C were similar or lower than at lower temperature treatments. Ecdysteroid titers had a significant increase of 15, 24 and 12-fold from intermolt to mid premolt and of 34, 44 and 14-fold from intermolt to late premolt, at each temperature respectively ($P<0.0001$). In the same way, ecdysteroid titers increased 11, 12, and 5-fold from early premolt to mid premolt and 25, 22 and 6-fold from early premolt to late premolt ($P<0.0001$). By contrast, postmolt at 35°C had the lowest concentration of titers with a decrease of ~163-fold ($P<0.0001$) and a concentration of 2±1 pg ul^{-1}. In intermolt, ecdysteroid titers varied from 17±2 pg ul^{-1} at 32°C to 25±4 pg ul^{-1} at 35°C. In early premolt stage, values increased to 26±2 pg ul^{-1} at 27°C,

32±4 pg ul^{-1} at 32°C and 59±7 pg ul^{-1} at 35°C. In mid premolt, titers ranged from 289±38 pg ul^{-1} at 27°C to 392±30 pg ul^{-1} at 32°C. Maximum concentrations at late premolt stage ranged from 353±37 pg ul^{-1} at 35°C to 724±68 pg ul^{-1} at 32°C.

In the MLA animals, *EF2*, *SIRT1*, *HSP70* and *HIFβ* were significantly affected by molt stage ($P<0.05$), and *AMPkα*, *AMPkγ*, *SIRT1*, *SIRT7*, *HSP60*, *HIFα* and *HIFβ* were significantly affected by temperature ($P<0.05$) (Table 3-2). Excepting *AMPkγ*, there was a significant interaction between molt stage and temperature in all the genes. When affected by molt stage, mRNA levels increased as animals approached ecdysis in late premolt. For *EF2* (Figure 3-1B), *SIRT1* (Figure 3-1E), *HSP70* (Figure 3-1H) and *HIFβ* (Figure 3-1J), there was a tendency for an increase with progressing molt stages and for higher expression at 32 and 35°C. These genes also showed higher mRNA levels in mid premolt and late premolt stages compared with intermolt, early premolt and postmolt stages. *EF2* mRNA levels increased significantly 48-fold in late premolt ($P=0.0181$). *SIRT1* expression decreased 9-fold from mid premolt to late premolt ($P=0.0008$). *HSP70* transcripts increased 51-fold from mid premolt to late premolt and decreased 25-fold from late premolt to postmolt ($P=0.0218$). *HIFβ* expression was lower in late premolt by 14-fold and increased to 11-fold from late premolt to postmolt ($P=0.0054$). When affected by temperature, mRNA levels increased with increasing temperature. For *AMPkα* (Figure 3-1C), *AMPkγ* (Figure 3-1G), *SIRT1* (Figure 3-1E), *SIRT7* (Figure 3-1F), *HSP60* (Figure 3-1G), *HIFα* (Figure 3-1I) and *HIFβ* (Figure 3-1J), there was a trend with progressing molt stages for a decrease at 27°C and for constant expression at 32°C. At 35°C, mRNA levels were mostly lower compared with lower temperatures and with higher mRNA levels in mid premolt, late premolt and postmolt stages compared with intermolt and early premolt stages. *AMPkα* mRNA levels were lower at 35°C compared with 27 and 32°C in intermolt, early premolt, mid premolt and

postmolt by 13, 48, 29 and 11-fold (P<0.0001). *AMPkγ* transcripts decreased at 35°C compared with 27°C in intermolt and early premolt by 24 and 42-fold, and by 22 and 18-fold in mid premolt and postmolt at 32°C (P<0.0001). *SIRT1* expression decreased by 6 and 4-fold from 27 to 35°C in intermolt and early premolt and were 9-fold higher in late premolt from 27°C (P=0.0026). *SIRT7* mRNA levels decreased in early premolt and mid premolt by 59 and 21-fold at 35°C and increased 37-fold in late premolt compared with 27°C (P=0.0235). *HSP60* transcripts decreased by 8 and 7-fold at 35°C compared with 32°C in intermolt and early premolt and was 46-fold higher in late premolt from 27°C (P=0.0455). *HIFα* expression was lower in early premolt and mid premolt by 52 and 18-fold from 27 to 35°C and increased 8-fold in late premolt from 27°C (P<0.0001). *HIFβ* mRNA levels increased 12-fold in late premolt from 27 to 32°C and decreased in intermolt, early premolt, mid premolt and postmolt by 34, 26, 29 and 24-fold from 32 to 35°C (P<0.0001).

Abundance and activity of AMPk and P70S6K proteins in the YO of MLA crabs under acute heat stress

In the YO, excluding *Gl*-p-AMPkα, molt stage, temperature and interaction of both significantly affected *Gl*-AMPkα, *Gl*-P70S6K and *Gl*-p-P70S6K (P<0.0004) (Table 3-3). *Gl*-p-AMPkα expression was only affected by temperature (P<0.0001). Ratios greater than 1 were considered as differences in affinity of antibodies against total and phosphorylated proteins but were not considered a fact for exclusion of proteomic data (Figure 3-3C and D).

A significant decrease of 6.5-fold in AMPk protein was detected from intermolt to postmolt at 27°C (P=0.0133) and between intermolt and late premolt at 35°C (4.8-fold, P=0.0040) (Figure 3-2A). Differences among temperature treatments at the same molt stage were

evident in all molt stages. Changes in expression respect to intermolt were mostly detected compared with early premolt (P=0.0123), late premolt (P=0.0251), and postmolt (P=0.0133) at 27°C. In contrast, changes among molt stages other than intermolt were mostly observable at 32 and 35°C. A significant decrease in mid premolt of 19.8-fold between 27 and 35°C (P<0.0001) and of 21.1-fold from 32 to 35°C (P<0.0001) were detected as well. Lower abundance of AMPk was observed in late premolt from 27 to 35°C (13.2-fold, P<0.0001) and from 32 to 35°C (11.2-fold, P<0.0001). A no significant increase of 2.6-fold in AMPk expression was detected between intermolt and postmolt at 32°C. Activity of p-AMPk was 10.9-fold lower from intermolt to mid premolt at 27°C (P=0.0003) (Figure 3-2B). Activity of p-AMPk among treatments was 7.1-fold higher from 27 to 35°C in mid premolt animals (P=0.0069) and in 2.9-fold higher in postmolt (P=0.0133).

Gl-p-AMPkα/*Gl*-AMPkα ratio showed a significant 8-fold increase from intermolt when late premolt animals were exposed to 35°C (P=0.0076, Figure 3-3A). Relation between total and active AMPK protein in late premolt was 27-fold significantly higher from 27 to 35°C (P=0.0012) and 36-fold greater at 35°C compared with 32°C (P=0.0017). In the Y-organ, a negative correlation between ecdysteroids and *Gl*-AMPkα (-0.1896) and *Gl*-p-AMPkα (-0.1323) was not significant at 27°C (P between 0.0852 and 0.2765). R^2 stayed between 1.9708 and 4.9387 (Figure 3-3C).

No significant differences in abundance of P70S6K were detected at 27 and 35°C when compared with intermolt animals (Figure 3-2C). Changes in P70S6K expression were significantly higher at 32°C in early premolt (P=0.0004), mid premolt (P=0.0001), and late premolt (P=0.0006). An increase of 7.3-fold in P70S6K was detected in intermolt from 32 to 35°C (P<0.0001). P70S6K abundance was 6.9 (P=0.0002, 32°C) and 12.1-fold (P<0.0001,

35°C) higher than at 27°C in mid premolt. At 35°C, P70S6K was 4-fold greater in postmolt than at 32°C (P=0.0026). Variation in p-P70S6K activity among molt stages other than intermolt were mostly detected at 27 and 32°C (P<0.0001, Figure 3-2D). Significant differences in p-P70S6K among temperature treatments at the same molt stage were mostly observed in intermolt, early premolt and mid premolt animals. No significant changes in abundance of p-P70S6K were detected at 35°C when compared with intermolt animals. Higher increases of p-P70S6K were observed in mid premolt animals at 32°C (2700-fold, P<0.0001) and 35°C (4710-fold, P<0.0001). Activity of p-P70S6K in early premolt decreased 4.8-fold at 32°C (P=0.0283). In intermolt crabs, p-P70S6K expression increased from 25.1 to 37.7-fold from 27°C (P<0.0001).

Gl-p-P70S6K/*Gl*-P70S6K ratio showed a significant 63-fold increase in intermolt at 32°C (P<0.0001, Figure 3-3B). Relation between total and active P70S6K protein was significantly 1.9 to 3-fold lower from 27°C in early premolt (P<0.0001). The most significant observation was a 448 to 500-fold increase in mid premolt when animals were exposed to 32°C (P=0.0178) and 35°C (P=0.0383). Also, a negative correlation between ecdysteroids and *Gl*-P70S6K (-0.2914) and *Gl*-p-P70S6K (-0.0930) was not significant at 27°C (P between 0.0711 and 0.3738). R^2 stayed between 1.3903 and 5.2417 (Figure 3-3D).

Effects of ESA and acute temperature stress on ecdysteroid titers and mRNA levels of stress response genes

Haemolymph ecdysteroid levels of *G. lateralis* ESA individuals exposed to 27 or 35°C changed by effect of ESA and temperature ($\chi^2(13) = 66.1$, P<0.0001, Figure 3-4A). ESA had a significant effect on ecdysteroid titers increasing from day 1 post-ESA and producing comparable concentrations until day 5 post-ESA (88±9 pg ul^{-1} at 27°C vs 81±12 pg ul^{-1} at 35°C,

P=1). At day 0, ecdysteroid titers were 1-fold higher at 27°C than 35°C group accumulating from 14±2 pg ul^{-1} to 19±3 pg ul^{-1}. ESA in animals at 27°C significantly increased ecdysteroid titers 12-fold by day 21 (*P*<0.0001). Ecdysteroid titers of animals at 35°C had a significant increase of 40-fold from day 0 to day 21 post-ESA (*P*<0.0001). At day 7 post-ESA, titers in the hemolymph varied between 77±7 pg ul^{-1} at 27°C and 132±26 pg ul^{-1} at 35°C. At day 14 post-ESA, values increased from 251±28 pg ul^{-1} at 27°C (maximum concentration) to 374±43 pg ul^{-1} at 35°C. Maximum concentrations at 35°C were detected by day 21 post-ESA with 554±43 pg ul^{-1}.

In the YO, *EF2*, *SIRT1*, *HSP60*, *HSP70*, *HIFα* and *HIFβ* were moderately affected by ESA (*P*<0.05) but not by temperature (Table 3-4). There was no significant interaction between ESA and temperature on expression of any of the genes. Nevertheless, *post-hoc* comparisons detected valid differences only for *SIRT1*, *HSP60*, *HSP70*, *HIFα* and *HIFβ*. *AMPkα*, *AMPkγ* and *SIRT7* showed no significant changes in expression. For *EF2* (Figure 3-4B), *SIRT1* (Figure 3-4E), *HSP60* (Figure 3-4G), *HSP70* (Figure 3-4H), *HIFα* (Figure 3-4I), and *HIFβ* (Figure 3-4J), there was a trend for constant expression with no significant variation at 27°C (Figure 3-5A). By contrast, expression of these genes had a tendency to increase at 35°C from day 0 to day 1 post-ESA and for higher expression from day 7 to 14 post-ESA, decreasing after 21 days post-ESA to values comparable with day 7 post-ESA (Figure 3-5B). *EF2* mRNA levels increased 15 and 24-fold at 27 and 35°C, respectively, by day 14 post-ESA. *SIRT1* expression was 6-fold higher by day 5 post-ESA at 27°C and 32-fold greater by day 14 post-ESA at 35°C; ESA significantly increased it 5-fold between 7- and 14-days post ESA at 35°C (*P*=0.0200). By day 7 post-ESA, *HSP60* mRNA levels at 27°C increased 29-fold and 66-fold at 35°C by day 14 post-ESA, significantly increasing 6-fold between 5- and 14-days post-ESA (*P*=0.0210). By day 14 post-ESA, *HSP70* mRNA were 22 and 68-fold higher at 27 and 35°C, respectively. Expression of

HSP70 at 35°C increased 8-fold from 5 to 14 days post-ESA (P=0.0290), then decreased 4-fold over the next 7 days to levels comparable at 7 days post-ESA. Levels of *HIFα* increased to its highest values by day 14 post-ESA at both temperatures with an increase of 6-fold at 27°C and 24-fold at 35°C. ESA had a significant effect on *HIFα* between 7- and 14-days post ESA and an increase of 2 to 4-fold (P=0.0170). *HIFβ* mRNA was 9 and 24-fold higher after 14 days post-ESA at 27 and 35°C, respectively, and 2 to 6-fold significantly greater from day 5 to 14 post-ESA (P=0.0340). By contrast, ESA had no effect on the expression of *AMPkα*, *AMPkγ* and *SIRT7* and there was no difference in mRNA levels between 27°C and 35°C animals.

In the thoracic ganglion, expression after 3 days post-ESA was not considered for analysis. Levels of mRNA in *AMPkα* had an increase of 1-fold by day 21 post-ESA (Figure 3-4C). Therefore, ESA had little effect on its mRNA levels with only a 3-fold increase from day 1 to 5 post-ESA (P=0.0212). *SIRT1* expression (Figure 3-4E) was 1-fold higher after 21 days post-ESA and increased 3-fold from day 1 to 5 post-ESA (P=0.0190). By day 21 post-ESA, *HSP60* mRNA (Figure 3-4G) was 1-fold higher and with a moderately significant increase of 2-fold after the first day post-ESA (P=0.0299). There were no significant differences in *EF2* (Figure 3-4B), *AMPkγ* (Figure 3-4D), *SIRT7* (Figure 3-4F), *HSP70* (Figure 3-4H), *HIFα* (Figure 3-4I),and *HIFβ* (Figure 3-4J) mRNA levels in *post hoc* comparisons of biological interest.

In the heart, the mRNA levels of all genes excluding *EF2* (Figure 3-4B) and *HSP70* (Figure 3-4H), were not significantly affected by ESA. *EF2* mRNA increased 4-fold to its higher level by day 21 post-ESA (P=0.0006) and was 3-fold greater from day 3 to 21 post-ESA (P=0.0086). Similarly, after 1- and 21-days post-ESA, *HSP70* mRNA levels were 2-fold significantly higher than day 0 (P=0.0313, P=0.0292).

DISCUSSION

In this study, the gene expression and protein abundance and activity of the stress responsive genes *AMPk*, *SIRT*, *HSP*, and *HIF* in the YOs, thoracic ganglion and heart of *G. lateralis* after acute heat shock were examined. Control (27°C) and 32°C temperatures correspond to the minimum and maximum mean temperature registered in Dominican Republic according to the Dominican Meteorological Office (ONAMET, in Spanish), which are the average daily temperatures *G. lateralis* population lives in naturally. 35°C was chosen as an extreme non-lethal temperature forcing a molecular and physiological response.

Response of MLA crabs to acute heat stress

In MLA, ecdysteroid titers had a typical pattern of lower values in intermolt and postmolt stages and increasing concentrations from early premolt to late premolt, prior to ecdysis (Abuhagr et al., 2016; Das et al., 2018; Mykles, 2011), with very similar values to other crustacean species (Durliat et al., 1988; Hopkins, 1983, 1986; Lee et al., 1998; Okumura et al., 1989; Soumoff and Skinner, 1983) or early developmental stages (Chang and Bruce, 1981; Johnson, 2003; Spindler and Anger, 1986). Gene expression showed a tendency for stable transcript concentrations of all genes at 27 and 32°C, particularly in intermolt, early premolt and mid premolt stages. Variations in mRNA levels were more evident in late premolt and postmolt stages with lower expression at 27°C. At 35°C, mRNA expression levels were mostly lower than those at the other two temperature treatments in the same molt stage, with similar or higher transcript concentration for *EF2*, *SIRT1*, *SIRT7*, *HSP60* and *HSP70* in late premolt and postmolt animals.

Since synthesis and function of new genes demand a large supply of ATP (Hofmann and Somero, 1995), it is expected to observe the upregulation of genes for energy production such as *AMPkα* and *AMPkγ* subunits following heat stress. Instead, these genes were regularly expressed at 27°C with lower levels in late molt stages, stable and slightly upregulated at 32°C and negatively regulated at 35°C when compared to lower temperatures. Frederich et al. (2009) proposed that *AMPk* subunits expression is an earlier indicator of heat stress than activation of chaperones. However, this condition may apply only for the rock crab *Cancer irroratus* and it was not demonstrated in subtidal organisms such as *Homarus americanus* (Jost et al., 2012), other intertidal species for instance *Carcinus maenas* (Jost et al., 2012) or in land decapod species like *G. lateralis* in the present study. *AMPk* is known by directly inhibiting mTOR through phosphorylation of Raptor and by indirect regulation of mTOR activity through phosphorylation of the Tuberous Sclerosis Protein 2 (TSC2) (Cetrullo et al., 2015; Haissaguerre et al., 2014; Mihaylova and Shaw, 2011). Considering that *AMPk* activity may regulate the energy source for ecdysteroid synthesis through mTOR, similarities between *AMPk* mRNA and ecdysteroid levels across temperatures would be expected, or similar ecdysteroid levels following a partial inhibition of protein synthesis (Wittmann et al., 2018). With invariable or lower mRNA levels at higher temperatures (32 and 35 °C), *AMPk* may not be a primary regulator or accurate indicator of early activation of a response system against thermal stress in *G. lateralis*. Since ecdysteroid titers increased constantly through the molt cycle at 27 and 32°C, but with significant lower concentrations at 35°C, the reduction in ecdysteroidgenesis is not mediated by *AMPk* through inhibition of the mTOR signaling network.

Expression of *SIRT1* was mostly consistent at 27 and 32°C and, although it was not highly upregulated at 35°C, it showed a sustained response from intermolt and reached

maximum mRNA levels when compared with late premolt at 27°C. *AMPk* and *SIRT1* are fuel-sensing molecules with a shared function on fat and glucose metabolism in response to physiological changes in energy levels (Canto et al., 2009; Ruderman et al., 2010). Similar to the expected for *AMPk* subunits, *SIRT1* might be a potential indicator of thermal stress in *G. lateralis* as well. As intermolt crabs transition into premolt and reach ecdysis in late premolt, energy-consuming arrangements such as protein synthesis and ecdysteroidgenesis need to be supplied in a mTOR-dependent manner and with activation of EF2 (Abuhagr et al., 2014b; Chang and Mykles, 2011). Production and relocation of energy in *G. lateralis* might be regulated by *SIRT1*, in terms of assisting catabolic and anabolic pathways (Figure 1-4) (Canto et al., 2009; Huang et al., 2015b). Under stress conditions, inhibition of mTOR by upregulation of *SIRT1* and a reciprocal activation with *AMPk* reduces protein synthesis and ecdysteroidgenesis (Ghosh et al., 2010; Giovannini and Bianchi, 2017). Different to the present study, Vasquez et al. (2017) found that SIRT1 was not triggered in gills of the heat-tolerant mollusk *Mytilus galloprovincialis* but HSP70 was significantly increased after critical exposure to 35°C. This can be related to the physiology and low or high tolerance of the species (Madeira et al., 2018; Verberk et al., 2018) and is also explained from the isolated activity of *SIRT1*. SIRT1 has been identified as a primary regulator of the heat shock response by deacetylating the heat shock factor 1 (HSF1), and delaying and sustaining transcription of HSP70 (Teigen et al., 2015; Westerheide et al., 2009). On the other hand, *SIRT7* expression was opposite to its function of regulating the expression of ribosomal RNA through RNA-PolI (Greiss and Gartner, 2009), thus mediating protein synthesis. Excepting in late premolt, mRNA levels of *SIRT7* were relatively constant between 27 and 32°C through all the stages, and significantly upregulated from early premolt to late premolt at 35°C. *SIRT7* expression was comparable with ecdysteroid titers at 27 and 32°C, but clearly inconsistent

with its values when exposed to 35°C. Ribosome biogenesis and protein synthesis are partly mediated by SIRT7 association with mTOR, which regulates RNA PolI, II, and III as well (Tsai et al., 2014). *SIRT7* expression inconsistent with ecdysteroids levels may be a result of an impaired association, after an inhibition of mTOR activity through the upregulation of *SIRT1* (Ghosh et al., 2010; Giovannini and Bianchi, 2017). To this date, this is the only characterization of SIRT family members in crustaceans other than the cladoceran *D. pulicaria* (Schumpert et al., 2016).

As expected, molecular chaperones (*HSP60* and *HSP70*) were upregulated, particularly in late premolt stage, when compared with intermolt animals as the temperature increased. Their reduction by early premolt and gradual increase through late premolt may indicate the gradual accumulation of these chaperones, acting as a buffer against heat shock and support for thermal protection (Buckley et al., 2001; Han et al., 2018). In fact, HSPs activity may trigger an early thermal protection through late premolt, without activation of a complex stress response, and may suggest recovery from the thermal stress in postmolt (Chen et al., 2018; Zheng et al., 2018). This is possible since some HSPs lack introns and do not undergo alternative splicing, generating a quicker stress response (Drozdova et al., 2019). *G. lateralis* were kept under simulated conditions of temperature and relative humidity in captivity. For this reason, some chaperones expression may be correlated to the exposure to thermal limits, rather than an actual thermal fluctuation of these genes (Tomanek, 2002). Chaperones structure and function are widely conserved and can be traced through different species of crustaceans (Baringou et al., 2016). The upregulated expression of a considerable number of stress proteins is an efficient but at the same time energy consuming system of lessening thermal stress (Tomanek and Zuzow, 2010). In mid premolt, *G. lateralis* reaches the highest peak of ecdysteroid titers in the hemolymph in a point of

no return or committed state (Chang and Mykles, 2011); a step partially mediated by the activity of mTOR and a crucial molting stage previous to ecdysis (Abuhagr et al., 2016). Under heat shock, translation of proteins other than chaperones may reduce and that energy shift to ecdysteroidgenesis and synthesis of few proteins essential for molt cycle (Han et al., 2013). In this manner the heat shock system in *G. lateralis* may be characterized by avoiding translation and exposure of new polypeptide chains to the denaturing conditions at 35°C (even at 32°C) (Holcik and Sonenberg, 2005), in especial, translation of genes of the mTOR signaling network necessary for protein synthesis (Abuhagr et al., 2014b; Abuhagr et al., 2016; see The mTOR Signaling Network, Saxton and Sabatini, 2017). It's hypothesized that premolt crabs will be less resistant to heat shock than intermolt animals and lower titers in the hemolymph may be the result of thermal stress. With high levels of chaperones and energy allocation for different activities, *G. lateralis* may decrease ecdysteroid levels and undergo negative effects in performance, growth and cell division (Madeira et al., 2018; Madeira et al., 2012).

Hypoxia inducible factor (*HIFα* and *β*) is a dimeric transcription factor, which regulates molecular and physiological responses to hypoxia and stays inactive in normoxia (Hardy et al., 2012; Soñanez-Organis et al., 2010; Sun et al., 2016; Thomas and Ashcroft, 2019; Zagorska and Dulak, 2004). mRNA levels of *HIFα* and *HIFβ* showed a downregulation pattern through late premolt at 27°C, constant expression at 32°C and lower transcripts concentration at 35°C. After exposure to 35°C, *HIFα* mRNA levels were upregulated by late premolt while *HIFβ* expression was stable. Under the controlled experimental conditions, hypoxia was avoided with a permanent air supply at different temperatures. It would be anticipated that under oxygen-reduced conditions of a thermal-induced hypoxia, metabolism will be depressed and consequently ATP reduced (Anestis et al., 2007; Inoki et al., 2012; Tan and Hagen, 2013), with regulation of energy

available for survival and response (Feder and Hofmann, 1999). Through the same response it would be possible to observe an upregulation of *HIF* subunits (primarily *HIFβ*) with higher temperatures (Velazque-Amado et al., 2012). Heat-induced hypoxia was not demonstrable in this experiment based on *HIF* activity, but it was already established that there is an early response at the transcriptional level in other crustaceans such as *Callinectes sapidus* (Hardy et al., 2012). Moreover, the thermal tolerance capacity of *G. lateralis* under resting conditions and higher temperatures is amplified by breathing air (Fusi et al., 2016). Ultimately, the thermal tolerance of an organism is related to the delivery capacity of oxygen to the cells (Portner et al., 2017; Pörtner et al., 2006). As previously mentioned, HSPs activity may indicate an early response system, *HIF* mRNA levels may obey to degradation of *HIFα* and suppression of its downstream transcriptional activity by the complex *HSP40-HSP70* (Velazque-Amado et al., 2012). A consequence is that *G. lateralis* will downregulate the aerobic energy metabolism, while energy-producing pathways are activated to respond to oxidative damage as an alternative for protein production and ecdysteroidgenesis (Madeira et al., 2018; Tomanek, 2015; Wittmann et al., 2018).

AMPK and P70S6K in MLA crabs under acute heat stress

Lower mRNA levels of the *AMPkα* and *AMPkγ* subunits were transcribed at 35°C which may indicate that less protein was produced at the same temperature. These data are consistent with increasing mRNA levels at the lowest and mid experimental temperature and mostly downregulated at the highest temperature in early, mid and late premolt juveniles of *Metacarcinus magister* (Wittmann et al., 2018). *AMPk* mRNA levels in *G. lateralis* were equivalent with total protein values. Higher p-AMPk values at 35 °C may result of stored protein

being activated, whereas the crabs increase transcriptional and translational levels as immediate response. By keeping lower and increasing *AMPk* mRNA levels in (early), mid and late premolt, may help in the reduction of the metabolic cost at higher temperatures (Wittmann et al., 2018). *AMPk* mRNA levels and protein activity in *G. lateralis* unequivocally support *AMPk* response in *C. irroratus*. Slower and increasing mRNA levels and therefore higher AMPk expression, and an immediate response to stress by phosphorylation of the AMPka subunit that increases AMPk within a short time (Frederich et al., 2009). During the heat-shock response, energy is required at different molt stages for the activation of transcription of stress responsive genes and synthesis of ecdysteroids, HSPs and other proteins (Somero, 2002). This validates that changes at the protein level are caused by a transcriptional and posttranscriptional regulation associated with ecdysteroidogenesis (Lee and Mykles, 2006).

Protein biosynthesis and ecdysteroidgenesis are energy-demanding processes that require synthesis of the machinery for biosynthesis, such as ribosomal proteins and synthesis of replacement proteins (Somero, 2002). There was not an evident pattern of total and active P70S6K protein expression after acute heat shock in *G. lateralis*. Although *P70S6K* mRNA expression was not measured, it is expected that with higher P70S6K protein levels, greater mRNA levels are evident in mid and late premolt crustaceans (Abuhagr et al., 2014b; Haj et al., 1996; Tian et al., 2019). In *H. americanus*, higher protein levels and ribosomal activity in claw, abdominal muscle and walking leg (El Haj, 1999; Haj et al., 1996) were closely related to molt stages with elevated ecdysteroid titers (Snyder and Chang, 1991). Correlated to these data, *P70S6K* mRNA levels were higher in claw, abdominal muscle and walking leg of *Eriocheir sinensis* in late premolt and lowest in intermolt and postmolt (Tian et al., 2019). However, similar values of the *Gl*-p-P70S6K/*Gl*-P70S6K ratio may indicate somehow that overall protein

synthesis at 32 and 35°C would decline and that some ribosomal proteins may be susceptible to denaturation and need replacement (Santiago and Sturgill, 2001; Teranishi and Stillman, 2007). In the giant isopod *Idotea resecata*, temperature related increases in ribosomal activity and protein synthesis did not turn into protein accumulation as protein degradation is a temperature-dependent consequence, which is more susceptible to higher temperatures (Whiteley et al., 1997). A low relationship between ecdysteroids and *Gl*-P70S6K and *Gl*-p-P70S6K expression may be explained by the temperature effect on ecdysteroidgenesis and proteins synthesis and/or ribosomal activity, as a minimum for MLA animals. Two possible ways to explain these results are that 1) the cost of protein synthesis has a fixed rate, especially at the optimum temperature of 27°C, and 2) a highly-consuming energy cost at lower synthesis rates at higher temperatures (Portner et al., 2017; Whiteley et al., 2001).

Response of ESA crabs to acute heat stress

Ecdysteroid titers had an opposite behavior to MLA animals, with higher titers in the hemolymph at 35°C and lower at 27°C. Animals responded to eyestalk removal by increasing circulating ecdysteroids in the hemolymph like other crustaceans (Abuhagr et al., 2016; Chang et al., 1976; Hopkins, 1986; Lee and Mykles, 2006; McDonald et al., 2011; Medler et al., 2005; Okumura and Aida, 2001; Okumura et al., 1989; Pitts et al., 2017; Tamone et al., 2005). Gene expression showed a similar pattern of expression in YO, thoracic ganglion and heart tissues. Levels of mRNA in the YO were mostly higher at 35°C after 7 days post-ESA and with notable difference between treatments at 14 days post-ESA for *AMPkα*, *SIRT1*, *SIRT7*, *HSP60*, *HSP70*, *HIFα* and *HIFβ*. Excepting *HIFα* and *HIFβ*, gene expression in thoracic ganglion and heart

samples showed no variation after 5 to 7 days post-ESA. Changes in mRNA levels until day 3 post-ESA were not significantly affected by ESA.

Not only gene expression and concentration of ecdysteroid titers in the hemolymph may direct the transition from intermolt to premolt and ecdysis in ESA *G. lateralis* (Pitts et al., 2017). Ecdysteroid titers were found to decrease with lower temperatures, which ultimately regulated the molt stage duration on a temperature-dependent change in *Calanus pacificus* (Johnson, 2003). High temperature delayed ecdysis while removal of a mild temperature stress favored the initiation of premolt in *G. lateralis* (Bliss and Boyer, 1964). A temperature-dependent regulation of ecdysteroid titers in premolt was found necessary to prevent premature death during molting in the lobster *H. americanus* as well (Aiken and Waddy, 1975). Also, in juveniles of *H. americanus*, ecdysteroids were higher at the maximum temperature after 21 days post-ESA, shortening the molt cycle (Chang and Bruce, 1980). If an inhibitory effect of MIH in the X-organ sinus gland complex (XOSG) is the only control of the YO secretory activity (Hopkins, 1986; Lee and Mykles, 2006), it would be expected to observe similar levels of ecdysteroid titers in the hemolymph regardless temperature. This was not the result. It has been stated that the pattern of ecdysteroid titers is independent of the eyestalks (Hopkins, 1983). This condition might be reinforced by 1) an increased secretion from the YO exceeding tissue storage capacity, 2) rate of excretion of *G. lateralis*, and 3) higher ecdysteroid peaks in late premolt following a positive feedback (Snyder and Chang, 1991).

An increase and sustained synthesis and secretion of ecdysteroids (considerably higher at 35°) indicates that proteomic changes in the YO after ESA, involve proteins for assimilation and metabolism of cholesterol (Lee and Mykles, 2006; Mykles, 2011). Considering the variation in temperature plus an exacerbated secretion of ecdysteroids (35°C) and the absence of a regulatory

XO/SG complex, ecdysteroids secretion during stress response in *G. lateralis* may be overall dependent on gene expression and somehow CHH activity. It is important to note that ESA is not a molt-inducing method taking place naturally and lacks specificity, in which inhibition of the MIH/CHH signaling pathway can affect several downstream targets and response to neuropeptides such as MIH does not involve changes in mRNA levels (Lee and Mykles, 2006). Eyestalk ablation does not only remove the primary source of CHH, but possibly causes changes in gene expression not solely related to the inhibition of CHH itself (Manfrin et al., 2013). Increasing levels of CHH are followed by hyperglycemia via glycolysis (Liu et al., 2019a; Vinagre and Chung, 2016). Catabolism of glucose will generate ATP and NAD^+, a product of AMPk activity and the SIRTs substrate, respectively. AMPk and SIRTs mRNA levels were unchanging in ESA crabs with no source of CHH. A cooperative effect of CHH in stress response may be increasing glucose levels via glycolysis but not through gluconeogenesis. For example, in ESA *Marsupenaeus japonicus*, some transcript levels of enzymes of the metabolic pathway of gluconeogenesis are invariable (Nagai et al., 2011; Webster et al., 2012). In case that CHH levels have an important effect on stress response by regulating energy in events of reduced ATP production and increased ATP demand, this process could require a compensatory action of normal cellular activity for certain tissues (Teranishi and Stillman, 2007). In females of *Macrobrachium rosenbergii*, levels of glucose in hemolymph tended to increase from intermolt to premolt and postmolt after ESA (Kamaruding et al., 2018). This may demonstrate that CHH improves stress response in MLA but is not detectable in ESA as a result of a compensatory activity, and that 35°C may not be an extreme temperature for *G. lateralis*. Opposite to ecdysteroids under thermal stress, CHH levels or hyperglycemia may not exhibit a pattern when crustaceans are exposed to hypothermic or hyperthermic stress. *C. maenas* exposed to increasing

temperature, showed increased CHH in the hemolymph and peaks at the maximum temperature (Chung and Webster, 2005). CHH mRNA levels in *M. magister* were downregulated from intermolt to late premolt and decreased at higher temperature (Wittmann et al., 2018). In both intact and ESA *M. rosenbergii*, lower temperatures triggered increasing values of glucose in hemolymph and showed a delayed peak after recovery up to two hours (Kuo and Yang, 1999). Even after one year, ESA *H. americanus* showed basal levels of CHH (Chang et al., 1998), which explain there is another source of CHH in some crustacean species (Fu et al., 2016; Tsai et al., 2008; Ventura-Lopez et al., 2016).

Additional and critical changes in gene expression are detected long time after an organism returns to normothermic temperatures. Sonna et al. (2002) mention that about 50 genes undergo changes in mRNA levels during or after heat shock, and that some of these non-HSP genes are important mediators in the cell stress response. An intrigued question remains on whether eyestalk ablation caused an energetic imbalance and altered gene expression and stress response.

CONCLUSIONS

Changes in gene expression induced by heat occur both during hyperthermia as well as after return to normothermia. An additional approach to the study of heat stress in *Gecarcinus lateralis* may elucidate the expression of stress responsive genes during acute heat shock and after recovery from the incident. Therefore, heat stress during and after heat shock can be established as a system of protection and recovery at the molecular and cellular level. Downregulation of genes at the optimal temperature range and stable and upregulated mRNA levels may explain the use and relocation of energy. The energy necessary for protein and

ecdysteroids synthesis in the molt cycle of *G. lateralis* may be locally reduced when, at higher temperatures and defined time points, the translation of specific genes during the incident is required. Expression of genes to respond to heat shock may be considered a universal defense system that is observed across arthropods and some very well studied crustacean taxa such as Cladoceran, Anomuran and Brachyuran. Genes of stress response involve in energy sensing and thermal stress seem to be closely connected through the use and demand of energy. As biomarkers, these genes provide molecular information of the intensity of the environmental stress.

G. lateralis populations inhabit coastal areas of continental landmasses and islands across the tropical zone. Since they are considered stenothermal tropical species, they might be affected for changes at the local and mesoscale climate level in the context of global climate change. It was defined by Janzen (1967) that temperate species in climates with high climate variability should exhibit broader thermal tolerances, compared to that tropical species evolve to have narrower thermal limits in low variability environments. Many studies on molting of crustaceans focus on gene characterization and expression through the molt cycle (Abehsera et al., 2014; Lv et al., 2017; Tarrant et al., 2014; Techa and Chung, 2013, 2015), but less or no attention is directed to the environmental effect on molting and physiology of crustacean species. The present study provided the basic data for understanding the mechanism of response of *G. lateralis* under heat stress and provided suggestions for investigating the role of AMPk, SIRT, HSP, and HIF in the stages of the molt cycle of *G. lateralis*.

Table 3-1. Oligonucleotide primer sequences used for expression analysis of *G. lateralis* genes. F, forward; R, reverse; Tm, melting temperature; bp, base pair

Primer	Sequence (5' – 3')	Tm (°C)	Product (bp)
Gl-EF2-F1	TTCTATGCCTTTGGCCGTGTC	60.7	227
Gl-EF2-R1	ATGGTGCCCGTCTTAACCA	60.2	227
Gl-AMPkα-F1	CTCAAGCTGTTTCGTCATCC	60.8	201
Gl-AMPkα-R1	CATATGTCTGTGGCAGTAGTCC	62.0	201
Gl-AMPkγ-F1	CTCACCATCACCGACTTC	59.2	235
Gl-AMPkγ-R1	ATAGCACATTCCCTGTAGCC	61.5	235
Gl-SIRT1-F1	TATCCAGGTGCTTTCAACCC	62.0	216
Gl-SIRT1-R1	TTCCTCTTTCATCCACTCTTGG	61.9	216
Gl-SIRT7-F1	TTGGGCAGAAGAGTGAAGG	61.6	215
Gl-SIRT7-R1	AGTGTCTACAGGGATGTTGC	61.6	215
Gl-HSP60-F1	GGATGACACACTGCTACTGAA	61.8	204
Gl-HSP60-R1	GGTAAACGAGAAGAAGGACCG	62.1	204
Gl-HSP70-F1	ACCCGTATTCCTAAGATTCAGC	61.8	215
Gl-HSP70-R1	ATCAGCTTGGTCATCACTCC	61.7	215
Gl-HIFα-F1	CCTTCGTCAGCAGACACTC	61.8	235
Gl-HIFα-R1	GTCACCAGCCACACATAGC	62.6	235
Gl-HIFβ-F1	CAGTCACACCAGTCCTTAACC	62.1	233
Gl-HIFβ-R1	GACCTTCATGCGGCAGAT	62.0	233

Table 3-2. Results of two-way ANOVA of gene expression in YO of MLA animals exposed to 27, 32, and 35°C. Level of significance set at α>0.05 in bold.

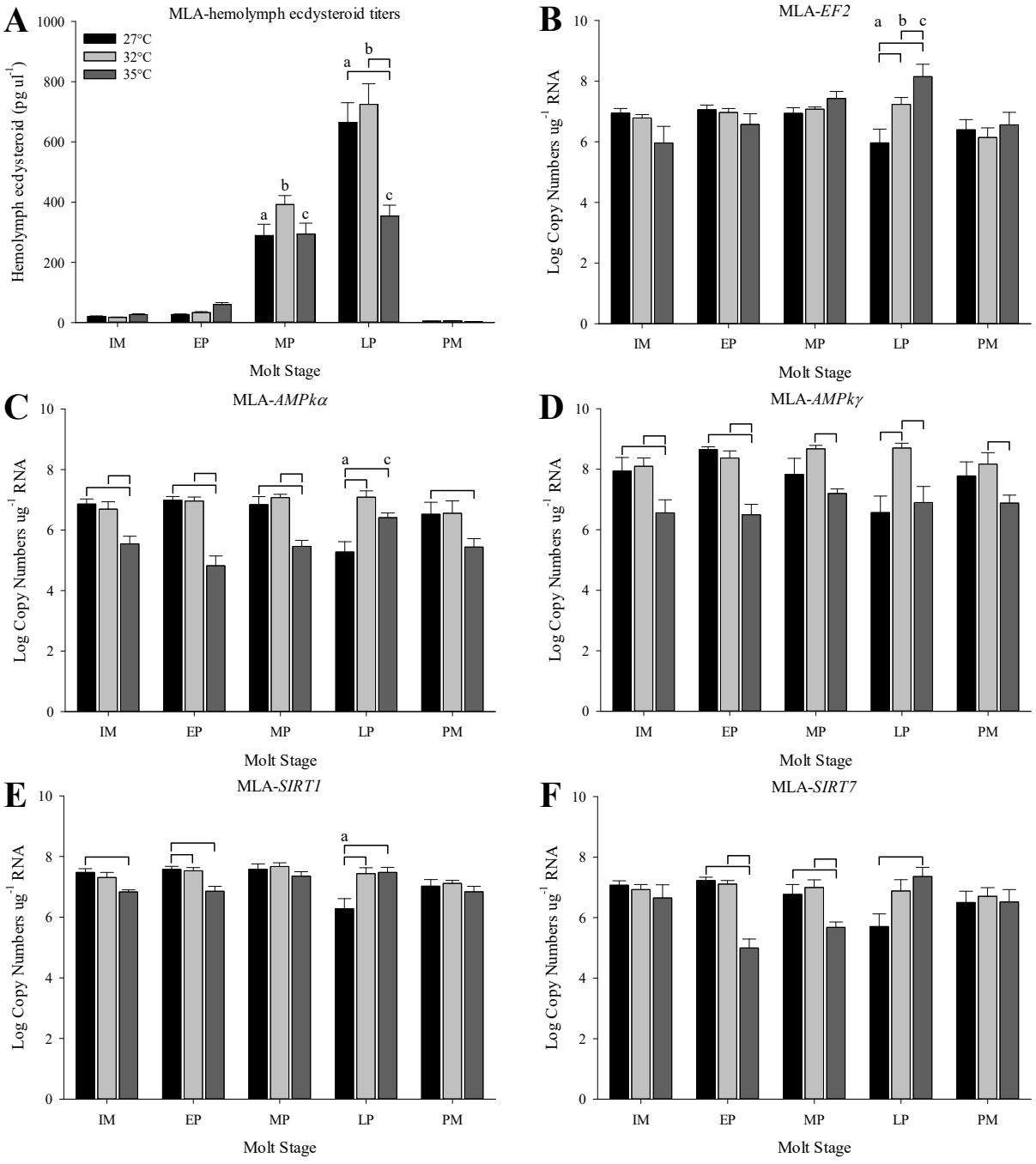
	Molt Stage			Temperature			Interaction		
	df	***F***	***P***	***df***	***F***	***P***	***df***	***F***	***P***
Gl-EF2	4	3.15	**0.0181**	2	0.80	0.4532	8	4.04	**0.0004**
Gl-AMPkα	4	0.98	0.4218	2	39.51	***P*<0.0001**	8	5.95	***P*<0.0001**
Gl-AMPkγ	4	1.61	0.1784	2	21.55	***P*<0.0001**	8	1.83	0.0788
Gl-SIRT1	4	5.19	**0.0008**	2	6.32	**0.0026**	8	6.11	***P*<0.0001**
Gl-SIRT7	4	0.74	0.5673	2	3.88	**0.0235**	8	5.28	***P*<0.0001**
Gl-HSP60	4	0.35	0.8461	2	3.18	**0.0455**	8	4.95	***P*<0.0001**
Gl-HSP70	4	3.00	**0.0218**	2	2.46	0.0904	8	5.75	***P*<0.0001**
Gl-HIFα	4	1.20	0.3175	2	13.32	***P*<0.0001**	8	3.35	**0.0019**
Gl-HIFβ	4	3.92	**0.0054**	2	114.90	***P*<0.0001**	8	7.82	***P*<0.0001**

Table 3-3. Results of two-way ANOVA of protein abundance and activity in YO of MLA animals exposed to 27, 32, and 35°C. Level of significance set at α>0.05 in bold.

	Molt Stage			**Temperature**			**Interaction**		
	df	***F***	***P***	***df***	***F***	***P***	***df***	***F***	***P***
Gl-AMPKα	4	12.51	***P*<0.0001**	2	93.17	***P*<0.0001**	8	4.48	**0.0001**
Gl-p-AMPKα	4	2.45	0.0509	2	11.66	***P*<0.0001**	8	2.91	**0.0058**
Gl-P70S6K	4	5.63	**0.0004**	2	18.03	***P*<0.0001**	8	8.23	***P*<0.0001**
Gl-p-P70S6K	4	3.71	***P*<0.0001**	2	60.7	***P*<0.0001**	8	49.1	***P*<0.0001**

Table 3-4. Results of two-way ANOVA of gene expression in YO of ESA animals exposed to 27 and 35°C. Level of significance set at α>0.05 in bold.

	Day post-ESA			**Temperature**			**Interaction**		
	df	***F***	***P***	***df***	***F***	***P***	***df***	***F***	***P***
Gl-EF2	6	2.24	**0.0493**	1	3.43	0.0683	5	0.33	0.8906
Gl-AMPkα	6	1.64	0.1514	1	0.41	0.5270	5	0.29	0.9178
Gl-AMPkγ	6	1.48	0.2010	1	0.94	0.3369	5	0.81	0.5487
Gl-SIRT1	6	2.95	**0.0130**	1	0.18	0.6699	5	0.55	0.7342
Gl-SIRT7	6	2.07	0.0685	1	0.01	0.9178	5	0.85	0.5181
Gl-HSP60	6	2.92	**0.0138**	1	0.09	0.7600	5	0.82	0.5395
Gl-HSP70	6	3.65	**0.0034**	1	0.27	0.6060	5	0.45	0.8135
Gl-HIFα	6	2.80	**0.0175**	1	0.71	0.4037	5	0.55	0.7345
Gl-HIFβ	6	2.21	**0.0381**	1	0.27	0.6041	5	0.32	0.8977


A
MLA-hemolymph ecdysteroid titers
27°C
32°C
35°C
Hemolymph ecdysteroid (pg ul-1)
Molt Stage
IM
EP
MP
LP
PM
B
MLA-EF2
Log Copy Numbers ug-1 RNA
C
MLA-AMPkα
D
MLA-AMPkγ
E
MLA-SIRT1
F
MLA-SIRT7

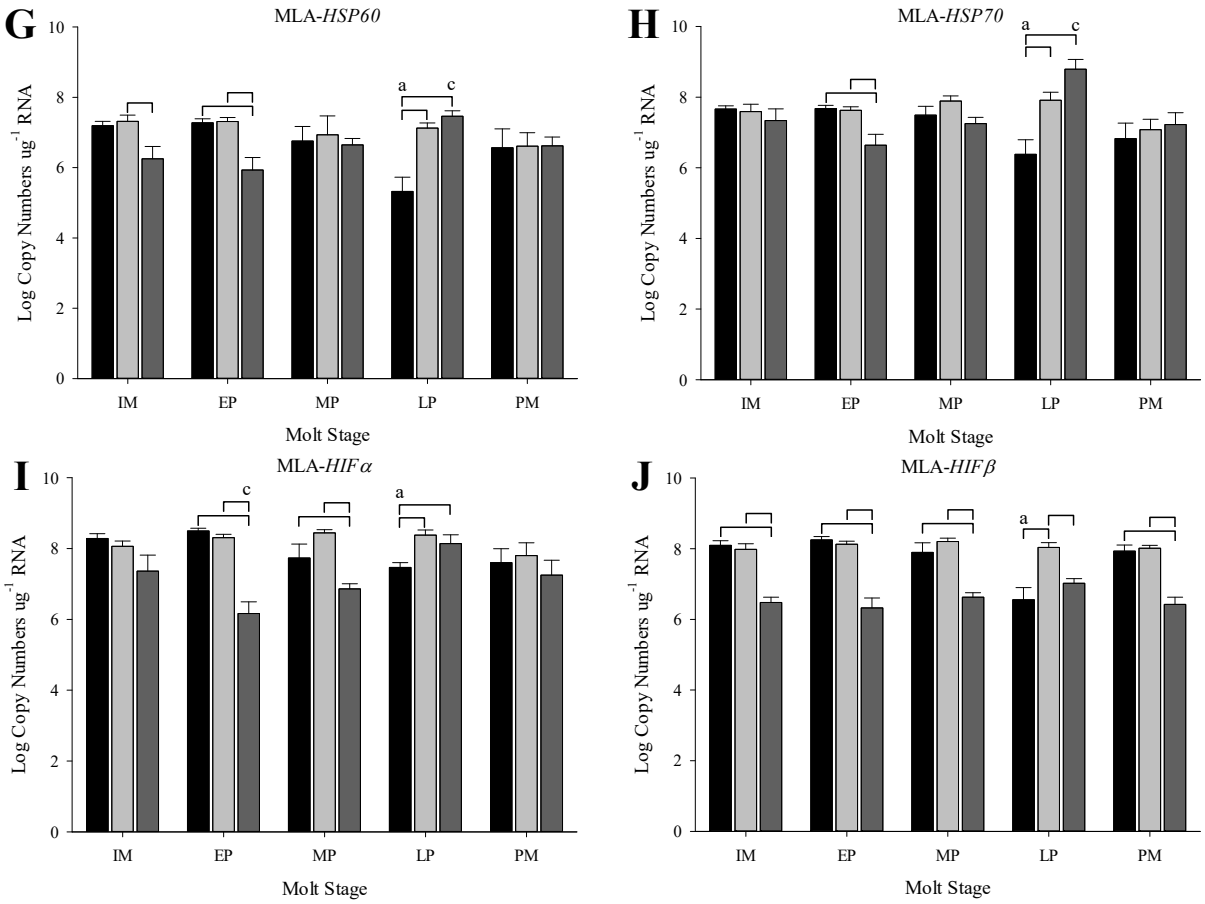


Figure 3-1. mRNA levels in MLA animals exposed to 27, 32 or 35°C for 1 h. (A) Ecdysteroid titers, (B) *EF2*, (C) *AMPkα*, (D) *AMPkγ*, (E) *SIRT1*, (F) *SIRT7*, (G) *HSP60*, (H) *HSP70*, (I) *HIFα*, (J) *HIFβ*. Letter indicates significant differences when compared with intermolt stages at specific temperature; brackets indicate significant differences according to Tukey-Kramer HSD *post hoc* tests at $P<0.05$ (n = 6-9).

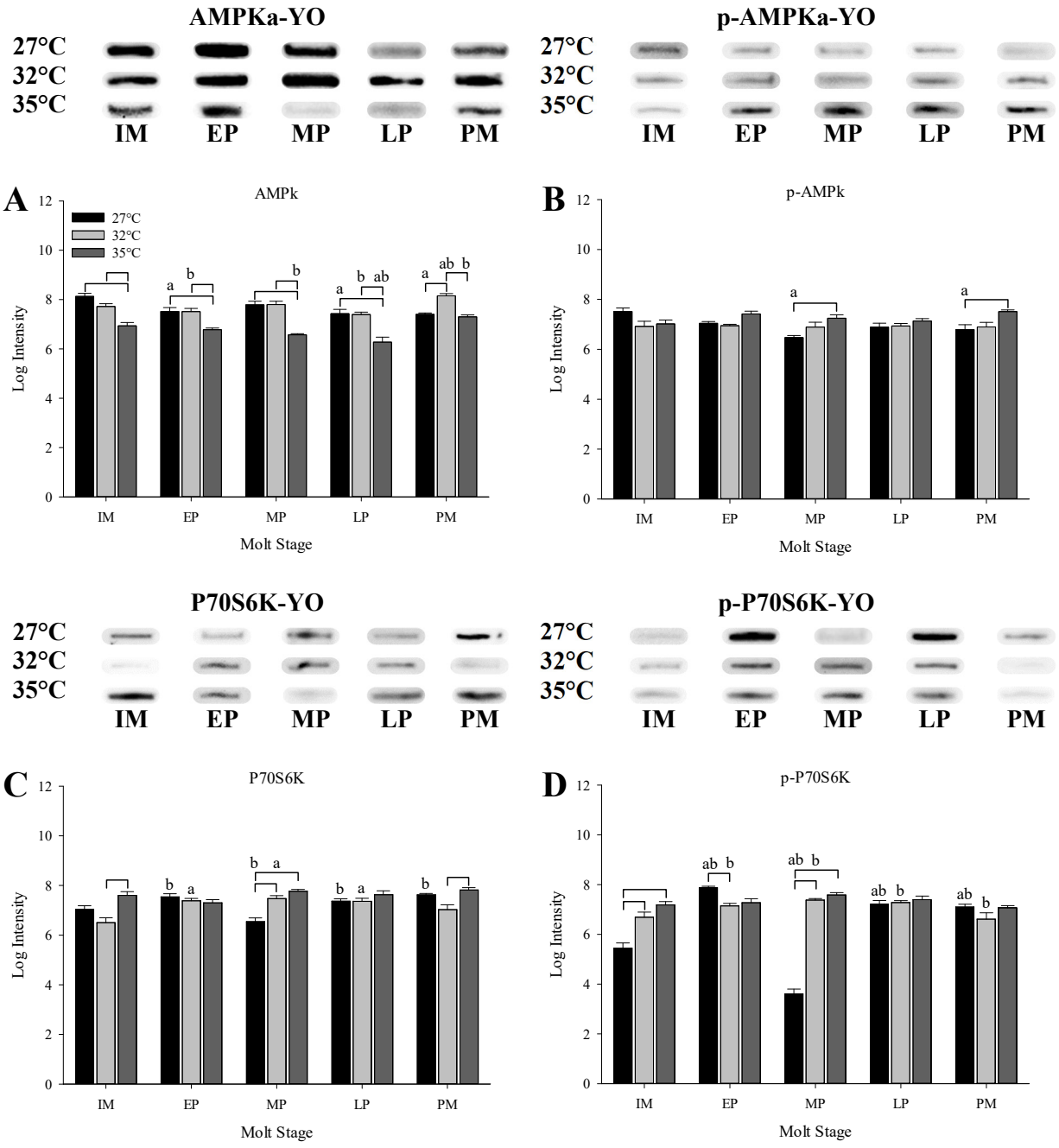
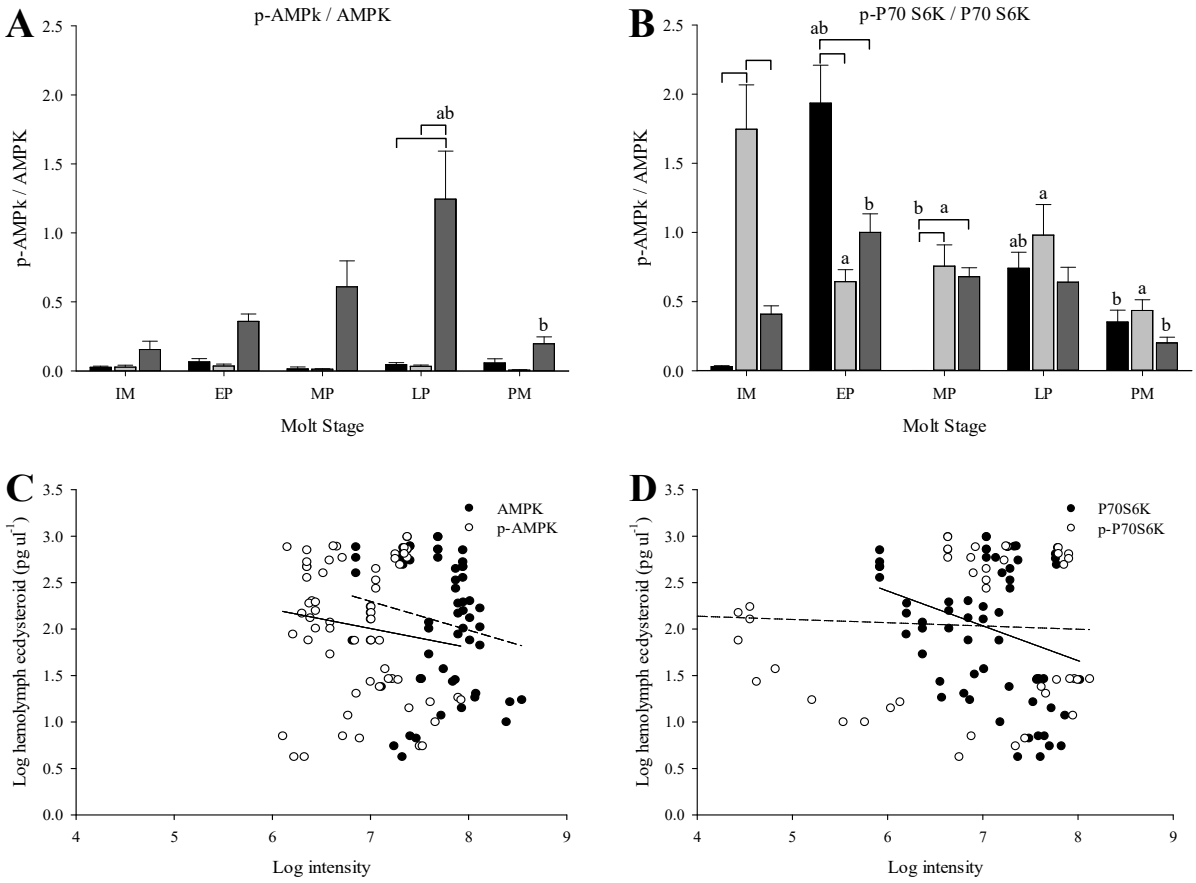
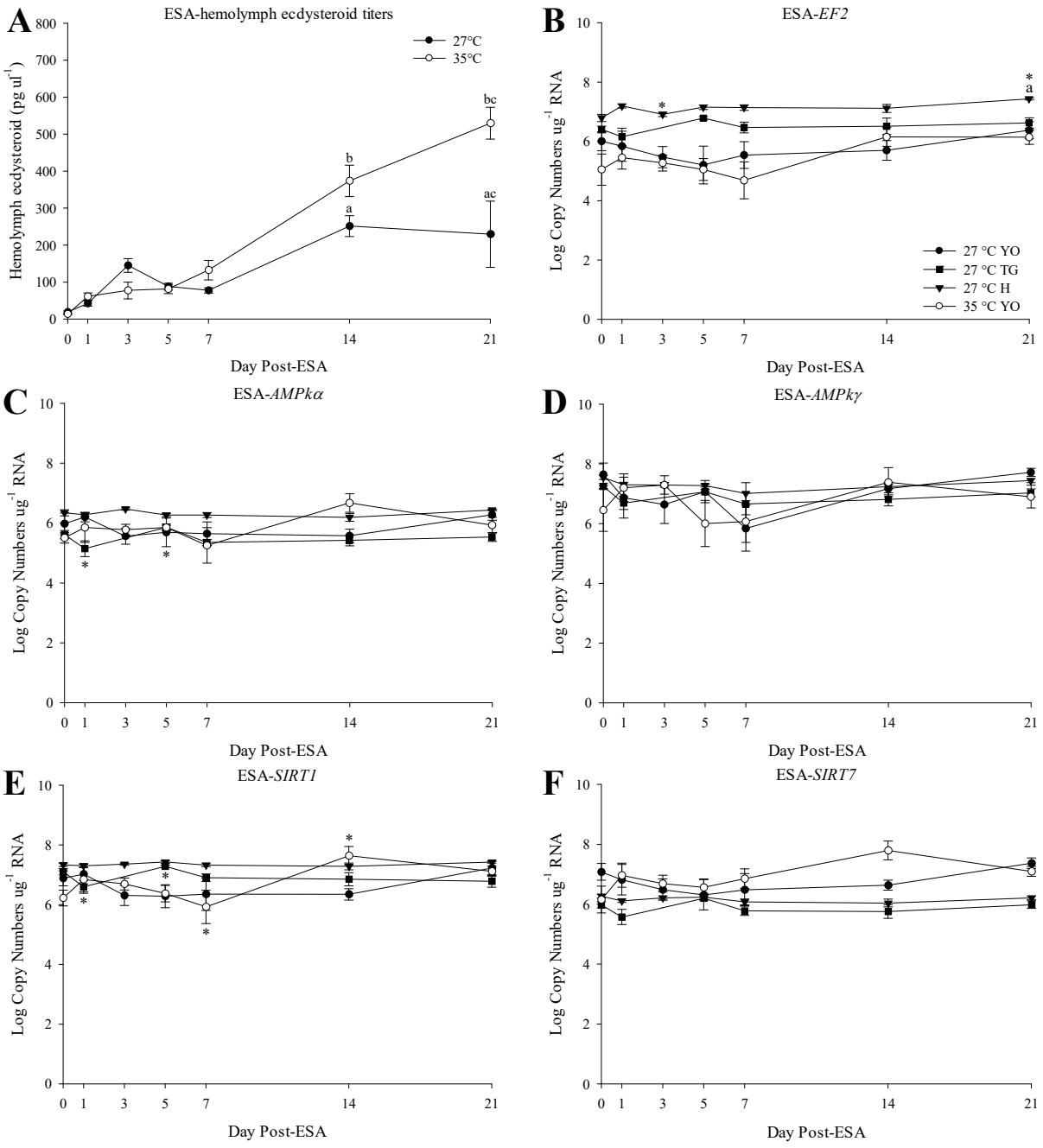


Figure 3-2. Western Blot analysis of protein abundance and activity in MLA animals exposed to 27, 32 or 35°C for 1 h. (A) Representative Western Blots of AMPKa (B) representative Western Blots of p-AMPKα, (C) representative Western Blots of P70S6K (D) representative Western Blots of p-P70S6K. *a* indicates significant differences when compared with intermolt stages at specific temperature; *b* indicates significant differences among molt stages at specific temperature, according to Tukey-Kramer HSD post hoc tests at $P<0.05$ (n = 5-8).



	27°C			
	AMPKα	**p-AMPKα**	**P70S6K**	**p-P70S6K**
Pearson (r)	-0.1896	-0.1323	-0.2914	-0.0930
R^2 (%)	4.9387	1.9708	5.2417	1.3903
***P*=**	0.0852	0.2765	0.0711	0.3738

Figure 3-3. Protein ratio in MLA animals exposed to 27, 32 or 35°C for 1 h. (A) Ratio of p-AMPKα / AMPKa, (B) Ratio of p-P70S6K / P70S6K, relationship at 27°C between ecdysteroid titers and (C) AMPKa (complete line) and p-AMPKa (dash line), (D) P70S6K(complete line) and p-P70S6K (dash line), table: relationship between ecdysteroid titers and *Gl*-AMPkα and *Gl*-P70S6K expression at 27°C. *a* indicates significant differences when compared with intermolt stages at specific temperature; *b* indicates significant differences among molt stages at specific temperature, according to Tukey-Kramer HSD post hoc tests at $P<0.05$ (n = 5-8).


A
ESA-hemolymph ecdysteroid titers
27°C
35°C
Hemolymph ecdysteroid (pg ul-1)
Day Post-ESA
B
ESA-EF2
27 °C YO
27 °C TG
27 °C H
35 °C YO
Log Copy Numbers ug-1 RNA
Day Post-ESA
C
ESA-AMPkα
Log Copy Numbers ug-1 RNA
Day Post-ESA
D
ESA-AMPkγ
Log Copy Numbers ug-1 RNA
Day Post-ESA
E
ESA-SIRT1
Log Copy Numbers ug-1 RNA
Day Post-ESA
F
ESA-SIRT7
Log Copy Numbers ug-1 RNA
Day Post-ESA

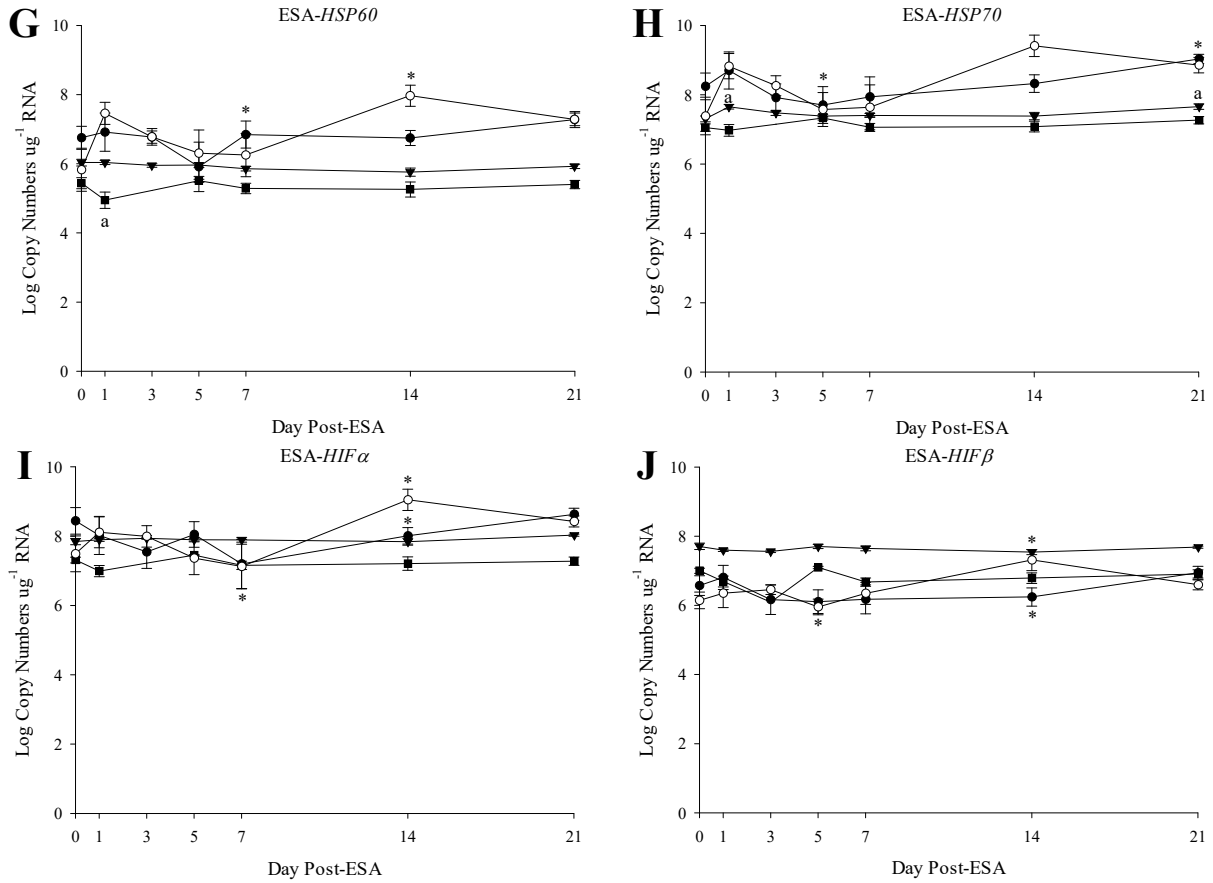


Figure 3-4. mRNA levels in ESA animals exposed to 27 or 35°C for 1 h. (A) Ecdysteroid titers, (B) *EF2*, (C) *AMPkα*, (D) *AMPkγ*, (E) *SIRT1*, (F) *SIRT7*, (G) *HSP60*, (H) *HSP70*, (I) *HIFα*, (J) *HIFβ*. *a* indicates significant differences when compared with day 0 post-ESA at specific temperature; *b* and asterisk indicate significant differences among days post-ESA at specific temperature, asterisk indicates significant differences between treatments at specific day post-ESA, according to Tukey-Kramer HSD *post hoc* tests at $P<0.05$ (n = 6-7).

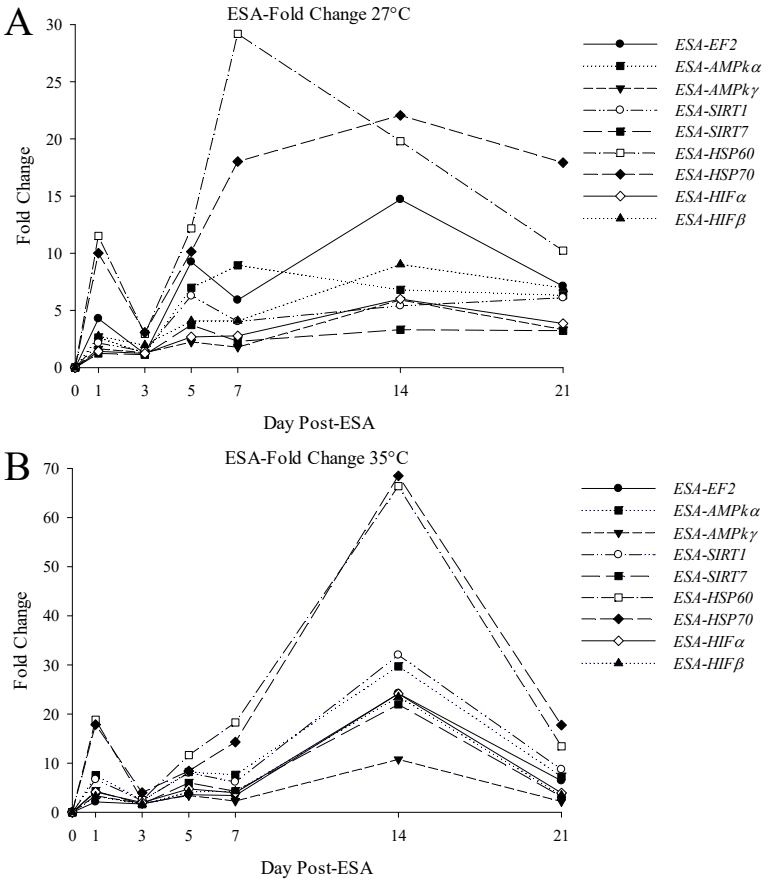


Figure 3-5. Fold Change of mRNA levels in Y-organ of ESA animals exposed to 27 or 35°C for 1 h. (A) 27°C, (B) 35°C.

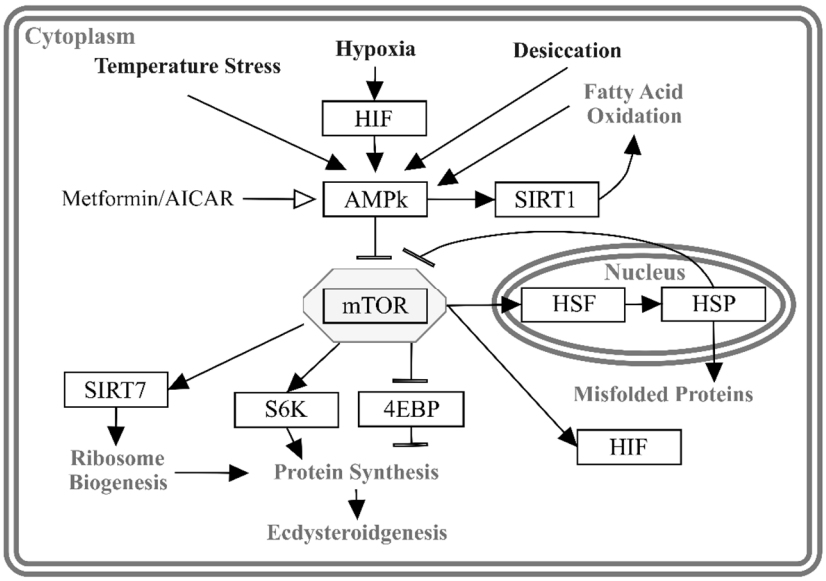


Figure 3-6. Model for stress response signaling in the crustacean YO. mTOR-dependent protein synthesis, which is regulated by environmental stresses via AMPk, is required for ecdysteroid biosynthesis. Downstream effectors are Silent Information Regulators (SIRTs), heat shock protein (HSPs), and hypoxia inducible factor (HIF). AICAR and Metformin activate AMPk.

REFERENCES

Abehsera, S., Glazer, L., Tynyakov, J., Plaschkes, I., Chalifa-Caspi, V., Khalaila, I., Aflalo, E. D. and Sagi, A. (2014). Binary gene expression patterning of the molt cycle: the case of chitin metabolism. *PLoS One* **10**, e0122602.

Abuhagr, A. M., Blindert, J. L., Nimitkul, S., Zander, I. A., Labere, S. M., Chang, S. A., Maclea, K. S., Chang, E. S. and Mykles, D. L. (2014a). Molt regulation in green and red color morphs of the crab *Carcinus maenas*: gene expression of molt-inhibiting hormone signaling components. *J Exp Biol* **217**, 796-808.

Abuhagr, A. M., Maclea, K. S., Chang, E. S. and Mykles, D. L. (2014b). Mechanistic target of rapamycin (mTOR) signaling genes in decapod crustaceans: cloning and tissue expression of mTOR, Akt, Rheb, and p70 S6 kinase in the green crab, *Carcinus maenas*, and blackback land crab, *Gecarcinus lateralis*. *Comp Biochem Physiol A Mol Integr Physiol* **168**, 25-39.

Abuhagr, A. M., MacLea, K. S., Mudron, M. R., Chang, S. A., Chang, E. S. and Mykles, D. L. (2016). Roles of mechanistic target of rapamycin and transforming growth factor-beta signaling in the molting gland (Y-organ) of the blackback land crab, *Gecarcinus lateralis*. *Comp Biochem Physiol A Mol Integr Physiol* **198**, 15-21.

Aiken, D. E. and Waddy, S. L. (1975). Temperature increase can cause hyperecdysonism in American lobsters (*Homarus americanus*) injected with ecdysterone. *Journal of the Fisheries Research Board of Canada* **32**, 1843-1845.

Aiken, D. E. and Waddy, S. L. (1995). Aquaculture. In *Biology of the lobster Homarus americanus*, (ed. J. R. Factor), pp. 153-175. San Diego: Academic Press.

Anestis, A., Lazou, A., Portner, H. O. and Michaelidis, B. (2007). Behavioral, metabolic, and molecular stress responses of marine bivalve *Mytilus galloprovincialis* during long-term acclimation at increasing ambient temperature. *Am J Physiol Regul Integr Comp Physiol* **293**, R911-21.

Baringou, S., Rouault, J. D., Koken, M., Hardivillier, Y., Hurtado, L. and Leignel, V. (2016). Diversity of cytosolic HSP70 Heat Shock Protein from decapods and their phylogenetic placement within Arthropoda. *Gene* **591**, 97-107.

Bliss, D. E. and Boyer, J. R. (1964). Environmental regulation of growth in the decapod crustacean *Gecarcinus lateralis*. *Gen Comp Endocrinol* **4**, 15-41.

Buckley, B. A., Owen, M. E. and Hofmann, G. E. (2001). Adjusting the thermostat: the threshold induction temperature for the heat-shock response in intertidal mussels (genus *Mytilus*) changes as a function of thermal history. *The Journal of Experimental Biology*.

Canto, C., Gerhart-Hines, Z., Feige, J. N., Lagouge, M., Noriega, L., Milne, J. C., Elliott, P. J., Puigserver, P. and Auwerx, J. (2009). AMPK regulates energy expenditure by modulating NAD+ metabolism and SIRT1 activity. *Nature* **458**, 1056-60.

Cetrullo, S., D'Adamo, S., Tantini, B., Borzi, R. M. and Flamigni, F. (2015). mTOR, AMPK, and Sirt1: key players in metabolic stress management. *Crit Rev Eukaryot Gene Expr* **25**, 59-75.

Chang, E. S. (1995). Physiological and biochemical changes during the molt cycle in decapod crustaceans: an overview. *Journal of Experimental Marine Biology and Ecology* **193**, 1-14.

Chang, E. S. and Bruce, M. J. (1980). Ecdysteroid titers of juvenile lobsters following molt induction. *Journal of Experimental Zoology* **214**, 157-160.

Chang, E. S. and Bruce, M. J. (1981). Ecdysteroid titers of larval lobsters. *Comparative Biochemistry and Physiology Part A: Physiology* **70**, 239-241.

Chang, E. S., Keller, R. and Chang, S. A. (1998). Quantification of crustacean hyperglycemic hormone by ELISA in hemolymph of the lobster, *Homarus americanus*, following various stresses. *Gen Comp Endocrinol* **111**, 359-66.

Chang, E. S. and Mykles, D. L. (2011). Regulation of crustacean molting: a review and our perspectives. *Gen Comp Endocrinol* **172**, 323-30.

Chang, E. S., Sage, B. A. and O'Connor, J. D. (1976). The qualitative and quantitative determinations of ecdysones in tissues of the crab, *Pachygrapsus crassipes*, following molt induction. *General and Comparative Endocrinology* **30**, 21-33.

Chen, T., Lin, T., Li, H., Lu, T., Li, J., Huang, W., Sun, H., Jiang, X., Zhang, J., Yan, A. et al. (2018). Heat shock protein 40 (HSP40) in Pacific white shrimp (*Litopenaeus vannamei*): molecular cloning, tissue distribution and ontogeny, response to temperature, acidity/alkalinity and salinity stresses, and potential role in ovarian development. *Front Physiol* **9**, 1784.

Chou, S. D., Prince, T., Gong, J. and Calderwood, S. K. (2012). mTOR is essential for the proteotoxic stress response, HSF1 activation and heat shock protein synthesis. *PLoS One* **7**, e39679.

Chung, J. S. and Webster, S. G. (2005). Dynamics of in vivo release of molt-inhibiting hormone and crustacean hyperglycemic hormone in the shore crab, *Carcinus maenas*. *Endocrinology* **146**, 5545-51.

Das, S., Vraspir, L., Zhou, W., Durica, D. S. and Mykles, D. L. (2018). Transcriptomic analysis of differentially expressed genes in the molting gland (Y-organ) of the

blackback land crab, *Gecarcinus lateralis*, during molt-cycle stage transitions. *Comp Biochem Physiol Part D Genomics Proteomics* **28**, 37-53.

Drozdova, P., Bedulina, D., Madyarova, E., Rivarola-Duarte, L., Schreiber, S., Stadler, P. F., Luckenbach, T. and Timofeyev, M. (2019). Description of strongly heat-inducible heat shock protein 70 transcripts from Baikal endemic amphipods. *Sci Rep* **9**, 8907.

Durliat, M., Moriniere, M. and Porcheron, P. (1988). Changes in ecdysteroids in *Astacus leptodactylus* during the molting cycle. *Comparative Biochemistry and Physiology Part A: Physiology* **89**, 223-229.

El Haj, A. J. (1999). Regulation of muscle growth and sarcomeric protein gene expression over the intermolt cycle. *American Zoologist* **39**, 570-579.

Feder, M. E. and Hofmann, G. E. (1999). Heat-shock proteins, molecular chaperones, and the stress response: evolutionary and ecological physiology. *Annu Rev Physiol* **61**, 243-82.

Frederich, M. and Balschi, J. A. (2002). The relationship between AMP-activated protein kinase activity and AMP concentration in the isolated perfused rat heart. *J Biol Chem* **277**, 1928-32.

Frederich, M., O'Rourke, M. R., Furey, N. B. and Jost, J. A. (2009). AMP-activated protein kinase (AMPK) in the rock crab, *Cancer irroratus*: an early indicator of temperature stress. *J Exp Biol* **212**, 722-30.

Fu, C., Huang, X., Gong, J., Chen, X., Huang, H. and Ye, H. (2016). Crustacean hyperglycaemic hormone gene from the mud crab, *Scylla paramamosain*: cloning, distribution and expression profiles during the moulting cycle and ovarian development. *Aquaculture Research* **47**, 2183-2194.

Fusi, M., Cannicci, S., Daffonchio, D., Mostert, B., Portner, H. O. and Giomi, F.

(2016). The trade-off between heat tolerance and metabolic cost drives the bimodal life strategy at the air-water interface. *Sci Rep* **6**, 19158.

Ghosh, H. S., McBurney, M. and Robbins, P. D. (2010). SIRT1 negatively regulates the mammalian target of rapamycin. *PLoS One* **5**, e9199.

Giovannini, L. and Bianchi, S. (2017). Role of nutraceutical SIRT1 modulators in AMPK and mTOR pathway: evidence of a synergistic effect. *Nutrition* **34**, 82-96.

Greiss, S. and Gartner, A. (2009). Sirtuin/Sir2 phylogeny, evolutionary considerations and structural conservation. *Mol Cells* **28**, 407-15.

Haissaguerre, M., Saucisse, N. and Cota, D. (2014). Influence of mTOR in energy and metabolic homeostasis. *Mol Cell Endocrinol* **397**, 67-77.

Haj, A., Clarke, S., Harrison, P. and Chang, E. (1996). In vivo muscle protein synthesis rates in the American lobster *Homarus americanus* during the moult cycle and in response to 20-hydroxyecdysone. *The Journal of Experimental Biology* **199**, 579-585.

Hammer, Ø., Harper, D. A. T. and Ryan, P. D. (2001). PAST: paleontological statistics software package for education and data analysis. *Palaeontologia Electronica* **4**, 9.

Han, G. D., Zhang, S., Marshall, D. J., Ke, C. H. and Dong, Y. W. (2013). Metabolic energy sensors (AMPK and SIRT1), protein carbonylation and cardiac failure as biomarkers of thermal stress in an intertidal limpet: linking energetic allocation with environmental temperature during aerial emersion. *J Exp Biol* **216**, 3273-82.

Han, J., Lee, M.-C., Park, J. C., Kim, S. and Lee, J.-S. (2018). Effects of temperature shifts on life parameters and expression of fatty acid synthesis and heat shock protein genes in temperate and Antarctic copepods *Tigriopus japonicus* and *Tigriopus kingsejongensis*. *Polar Biology* **41**, 2459-2466.

Hardy, K. M., Follett, C. R., Burnett, L. E. and Lema, S. C. (2012). Gene transcripts encoding hypoxia-inducible factor (HIF) exhibit tissue- and muscle fiber type-dependent responses to hypoxia and hypercapnic hypoxia in the Atlantic blue crab, *Callinectes sapidus*. *Comp Biochem Physiol A Mol Integr Physiol* **163**, 137-46.

Hartnoll, R. G. (1988). Growth and molting. In *Biology of the Land Crabs*, (ed. W. Burggren and B. Mcmahon), pp. 186-210. Cambridge: Cambridge University Press.

Hartnoll, R. G. (2001). Growth in Crustacea - twenty years on. *Hydrobiologia* **449**, 111-122.

Hofmann, G. and Somero, G. (1995). Evidence for protein damage at environmental temperatures: seasonal changes in levels of ubiquitin conjugates and hsp70 in the intertidal mussel *Mytilus trossulus*. *J Exp Biol* **198**, 1509-18.

Holcik, M. and Sonenberg, N. (2005). Translational control in stress and apoptosis. *Nat Rev Mol Cell Biol* **6**, 318-27.

Hopkins, P. M. (1983). Patterns of serum ecdysteroids during induced and uninduced proecdysis in the fiddler crab, *Uca pugilator*. *Gen Comp Endocrinol* **52**, 350-6.

Hopkins, P. M. (1986). Ecdysteroid titers and Y-organ activity during late anecdysis and proecdysis in the fiddler crab, *Uca pugilator*. *Gen Comp Endocrinol* **63**, 362-73.

Hopkins, P. M. (2001). Limb regeneration in the fiddler crab, *Uca pugilator*: hormonal and growth factor control. *American Zoologist* **41**, 389-398.

Hopkins, P. M. (2012). The eyes have it: a brief history of crustacean neuroendocrinology. *Gen Comp Endocrinol* **175**, 357-66.

Huang, X., Wang, T., Ye, Z., Han, G. and Dong, Y. (2015). Temperature relations of aerial and aquatic physiological performance in a mid-intertidal limpet *Cellana toreuma*:

adaptation to rapid changes in thermal stress during emersion. *Integr Zool* **10**, 159-70.

Inoki, K., Kim, J. and Guan, K. L. (2012). AMPK and mTOR in cellular energy homeostasis and drug targets. *Annu Rev Pharmacol Toxicol* **52**, 381-400.

Janzen, D. H. (1967). Why mountain passes are higher in the tropics. *The American Naturalist* **101**, 233-249.

Johnson, C. L. (2003). Ecdysteroids in the oceanic copepod *Calanus pacificus*: variation during molt cycle and change associated with diapause. *Marine Ecology Progress Series* **257**, 159-165.

Jost, J. A., Podolski, S. M. and Frederich, M. (2012). Enhancing thermal tolerance by eliminating the pejus range: a comparative study with three decapod crustaceans. *Marine Ecology Progress Series* **444**, 263-274.

Kamaruding, N. A., Ismail, N. and Ikhwanuddin, M. (2018). Physiological effect of eyestalk ablation on nutrient utilization and plasma protein expression in the female giant freshwater prawn (*Macrobrachium rosenbergii*) during different molting cycles. *Journal of Shellfish Research* **37**, 1113-1120.

Kuo, C. M. and Yang, Y. H. (1999). Hyperglycemic responses to cold shock in the freshwater giant prawn, *Macrobrachium rosenbergii*. *Journal of Comparative Physiology B* **169**, 49-54.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-5.

Larkin, M. A., Blackshields, G., Brown, N. P., Chenna, R., McGettigan, P. A., McWilliam, H., Valentin, F., Wallace, I. M., Wilm, A., Lopez, R. et al. (2007). Clustal W and Clustal X version 2.0. *Bioinformatics* **23**, 2947-8.

Lee, K. J., Watson, R. D. and Roer, R. D. (1998). Molt-inhibiting hormone mRNA levels and ecdysteroid titer during a molt cycle of the blue crab, *Callinectes sapidus*. *Biochem Biophys Res Commun* **249**, 624-7.

Lee, S. G. and Mykles, D. L. (2006). Proteomics and signal transduction in the crustacean molting gland. *Integr Comp Biol* **46**, 965-77.

Liu, A., Liu, J., Chen, X., Lu, B., Zeng, C. and Ye, H. (2019). A novel crustacean hyperglycemic hormone (CHH) from the mud crab *Scylla paramamosain* regulating carbohydrate metabolism. *Comp Biochem Physiol A Mol Integr Physiol* **231**, 49-55.

Lv, J., Zhang, L., Liu, P. and Li, J. (2017). Transcriptomic variation of eyestalk reveals the genes and biological processes associated with molting in *Portunus trituberculatus*. *PLoS One* **12**, e0175315.

Madeira, C., Leal, M. C., Diniz, M. S., Cabral, H. N. and Vinagre, C. (2018). Thermal stress and energy metabolism in two circumtropical decapod crustaceans: responses to acute temperature events. *Mar Environ Res* **141**, 148-158.

Madeira, D., Narciso, L., Cabral, H. N., Diniz, M. S. and Vinagre, C. (2012). Thermal tolerance of the crab *Pachygrapsus marmoratus*: intraspecific differences at a physiological (CTMax) and molecular level (Hsp70). *Cell Stress Chaperones* **17**, 707-16.

Manfrin, C., Tom, M., De Moro, G., Gerdol, M., Guarnaccia, C., Mosco, A., Pallavicini, A. and Giulianini, P. G. (2013). Application of d-crustacean hyperglycemic hormone induces peptidases transcription and suppresses glycolysis-related transcripts in the hepatopancreas of the crayfish *Pontastacus leptodactylus* - results of a transcriptomic study. *PLoS One* **8**, e65176.

McDonald, A. A., Chang, E. S. and Mykles, D. L. (2011). Cloning of a nitric oxide

synthase from green shore crab, C*arcinus maenas*: a comparative study of the effects of eyestalk ablation on expression in the molting glands (Y-organs) of *C. maenas*, and blackback land crab, *Gecarcinus lateralis*. *Comp Biochem Physiol A Mol Integr Physiol* **158**, 150-62.

Medler, S., Brown, K. J., Chang, E. S. and Mykles, D. L. (2005). Eyestalk ablation has little effect on actin and myosin heavy chain gene expression in adult lobster skeletal muscles. *Biol Bull* **208**, 127-37.

Mihaylova, M. M. and Shaw, R. J. (2011). The AMPK signalling pathway coordinates cell growth, autophagy and metabolism. *Nat Cell Biol* **13**, 1016-23.

Mykles, D. L. (2001). Interactions between limb regeneration and molting in decapod crustaceans. *American Zoologist* **41**, 399-406.

Mykles, D. L. (2011). Ecdysteroid metabolism in crustaceans. *J Steroid Biochem Mol Biol* **127**, 196-203.

Nagai, C., Nagata, S. and Nagasawa, H. (2011). Effects of crustacean hyperglycemic hormone (CHH) on the transcript expression of carbohydrate metabolism-related enzyme genes in the kuruma prawn, *Marsupenaeus japonicus*. *General and Comparative Endocrinology* **172**, 293-304.

Nicholas, K. B. and Nicholas, H. B. J. (1997). GeneDoc: a tool for editing and annotating multiple sequence alignments. *Distributed by the author*.

Okumura, t. and Aida, k. (2001). Effects of bilateral eyestalk ablation on molting and ovarian development in the giant freshwater prawn, *Macrobrachium rosenbergii*. *Fisheries Science* **67**, 1125-1135.

Okumura, T., Nakamura, K., Aida, K. and Hanyu, I. (1989). Hemolymph ecdysteroid levels during the molt cycle in the kuruma prawn *Penaeus japonicus*. *Nippon Suisan Gakkaishi*

55, 2091-2098.

Pitts, N. L., Schulz, H. M., Oatman, S. R. and Mykles, D. L. (2017). Elevated expression of neuropeptide signaling genes in the eyestalk ganglia and Y-organ of *Gecarcinus lateralis* individuals that are refractory to molt induction. *Comp Biochem Physiol A Mol Integr Physiol* **214**, 66-78.

Portner, H. O., Bock, C. and Mark, F. C. (2017). Oxygen- and capacity-limited thermal tolerance: bridging ecology and physiology. *J Exp Biol* **220**, 2685-2696.

Pörtner, H. O., Peck, L. S. and Hirse, T. (2006). Hyperoxia alleviates thermal stress in the Antarctic bivalve, *Laternula elliptica*: evidence for oxygen limited thermal tolerance. *Polar Biology* **29**, 688-693.

Ruderman, N. B., Xu, X. J., Nelson, L., Cacicedo, J. M., Saha, A. K., Lan, F. and Ido, Y. (2010). AMPK and SIRT1: a long-standing partnership? *Am J Physiol Endocrinol Metab* **298**, E751-60.

Santiago, J. and Sturgill, T. (2001). Identification of the S6 kinase activity stimulated in quiescent brine shrimp embryos upon entry to preemergence development as p70 ribosomal protein S6 kinase: isolation of *Artemia franciscana* p70S6k cDNA. *Biochem Cell Biol* **79**, 141 - 52.

Saxton, R. A. and Sabatini, D. M. (2017). mTOR Signaling in growth, metabolism, and disease. *Cell* **168**, 960-976.

Schumpert, C. A., Anderson, C., Dudycha, J. L. and Patel, R. C. (2016). Involvement of *Daphnia pulicaria* Sir2 in regulating stress response and lifespan. *Aging (Albany NY)* **8**, 402-17.

Sheng, B., Liu, J. and Li, G. H. (2012). Metformin preconditioning protects Daphnia

pulex from lethal hypoxic insult involving AMPK, HIF and mTOR signaling. *Comp Biochem Physiol B Biochem Mol Biol* **163**, 51-8.

Skinner, D. M. (1985). Molting and Regeneration. In *The Biology of Crustacea*, vol. 9: *Integument, Pigments and Hormonal Processes*. (ed. D. E. Bliss and L. H. Mantel), pp. 43-146. San Diego: Academic Press.

Skinner, D. M. and Graham, D. E. (1972). Loss of limbs as a stimulus to ecdysis in brachyura (true crabs). *The Biological Bulletin* **143**, 222-233.

Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J. and Klenk, D. C. (1985). Measurement of protein using bicinchoninic acid. *Analytical Biochemistry* **150**, 76-85.

Snyder, M. J. and Chang, E. S. (1991). Ecdysteroids in relation to the molt cycle of the American lobster, *Homarus americanus* I. Hemolymph Titers and Metabolites. *General and Comparative Endocrinology* **81**, 133-145.

Somero, G. N. (2002). Thermal physiology and vertical zonation of intertidal animals: optima, limits, and costs of living. *Integr Comp Biol* **42**, 780-9.

Soñanez-Organis, J. G., Racotta, I. S. and Yepiz-Plascencia, G. (2010). Silencing of the hypoxia inducible factor 1 -HIF-1- obliterates the effects of hypoxia on glucose and lactate concentrations in a tissue-specific manner in the shrimp *Litopenaeus vannamei*. *Journal of Experimental Marine Biology and Ecology* **393**, 51-58.

Sonna, L. A., Fujita, J., Gaffin, S. L. and Lilly, C. M. (2002). Invited review: effects of heat and cold stress on mammalian gene expression. *J Appl Physiol (1985)* **92**, 1725-42.

Soumoff, C. and Skinner, D. M. (1983). Ecdysteroid titers during the molt cycle of the blue crab resemble those of other crustacea. *The Biological Bulletin* **165**, 321-329.

Spindler, K.-D. and Anger, K. (1986). Ecdysteroid levels during the larval development of the spider crab *Hyas araneus*. *General and Comparative Endocrinology* **64**, 122-128.

Sun, S., Xuan, F., Fu, H., Ge, X., Zhu, J., Qiao, H., Jin, S. and Zhang, W. (2016). Molecular characterization and mRNA expression of hypoxia inducible factor-1 and cognate inhibiting factor in *Macrobrachium nipponense* in response to hypoxia. *Comp Biochem Physiol B Biochem Mol Biol* **196-197**, 48-56.

Tamone, S. L., Adams, M. M. and Dutton, J. M. (2005). Effect of eyestalk-ablation on circulating ecdysteroids in hemolymph of snow crabs, *Chionoecetes opilio*: physiological evidence for a terminal molt. *Integr Comp Biol* **45**, 166-71.

Tan, C. Y. and Hagen, T. (2013). Post-translational regulation of mTOR complex 1 in hypoxia and reoxygenation. *Cell Signal* **25**, 1235-44.

Tarrant, A. M., Baumgartner, M. F., Hansen, B. H., Altin, D., Nordtug, T. and Olsen, A. J. (2014). Transcriptional profiling of reproductive development, lipid storage and molting throughout the last juvenile stage of the marine copepod *Calanus finmarchicus*. *Front Zool* **11**, 91.

Techa, S. and Chung, J. S. (2013). Ecdysone and retinoid-X receptors of the blue crab, *Callinectes sapidus*: cloning and their expression patterns in eyestalks and Y-organs during the molt cycle. *Gene* **527**, 139-53.

Techa, S. and Chung, J. S. (2015). Ecdysteroids regulate the levels of molt-inhibiting hormone (MIH) expression in the blue crab, *Callinectes sapidus*. *PLoS One* **10**, e0117278.

Teigen, L. E., Orczewska, J. I., McLaughlin, J. and O'Brien, K. M. (2015). Cold acclimation increases levels of some heat shock protein and sirtuin isoforms in threespine stickleback. *Comp Biochem Physiol A Mol Integr Physiol* **188**, 139-47.

Teranishi, K. S. and Stillman, J. H. (2007). A cDNA microarray analysis of the response to heat stress in hepatopancreas tissue of the porcelain crab *Petrolisthes cinctipes*. *Comp Biochem Physiol Part D Genomics Proteomics* **2**, 53-62.

Thomas, L. W. and Ashcroft, M. (2019). Exploring the molecular interface between hypoxia-inducible factor signalling and mitochondria. *Cell Mol Life Sci* **76**, 1759-1777.

Tian, Z., Lin, G. and Jiao, C. (2019). Identification of a S6 kinase transcript in the Chinese mitten crab *Eriocheir sinensis* and its molting-related expression in muscle tissues. *Fisheries Science* **85**, 737-746.

Tomanek, L. (2002). The heat-shock response: its variation, regulation and ecological importance in intertidal gastropods (genus *Tegula*). *Integr Comp Biol* **42**, 797-807.

Tomanek, L. (2015). Proteomic responses to environmentally induced oxidative stress. *J Exp Biol* **218**, 1867-79.

Tomanek, L. and Zuzow, M. J. (2010). The proteomic response of the mussel congeners *Mytilus galloprovincialis* and *M. trossulus* to acute heat stress: implications for thermal tolerance limits and metabolic costs of thermal stress. *J Exp Biol* **213**, 3559-74.

Tsai, K.-W., Chang, S.-J., Wu, H.-J., Shih, H.-Y., Chen, C.-H. and Lee, C.-Y. (2008). Molecular cloning and differential expression pattern of two structural variants of the crustacean hyperglycemic hormone family from the mud crab *Scylla olivacea*. *Gen Comp Endocrinol* **159**, 16-25.

Tsai, Y. C., Greco, T. M. and Cristea, I. M. (2014). Sirtuin 7 plays a role in ribosome biogenesis and protein synthesis. *Mol Cell Proteomics* **13**, 73-83.

Vasquez, M. C., Beam, M., Blackwell, S., Zuzow, M. J. and Tomanek, L. (2017). Sirtuins regulate proteomic responses near thermal tolerance limits in the blue mussels *Mytilus*

galloprovincialis and *Mytilus trossulus*. *J Exp Biol* **220**, 4515-4534.

Velazque-Amado, R. M., Escamilla-Chimal, E. G. and Fanjul-Moles, M. L. (2012). Daily light-dark cycles influence hypoxia-inducible factor 1 and heat shock protein levels in the pacemakers of crayfish. *Photochem Photobiol* **88**, 81-9.

Ventura-Lopez, C., Gomez-Anduro, G., Arcos, F. G., Llera-Herrera, R., Racotta, I. S. and Ibarra, A. M. (2016). A novel CHH gene from the Pacific white shrimp *Litopenaeus vannamei* was characterized and found highly expressed in gut and less in eyestalk and other extra-eyestalk tissues. *Gene* **582**, 148-60.

Verberk, W., Leuven, R., van der Velde, G. and Gabel, F. (2018). Thermal limits in native and alien freshwater peracarid Crustacea: The role of habitat use and oxygen limitation. *Funct Ecol* **32**, 926-936.

Vinagre, A. S. and Chung, J. S. (2016). Effects of starvation on energy metabolism and crustacean hyperglycemic hormone (CHH) of the Atlantic ghost crab *Ocypode quadrata* (Fabricius, 1787). *Marine Biology* **163**.

Webster, S. G., Keller, R. and Dircksen, H. (2012). The CHH-superfamily of multifunctional peptide hormones controlling crustacean metabolism, osmoregulation, moulting, and reproduction. *Gen Comp Endocrinol* **175**, 217-33.

Westerheide, S. D., Anckar, J., Stevens, S. M., Jr., Sistonen, L. and Morimoto, R. I. (2009). Stress-inducible regulation of heat shock factor 1 by the deacetylase SIRT1. *Science* **323**, 1063-6.

Whiteley, N. M., Robertson, R. F., Meagor, J., El Haj, A. J. and Taylor, E. W. (2001). Protein synthesis and specific dynamic action in crustaceans: effects of temperature. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology* **128**,

593-604.

Whiteley, N. M., Taylor, E. W. and El Haj, A. J. (1997). Seasonal and latitudinal adaptation to temperature in crustaceans. *Journal of Thermal Biology* **22**, 419-427.

Wittmann, A. C., Benrabaa, S. A. M., Lopez-Ceron, D. A., Chang, E. S. and Mykles, D. L. (2018). Effects of temperature on survival, moulting, and expression of neuropeptide and mTOR signalling genes in juvenile Dungeness crab (*Metacarcinus magister*). *J Exp Biol* **221**.

Yorde, D. E., Sasse, E. A., Wang, T. Y., Hussa, R. O. and Garancis, J. C. (1976). Competitive enzyme-liked immunoassay with use of soluble enzyme/antibody immune complexes for labeling. I. Measurement of human choriogonadotropin. *Clin Chem* **22**, 1372-7.

Yu, X., Chang, E. S. and Mykles, D. L. (2002). Characterization of limb autotomy factor-proecdysis (LAF(pro)), isolated from limb regenerates, that suspends molting in the land crab *Gecarcinus lateralis*. *Biol Bull* **202**, 204-12.

Zagorska, A. and Dulak, J. (2004). HIF-1: the knowns and unknowns of hypoxia sensing. *Acta Biochim Pol* **51**, 563-85.

Zhang, D., Guo, X., Wang, F. and Dong, S. (2016). Effects of periodical salinity fluctuation on the growth, molting, energy homeostasis and molting-related gene expression of *Litopenaeus vannamei*. *Journal of Ocean University of China* **15**, 911-917.

Zheng, J., Li, L., Dong, H., Mao, Y., Su, Y. and Wang, J. (2018). Molecular cloning of heat shock protein 60 from *Marsupenaeus japonicus* and its expression profiles at early developmental stages and response to heat stress. *Aquaculture Research* **49**, 301-312.

CHAPTER FOUR: EFFECTS OF EMERSION AND TEMPERATURE ON EXPRESSION OF STRESS RESPONSE GENES IN THE GREEN SHORE CRAB, *CARCINUS MAENAS*

SUMMARY

The effects of temperature change and emersion on *Carcinus maenas* to acute were determined using genetic markers for stress proteins in Y-organ, heart, gill, claw muscle, thoracic ganglion, eyestalk ganglion and brain. The HSP70 chaperone gene was used as a proxy for the upper limit of the pejus temperature range. AMPKγ, mTOR, and Rheb genes were selected as markers for temperature and emersion-induced metabolic stress. Animals were exposed to temperatures between 5° and 30°C for 1 or 2 hours. Similarly, green crabs were exposed to emersion in non-desiccated-desiccated conditions for 2, 4, or 6 hours. *Cm*-EF2, a constitutively expressed gene required for translation, *Cm-HSP70*, *Cm-AMPKγ*, *Cm-mTOR*, and *Cm-Rheb* mRNA levels were quantified by quantitative RT-PCR (qPCR). The possible involvement of eyestalk neuropeptides was assessed by quantifying *Cm-HSP70*, *Cm-AMPKγ*, *Cm-mTOR*, and *Cm-Rheb* mRNA levels in five tissues from eyestalk-ablated animals. The results indicate that *C. maenas* tolerated a wide temperature range, requiring 2 hour exposures at 5°C and 30°C to affect tissue-specific changes in gene expression. The results indicate that *C. maenas* tolerates emersion stress under non-desiccated conditions. Prolonged emersion under desiccated conditions mostly affected heart and nervous tissue, showing large decreases in both *Cm-EF2* and *Cm-AMPKγ* expression at 4 and 6 hours. It is hypothesized proteins expression may be affected by temperature and emersion stress. An increase in temperature and emersion time upregulates *Cm-AMPKγ* and downregulates *Cm-mTOR* and *Cm-Rheb*, which would repress translation and allow cellular energy relocation for repair.

INTRODUCTION

The green shore crab, *Carcinus maenas*, is a highly invasive species in temperate coastal regions worldwide (OBIS, 2019). This decapod is considered an "ecosystem engineer" that modifies new environment where it inhabits through consumption of and competition with native fauna (Burden et al., 2014; Grosholz and Ruiz, 1996; Klassen and Locke, 2007; McGaw et al., 2011). Originating from native populations in Western Europe and Northern Africa, there are established populations on the East and West Coasts of North America. The East Coast population was established in 1817 in New York and currently ranges from Nova Scotia to Virginia (Carlton and Cohen, 2003; Tepolt and Somero, 2014). West Coast populations have the least genetic diversity, as the species was only introduced around 1990 from a single introduction event in San Francisco Bay, California (Darling et al., 2008). Their geographic expansion is attributed to the ability to tolerate wide ranges in temperature, salinity, oxygen level and pH (Cuculescu et al., 1998; Jost et al., 2012; Roman and Palumbi, 2004; Taylor et al., 1973). Since temperature is a determinant in its biogeographic distribution (Compton et al., 2010), *C. maenas* may continue to expand its current range into warming waters, and it will compete ecologically and affect native biodiversity of coastal communities (Worm et al., 2006).

C. maenas is an intertidal species that tolerates short-term temperature stress and can acclimate to a wide range of temperatures (McGaw and Whiteley, 2012; Taylor et al., 1977). In comparison to *C. maenas*, other decapods such as *Cancer irroratus*, *Homarus americanus*, *Portunus marmoreus* and *Cancer pagurus* have a lower tolerance to thermal stress at high temperatures (Ahsanullah and Newell, 1977; Cuculescu et al., 1998; Jost et al., 2012). With a wider range of thermal acclimation, green crab tends to tolerate acute changes in temperature

more readily than not being acclimated (Tepolt and Somero, 2014). When environmental temperature is in the optimum range of an organism, ATP supply is sufficient to maintain all the physiological demands (Rodríguez-Fuentes et al., 2017). Beyond the optimal conditions, the cost of maintenance increases and ATP does not cover those physiological demands leading the organism to a *pejus* limit (Pörtner, 2010). *C. maenas* may be more tolerant to thermal stress as it does not exhibit a *pejus* range or "turning worse" point (Frederich et al., 2009; Frederich and Portner, 2000), but rather enters into a *pessimum* range at temperatures between 34-37°C (Jost et al., 2012). The term *pejus* range, refers to the point where an animal's performance begins to decline (Frederich et al., 2009; Frederich and Portner, 2000). Once the animal reaches a critical point or *pessimum* range, there is a switch into anaerobic metabolism and performance drops precipitously making recovery unlikely (Jost et al., 2012). This mechanism of response is found in species that experience changes in temperature or humidity, especially species located in the intertidal zone (Burggren and McMahon, 1981; Dong et al., 2014; Paganini et al., 2014).

Intertidal species are constantly exposed to rapidly changing conditions, especially in temperature, salinity, and oxygen level fluctuate constantly (Truchot, 1990). *C. maenas* can survive emersion for several hours and, in combination with resistance to high temperatures, this crab has a tolerance to invade and disturb the natural balance of ecosystems it invades (Ahsanullah and Newell, 1977; Cuculescu et al., 1998). During low-tide cycles in summer months, green crabs undergo an average of 6 h of exposure to air or emersion periods (Simonik and Henry, 2014). As crustaceans are less resistant to lethal conditions of internal low oxygen in comparison to fishes and mollusks, they exhibit a strong threshold to extreme low oxygen concentrations (Bell and Eggleston, 2004; Geihs et al., 2013). Organisms coping with emersion-induced oxygen fluctuations develop a behavioral strategy to survive transitory hypoxic events

for prolonged periods (Defur, 1988). However, response to induced hypoxia is linked to severity of prior and actual stress exposure (Dong et al., 2016; Dong et al., 2019), but also to a lower tolerance to hypoxia, longer exposure to emersion and a subsequent damage during reoxygenation cycle (de Lima et al., 2015). When exposed to hypoxic shallow water, green crabs emerge to extract oxygen from the air and exhibited normoxia and unchanged respiratory rate (Taylor et al., 1977; Taylor et al., 1973). To determine the molecular biology of the response to emersion-induced hypoxia, an estuarine or intertidal species undergoing frequent oxygen variations and emersion episodes should be used as an ideal model organism (Brown-Peterson et al., 2005). Multiple stress proteins have been shown to increase in various invertebrates after exposure to acute and/or prolonged environmental stressors (Han et al., 2013; Huang et al., 2015b; Snyder and Rossi, 2007).

Heat shock proteins (HSP) display a classic upregulation in response to elevated temperatures (Zhang et al., 2015). This highly conserved protein, among the most abundant superfamily present in all organisms (reviewed in Jung et al., 2013; see HSP database in Ratheesh et al., 2012), acts as a molecular chaperone, binding to existing functioning proteins and preventing them from unfolding and misfolding (reviewed in De Maio, 1999; Morrow et al., 2015; Sørensen et al., 2003). In previous studies, crustaceans subjected to acute and chronic thermal stress had increased HSP's isoforms expression, mostly upregulated when transferred from ambient conditions to experimental temperatures (Chang et al., 1999; Chen et al., 2018; Cimino et al., 2002; Cottin et al., 2015; Drozdova et al., 2019; Gusev et al., 2006; Jost et al., 2012; Spees et al., 2002). Adenosine Monophosphate-activated protein kinase (AMPk) is a heterotrimer regulated by AMP/ADP:ATP levels in the cell and formed by an α-catalytic subunit and two non-catalytic β- and γ-subunits (Jeon, 2016; Kim et al., 2016; Olivier et al., 2018;

Stapleton et al., 1997; Willows et al., 2017). Commonly used as a stress biomarker in crustaceans and other species, AMPk activity has shown to increase due to emersion-induced hypoxia and heat shock, and to reduce anabolic activity (Frederich et al., 2009; Guevelou et al., 2013; Han et al., 2013; Huang et al., 2015b; Jibb and Richards, 2008; Jost et al., 2012; Lu et al., 2016; Ramnanan et al., 2010; Wittmann et al., 2018).

The mechanistic Target of Rapamycin (mTOR) stimulates protein synthesis through phosphorylation of P70 S6K (ribosomal S6 kinase) and eIF4E-binding protein (Abuhagr et al., 2014b; Abuhagr et al., 2016; MacLea et al., 2012; Saxton and Sabatini, 2017). The mTOR signaling pathway plays a critical role in regulating molting and tissue growth in decapod crustaceans. Under conditions of stress and depletion of energy, AMPk can inhibit mTOR activity by direct phosphorylation of Raptor, and by indirect phosphorylation and activation of the Tuberous Sclerosis Complex (TSC) and suppression of Rheb (Ras homolog enriched in brain) (Faubert et al., 2015; Inoki et al., 2012; MacLea et al., 2012; Wittmann et al., 2018). Orthologs of mTOR and Rheb have been characterized in *C. maenas* and *Gecarcinus lateralis* (Abuhagr et al., 2014b; MacLea et al., 2012). While AMPk and mTOR are regulated by phosphorylation, Rheb protein level is correlated with mRNA level and activity. In the molting gland, or Y-organ (YO), mTOR-dependent protein synthesis is required for hypertrophy and increased steroid molting hormone (ecdysteroid) synthesis during premolt (Mykles, 2011; Wittmann et al., 2018).

The purpose of this study was to determine the effects of acute temperature change and emersion in non-desiccated-desiccated conditions, on mRNA expression of genetic markers for stress proteins as a measure of the response of the YO, heart, gill, claw muscle, thoracic ganglion, eyestalk ganglion and brain to stress in *C. maenas*. The HSP70 chaperone gene was

used as a proxy for the upper limit of the pejus temperature range (Frederich et al., 2009; Jost et al., 2012). AMPkγ, mTOR, and Rheb genes were selected as markers for temperature and emersion metabolic stress (Abuhagr et al., 2014b; Frederich et al., 2009; Han et al., 2013; Madeira et al., 2018). As the three proteins regulate global translation of mRNAs, it is hypothesized that their expression may be affected by temperature and emersion stress. The hypothesis predicts that an increase in temperature and emersion time upregulates AMPkγ and downregulates mTOR and Rheb, which would repress translation and allow cellular energy to be redirected for repair and essential cellular functions. However, the response would likely vary between tissues, as tissues with higher metabolic activity (e.g., heart, thoracic ganglion and eyestalk ganglia) may be more sensitive to temperature than tissues with lower metabolic activity (e.g., YO, gill and claw skeletal muscle).

MATERIALS AND METHODS

Experimental animals

Carcinus maenas were collected from the harbor at Bodega Bay, California. Animals used for experiments in the facilities of the Bodega Marine Laboratory were maintained under flow-through water at ±15°C and fed squid twice per week. Y-organs (YO, molting gland) harvested from individuals of *C. maenas* at intermolt (IM), early premolt (EP), mid premolt (MP), late premolt (LP) and postmolt (PM) stages captured at Bodega Bay in spring 2016 and 2017, were used in RNA isolation and mRNA analysis. Another group of animals was shipped to Colorado, kept in aerated water at a salinity of 30 ppt Instant Ocean (Aquarium Systems, Inc., Mentor, OH) and room temperature on a 12 h:12 h dark:light cycle. They were fed cooked chicken liver twice a week.

Experimental treatments

Three different experiments in the Bodega Marine Laboratory determined the effects of acute temperature shifts. For the first experiment, intact intermolt animals were exposed to 5, 10, 20, 25 or 30°C during 1 h. A second experiment exposed intact animals to either 5 or 30°C during 2 h. The last experiment consisted of eyestalk-ablated (ESA) animals exposed to either 5 or 30°C during 1 h. Intact animals kept at ±15°C were used as controls.

A preliminary test was conducted to determine the tolerance limits to emersion. Two small (carapace size <50 mm) green morphs were removed from a water tank and completely dried, including at the junction of the carapace and legs and at the mandibles to remove water from the branchial chamber. Crabs were placed in a dry tank and separated to prevent interactions. Animals were checked at 1, 3, 6, and 9 hours post-emersion. Animals were placed back in their tanks at the end of 9 hours. Two experiments were conducted to study the effect of emersion effect. Animals were removed randomly from water tanks and their branchial chambers were dried with KimWipe (Kimberly-Clark, Roswell, GA) to remove excess of water. Dried animals were placed in empty tanks for 2, 4 or 6 h at room temperature (~22°C), and non-extracted animals were considered as control (0 h, no emersion). One treatment intended to reduce desiccation effects by placing wet towels on the bottom of the tank, while a second treatment exposed animals to an absolute dried out container.

Temperature and emersion in desiccation conditions data were principally obtained by Megan Mudron as part of the Master of Science thesis on *Molecular Regulation of Growth and Molting in Decapod Crustaceans* (2014) at Colorado State University (CSU), Fort Collins, CO. mRNA analysis of Y-organ in the molt cycle and emersion in non-desiccated conditions

experiment were completed by Diego Alejandro López-Cerón as part of the doctoral dissertation on the *Role of Stress-Responsive Genes in the Molt Cycle of Decapod Crustaceans* (2019).

Prior to dissection, each animal was anesthetized by placing it on crushed ice. After exposure, 100 µl of hemolymph was combined with 300 µl MeOH and ecdysteroids titers in the hemolymph were quantified by an competitive enzyme linked immunosorbent assay (ELISA) (Kingan, 1989; Yorde et al., 1976), to determine only intermolt crabs were used for cDNA cloning and tissue expression analysis. YOs, heart (H), gill (G, right side), claw muscle (CM), thoracic ganglion (TG), eyestalk ganglion (ESG) and brain (B), were harvested after either temperature or emersion treatment. Tissues were preserved in 1 µl RNA Later (Life Technologies, Grand Island, NY) and stored at -20°C.

RNA isolation and cDNA synthesis

In 1 ml TRIzol (Life Technologies, Grand Island, NY), YOs were homogenized with a pestle mixer (VWR, Denver, CO). Heart, gill, claw muscle, thoracic ganglion, eyestalk ganglion and brain tissues were homogenized using a TissueLyser II (QIAGEN, Germantown, MD). Samples were run at 180 oscillations min^{-1} for 2 min and 1 ml TRIzol (Life Technologies, Grand Island, NY) was added to resuspend the tissue. Homogenized tissues were centrifuged at 16,000 g for 15 min to extract 1 ml of supernatant. Total RNA was treated with chloroform/isopropanol/ethanol (70%) and pellets were resuspended in 20 µl of RNase/DNase free water. Samples were treated with 3 µl 10x DNase buffer, 0.75 µl Ribolock, 3.25 µl RNase/DNase free water and 3 µl DNase I (Life Technologies, Grand Island, NY) to remove genomic DNA. A second RNA extraction was done by using acidic phenol/chloroform isoamyl alcohol, and RNA was precipitated with isopropanol/sodium acetate (pH = 5.2). Pellets were

rehydrated in 22 µl RNase/DNase free water and RNA purity and concentration was determined by measuring absorbance at 260 and 280 nm using a Nanodrop ND1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE). Total RNA was reverse transcribed to cDNA using SuperScript IV Reverse Transcriptase (Thermo Fisher Scientific, Wilmington, DE), according to the manufacturer's instructions.

Gene isolation

Nine mRNA sequences encoding six proteins were obtained from two *C. maenas de novo* transcriptomes (Tepolt and Palumbi, 2015; Verbruggen et al., 2015). Sequences encoding *AMPkα*-, *β*-, and *γ*-subunits, *SIRT1*, *SIRT7*, *HSP60*, *HSP70*, *HIFα*-, and *β*-subunits, were extracted using the stand-alone software prfectBLAST 2.0 (Santiago-Sotelo and Ramirez-Prado, 2012). To verify the correct identification of the genes, both nucleotide sequences and predicted protein sequences were blasted against the National Center for Biotechnology Information (NCBI) database. To confirm the isolation of full-length encoding contigs, open reading frames (ORF) of the nucleotide sequences were translated to six reading frames. Basic protein structure and conserved domains were obtained from the Conserved Domain Database (CDD) on NCBI and InterPro on the European Bioinformatics Institute (EBI). ClustalX 2.0 (Larkin et al., 2007) and GeneDoc (Nicholas and Nicholas, 1997) were used to create multiple alignments with orthologs from other crustacean species on the NCBI database to reveal sequence identity. The identified nucleotide and deduced amino acid sequences from *C. maenas* isolated in this chapter, were deposited in the GenBank nucleotide sequence database. Sequences record will be held confidential by GenBank until their accession numbers or sequence data appear in a peer-reviewed publication in a biological journal.

Quantitative PCR

Specific primers were designed for *Cm-EF2* (Elongation Factor 2, GenBank accession GU808334.1), *Cm-AMPkα* (GenBank accession BK011189), *Cm-AMPkβ* (GenBank accession BK011188), *Cm-AMPKγ* (GenBank accession BK011184), *Cm-SIRT1* (GenBank accession BK011191), *Cm-SIRT7* (GenBank accession BK011193), *Cm-HSP60* (GenBank accession BK011187), *Cm-HSP70* (GenBank accession BK011185), *Cm-HIFα* (GenBank accession BK011190), and *Cm-HIFβ* (GenBank accession BK011192), *Cm-mTOR* (Mechanistic Target Of Rapamycin, GenBank accession JQ864248) and *Cm-Rheb* (Ras Homolog Enriched in Brain, GenBank accession HM989970) (Table 4-1). *Cm-EF2* was used as control of quality in mRNA isolation and cDNA synthesis. Quantitative PCR (qPCR) was developed using a LightCycler 480 thermal cycler (Roche Applied Science, Indianapolis, IN). qPCR reactions consisted of 0.5 µl each of 10 µmol l^{-1} forward and reverse primer (Table 4-1), 3 µl PCR-grade water, 5 µl SYBR Green I Master Mix, and 1 µl first strand cDNA. PCR conditions were set in an initial denaturation at 95°C for 5 min, 45 cycles at 95°C for 5 s, annealing at 60°C for 5 s, and extension at 72°C for 20 s. Absolute concentration of transcripts was calculated using a standard curve generated by serial dilutions ranging 10^{-9} to 10^{-18} ng μl^{-1} of purified cDNA product of each gene. Final qPCR transcript concentration was standardized to copy number per µg of total RNA used in the cDNA synthesis, and $\log_{10}$ transformed prior to analysis.

Statistical analysis

Statistical analysis was performed on Statgraphics 18 (The Plains, VA) and SPSS Statistics 22 (IBM Software). Graphs were created on SigmaPlot 12 (Systat Software, San Jose,

CA). Outliers were detected using the Grubbs' Test, and removed. One-way ANOVA and Tukey-Kramer HSD *post-hoc* comparisons were used to test differences on ecdysteroid titers and transcript concentration among molt stages and temperature and emersion time treatments. Data were plotted as mean ±1 S.E.M. and all statistical analysis were set at $\alpha>0.05$ level of significance.

RESULTS

Genes of stress response in the molt cycle of *Carcinus maenas*

Ecdysteroid titers in hemolymph depicted a typical pattern of the *Carcinus maenas* molt cycle: low titers at intermolt, increasing values from early premolt to late premolt, and the lowest levels at postmolt stage (Figure 4-1A). Compared with intermolt stage (9.3 pg ul^{-1}), significantly higher ecdysteroid titers were detected in mid (60.3 pg ul^{-1}) and late premolt (323.1 pg ul^{-1}) crabs ($F_{4,80}$=100.69; P<0.0001). *Cm-EF2* mRNA levels were 3.8-fold downregulated at postmolt stage ($F_{4,78}$=3.01; P=0.0230, Figure 4-1B). Expression of *Cm-AMPkα* ($F_{4,76}$=3.35; P=0.0139) and *Cm-AMPkβ* ($F_{4,72}$=7.97; P<0.0001) was downregulated 10.7- and 69.3-fold at postmolt stage, respectively (Figure 4-1C). *Cm-SIRT1* was upregulated 7.2-fold by mid premolt with a significant decrease at late (8.8-fold) and postmolt (3.8-fold) stages ($F_{4,77}$=15.81; P<0.0001, Figure 4-1D). By mid premolt, mRNA levels of *Cm-HSP60* (Figure 4-1E) were 208.9-fold considerably higher ($F_{4,75}$=4.20; P=0.0040), while *Cm-HSP70* (Figure 4-1E) expression decreased 48.8-fold ($F_{4,75}$=5.24; P=0.0009). *Cm-HIFα* expression was upregulated 113.4-fold in mid premolt after no detectable expression in early premolt ($F_{4,73}$=4.50; P=0.0026, Figure 4-1F).

Effects of acute temperature change

C. maenas had a wide tolerance range and all animals tolerated the 1 or 2 h exposures over the 5°C to 30°C range of temperatures used in this study. While not quantified, it was observed that animals had more difficulty righting themselves when submerged in 5°C or 30°C, and occasionally were not able to right themselves at any point during the experiment (these animals were assisted after ~5 minutes).

Temperature had no substantial effect on hemolymph ecdysteroid titers and levels were stable before and after exposure to each temperature treatment. No significant variations ($F_{11,132}$=1.10; P=0.3691) between hemolymph concentrations of intact animals before and after 1 h in the range of 5 to 30°C (Figure 4-2) were found. Higher ecdysteroid titers were detected in intact animals at 30°C than those at 5°C before 2 h exposure, with no substantial changes in mean ecdysteroid concentrations at both treatments ($F_{5,66}$=2.23; P=0.0616, Figure 4-3). Higher ecdysteroid concentrations at 30°C were detected in eyestalk-ablated animals both before and after exposure to emersion, with significant variations from 15 to 30°C after exposure ($F_{5,41}$=4.41; P=0.0027, Figure 4-4).

After 1 h of exposure, *Cm-EF2* varied from 1.30×10^6 to 7.05×10^7 copies μg^{-1} mRNA with no significant effect of temperature on its expression in general (Figure 4-2B). YO increased 3.2-fold at 20°C (P=0.0002) and gill showed a 3.2-fold rising at 25°C (P=0.0108). *Cm-HSP70* expression was highly influenced in the six tissues studied (P=0.0142 in gill, P<0.0001 in other tissues). Y-organ, heart, claw muscle, thoracic ganglion and eyestalk ganglion had the highest increment of 749.1, 88.3-, 20.1, 106.5-, and 715.2-fold at 30°C, respectively (Figure 4-2C). By contrast, temperature had a slight effect on *Cm-AMPKγ* expression (Figure 4-2D), where mRNA levels were not meaningfully different from control in Y-organ, heart, and claw muscle.

However, differences were present at 25°C in gill (7.7-fold higher, $P<0.0001$) and a decrease of 11.5-fold at 25°C in thoracic ganglion ($P<0.0001$). *Cm-mTOR* mRNA levels increased 7.6-fold ($P=0.0150$) in gill and 2.6-fold after 30°C exposure in eyestalk ganglion ($P<0.0001$), while mRNA values decreased 23.3-fold in thoracic ganglion ($P<0.0001$) at 25°C (Figure 4-2E). *Cm-Rheb* expression in Y-organ slightly decreased 2.1-fold at 20°C ($P=0.0011$) and 16.9-fold in thoracic ganglion at 25°C ($P<0.001$). After a reduction of 35.1-fold from 5 to 15°C, remarkable increments were detected in gill between 15 ($9.13x10^3$ copies μg^{-1} mRNA) and 30°C (56.1-fold higher), with the highest expression at 25°C (109.9-fold higher, $1.00x10^6$ copies μg^{-1} mRNA, $P<0.0001$, Figure 4-2F).

After 2 h of exposure, *Cm-EF2* mRNA levels had changes between 5 and 30°C in YO, gill and thoracic ganglion (Figure 4-3B). A significant increase in YO (2.4-fold, $P=0.0388$) and gill (2.9-fold, $P=0.0106$) was detected. By contrast, an increase of 5.2-fold from 5 to 15°C ($P=0.0001$) and expression levels 2.4-fold lower at 30°C were detected in thoracic ganglion. In all the six tissues, *Cm-HSP70* displayed substantially higher mRNA levels at 30°C ($P=0.0010$ in gill, $P<0.0001$ in other tissues, Figure 4-3C). Highest levels were observed in YO (90.5-fold, $1.71x10^4$ to $1.55x10^6$ copies μg^{-1} mRNA), heart (42.3-fold, $2.52x10^5$ to $1.06x10^7$ copies μg^{-1} mRNA), and eyestalk ganglion (308.6-fold, $6.66x10^4$ to $2.06x10^7$ copies μg^{-1} mRNA). Thoracic ganglion had 3.2- to 4.9-fold lower mRNA levels at 5°C and 30°C, as the only relevant change for *Cm-AMPKγ* ($P<0.0001$, Figure 4-3D). *Cm-mTOR* mRNA levels were up to 2.5-fold significantly higher in Y-organ ($P=0.0003$) and up to 3.1-fold higher in eyestalk ganglion ($P=0.0005$) at 5 and 30°C. Oppositely, thoracic ganglion showed mRNA levels 9.5- to 12.7-fold lower compared to control group ($P<0.0001$, Figure 4-3E). Similar to 1 h treatment, *Cm-Rheb* expression in gill had an increment at 30°C of 2-fold ($9.13x10^3$ to $2.12x10^5$ copies μg^{-1} mRNA).

In contrast, thoracic ganglion showed a reduction of 5.9-fold at 5°C and 2.4-fold at 30°C (P=0.0003, Figure 4-3F).

Eyestalk-ablated animals showed significant changes in *Cm-EF2* expression only in claw muscle at 30°C (2.1-fold lower, P=0.0007, Figure 4-4B). Animals exposed to 30°C had higher levels of *Cm-HSP70* in heart (33.8-fold, P=0.0236), thoracic ganglion (41.2-fold, P=0.0128), with the highest expression level of 998.4-fold in YO ($1.71x10^4$ to $1.71x10^7$ copies μg^{-1} mRNA, Figure 4-4C). A reduction of ~3.4-fold in *Cm-AMPKγ* and ~6.2-fold in *Cm-mTOR* mRNA levels was measured in thoracic ganglion at both experimental temperatures (P<0.0001, Figure 4-4D and E). *Cm-Rheb* mRNA level showed no significant changes in expression levels compared to control group (Figure 4-4F).

Effects of emersion exposure

Immediately after removal from water and drying of branchial chambers, the crabs were not as active as normally seen in water. The animals were active through the first hour. After 3 h, each animal was found in the corner of the empty tank with its legs tucked in close to its body. However, gentle prodding raised a response of movement away from the corner and animals were able to right themselves after being overturned. After 6 h, the animals were less responsive when touched and were not able to right themselves when placed on their backs. After 9 h, the animals would not move when touched, nor were they able to flip over when placed on their backs. Placement back in the water induced more movements that are normal; however, they were still slow. Crabs were found dead in their tanks 12 h later. Animals exposed to 6 hours or less of emersion were able to recover when returned to seawater. Maximum emersion time for

the experiments to simulate an acute emersion stress that an animal could still recover from was 6 hours.

No changes in mean ecdysteroid levels were determined between non-desiccated and desiccated conditions ($F_{3,36}$=0.62; P=0.7393, Figure 4-5A). Under non-desiccated conditions, crabs did not show variation in ecdysteroid titers ($F_{3,18}$=1.04; P=0.3998). An increase of 22-fold ($2.59x10^5$ to $5.67x10^6$ copies μg^{-1} mRNA) in *Cm-EF2* transcription was detected in gill after 4 h of emersion (P=0.0187, Figure 4-5B). A slight increase of 4-fold ($1.02x10^6$ to $4.08x10^6$ copies μg^{-1} mRNA) in *Cm-HSP70* mRNA levels in heart was detected after 6 h of emersion (P=0.0161, Figure 4-5D). No significant changes were detected for *Cm-AMPKγ* (Figure 4-5D). *Cm-mTOR* mRNA levels showed significant increases of 2-fold (from $7.50x10^5$ at 0 h to $1.67x10^6$ (4 h) and $1.76x10^6$ (6 h) copies μg^{-1} mRNA) in YO after 4 and 6 h of exposure to emersion (P=0.0007). Expression of *Cm-mTOR* in claw muscle significantly decreased between 2- and 4-fold 4 and 2 h post-emersion, respectively (P=0.0011, Figure 4-5H). Lastly, *Cm-Rheb* mRNA levels in brain substantially decreased between 7- and 20-fold (2 to 6 h of emersion, P=0.0067, Figure 4-5J).

Under desiccated conditions, there was not significant variations in mean ecdysteroid levels in animals ($F_{3,18}$=0.40; P=0.7526, Figure 4-5A). Four of the six tissues studied showed important changes in *Cm-EF2* mRNA levels. Expression levels in heart had a reduction up to 21-fold after 6 h of emersion (P=0.0005, $8.90x10^6$ at 0 h to $2.04x10^6$ (4 h) and $4.34x10^5$ copies μg^{-1} mRNA (6 h), Figure 4-5C). Following 6 h of emersion, *Cm-EF2* mRNA levels in gill reduced 3-fold (P=0.0352, $1.94x10^6$ to $6.16x10^5$ copies μg^{-1} mRNA) and 19-fold in thoracic ganglion (P=0.0306, $1.77x10^6$ to $9.53x10^4$ copies μg^{-1} mRNA). Brain samples showed decreased of *Cm-EF2* expression in all emersion periods, reducing from 4 to 8-fold (P=0.0011, $3.92x10^5$ at 0 h to $5.07x10^4$ copies μg^{-1} mRNA at 6 h). *Cm-HSP70* expression was also reduced by 2- to 3-fold in

heart under desiccation conditions 2 to 6 h post-emersion (P=0.0021, Figure 4-5E). After 4 and 6 h of emersion, *Cm-AMPKγ* levels in heart reduced 11-fold (P=0.0001, Figure 4-5G), 15- to 39-fold in thoracic ganglion (P=0.0104) and 58-fold in brain (P=0.0177). Expression levels of *Cm-mTOR* in heart (P=0.0038) and gill (P=0.0132) were 9- to 12-fold lower after 4 and 6 h, respectively (Figure 4-5I). By contrast, *Cm-mTOR* in YO was 2-fold upregulated after 4 h (P=0.0066). Finally, *Cm-Rheb* mRNA levels (Figure 4-5K) were significantly downregulated 11 to 20-fold in heart after 4 and 6 h (P=0.0004) and 8-fold in gill samples after 6 h (P=0.0221).

DISCUSSION

Green crabs are continuously exposed to changing temperatures and humidity levels that can lead to cellular and physiological stress representative of their intertidal habitat. Since this species is more tolerant of habitat variability, it may have alternative mechanisms to regulate stress at the molecular level (Tepolt and Somero, 2014).

Through the molt cycle of *Carcinus maenas*, ecdysteroid titers exhibited a pattern of lower concentrations in intermolt and postmolt stages and increasing concentrations from early premolt to late premolt, prior to ecdysis, with very similar values to other crustacean species (Abuhagr et al., 2016; Das et al., 2018; Durliat et al., 1988; Hopkins, 1983, 1986; Kappalli et al., 2012; Lee et al., 1998; Mykles, 2011; Okumura and Aida, 2000; Okumura et al., 1989; Soumoff and Skinner, 1983; Thomton et al., 2006). Absolute expression of *Cm-AMPk*, *Cm-SIRT1*, *Cm-SIRT7*, *Cm-HSP60*, *Cm-HSP70*, *Cm-HIF*, *Cm-mTOR* and *Cm-Rheb* in the YO of *C. maenas* represents a baseline of biomarkers essential for growth, development and response to extreme environmental conditions. It may complement the understanding of ecdysis regulation by the

synthesis of ecdysteroids in the YO of green crabs and other decapods (Mykles, 2011; Oliphant et al., 2018; Techa et al., 2015).

Response to acute temperature change

Ecdysteroid synthesis in *C. maenas* seemed unaffected by temperature stress with similar concentrations and irrelevant variations before and after treatments. Animals did not experience heat stress until a 1 h exposure in the range of 25 and 30°C and with minor changes after 2 h of exposure. Differential expression of *Cm-EF2*, *Cm-AMPKγ*, *Cm-mTOR* and *Cm-Rheb* is probably an early response mechanism stimulated prior to the typical heat shock response (Aramburu et al., 2014; Chou et al., 2012) and indicated by *Cm-HSP70* activation, since this protein is regulated by inducible transcription in response to environmental stress (Chou et al., 2012; Mohamad et al., 2018). It has been stablished that these genes are involved in the regulation of translation (Kenney et al., 2014; Proud, 2015), maintenance of cell energy and cell survival (Kim et al., 2016; Olivier et al., 2018), maintenance of metabolism and cell growth (Kaur and Sharma, 2017; Saxton and Sabatini, 2017), and control and regulation of protein synthesis (Abuhagr et al., 2014b; MacLea et al., 2012). All of these activities respond to any changes in energy levels during stress and, consequently, ATP is conserved by reducing gene expression and protein synthesis (Cetrullo et al., 2015; Kaur and Sharma, 2017). Although some significant differences were found in *Cm-AMPKγ*, *Cm-mTOR* and *Cm-Rheb* mRNA levels in intact animals at high temperatures, there was no consistent tissue-specific response to stress by temperature. The changes in expression were relatively modest, even at the extremes of the temperature range. Expression levels of these genes were mostly altered in gill and thoracic ganglion after 1 h, but can be related to their anatomical proximity as thoracic ganglion generally innervates gills and

musculature around it resides (Beltz, 1995). *Cm-EF2*, a constitutively gene involved in RNA translation (Kenney et al., 2014; Pitts et al., 2017), varied with only significant thermal effect on its expression in gill tissues of intact animals.

Cm-HSP70 exhibited a typical heat shock response (Harada and Burton, 2019; Liu et al., 2019b; Yi et al., 2018) with significantly increased expression at 25°C in some tissues and the highest levels at 30°C in all tissues, after both 1 and 2 h treatments. While expression was variable between tissue types, there was generally very little variation between temperatures at 1 and 2 h exposure times. Green crabs may have reached a *pessimum* temperature, which suggests that *C. maenas* can tolerate exposures to 25-30°C without the induction of chaperones to stabilize intracellular proteins (Jost et al., 2012; Portner et al., 2017). Contrary to the hypothesis, *Cm-AMPKγ* mRNA expression was not increased at high temperature, which also indicates our results do not support Frederich et al. (2009) proposition that *Cm-AMPKγ* gene expression is an earlier indicator of heat stress than activation of chaperones. With an increment of *Cm-HSP70* expression and a reduced *Cm-AMPKγ* expression, *Cm-HSP70* may be beneficial to cell survival through re-folding of proteins (Wang et al., 2010) and *Cm-AMPKγ* activation would reduce energy-consuming pathways (Olivier et al., 2018; Willows et al., 2017). Intact and ESA animals showed similar *Cm-HSP70* and *Cm-AMPKγ* mRNA levels after 1 h at 30°C. Although *Cm-AMPKγ* expression was not an indicator of stress in *C. maenas* under the tested parameters, it was consistent with no detected increment of AMPk in a comparative heat shock study in three decapods (Jost et al., 2012) and the AMPk activation before the heat shock response triggering HSP70 expression in *Cancer irroratus* (Frederich et al., 2009). It was observed that *Cm-HSP70* expression was robustly increased at 30°C but *Cm-AMPKγ* expression changed little in most tissues, suggesting that *Cm-HSP70* is an indicator of early response to temperature stress in *C.*

maenas (Buckley et al., 2001; Chen et al., 2018; Han et al., 2018; Zheng et al., 2018). Perhaps crabs were not exposed to temperatures high or long enough to cause an increase in *Cm-AMPKγ* mRNA levels. Reported results are consistent to an increase of the *Cm-HSP70* mRNA levels after heat shock under similar conditions of time and temperature (Cottin et al., 2015; Frederich et al., 2009; Kelley et al., 2011; Madeira et al., 2012; Oksala et al., 2014; Rungrassamee et al., 2010; Spees et al., 2002). No significant changes in *Cm-AMPKγ* expression would demonstrate an adaptation to subtle energy demand at lower temperatures (Dong et al., 2016; Lu et al., 2016). Contrary to our hypothesis, while *Cm-HSP70* expression was significantly higher in all tissues starting 10-15°C higher than ambient water temperature, *Cm-AMPKγ*, *Cm-mTOR*, and *Cm-Rheb* expression showed no consistent response.

Increased *Cm-Rheb* expression in gill in response to higher temperatures in intact animals was an exception and may indicate an early response as well (Tyagi et al., 2015). Binding and hydrolysis of GTP allows Rheb to regulate mTOR expression (MacLea et al., 2012) through the AMPk/TSC1/TSC2 pathway (Abuhagr et al., 2014b; Duan et al., 2015). This suggests that *Cm-Rheb* expression would decrease with temperature stress, so that mTOR activity and protein synthesis were reduced as the AMP:ADP/ATP ratio increased (Kim et al., 2013; Wittmann et al., 2018). An increased synthesis of *Cm-Rheb* during increased metabolism and stress can decrease cellular protein synthesis (Tyagi et al., 2015), and can explain the opposite response observed at least in gill. However, this trend in expression cannot be generalized among all tissues, as gill should have shown a response due to its function in gas exchange and ion transport (Chen et al., 2019; Greenaway, 1988). After 1 h treatment, a decreased *Cm-Rheb* expression consistent with reduced *Cm-mTOR* levels may be related to compensate lower mTOR activity during low cellular energy supplies (Tee et al., 2005; Wittmann et al., 2018). *Cm-mTOR* expression was

generally stable in most tissues in response to temperature stress. mTOR is regulated by phosphorylation and is altered primarily at the protein level to allow rapid response to environmental conditions (Dalle Pezze et al., 2016; Giovannini and Bianchi, 2017; Huang and Fingar, 2014). Therefore, mRNA levels may not accurately reflect mTOR activity, especially after such a short exposure time (Abuhagr et al., 2014b; Dalle Pezze et al., 2016). However, heat stress is not always effective and may not affect mTOR activation by phosphorylation, since mTOR has several phosphorylation sites (Reiling and Sabatini, 2006; Yoshihara et al., 2013).

Response to emersion exposure

In the same way to the thermal response, ecdysteroid titers were not significantly influenced by emersion and air exposure. Changes in ecdysteroid concentration were observable after 2 and 4 h of exposure and similar between 0-h (control) and 6 h treatments. Humidity level may be an important stressor for aquatic organisms (Dong et al., 2016; Duan et al., 2016; Radzikowski, 2013; Thorat and Nath, 2015), but it was not confirmed for ecdysteroid titers in hemolymph. Although animals in non-desiccated treatment had no variation in *Cm-EF2* expression (except for gill after 4 h), green crabs in desiccated conditions showed strong variation in heart, gill, thoracic ganglion and brain. This variation resembles the response exhibited by the same tissues after 2 h either at 5 or 30°C during temperature stress. It may also be that both temperature and humidity have the same effect over *Cm-EF2* expression on specific cardiac, respiratory and neuronal tissues (Greenaway, 1988).

In contrast to temperature stress, emersion was not an effective stressor over *Cm-HSP70* expression, which only exhibited variation in heart. Green crabs in the desiccated treatment showed 1 to 3 orders of magnitude less than crabs in a non-desiccated treatment, especially for

gill, thoracic ganglion and brain. Emersion can produce a lesser response on *Cm-HSP70* expression than the sum of temperature and emersion or simply exposure to extreme temperatures in the same period (Clark and Peck, 2009; Snyder and Rossi, 2007). *Cm-HSP70* expression might be commonly downregulated after air exposure in decapods with no effect of temperature (Lu et al., 2016). However, captivity stress might be another possible explanation to differences in magnitude of *Cm-HSP70* expression between the non-desiccated and desiccated treatments in *C. maenas* (Brown-Peterson et al., 2005). Expression of heat shock proteins may be correlated to the exposure of green crabs to its thermal limits, rather than an actual fluctuation of these genes (Tomanek, 2002). *Cm-HSP70* expression may be also regulated by nutritional status of *C. maenas*, as depletion of nutrients decrease the ability of protein synthesis in response to thermal stress (Jeno and Brokordt, 2014). Crabs were fed twice a week and starvation was not a variable influencing *Cm-HSP70* synthesis in this study. *Cm-AMPkγ* expression showed an opposed pattern to *Cm-HSP70* and was significantly affected by desiccation in specific tissues. Brain, heart, and thoracic ganglion tissues decreased from 10- up to 58-fold when animals were exposed to a desiccated ambient but, contrarily, gill samples modestly increased 3-fold with humidity. In presence of moisture, water kept into the gills should have functioned as substrate to continue with regular metabolic activities, such as respiration and protein synthesis, and to maintain cellular energy balance (Chen et al., 2019). During absence of moisture, we predict green crabs can exhibit a repression of *Cm-AMPkγ* expression in specific tissues, leading to overproduction of others essential proteins such as *Cm-EF2* (Hochachka et al., 1996; Kenney et al., 2014). Although not tested, hypoxia might be a secondary source of stress after emersion and leading *Cm-AMPkγ* to be upregulated under conditions of anaerobiosis (Hand et al., 2011; Pinz et al., 2005). *Cm-AMPkγ* upregulation indicates that under physiological and metabolic stress

conditions, transcription of metabolic regulators of energy-depleting and energy-producing pathways is induced instead of heat shock proteins at the transcriptional level (Dong et al., 2014; Han et al., 2013).

Cm-mTOR and *Cm-Rheb* expression was mostly lower after 4 and 6 hours in claw muscle and brain under non-desiccated conditions and heart and gill under desiccation. Contrarily, *Cm-mTOR* expression in YO increased 2-fold between 4 and 6 hours. As crabs cope with an energy-demanding situation in desiccation, downregulation of *Cm-mTOR* may allow the heart and gill to function efficiently for a short initial time while energy is relocated to transcription and translation of metabolic sensors (Wittmann et al., 2018; Xu et al., 2012). This observation supports that 1) the shutdown of metabolic mRNA levels was seen in highly oxygen dependent tissues (Aagaard, 1996; Cieluch et al., 2004; Pennoyer et al., 2016) and 2) although emersion-induced hypoxia was not determined, possible low oxygen levels would activate *Cm-AMPkγ* inhibiting *Cm-mTOR* in a *Cm-Rheb*-regulated manner (Liu et al., 2006; Tan and Hagen, 2013). By contrasting ecdysteroids levels and *Cm-mTOR* expression during hypoxia, there was an inversed relation between its activity in Y-organ and ecdysteroid synthesis in both treatments. *Cm-mTOR* expression was higher during longer periods in non-desiccation conditions than that in desiccation. In specific cases, mTOR may be attenuated during hypoxic events with a sustained ecdysteroid production without entering to the hypoxia-induced pathway (Abuhagr et al., 2014b; Laplante and Sabatini, 2012; Saxton and Sabatini, 2017). In fact, *Cm-mTOR* would need a longer exposure or more extreme conditions (e.g. dryness) for total inhibition, going from cell maintenance to diminished cell proliferation rates during stress events (Kaper et al., 2006).

CONCLUSIONS

Some physiological and ecological reviews (Baillie and Grabowski, 2018; Lehnert et al., 2018; Newsom and Williams, 2014; Rayner et al., 2019; Rayner and McGaw, 2019; Tepolt and Palumbi, 2015; Torres and González-Pisani, 2016; Watts et al., 2014) and ecotoxicology studies (Rodrigues et al., 2014; Rodrigues et al., 2015a; Rodrigues et al., 2015b; Rodrigues and Pardal, 2014) suggest that green crabs would be able to expand its current range into new areas and, grow and reproduce at comparatively higher rates. While bioassays are crucial to determine range of response and physiological limits during stress response, actual physical changes may be considerably different in the animal living in natural habitat. Experiments at higher temperatures and longer emersion exposure would produce greater physiological responses, but they might not be necessarily the situation in all environments. The combination of several environmental variables forcing a response instantly can reflect the actual adjustment in the physiology of *Carcinus maenas*.

C. maenas was highly tolerant to acute temperature changes, meaning its highly invasive capacity is consequence of its thermal flexibility and tolerance to changes at the molecular and physiological level. Our findings represent some change in overall gene expression in response to acute temperature changes in either intact or ESA animals; unlike the changes that have been observed in other decapod crustaceans. As a species that appears to be highly tolerant to stressful temperatures, it is likely *C. maenas* employs a variety of mechanisms that enable it to adapt quickly new environments. Its tissues can tolerate a wide range of temperatures without altering mRNA levels of metabolic genes and appears the upregulation of some genes allows to cope with short-term exposures to temperatures approaching its *pessimus* range.

Inhibition of protein synthesis could occur at the transcriptional and/or translational level by a hypoxia-induced event (Hochachka et al., 1996). Apparently, it did not happen at neither one of these levels during the experiments. Ecdysteroids titers were comparable and *Cm-EF2* expression had no important variations after thermal stress and emersion. Less metabolically active tissues, such as YO, gill and claw muscle were refractory to emersion. The differential response of tissues to emersion stress may indicate that the failure of key organ systems, such as the nervous and circulatory systems, would contribute to limit the stress response in decapods. Although laboratory experiments represent a broad point of view in physiology of the green crabs facing constant stressors, studies in natural habitat would explain relevant and much natural adaptations (Behrens Yamada et al., 2015; Cosham et al., 2016; Simonik and Henry, 2014; Stevens et al., 2014). Intertidal species deal with more than one stressor at the same period. New bioassays will elucidate interaction between very important factors such as lower sea and air temperatures, emersion and high salinities and temperatures, and effects of periodic stresses by simulating tidal variation. Green crabs are a reliable model organism to examine molecular biomarkers on intertidal species frequently exposed to temperature and oxygen fluctuations.

Table 4-1. Oligonucleotide primer sequences used for expression analysis of *C. maenas* genes. F, forward; R, reverse; Tm, melting temperature; bp, base pair; MC: molt cycle; E: emersion. *EF2*, elongation factor 2; *AMPkαβγ*: AMP-activated protein kinase alpha, beta, gamma subunit; *SIRT*: Silent Information Regulator 2; *HSP*: Heat Shock Protein; *HIFαβ*: Hypoxia Inducible Factor alpha, beta subunit; *mTOR*: mammalian mechanistic Target Of Rapamycin; *Rheb*: Ras homolog enriched in brain.

Primer	Sequence (5' – 3')	Tm (°C)	Product (bp)	Assay
Cm-EF2-F1	CCATCAAGAGCTCCGACAATG	61.6	278	MC,E
Cm-EF2-R1	CATTTCGGCACGGTACTTCTG	61.8	278	MC,E
Cm-AMPkα-F1	TTGCACCACTGAGAGAACG	61.9	219	MC
Cm-AMPkα-R1	AACCTCCTGTTATTGGGTTCC	62.0	219	MC
Cm-AMPkβ-F1	CTCCCACCAGTACAAGTTCC	61.7	225	MC
Cm-AMPkβ-R1	TCATCTTCCTCTTGCATACCG	61.7	225	MC
Cm-AMPkγ-F1	TGGAGGACCATCGCTTGGAGACT	61.9	247	MC,E
Cm-AMPkγ-R1	GGCTTGTGTAGGATTGACGGCT	59.5	247	MC,E
Cm-SIRT1-F1	CTTTCTTTGTGCTGGCTAAGG	61.4	240	MC
Cm-SIRT1-R1	CCTCCTTCATCCAGTCTTGG	61.5	240	MC
Cm-SIRT7-F1	GGTTATGTCCAGTACGTGGT	61.3	249	MC
Cm-SIRT7-R1	CCAGTCCAGGATGTTGTCAT	61.7	249	MC
Cm-HSP60-F1	ATTGCCGAGGATGTTGATGG	62.6	227	MC
Cm-HSP60-R1	TGAATCTCGCCAACCATTCC	62.5	227	MC
Cm-HSP70-F1	GACAACAGAATGGTGAATCACTTCGCT C	59.6	160	MC,E
Cm-HSP70-R1	GAGAGTCGATCTCTATGCTGGCCTG	60.3	160	MC,E
Cm-HIFα-F1	CCTTCGTGAGCAGACACTC	61.8	235	MC
Cm-HIFα-R1	GTCACCAGCCACACGTAAC	62.6	235	MC
Cm-HIFβ-F1	TATTCTTGAGGCTGCTGATGG	62.0	224	MC
Cm-HIFβ-R1	CGGTCTTGAGGTCCAATATACG	62.0	224	MC
Cm-mTOR-F2	CATCCCTCAAACCTCATGCT	54.9	319	E
Cm-mTOR-R2	CACCCACCACAGAACGCTTT	58.3	319	E
Cm-Rheb-F1	ATGGGCAAAGTCACAGTTCC	55.6	281	E
Cm-Rheb-R1	GTCAGGAAGATGGTGGCAAT	55.1	281	E

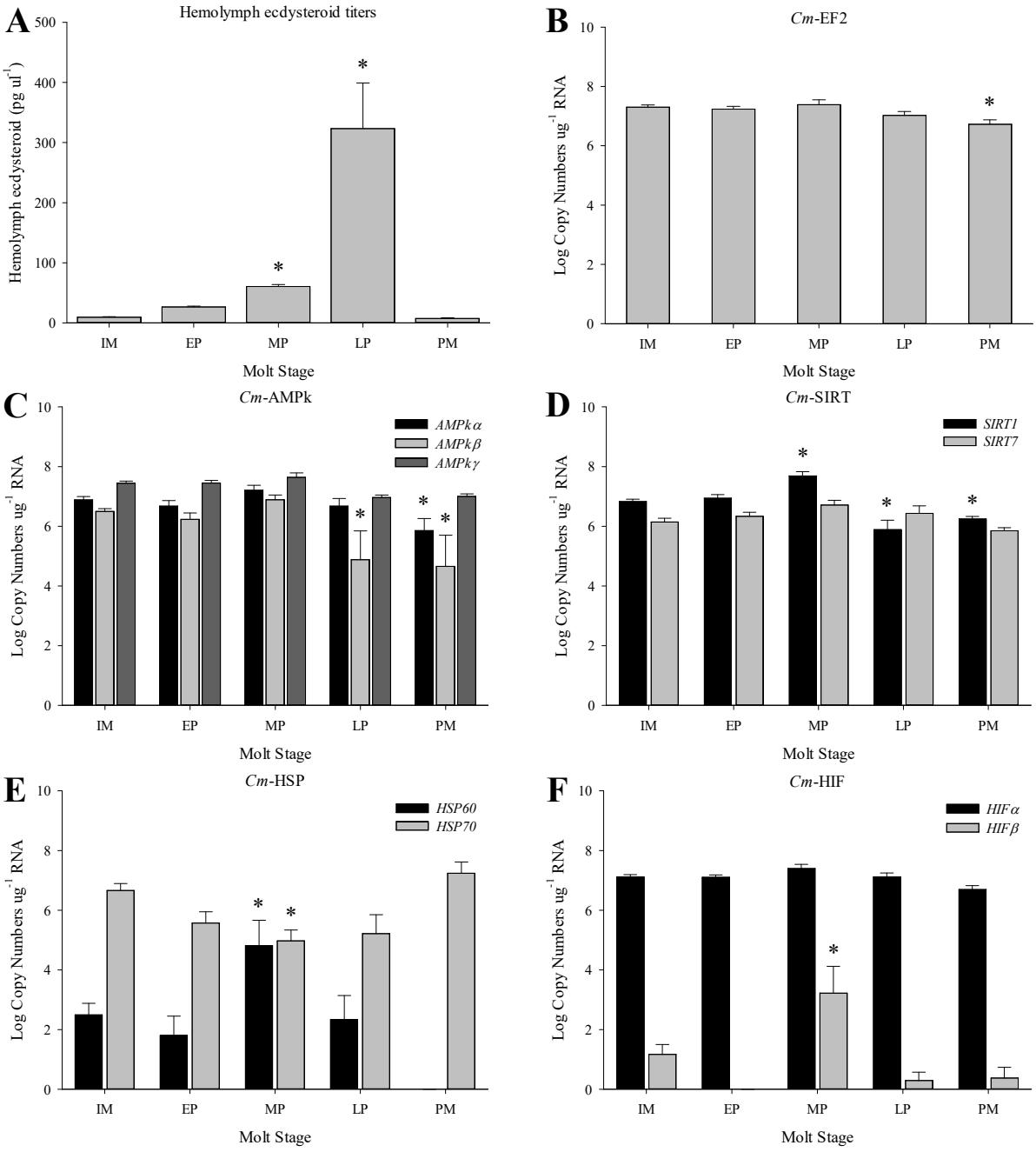


Figure 4-1. Ecdysteroid titers and mRNA levels in the YO through the molt cycle of *C. maenas*. (A) ecdysteroid titers, (B) *Cm*-EF2, (C) *Cm*-AMPk, (D) *Cm*-SIRT, (E) *Cm-HSP70*, (F) Cm-HIF. *: indicates significant differences when compared to intermolt according to Tukey-Kramer HSD post hoc tests at $P<0.05$ (n = 4-43).

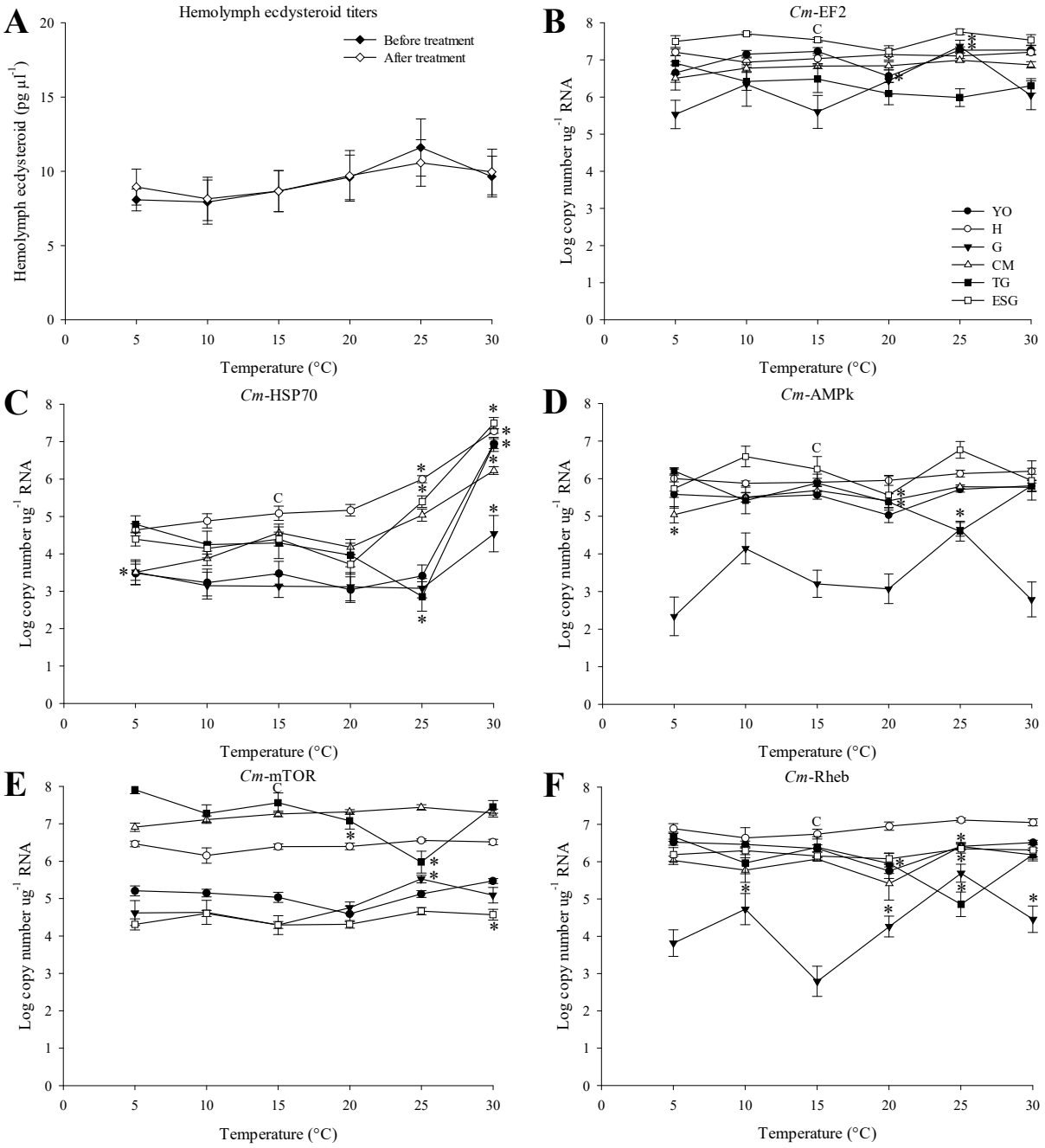


Figure 4-2. Effects of 1 h temperature treatment on hemolymph ecdysteroid levels and mRNA levels in *C. maenas* tissues. (A) ecdysteroid titers, (B) *Cm*-EF2, (C) *Cm-HSP70*, (D) *Cm*-AMPkγ, (E) *Cm*-mTOR, (F) *Cm*-Rheb. YO: Y-organ; H: heart; G: gill; CM: claw muscle; TG: thoracic ganglion; ESG: eyestalk ganglion. C: control (15°C), *: indicates significant differences from the control (C) temperature according to Tukey-Kramer HSD post hoc tests at $P<0.05$ (n = 6).

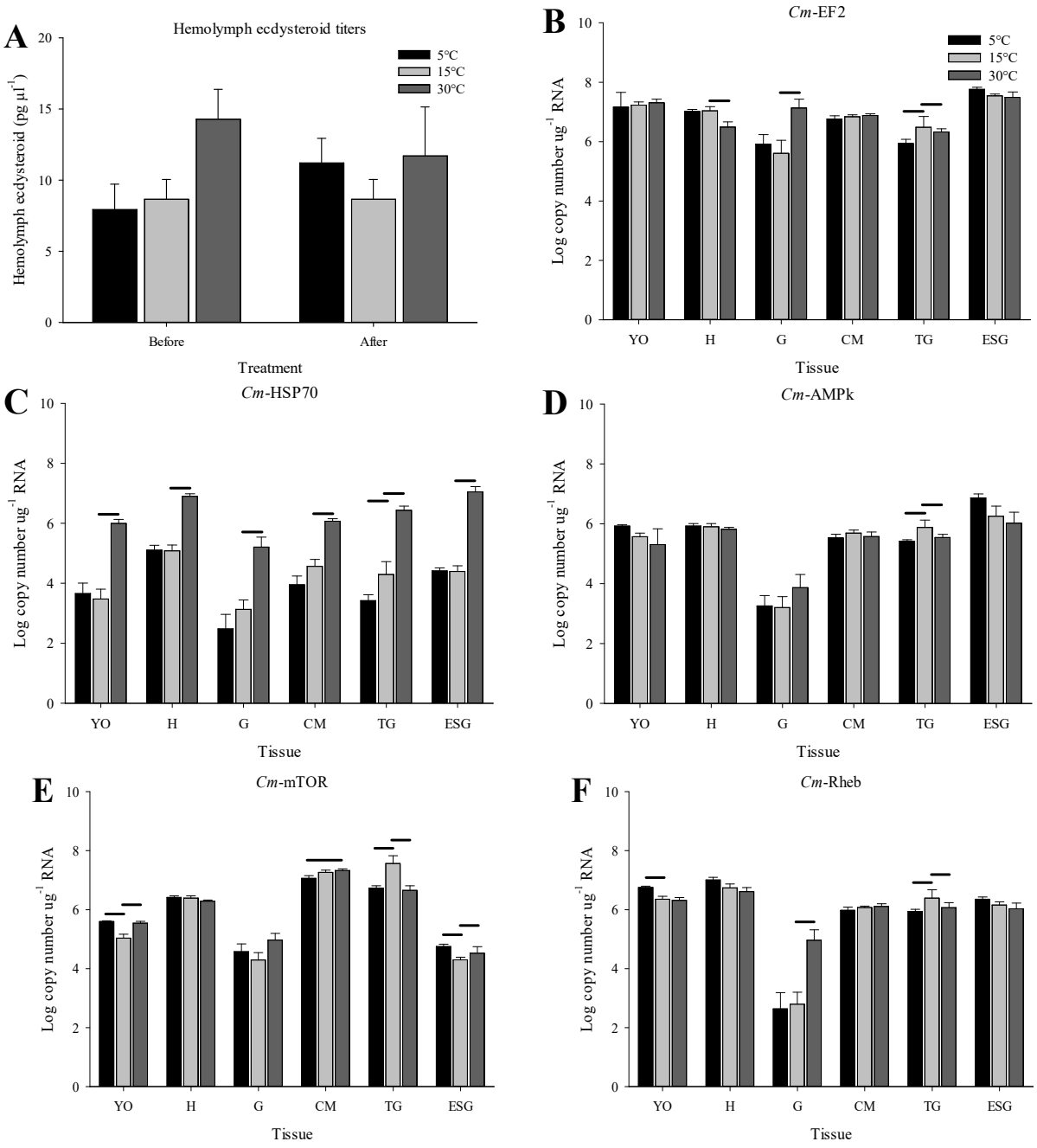


Figure 4-3. Effects of 2 h temperature treatment on hemolymph ecdysteroid levels and mRNA levels in *C. maenas* tissues. (A) ecdysteroid titers, (B) *Cm*-EF2, (C) *Cm-HSP70*, (D) *Cm*-AMPkγ, (E) *Cm*-mTOR, (F) *Cm*-Rheb. YO: Y-organ; H: heart; G: gill; CM: claw muscle; TG: thoracic ganglion; ESG: eyestalk ganglion. Black bars: 5°C, light gray bars: 15°C (Control), dark gray bars: 30°C. Black horizontal line: indicates significant differences from 15°C (Control) according to Tukey-Kramer HSD post hoc tests at $P<0.05$ (n = 6).

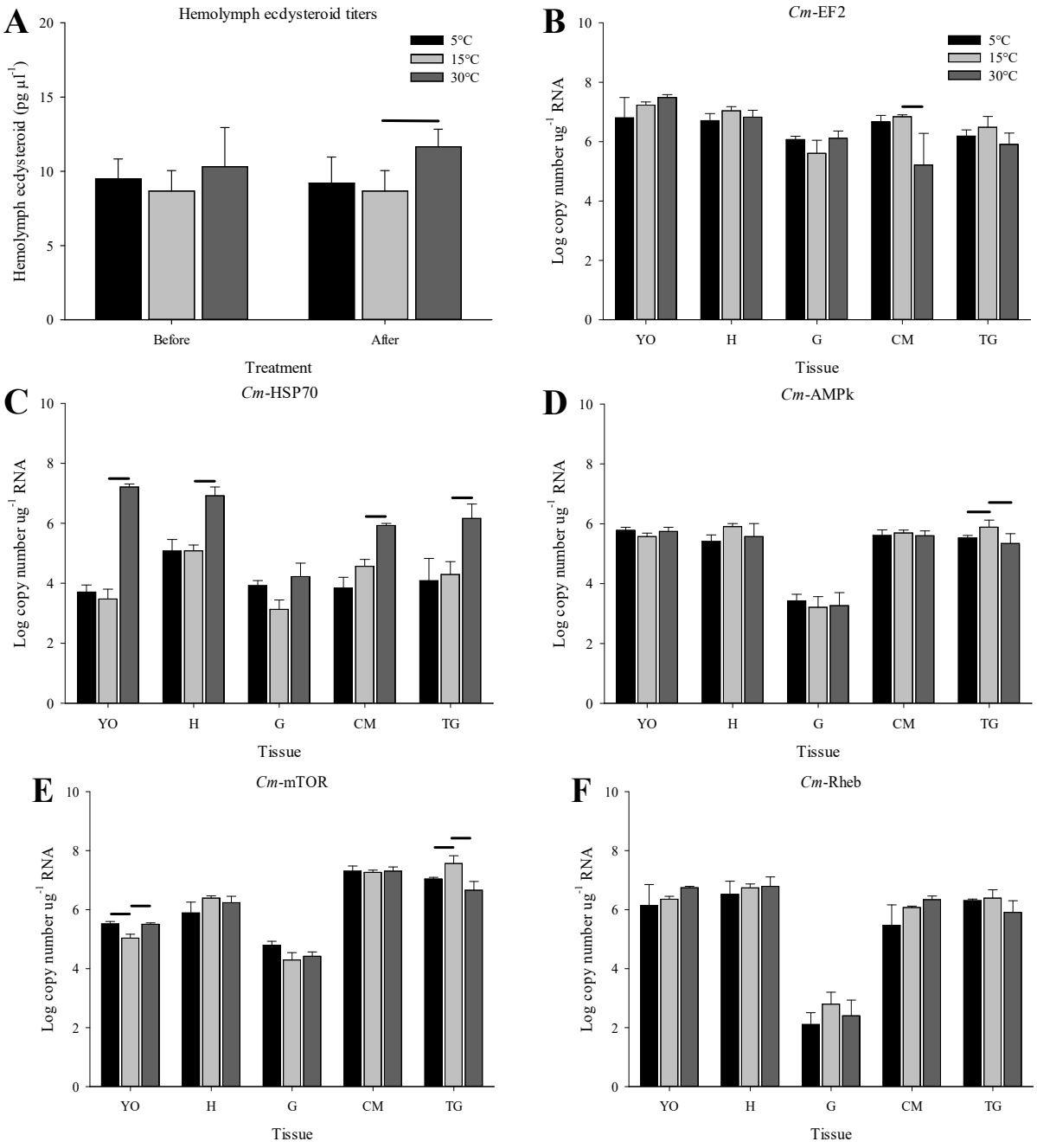
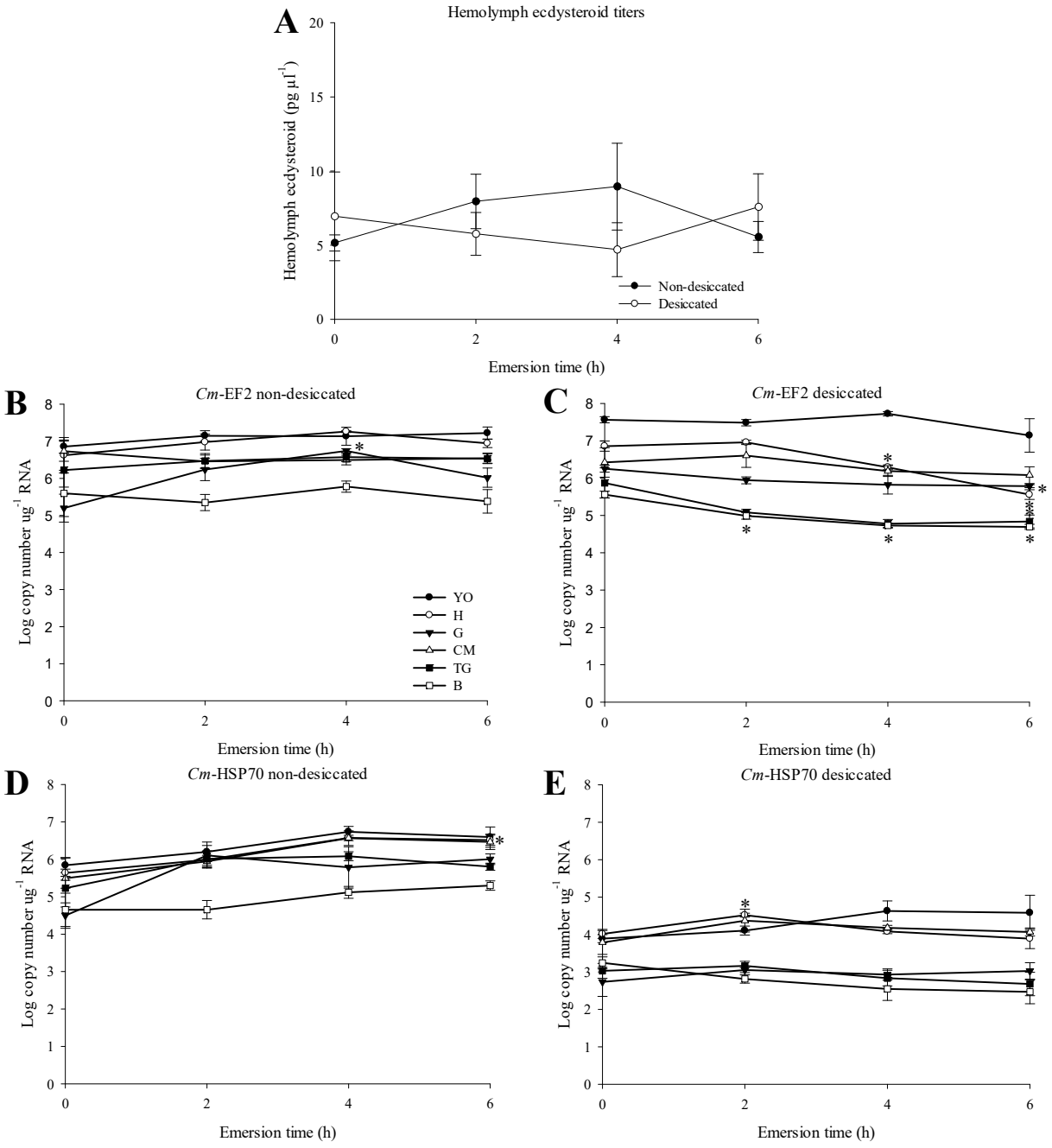


Figure 4-4. Effects of 1 h temperature treatment on hemolymph ecdysteroid levels and mRNA levels in ESA *C. maenas* tissues. (A) ecdysteroid titers, (B) *Cm*-EF2, (C) *Cm-HSP70*, (D) *Cm*-AMPkγ, (E) *Cm*-mTOR, (F) *Cm*-Rheb. YO: Y-organ; H: heart; G: gill; CM: claw muscle; TG: thoracic ganglion. Black bars: 5°C, light gray bars: 15°C (Control), dark gray bars: 30°C. Black horizontal line: indicates significant differences from 15°C (Control) according to Tukey-Kramer HSD post hoc tests at $P<0.05$ (n = 6).


A
Hemolymph ecdysteroid titers
Hemolymph ecdysteroid (pg µl-1)
Non-desiccated
Desiccated
Emersion time (h)
B
Cm-EF2 non-desiccated
Log copy number ug-1 RNA
YO
H
G
CM
TG
B
Emersion time (h)
C
Cm-EF2 desiccated
Log copy number ug-1 RNA
Emersion time (h)
D
Cm-HSP70 non-desiccated
Log copy number ug-1 RNA
Emersion time (h)
E
Cm-HSP70 desiccated
Log copy number ug-1 RNA
Emersion time (h)

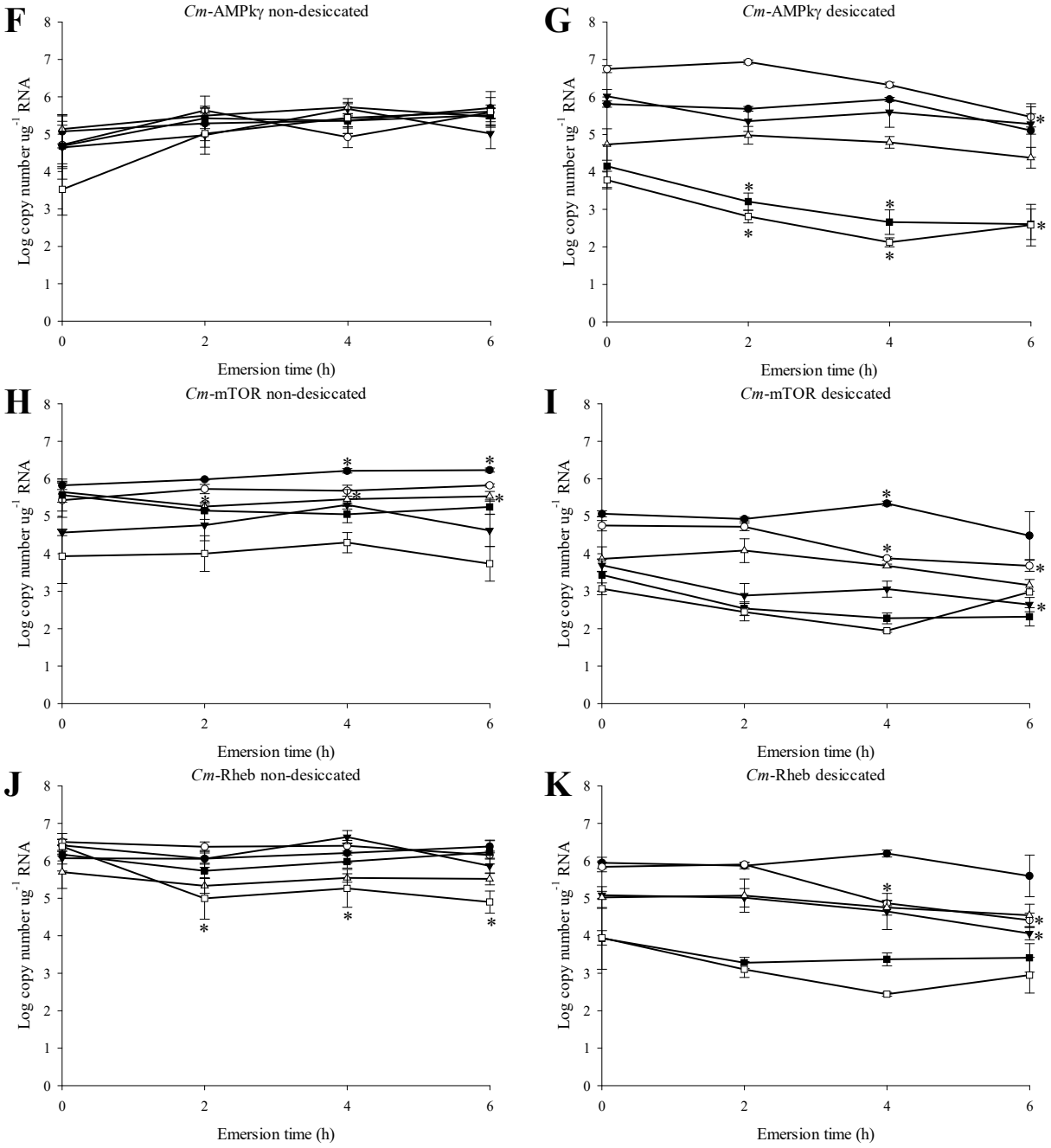


Figure 4-5. Effects of emersion and non-desiccated and desiccated conditions on hemolymph ecdysteroid levels and mRNA levels in *C. maenas* tissues. (A) ecdysteroid titers, (B,C) *Cm*-EF2, (D,E) *Cm-HSP70*, (F,G) *Cm*-AMPkγ, (H,I) *Cm*-mTOR, (J,K) *Cm*-Rheb. *: indicates significant differences when compared to control (0 h, no emersion) according to Tukey-Kramer HSD post hoc tests at $P<0.05$ (n = 6).

REFERENCES

Aagaard, A. (1996). In situ variation in heart rate of the shore crab *Carcinus maenas* in relation to environmental factors and physiological condition. *Marine Biology* **125**, 765-772.

Abehsera, S., Glazer, L., Tynyakov, J., Plaschkes, I., Chalifa-Caspi, V., Khalaila, I., Aflalo, E. D. and Sagi, A. (2014). Binary gene expression patterning of the molt cycle: the case of chitin metabolism. *PLoS One* **10**, e0122602.

Abuhagr, A. M., Blindert, J. L., Nimitkul, S., Zander, I. A., Labere, S. M., Chang, S. A., Maclea, K. S., Chang, E. S. and Mykles, D. L. (2014a). Molt regulation in green and red color morphs of the crab *Carcinus maenas*: gene expression of molt-inhibiting hormone signaling components. *J Exp Biol* **217**, 796-808.

Abuhagr, A. M., Maclea, K. S., Chang, E. S. and Mykles, D. L. (2014b). Mechanistic target of rapamycin (mTOR) signaling genes in decapod crustaceans: cloning and tissue expression of mTOR, Akt, Rheb, and p70 S6 kinase in the green crab, *Carcinus maenas*, and blackback land crab, *Gecarcinus lateralis*. *Comp Biochem Physiol A Mol Integr Physiol* **168**, 25-39.

Abuhagr, A. M., MacLea, K. S., Mudron, M. R., Chang, S. A., Chang, E. S. and Mykles, D. L. (2016). Roles of mechanistic target of rapamycin and transforming growth factor-beta signaling in the molting gland (Y-organ) of the blackback land crab, *Gecarcinus lateralis*. *Comp Biochem Physiol A Mol Integr Physiol* **198**, 15-21.

Ahsanullah, M. and Newell, R. C. (1977). The effects of humidity and temperature on water loss in *Carcinus maenas* (L) and *Portunus marmoreus* (Leach). *Comparative Biochemistry and Physiology Part A: Physiology* **56**, 593-599.

Aiken, D. E. and Waddy, S. L. (1975). Temperature increase can cause hyperecdysonism in American lobsters (*Homarus americanus*) injected with ecdysterone. *Journal of the Fisheries Research Board of Canada* **32**, 1843-1845.

Aiken, D. E. and Waddy, S. L. (1995). Aquaculture. In *Biology of the lobster Homarus americanus*, (ed. J. R. Factor), pp. 153-175. San Diego: Academic Press.

Alter, K., Paschke, K., Gebauer, P., Cumillaf, J.-P. and Pörtner, H.-O. (2015). Differential physiological responses to oxygen availability in early life stages of decapods developing in distinct environments. *Marine Biology* **162**, 1111-1124.

Anestis, A., Lazou, A., Portner, H. O. and Michaelidis, B. (2007). Behavioral, metabolic, and molecular stress responses of marine bivalve *Mytilus galloprovincialis* during long-term acclimation at increasing ambient temperature. *Am J Physiol Regul Integr Comp Physiol* **293**, R911-21.

Aramburu, J., Ortells, M. C., Tejedor, S., Buxade, M. and Lopez-Rodriguez, C. (2014). Transcriptional regulation of the stress response by mTOR. *Sci Signal* **7**, re2.

Baillie, C. and Grabowski, J. H. (2018). Invasion dynamics: interactions between the European green crab *Carcinus maenas* and the asian shore crab *Hemigrapsus sanguineus*. *Biological Invasions* **21**, 787-802.

Baringou, S., Rouault, J. D., Koken, M., Hardivillier, Y., Hurtado, L. and Leignel, V. (2016). Diversity of cytosolic HSP70 Heat Shock Protein from decapods and their phylogenetic placement within Arthropoda. *Gene* **591**, 97-107.

Behrens Yamada, S., Peterson, W. T. and Kosro, P. M. (2015). Biological and physical ocean indicators predict the success of an invasive crab, *Carcinus maenas*, in the northern California Current. *Marine Ecology Progress Series* **537**, 175-189.

Bell, G. W. and Eggleston, D. B. (2004). Species-specific avoidance responses by blue crabs and fish to chronic and episodic hypoxia. *Marine Biology* **146**, 761-770.

Beltz, B. S. (1995). Neurobiology and Neuroendocrinology. In *Biology of the lobster Homarus americanus*, (ed. J. R. Factor), pp. 217-266. San Diego: Academic Press.

Bernasconi, C. J. and Uglow, R. F. (2008). Effects of emersion-induced hypoxia on some haemolymph constituents of *Nephrops norvegicus*. *Dis Aquat Organ* **82**, 135-43.

Bliss, D. E. (1968). Transition from water to land in decapod crustaceans. *American Zoologist* **8**, 355-392.

Bliss, D. E. (1979). From sea to tree - saga of a land crab. *American Zoologist* **19**, 385-410.

Bliss, D. E. and Boyer, J. R. (1964). Environmental regulation of growth in the decapod crustacean *Gecarcinus lateralis*. *Gen Comp Endocrinol* **4**, 15-41.

Bliss, D. E., van Montfrans, J., van Montfrans, M. and Boyer, J. R. (1978). Behavior and growth of the land crab *Gecarcinus lateralis* (Fréminville) in southern Florida. *Bull. Am. Mus. Nat. Hist* **160**, 111-152.

Bosch-Presegue, L. and Vaquero, A. (2014). Sirtuins in stress response: guardians of the genome. *Oncogene* **33**, 3764-75.

Brown-Peterson, N. J., Larkin, P., Denslow, N., King, C., Manning, S. and Brouwer, M. (2005). Molecular indicators of hypoxia in the blue crab *Callinectes sapidus*. *Marine Ecology Progress Series* **286**, 203-215.

Buckley, B. A., Owen, M. E. and Hofmann, G. E. (2001). Adjusting the thermostat: the threshold induction temperature for the heat-shock response in intertidal mussels (genus *Mytilus*) changes as a function of thermal history. *The Journal of Experimental Biology*.

Burden, C. T., Stow, A. J., Hoggard, S. J., Coleman, M. A. and Bishop, M. J. (2014). Genetic structure of *Carcinus maenas* in southeast Australia. *Marine Ecology Progress Series* **500**, 139-147.

Burggren, W. W. and McMahon, B. R. (1981). Oxygen uptake during environmental temperature change in hermit crabs: adaptation to subtidal, intertidal, and supratidal habitats. *Physiological Zoology* **54**, 325-333.

Burggren, W. W. and McMahon, B. R. (1988). Biology of the land crabs. New York, US: Cambridge University Press.

Cameron, J. N. (1975). Aerial gas exchange in the terrestrial Brachyura *Gecarcinus lateralis* and *Cardisoma guanhumi*. *Comp Biochem Physiol A Comp Physiol* **52**, 129-34.

Canto, C., Gerhart-Hines, Z., Feige, J. N., Lagouge, M., Noriega, L., Milne, J. C., Elliott, P. J., Puigserver, P. and Auwerx, J. (2009). AMPK regulates energy expenditure by modulating NAD+ metabolism and SIRT1 activity. *Nature* **458**, 1056-60.

Capistran-Barradas, A., Defeo, O. and Moreno-Casasola, P. (2003). Density and population structure of the red land crab *Gecarcinus lateralis* in a tropical semi-deciduous forest in Veracruz, Mexico. *Interciencia* **28**, 323-+.

Carafa, V., Nebbioso, A. and Altucci, L. (2012). Sirtuins and disease: the road ahead. *Front Pharmacol* **3**, 4.

Carls, M. G. and Oclair, C. E. (1995). Responses of tanner crabs, *Chionoecetes-Bairdi*, exposed to cold-air. *Fishery Bulletin* **93**, 44-56.

Carlton, J. T. and Cohen, A. N. (2003). Episodic global dispersal in shallow water marine organisms: the case history of the European shore crabs *Carcinus maenas* and *C. aestuarii*. *Journal of Biogeography* **30**, 1809-1820.

Carroll, V. A. and Ashcroft, M. (2005). Targeting the molecular basis for tumour hypoxia. *Expert Rev Mol Med* **7**, 1-16.

Cetrullo, S., D'Adamo, S., Tantini, B., Borzi, R. M. and Flamigni, F. (2015). mTOR, AMPK, and Sirt1: key players in metabolic stress management. *Crit Rev Eukaryot Gene Expr* **25**, 59-75.

Chan, S. M., Gu, P. L., Chu, K. H. and Tobe, S. S. (2003). Crustacean neuropeptide genes of the CHH/MIH/GIH family: implications from molecular studies. *Gen Comp Endocrinol* **134**, 214-9.

Chang, E. S. (1995). Physiological and biochemical changes during the molt cycle in decapod crustaceans: an overview. *Journal of Experimental Marine Biology and Ecology* **193**, 1-14.

Chang, E. S. (2005). Stressed-out lobsters: crustacean hyperglycemic hormone and stress proteins. *Integr Comp Biol* **45**, 43-50.

Chang, E. S. and Bruce, M. J. (1980). Ecdysteroid titers of juvenile lobsters following molt induction. *Journal of Experimental Zoology* **214**, 157-160.

Chang, E. S. and Bruce, M. J. (1981). Ecdysteroid titers of larval lobsters. *Comparative Biochemistry and Physiology Part A: Physiology* **70**, 239-241.

Chang, E. S., Chang, S. A., Keller, R., Reddy, P. S., Snyder, M. J. and Spees, J. L. (1999). Quantification of stress in lobsters: crustacean hyperglycemic hormone, stress proteins, and gene expression. *American Zoologist* **39**, 487-495.

Chang, E. S., Keller, R. and Chang, S. A. (1998). Quantification of crustacean hyperglycemic hormone by ELISA in hemolymph of the lobster, *Homarus americanus*, following various stresses. *Gen Comp Endocrinol* **111**, 359-66.

Chang, E. S. and Mykles, D. L. (2011). Regulation of crustacean molting: a review and our perspectives. *Gen Comp Endocrinol* **172**, 323-30.

Chang, E. S., Prestwich, G. D. and Bruce, M. J. (1990). Amino acid sequence of a peptide with both molt-inhibiting and hyperglycemic activities in the lobster, *Homarus americanus*. *Biochemical and Biophysical Research Communications* **171**, 818-826.

Chang, E. S., Sage, B. A. and O'Connor, J. D. (1976). The qualitative and quantitative determinations of ecdysones in tissues of the crab, *Pachygrapsus crassipes*, following molt induction. *General and Comparative Endocrinology* **30**, 21-33.

Chaves, M. L., Horta, M. S., Chainho, P., Costa, M. J. and Costa, J. L. (2010). New additions to the feeding ecology of Carcinus maenas (L., 1758) in a South-western Europe estuary (Portugal). *Cahiers De Biologie Marine* **51**, 229-238.

Chen, T., Lin, T., Li, H., Lu, T., Li, J., Huang, W., Sun, H., Jiang, X., Zhang, J., Yan, A. et al. (2018). Heat shock protein 40 (HSP40) in Pacific white shrimp (*Litopenaeus vannamei*): molecular cloning, tissue distribution and ontogeny, response to temperature, acidity/alkalinity and salinity stresses, and potential role in ovarian development. *Front Physiol* **9**, 1784.

Chen, X., Wang, J., Hou, X., Yue, W., Li, Z. and Wang, C. (2019). Gene expression profiles of gill provide insights into the aerial respiration capacity of the Chinese mitten crab, *Eriocheir sinensis*. *Aquaculture* **506**, 148-153.

Chou, S. D., Prince, T., Gong, J. and Calderwood, S. K. (2012). mTOR is essential for the proteotoxic stress response, HSF1 activation and heat shock protein synthesis. *PLoS One* **7**, e39679.

Christie, A. E., Stemmler, E. A. and Dickinson, P. S. (2010). Crustacean

neuropeptides. *Cell Mol Life Sci* **67**, 4135-69.

Chung, J. S. and Webster, S. G. (2003). Moult cycle-related changes in biological activity of moult-inhibiting hormone (MIH) and crustacean hyperglycaemic hormone (CHH) in the crab, *Carcinus maenas*. From target to transcript. *Eur J Biochem* **270**, 3280-8.

Chung, J. S. and Webster, S. G. (2005). Dynamics of in vivo release of molt-inhibiting hormone and crustacean hyperglycemic hormone in the shore crab, *Carcinus maenas*. *Endocrinology* **146**, 5545-51.

Chung, J. S. and Zmora, N. (2008). Functional studies of crustacean hyperglycemic hormones (CHHs) of the blue crab, *Callinectes sapidus* - the expression and release of CHH in eyestalk and pericardial organ in response to environmental stress. *FEBS J* **275**, 693-704.

Chung, J. S., Zmora, N., Katayama, H. and Tsutsui, N. (2010). Crustacean hyperglycemic hormone (CHH) neuropeptides family: Functions, titer, and binding to target tissues. *Gen Comp Endocrinol* **166**, 447-54.

Cieluch, U., Anger, K., Aujoulat, F., Buchholz, F., Charmantier-Daures, M. and Charmantie, G. (2004). Ontogeny of osmoregulatory structures and functions in the green crab *Carcinus maenas* (Crustacea, Decapoda). *J Exp Biol* **207**, 325-36.

Cimino, E. J., Owens, L., Bromage, E. and Anderson, T. A. (2002). A newly developed ELISA showing the effect of environmental stress on levels of hsp86 in *Cherax quadricarinatus* and *Penaeus monodon*. *Comp Biochem Physiol A Mol Integr Physiol* **132**, 591-8.

Clark, M. S. and Peck, L. S. (2009). Triggers of the HSP70 stress response: environmental responses and laboratory manipulation in an Antarctic marine invertebrate (*Nacella concinna*). *Cell Stress Chaperones* **14**, 649-60.

Compton, T. J., Leathwick, J. R. and Inglis, G. J. (2010). Thermogeography predicts the potential global range of the invasive European green crab (*Carcinus maenas*). *Diversity and Distributions* **16**, 243-255.

Cosham, J. A., Beazley, K. F. and McCarthy, C. (2016). Local knowledge of distribution of European green crab (*Carcinus maenas*) in southern Nova Scotian coastal waters. *Human Ecology* **44**, 409-424.

Costantini, S., Sharma, A., Raucci, R., Costantini, M., Autiero, I. and Colonna, G. (2013). Genealogy of an ancient protein family: the Sirtuins, a family of disordered members. *BMC Evol Biol* **13**, 60.

Cottin, D., Foucreau, N., Hervant, F. and Piscart, C. (2015). Differential regulation of hsp70 genes in the freshwater key species *Gammarus pulex* (Crustacea, Amphipoda) exposed to thermal stress: effects of latitude and ontogeny. *J Comp Physiol B* **185**, 303-13.

Cuculescu, M., Hyde, D. and Bowler, K. (1998). Thermal tolerance of two species of marine crab, *Cancer pagurus* and *Carcinus maenas*. *Journal of Thermal Biology* **23**, 107-110.

D'Incao, F., Ruffino, M. L., da Silva, K. G. and da Costa Braga, A. (1992). Responses of *Chasmagnathus granulata* Dana (Decapoda: Grapsidae) to salt-marsh environmental variations. *Journal of Experimental Marine Biology and Ecology* **161**, 179-188.

Dalle Pezze, P., Ruf, S., Sonntag, A. G., Langelaar-Makkinje, M., Hall, P., Heberle, A. M., Razquin Navas, P., van Eunen, K., Tolle, R. C., Schwarz, J. J. et al. (2016). A systems study reveals concurrent activation of AMPK and mTOR by amino acids. *Nat Commun* **7**, 13254.

Darling, J. A., Bagley, M. J., Roman, J., Tepolt, C. K. and Geller, J. B. (2008). Genetic patterns across multiple introductions of the globally invasive crab genus *Carcinus*. *Mol*

Ecol **17**, 4992-5007.

Das, S. and Mykles, D. L. (2016). A comparison of resources for the annotation of a de novo assembled transcriptome in the molting gland (Y-organ) of the blackback land crab, *Gecarcinus lateralis*. *Integr Comp Biol* **56**, 1103-1112.

Das, S., Pitts, N. L., Mudron, M. R., Durica, D. S. and Mykles, D. L. (2016). Transcriptome analysis of the molting gland (Y-organ) from the blackback land crab, *Gecarcinus lateralis*. *Comp Biochem Physiol Part D Genomics Proteomics* **17**, 26-40.

Das, S., Vraspir, L., Zhou, W., Durica, D. S. and Mykles, D. L. (2018). Transcriptomic analysis of differentially expressed genes in the molting gland (Y-organ) of the blackback land crab, *Gecarcinus lateralis*, during molt-cycle stage transitions. *Comp Biochem Physiol Part D Genomics Proteomics* **28**, 37-53.

de la Vega, E., Hall, M. R., Wilson, K. J., Reverter, A., Woods, R. G. and Degnan, B. M. (2007). Stress-induced gene expression profiling in the black tiger shrimp *Penaeus monodon*. *Physiol Genomics* **31**, 126-38.

de Lima, T. M., Geihs, M. A., Nery, L. E. and Maciel, F. E. (2015). Air exposure behavior of the semiterrestrial crab *Neohelice granulata* allows tolerance to severe hypoxia but not prevent oxidative damage due to hypoxia-reoxygenation cycle. *Physiol Behav* **151**, 97-101.

De Maio, A. (1999). Heat shock proteins: facts, thoughts, and dreams. *Shock* **11**, 1-12.

Defur, P. L. (1988). Systemic respiratory adaptations to air exposure in intertidal decapod crustaceans. *American Zoologist* **28**, 115-124.

Defur, P. L., McMahon, B. R. and Booth, C. E. (1983). Analysis of hemolymph oxygen levels and acid-base status during emersion 'in situ' in the red rock crab, *Cancer productus*. *Biol Bull* **165**, 582-590.

Devaraj, H. and Natarajan, A. (2006). Molecular mechanisms regulating molting in a crustacean. *FEBS J* **273**, 839-46.

Dioum, E. M., Chen, R., Alexander, M. S., Zhang, Q., Hogg, R. T., Gerard, R. D. and Garcia, J. A. (2009). Regulation of hypoxia-inducible factor 2a signaling by the stress-responsive deacetylase sirtuin 1. *Science* **324**, 1289-93.

Dong, Y.-w., Zhang, S. and Woods, A. (2016). Ecological relevance of energy metabolism: transcriptional responses in energy sensing and expenditure to thermal and osmotic stresses in an intertidal limpet. *Functional Ecology* **30**, 1539-1548.

Dong, Y. W., Han, G. D. and Huang, X. W. (2014). Stress modulation of cellular metabolic sensors: interaction of stress from temperature and rainfall on the intertidal limpet *Cellana toreuma*. *Mol Ecol* **23**, 4541-54.

Dong, Z., Mao, S., Chen, Y., Ge, H., Li, X., Wu, X., Liu, D., Zhang, K., Bai, C. and Zhang, Q. (2019). Effects of air-exposure stress on the survival rate and physiology of the swimming crab *Portunus trituberculatus*. *Aquaculture* **500**, 429-434.

Drozdova, P., Bedulina, D., Madyarova, E., Rivarola-Duarte, L., Schreiber, S., Stadler, P. F., Luckenbach, T. and Timofeyev, M. (2019). Description of strongly heat-inducible heat shock protein 70 transcripts from Baikal endemic amphipods. *Sci Rep* **9**, 8907.

Duan, Y., Li, F., Tan, K., Liu, H., Li, Y., Liu, Y., Kong, X., Tang, Y., Wu, G. and Yin, Y. (2015). Key mediators of intracellular amino acids signaling to mTORC1 activation. *Amino Acids* **47**, 857-67.

Duan, Y., Zhang, Y., Dong, H. and Zhang, J. (2016). Effect of desiccation on oxidative stress and antioxidant response of the black tiger shrimp *Penaeus monodon*. *Fish Shellfish Immunol* **58**, 10-17.

Durliat, M., Moriniere, M. and Porcheron, P. (1988). Changes in ecdysteroids in *Astacus leptodactylus* during the molting cycle. *Comparative Biochemistry and Physiology Part A: Physiology* **89**, 223-229.

El Haj, A. J. (1999). Regulation of muscle growth and sarcomeric protein gene expression over the intermolt cycle. *American Zoologist* **39**, 570-579.

Factor, J. R. (1995). Biology of the lobster *Homarus americanus*, vol. 1, pp. 528: Academic Press.

Faubert, B., Vincent, E. E., Poffenberger, M. C. and Jones, R. G. (2015). The AMP-activated protein kinase (AMPK) and cancer: many faces of a metabolic regulator. *Cancer Lett* **356**, 165-70.

Feder, M. E. and Hofmann, G. E. (1999). Heat-shock proteins, molecular chaperones, and the stress response: evolutionary and ecological physiology. *Annu Rev Physiol* **61**, 243-82.

Frederich, M. and Balschi, J. A. (2002). The relationship between AMP-activated protein kinase activity and AMP concentration in the isolated perfused rat heart. *J Biol Chem* **277**, 1928-32.

Frederich, M., O'Rourke, M. R., Furey, N. B. and Jost, J. A. (2009). AMP-activated protein kinase (AMPK) in the rock crab, *Cancer irroratus*: an early indicator of temperature stress. *J Exp Biol* **212**, 722-30.

Frederich, M., O'Rourke, M. and Towle, D. (2006). Is AMP activated protein kinase expression in *Cancer irroratus* a better signal for temperature stress then HSP70? *The MDIBL Bulletin* **45**, 37-39.

Frederich, M. and Portner, H. O. (2000). Oxygen limitation of thermal tolerance defined by cardiac and ventilatory performance in spider crab, *Maja squinado*. *Am J Physiol*

Regul Integr Comp Physiol **279**, R1531-8.

Frye, R. A. (2000). Phylogenetic classification of prokaryotic and eukaryotic Sir2-like proteins. *Biochem Biophys Res Commun* **273**, 793-8.

Fu, C., Huang, X., Gong, J., Chen, X., Huang, H. and Ye, H. (2016). Crustacean hyperglycaemic hormone gene from the mud crab, *Scylla paramamosain*: cloning, distribution and expression profiles during the moulting cycle and ovarian development. *Aquaculture Research* **47**, 2183-2194.

Fusi, M., Cannicci, S., Daffonchio, D., Mostert, B., Pörtner, H. O. and Giomi, F. (2016). The trade-off between heat tolerance and metabolic cost drives the bimodal life strategy at the air-water interface. *Sci Rep* **6**, 19158.

Gasser, S. M. and Cockell, M. M. (2001). The molecular biology of the SIR proteins. *Gene* **279**, 1-16.

Geihs, M. A., Maciel, F. E., Vargas, M. A., Cruz, B. P. and Nery, L. E. M. (2013). Effects of hypoxia and reoxygenation on the energetic metabolism of the crab *Neohelice granulata* (Decapoda, Varunidae). *Journal of Experimental Marine Biology and Ecology* **445**, 69-78.

Ghosh, H. S., McBurney, M. and Robbins, P. D. (2010). SIRT1 negatively regulates the mammalian target of rapamycin. *PLoS One* **5**, e9199.

Gillum, M. P., Erion, D. M. and Shulman, G. I. (2011). Sirtuin-1 regulation of mammalian metabolism. *Trends Mol Med* **17**, 8-13.

Giovannini, L. and Bianchi, S. (2017). Role of nutraceutical SIRT1 modulators in AMPK and mTOR pathway: evidence of a synergistic effect. *Nutrition* **34**, 82-96.

Gonzalez-Aravena, M., Calfio, C., Mercado, L., Morales-Lange, B., Bethke, J., De

Lorgeril, J. and Cardenas, C. A. (2018). HSP70 from the Antarctic sea urchin Sterechinus neumayeri: molecular characterization and expression in response to heat stress. *Biol Res* **51**, 8.

Gray, I. E. (1957). A comparative study of the gill area of crabs. *The Biological Bulletin* **112**, 34-42.

Greenaway, P. (1988). Ion and water balance. In *Biology of the Land Crabs*, (ed. W. Burggren and B. Mcmahon), pp. 211-246. Cambridge: Cambridge University Press.

Greiss, S. and Gartner, A. (2009). Sirtuin/Sir2 phylogeny, evolutionary considerations and structural conservation. *Mol Cells* **28**, 407-15.

Grosholz, E. D. and Ruiz, G. M. (1996). Predicting the impact of introduced marine species: lessons from the multiple invasions of the European green crab *Carcinus maenas*. *Biological Conservation* **78**, 59-66.

Gu, S. H., Chen, C. H., Hsieh, Y. C., Lin, P. L. and Young, S. C. (2015). Modulatory effects of bombyxin on ecdysteroidogenesis in Bombyx mori prothoracic glands. *J Insect Physiol* **72**, 61-69.

Guevelou, E., Huvet, A., Sussarellu, R., Milan, M., Guo, X., Li, L., Zhang, G., Quillien, V., Daniel, J. Y., Quere, C. et al. (2013). Regulation of a truncated isoform of AMP-activated protein kinase alpha (AMPKalpha) in response to hypoxia in the muscle of Pacific oyster *Crassostrea gigas*. *J Comp Physiol B* **183**, 597-611.

Gunderson, A. R. and Stillman, J. H. (2015). Plasticity in thermal tolerance has limited potential to buffer ectotherms from global warming. *Proc Biol Sci* **282**, 20150401.

Gusev, O., Ziegler, T. and Saigusa, M. (2006). Expression and structure of stress chaperon hsp90 in terrestrial decapods, *Coenobita* (Anomura: Coenobitidae) and *Chiromantes* (Brachyura: Sesarmidae). In *Biology of Anomura II – Proceedings of the Symposium. Sixth*

International Crustacean Congress, vol. 6 (ed. A. Asakura), pp. 109. Glasgow, Scotland.

Haissaguerre, M., Saucisse, N. and Cota, D. (2014). Influence of mTOR in energy and metabolic homeostasis. *Mol Cell Endocrinol* **397**, 67-77.

Haj, A., Clarke, S., Harrison, P. and Chang, E. (1996). In vivo muscle protein synthesis rates in the American lobster *Homarus americanus* during the moult cycle and in response to 20-hydroxyecdysone. *The Journal of Experimental Biology* **199**, 579-585.

Hammer, Ø., Harper, D. A. T. and Ryan, P. D. (2001). PAST: paleontological statistics software package for education and data analysis. *Palaeontologia Electronica* **4**, 9.

Han, G. D., Zhang, S., Marshall, D. J., Ke, C. H. and Dong, Y. W. (2013). Metabolic energy sensors (AMPK and SIRT1), protein carbonylation and cardiac failure as biomarkers of thermal stress in an intertidal limpet: linking energetic allocation with environmental temperature during aerial emersion. *J Exp Biol* **216**, 3273-82.

Han, J., Lee, M.-C., Park, J. C., Kim, S. and Lee, J.-S. (2018). Effects of temperature shifts on life parameters and expression of fatty acid synthesis and heat shock protein genes in temperate and Antarctic copepods *Tigriopus japonicus* and *Tigriopus kingsejongensis*. *Polar Biology* **41**, 2459-2466.

Hand, S. C., Menze, M. A., Borcar, A., Patil, Y., Covi, J. A., Reynolds, J. A. and Toner, M. (2011). Metabolic restructuring during energy-limited states: insights from *Artemia franciscana* embryos and other animals. *J Insect Physiol* **57**, 584-94.

Harada, A. E. and Burton, R. S. (2019). Ecologically relevant temperature ramping rates enhance the protective heat shock response in an intertidal ectotherm. *Physiol Biochem Zool* **92**, 152-162.

Hardy, K. M., Follett, C. R., Burnett, L. E. and Lema, S. C. (2012). Gene transcripts

encoding hypoxia-inducible factor (HIF) exhibit tissue- and muscle fiber type-dependent responses to hypoxia and hypercapnic hypoxia in the Atlantic blue crab, *Callinectes sapidus*. *Comp Biochem Physiol A Mol Integr Physiol* **163**, 137-46.

Harms, L., Frickenhaus, S., Schiffer, M., Mark, F. C., Storch, D., Held, C., Portner, H. O. and Lucassen, M. (2014). Gene expression profiling in gills of the great spider crab *Hyas araneus* in response to ocean acidification and warming. *BMC Genomics* **15**, 789.

Hartnoll, R. G. (1988). Growth and molting. In *Biology of the Land Crabs*, (ed. W. Burggren and B. Mcmahon), pp. 186-210. Cambridge: Cambridge University Press.

Hartnoll, R. G. (2001). Growth in Crustacea - twenty years on. *Hydrobiologia* **449**, 111-122.

Haukenes, A. H., Buck, C. L. and El Mejjati, S. Y. (2009). Effects of emersion temperature on the oxygen consumption rates of male tanner crabs, *Chionoecetes Bairdi*. *Journal of Crustacean Biology* **29**, 91-95.

Hill, R., Wyse, G. and Anderson, M. (2012). Animal Physiology. Sunderland, Massachusetts: Sinauer Associates.

Hines, D. J., Clark, K. F. and Greenwood, S. J. (2013). Global gene expression profiling of *Homarus americanus* (Crustacea) larval stages during development and metamorphosis. *Invertebrate Reproduction & Development* **58**, 97-107.

Hochachka, P. W., Buck, L. T., Doll, C. J. and Land, S. C. (1996). Unifying theory of hypoxia tolerance: molecular/metabolic defense and rescue mechanisms for surviving oxygen lack. *Proc Natl Acad Sci U S A* **93**, 9493-8.

Hofmann, G. and Somero, G. (1995). Evidence for protein damage at environmental temperatures: seasonal changes in levels of ubiquitin conjugates and hsp70 in the intertidal

mussel *Mytilus trossulus*. *J Exp Biol* **198**, 1509-18.

Holcik, M. and Sonenberg, N. (2005). Translational control in stress and apoptosis. *Nat Rev Mol Cell Biol* **6**, 318-27.

Hopkins, P. M. (1983). Patterns of serum ecdysteroids during induced and uninduced proecdysis in the fiddler crab, *Uca pugilator*. *Gen Comp Endocrinol* **52**, 350-6.

Hopkins, P. M. (1986). Ecdysteroid titers and Y-organ activity during late anecdysis and proecdysis in the fiddler crab, *Uca pugilator*. *Gen Comp Endocrinol* **63**, 362-73.

Hopkins, P. M. (2001). Limb regeneration in the fiddler crab, *Uca pugilator*: hormonal and growth factor control. *American Zoologist* **41**, 389-398.

Hopkins, P. M. (2012). The eyes have it: a brief history of crustacean neuroendocrinology. *Gen Comp Endocrinol* **175**, 357-66.

Hsu, Y. W., Messinger, D. I., Chung, J. S., Webster, S. G., de la Iglesia, H. O. and Christie, A. E. (2006). Members of the crustacean hyperglycemic hormone (CHH) peptide family are differentially distributed both between and within the neuroendocrine organs of *Cancer crabs*: implications for differential release and pleiotropic function. *J Exp Biol* **209**, 3241-56.

Huang, H., Fu, C., Chen, X., Gong, J., Huang, X. and Ye, H. (2015a). Molt-inhibiting hormone (MIH) gene from the green mud crab *Scylla paramamosain* and its expression during the molting and ovarian cycle. *Aquaculture Research* **46**, 2665-2675.

Huang, K. and Fingar, D. C. (2014). Growing knowledge of the mTOR signaling network. *Semin Cell Dev Biol* **36**, 79-90.

Huang, X., Wang, T., Ye, Z., Han, G. and Dong, Y. (2015b). Temperature relations of aerial and aquatic physiological performance in a mid-intertidal limpet *Cellana toreuma*:

adaptation to rapid changes in thermal stress during emersion. *Integr Zool* **10**, 159-70.

Huang, X., Ye, H., Huang, H., Yang, Y. and Gong, J. (2014). An insulin-like androgenic gland hormone gene in the mud crab, *Scylla paramamosain*, extensively expressed and involved in the processes of growth and female reproduction. *Gen Comp Endocrinol* **204**, 229-38.

Huberman, A., Aguilar, M. B. and Quackenbush, L. S. (1995). A neuropeptide family from the sinus gland of the Mexican crayfish, *Procambarus bouvieri* (Ortmann). *Aquaculture* **135**, 149-160.

Inoki, K., Kim, J. and Guan, K. L. (2012). AMPK and mTOR in cellular energy homeostasis and drug targets. *Annu Rev Pharmacol Toxicol* **52**, 381-400.

Janzen, D. H. (1967). Why mountain passes are higher in the tropics. *The American Naturalist* **101**, 233-249.

Jeno, K. and Brokordt, K. (2014). Nutritional status affects the capacity of the snail *Concholepas concholepas* to synthesize Hsp70 when exposed to stressors associated with tidal regimes in the intertidal zone. *Marine Biology* **161**, 1039-1049.

Jensen, G. C. and Armstrong, D. A. (1991). Intertidal zonation among congeners: factors regulating distribution of porcelain crabs *Petrolisthes spp.* (Anomura: Porcellanidae). *Marine Ecology Progress Series* **73**, 47-60.

Jeon, J. M., Kim, B. K., Lee, J. H., Kim, H. J., Kang, C. K., Mykles, D. L. and Kim, H. W. (2012). Two type I crustacean hyperglycemic hormone (CHH) genes in Morotoge shrimp (*Pandalopsis japonica*): cloning and expression of eyestalk and pericardial organ isoforms produced by alternative splicing and a novel type I CHH with predicted structure shared with type II CHH peptides. *Comp Biochem Physiol B Biochem Mol Biol* **162**, 88-99.

Jeon, S. M. (2016). Regulation and function of AMPK in physiology and diseases. *Exp Mol Med* **48**, e245.

Jibb, L. A. and Richards, J. G. (2008). AMP-activated protein kinase activity during metabolic rate depression in the hypoxic goldfish, Carassius auratus. *J Exp Biol* **211**, 3111-22.

Johnson, C. L. (2003). Ecdysteroids in the oceanic copepod *Calanus pacificus*: variation during molt cycle and change associated with diapause. *Marine Ecology Progress Series* **257**, 159-165.

Johnson, I. and Uglow, R. F. (1985). Some effects of aerial exposure on the respiratory physiology and blood chemistry of *Carcinus maenas* (L.) and *Liocarcinus puber* (L.). *Journal of Experimental Marine Biology and Ecology* **94**, 151-165.

Jost, J. A., Podolski, S. M. and Frederich, M. (2012). Enhancing thermal tolerance by eliminating the pejus range: a comparative study with three decapod crustaceans. *Marine Ecology Progress Series* **444**, 263-274.

Jung, H., Lyons, R. E., Hurwood, D. A. and Mather, P. B. (2013). Genes and growth performance in crustacean species: a review of relevant genomic studies in crustaceans and other taxa. *Reviews in Aquaculture* **5**, 77-110.

Kamaruding, N. A., Ismail, N. and Ikhwanuddin, M. (2018). Physiological effect of eyestalk ablation on nutrient utilization and plasma protein expression in the female giant freshwater prawn (*Macrobrachium rosenbergii*) during different molting cycles. *Journal of Shellfish Research* **37**, 1113-1120.

Kaper, F., Dornhoefer, N. and Giaccia, A. J. (2006). Mutations in the PI3K/PTEN/TSC2 pathway contribute to mammalian target of rapamycin activity and increased translation under hypoxic conditions. *Cancer Res* **66**, 1561-9.

Kappalli, S., Supriya, N. T., Krishnakumar, V., Gopinathan, A. and Chang, E. S. (2012). Hemolymph ecdysteroid titers in a brachyuran crab *Uca triangularis* that concomitantly undergoes molting and reproduction. *Zoological Studies* **51**, 966-976.

Kaur, A. and Sharma, S. (2017). Mammalian target of rapamycin (mTOR) as a potential therapeutic target in various diseases. *Inflammopharmacology* **25**, 293-312.

Ke, Q. and Costa, M. (2006). Hypoxia-inducible factor-1 (HIF-1). *Mol Pharmacol* **70**, 1469-80.

Kelley, A. L., de Rivera, C. E. and Buckley, B. A. (2011). Intraspecific variation in thermotolerance and morphology of the invasive European green crab, *Carcinus maenas*, on the west coast of North America. *Journal of Experimental Marine Biology and Ecology* **409**, 70-78.

Kenney, J. W., Moore, C. E., Wang, X. and Proud, C. G. (2014). Eukaryotic elongation factor 2 kinase, an unusual enzyme with multiple roles. *Adv Biol Regul* **55**, 15-27.

Kim, J., Yang, G., Kim, Y., Kim, J. and Ha, J. (2016). AMPK activators: mechanisms of action and physiological activities. *Exp Mol Med* **48**, e224.

Kim, S. G., Buel, G. R. and Blenis, J. (2013). Nutrient regulation of the mTOR complex 1 signaling pathway. *Mol Cells* **35**, 463-73.

King, A. M. and MacRae, T. H. (2015). Insect Heat Shock Proteins During Stress and Diapause. In *Annual Review of Entomology, Vol 60*, vol. 60: *Annual Review of Entomology*. (ed. M. R. Berenbaum), pp. 59-75.

Kingan, T. G. (1989). A competitive enzyme-linked immunosorbent assay: applications in the assay of peptides, steroids, and cyclic nucleotides. *Anal Biochem* **183**, 283-9.

Klassen, G. and Locke, A. (2007). A Biological Synopsis of the European Green Crab, *Carcinus maenas*. *Canadian Manuscript Report of Fisheries and Aquatic Sciences 2818*, 75.

Klumpen, E., Hoffschroer, N., Zeis, B., Gigengack, U., Dohmen, E. and Paul, R. J. (2017). Reactive oxygen species (ROS) and the heat stress response of *Daphnia pulex*: ROS-mediated activation of hypoxia-inducible factor 1 (HIF-1) and heat shock factor 1 (HSF-1) and the clustered expression of stress genes. *Biol Cell* **109**, 39-64.

Kuo, C. M. and Yang, Y. H. (1999). Hyperglycemic responses to cold shock in the freshwater giant prawn, *Macrobrachium rosenbergii*. *Journal of Comparative Physiology B* **169**, 49-54.

Laemmle, A., Lechleiter, A., Roh, V., Schwarz, C., Portmann, S., Furer, C., Keogh, A., Tschan, M. P., Candinas, D., Vorburger, S. A. et al. (2012). Inhibition of SIRT1 impairs the accumulation and transcriptional activity of HIF-1alpha protein under hypoxic conditions. *PLoS One* **7**, e33433.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-5.

Laplante, M. and Sabatini, D. M. (2012). mTOR signaling in growth control and disease. *Cell* **149**, 274-93.

Larkin, M. A., Blackshields, G., Brown, N. P., Chenna, R., McGettigan, P. A., McWilliam, H., Valentin, F., Wallace, I. M., Wilm, A., Lopez, R. et al. (2007). Clustal W and Clustal X version 2.0. *Bioinformatics* **23**, 2947-8.

Lee, K. J., Kim, H. W., Gomez, A. M., Chang, E. S., Covi, J. A. and Mykles, D. L. (2007). Molt-inhibiting hormone from the tropical land crab, *Gecarcinus lateralis*: cloning, tissue expression, and expression of biologically active recombinant peptide in yeast. *Gen Comp Endocrinol* **150**, 505-13.

Lee, K. J., Watson, R. D. and Roer, R. D. (1998). Molt-inhibiting hormone mRNA

levels and ecdysteroid titer during a molt cycle of the blue crab, *Callinectes sapidus*. *Biochem Biophys Res Commun* **249**, 624-7.

Lee, S. G. and Mykles, D. L. (2006). Proteomics and signal transduction in the crustacean molting gland. *Integr Comp Biol* **46**, 965-77.

Lehnert, S. J., DiBacco, C., Jeffery, N. W., Blakeslee, A. M. H., Isaksson, J., Roman, J., Wringe, B. F., Stanley, R. R. E., Matheson, K., McKenzie, C. H. et al. (2018). Temporal dynamics of genetic clines of invasive European green crab (*Carcinus maenas*) in eastern North America. *Evol Appl* **11**, 1656-1670.

Li, S., Li, F., Wang, B., Xie, Y., Wen, R. and Xiang, J. (2010). Cloning and expression profiles of two isoforms of a CHH-like gene specifically expressed in male Chinese shrimp, *Fenneropenaeus chinensis*. *Gen Comp Endocrinol* **167**, 308-16.

Li, T. and Brouwer, M. (2007). Hypoxia-inducible factor, gsHIF, of the grass shrimp *Palaemonetes pugio*: molecular characterization and response to hypoxia. *Comp Biochem Physiol B Biochem Mol Biol* **147**, 11-9.

Lindquist, S. and Craig, E. A. (1988). The heat-shock proteins. *Annu Rev Genet* **22**, 631-77.

Liu, A., Liu, J., Chen, X., Lu, B., Zeng, C. and Ye, H. (2019a). A novel crustacean hyperglycemic hormone (CHH) from the mud crab *Scylla paramamosain* regulating carbohydrate metabolism. *Comp Biochem Physiol A Mol Integr Physiol* **231**, 49-55.

Liu, L., Cash, T. P., Jones, R. G., Keith, B., Thompson, C. B. and Simon, M. C. (2006). Hypoxia-induced energy stress regulates mRNA translation and cell growth. *Mol Cell* **21**, 521-31.

Liu, Y., Li, L., Huang, B., Wang, W. and Zhang, G. (2019b). RNAi based

transcriptome suggests genes potentially regulated by HSF1 in the Pacific oyster *Crassostrea gigas* under thermal stress. *BMC Genomics* **20**, 639.

Lu, Y., Zhang, D., Wang, F. and Dong, S. (2016). Hypothermal effects on survival, energy homeostasis and expression of energy-related genes of swimming crabs *Portunus trituberculatus* during air exposure. *J Therm Biol* **60**, 33-40.

Luo, X., Chen, T., Zhong, M., Jiang, X., Zhang, L., Ren, C. and Hu, C. (2015). Differential regulation of hepatopancreatic vitellogenin (VTG) gene expression by two putative molt-inhibiting hormones (MIH1/2) in Pacific white shrimp (*Litopenaeus vannamei*). *Peptides* **68**, 58-63.

Lv, J., Liu, P., Gao, B., Wang, Y., Wang, Z., Chen, P. and Li, J. (2014). Transcriptome analysis of the Portunus trituberculatus: de novo assembly, growth-related gene identification and marker discovery. *PLoS One* **9**, e94055.

Lv, J., Zhang, L., Liu, P. and Li, J. (2017). Transcriptomic variation of eyestalk reveals the genes and biological processes associated with molting in *Portunus trituberculatus*. *PLoS One* **12**, e0175315.

MacLea, K. S., Abuhagr, A. M., Pitts, N. L., Covi, J. A., Bader, B. D., Chang, E. S. and Mykles, D. L. (2012). Rheb, an activator of target of rapamycin, in the blackback land crab, *Gecarcinus lateralis:* cloning and effects of molting and unweighting on expression in skeletal muscle. *J Exp Biol* **215**, 590-604.

Madeira, C., Leal, M. C., Diniz, M. S., Cabral, H. N. and Vinagre, C. (2018). Thermal stress and energy metabolism in two circumtropical decapod crustaceans: responses to acute temperature events. *Mar Environ Res* **141**, 148-158.

Madeira, D., Narciso, L., Cabral, H. N., Diniz, M. S. and Vinagre, C. (2012). Thermal

tolerance of the crab *Pachygrapsus marmoratus*: intraspecific differences at a physiological (CTMax) and molecular level (Hsp70). *Cell Stress Chaperones* **17**, 707-16.

Manfrin, C., Tom, M., De Moro, G., Gerdol, M., Guarnaccia, C., Mosco, A., Pallavicini, A. and Giulianini, P. G. (2013). Application of d-crustacean hyperglycemic hormone induces peptidases transcription and suppresses glycolysis-related transcripts in the hepatopancreas of the crayfish *Pontastacus leptodactylus* - results of a transcriptomic study. *PLoS One* **8**, e65176.

Mann, K. H. and Lazier, J. R. N. (2006). Dynamics of marine ecosystems: biological-physical interactions in the oceans. Malden, MA, USA/Oxford, UK/Victoria, Australia: Wiley-Blackwell.

Mattson, M. P. and Spaziani, E. (1986). Evidence for ecdysteroid feedback on release of molt-inhibiting hormone from crab eyestalk ganglia. *The Biological Bulletin* **171**, 264-273.

Mayer, M. P., Brehmer, D., Gassler, C. S. and Bukau, B. (2001). Hsp70 chaperone machines. *Adv Protein Chem* **59**, 1-44.

Mayer, M. P. and Bukau, B. (2005). Hsp70 chaperones: cellular functions and molecular mechanism. *Cell Mol Life Sci* **62**, 670-84.

McDonald, A. A., Chang, E. S. and Mykles, D. L. (2011). Cloning of a nitric oxide synthase from green shore crab, C*arcinus maenas*: a comparative study of the effects of eyestalk ablation on expression in the molting glands (Y-organs) of *C. maenas*, and blackback land crab, *Gecarcinus lateralis*. *Comp Biochem Physiol A Mol Integr Physiol* **158**, 150-62.

McGaw, I. J., Edgell, T. C. and Kaiser, M. J. (2011). Population demographics of native and newly -invasive populations of the green crab *Carcinus maenas*. *Marine Ecology Progress Series* **430**, 235-240.

McGaw, I. J. and Whiteley, N. M. (2012). Effects of acclimation and acute temperature change on specific dynamic action and gastric processing in the green shore crab, *Carcinus maenas*. *Journal of Thermal Biology* **37**, 570-578.

McLaughlin, P. A. (1980). Comparative morphology of recent crustacea: W. H. Freeman.

McMahon, B. R. and Wilkes, P. R. H. (1983). Emergence responses and aerial ventilation in normoxic and hypoxic crayfish *Orconectes rusticus*. *Physiological Zoology* **56**, 133-141.

Medler, S., Brown, K. J., Chang, E. S. and Mykles, D. L. (2005). Eyestalk ablation has little effect on actin and myosin heavy chain gene expression in adult lobster skeletal muscles. *Biol Bull* **208**, 127-37.

Mihaylova, M. M. and Shaw, R. J. (2011). The AMPK signalling pathway coordinates cell growth, autophagy and metabolism. *Nat Cell Biol* **13**, 1016-23.

Mohamad, A., Arshad, A., Sung, Y. Y. and Jasmani, S. (2018). Effect of thermal stress on Hsp70 gene expression and female reproductive performance of giant freshwater prawn, *Macrobrachium rosenbergii*. *Aquaculture Research* **49**, 135-150.

Moniot, S., Weyand, M. and Steegborn, C. (2012). Structures, substrates, and regulators of Mammalian sirtuins - opportunities and challenges for drug development. *Front Pharmacol* **3**, 16.

Morrow, G., Hightower, L. E. and Tanguay, R. M. (2015). Small heat shock proteins: big folding machines. *Cell Stress Chaperones* **20**, 207-12.

Mykles, D. L. (1980). Mechanism of Fluid Absorption at Ecdysis in the American Lobster, Homarus-Americanus. *Journal of Experimental Biology* **84**, 89-&.

Mykles, D. L. (2001). Interactions between limb regeneration and molting in decapod crustaceans. *American Zoologist* **41**, 399-406.

Mykles, D. L. (2011). Ecdysteroid metabolism in crustaceans. *J Steroid Biochem Mol Biol* **127**, 196-203.

Nagai, C., Nagata, S. and Nagasawa, H. (2011). Effects of crustacean hyperglycemic hormone (CHH) on the transcript expression of carbohydrate metabolism-related enzyme genes in the kuruma prawn, *Marsupenaeus japonicus*. *General and Comparative Endocrinology* **172**, 293-304.

Newsom, A. J. and Williams, S. L. (2014). Predation and functional responses of *Carcinus maenas* and *Cancer magister* in the presence of the introduced cephalaspidean *Philine orientalis*. *Estuaries and Coasts* **37**, 1582-1582.

Nicholas, K. B. and Nicholas, H. B. J. (1997). GeneDoc: a tool for editing and annotating multiple sequence alignments. *Distributed by the author*.

OBIS. (2019). Distribution records of *Carcinus maenas* (Linnaeus, 1758). Ocean Biogeographic Information System. Intergovernmental Oceanographic Commission of UNESCO. Available on https://obis.org/taxon/107381. Access Date: October 18, 2019.

Oksala, N. K., Ekmekci, F. G., Ozsoy, E., Kirankaya, S., Kokkola, T., Emecen, G., Lappalainen, J., Kaarniranta, K. and Atalay, M. (2014). Natural thermal adaptation increases heat shock protein levels and decreases oxidative stress. *Redox Biol* **3**, 25-8.

Okumura, T. and Aida, K. (2000). Fluctuations in hemolymph ecdysteroid levels during the reproductive and non-reproductive molt cycles in the giant freshwater prawn *Macrobrachium rosenbergii*. *Fisheries Science* **66**, 876-883.

Okumura, t. and Aida, k. (2001). Effects of bilateral eyestalk ablation on molting and

ovarian development in the giant freshwater prawn, *Macrobrachium rosenbergii*. *Fisheries Science* **67**, 1125-1135.

Okumura, T., Nakamura, K., Aida, K. and Hanyu, I. (1989). Hemolymph ecdysteroid levels during the molt cycle in the kuruma prawn *Penaeus japonicus*. *Nippon Suisan Gakkaishi* **55**, 2091-2098.

Oligschlaeger, Y., Miglianico, M., Chanda, D., Scholz, R., Thali, R. F., Tuerk, R., Stapleton, D. I., Gooley, P. R. and Neumann, D. (2015). The recruitment of AMP-activated protein kinase to glycogen is regulated by autophosphorylation. *J Biol Chem* **290**, 11715-28.

Oliphant, A., Alexander, J. L., Swain, M. T., Webster, S. G. and Wilcockson, D. C. (2018). Transcriptomic analysis of crustacean neuropeptide signaling during the moult cycle in the green shore crab, *Carcinus maenas*. *BMC Genomics* **19**, 711.

Olivier, S., Foretz, M. and Viollet, B. (2018). Promise and challenges for direct small molecule AMPK activators. *Biochem Pharmacol* **153**, 147-158.

Paganini, A. W., Miller, N. A. and Stillman, J. H. (2014). Temperature and acidification variability reduce physiological performance in the intertidal zone porcelain crab *Petrolisthes cinctipes*. *J Exp Biol* **217**, 3974-80.

Pennoyer, K. E., Himes, A. R. and Frederich, M. (2016). Effects of sex and color phase on ion regulation in the invasive European green crab, *Carcinus maenas*. *Marine Biology* **163**, 1-15.

Perez, T. M., Stroud, J. T. and Feeley, K. J. (2016). Thermal trouble in the tropics. *Science* **351**, 1392-3.

Pinz, I., Perry, D. and Frederich, M. (2005). Activation of 5'-AMP activated protein kinase during anaerobiosis in the rock crab, *Cancer irroratus*. *The MDIBL Bulletin* **44**, 31-32.

Pitts, N. L., Schulz, H. M., Oatman, S. R. and Mykles, D. L. (2017). Elevated expression of neuropeptide signaling genes in the eyestalk ganglia and Y-organ of *Gecarcinus lateralis* individuals that are refractory to molt induction. *Comp Biochem Physiol A Mol Integr Physiol* **214**, 66-78.

Pörtner, H.-O. (2010). Oxygen- and capacity-limitation of thermal tolerance: a matrix for integrating climate-related stressor effects in marine ecosystems. *The Journal of Experimental Biology* **213**, 881-893.

Portner, H. O., Bock, C. and Mark, F. C. (2017). Oxygen- and capacity-limited thermal tolerance: bridging ecology and physiology. *J Exp Biol* **220**, 2685-2696.

Pörtner, H. O., Peck, L. S. and Hirse, T. (2006). Hyperoxia alleviates thermal stress in the Antarctic bivalve, *Laternula elliptica*: evidence for oxygen limited thermal tolerance. *Polar Biology* **29**, 688-693.

Proud, C. G. (2015). Regulation and roles of elongation factor 2 kinase. *Biochem Soc Trans* **43**, 328-32.

Quackenbush, L. S. (1986). Crustacean endocrine toxicology: a review. *Canadian Journal of Fisheries and Aquatic Sciences* **43**, 2271-2282.

Quijón, P., Jaramillo, E. and Contreras, H. (2001). Distribution and habitat structure of *Ocypode gaudichaudii* H. Milne Edwards & Lucas, 1843, in sandy beaches of Northern Chile. *Crustaceana* **74**, 91-103.

Radzikowski, J. (2013). Resistance of dormant stages of planktonic invertebrates to adverse environmental conditions. *Journal of Plankton Research* **35**, 707-723.

Ramnanan, C. J., McMullen, D. C., Groom, A. G. and Storey, K. B. (2010). The regulation of AMPK signaling in a natural state of profound metabolic rate depression. *Mol Cell*

Biochem **335**, 91-105.

Rastrick, S. P., Calosi, P., Calder-Potts, R., Foggo, A., Nightingale, G., Widdicombe, S. and Spicer, J. I. (2014). Living in warmer, more acidic oceans retards physiological recovery from tidal emersion in the velvet swimming crab, *Necora puber*. *J Exp Biol* **217**, 2499-508.

Ratheesh, R. K., Nagarajan, N., Arunraj, S. P., Sinha, D., Veedin Rajan, V. B., Esthaki, V. K. and D'Silva, P. (2012). HSPIR: a manually annotated heat shock protein information resource. *Bioinformatics* **28**, 2853-5.

Ravaux, J., Gaill, F., Le Bris, N., Sarradin, P. M., Jollivet, D. and Shillito, B. (2003). Heat-shock response and temperature resistance in the deep-sea vent shrimp *Rimicaris exoculata*. *J Exp Biol* **206**, 2345-54.

Rayner, G., Bird, T. J. and McGaw, I. J. (2019). Quantifying factors influencing American lobster (*Homarus americanus*) predation on the invasive green crab (*Carcinus maenas*) in Newfoundland, Canada. *Invertebrate Biology* **138**.

Rayner, G. and McGaw, I. J. (2019). Effects of the invasive green crab (*Carcinus maenas*) on American lobster (*Homarus americanus*): Food acquisition and trapping behaviour. *Journal of Sea Research* **144**, 95-104.

Reid, D. G. and Aldrich, J. C. (1989). Variations in response to environmental hypoxia of different colour forms of the shore crab, *Carcinus maenas*. *Comparative Biochemistry and Physiology Part A: Physiology* **92**, 535-539.

Reiling, J. H. and Sabatini, D. M. (2006). Stress and mTORture signaling. *Oncogene* **25**, 6373-83.

Robert, A., Monsinjon, T., Delbecque, J. P., Olivier, S., Poret, A., Foll, F. L., Durand, F. and Knigge, T. (2016). Neuroendocrine disruption in the shore crab *Carcinus*

maenas: Effects of serotonin and fluoxetine on chh and mih-gene expression, glycaemia and ecdysteroid levels. *Aquat Toxicol* **175**, 192-204.

Rodrigues, A. P., Oliva-Teles, T., Mesquita, S. R., Delerue-Matos, C. and Guimaraes, L. (2014). Integrated biomarker responses of an estuarine invertebrate to high abiotic stress and decreased metal contamination. *Mar Environ Res* **101**, 101-114.

Rodrigues, A. P., Santos, L. H., Ramalhosa, M. J., Delerue-Matos, C. and Guimaraes, L. (2015a). Sertraline accumulation and effects in the estuarine decapod *Carcinus maenas*: importance of the history of exposure to chemical stress. *J Hazard Mater* **283**, 350-8.

Rodrigues, E. T., Moreno, A., Mendes, T., Palmeira, C. and Pardal, M. A. (2015b). Biochemical and physiological responses of *Carcinus maenas* to temperature and the fungicide azoxystrobin. *Chemosphere* **132**, 127-34.

Rodrigues, E. T. and Pardal, M. A. (2014). The crab *Carcinus maenas* as a suitable experimental model in ecotoxicology. *Environ Int* **70**, 158-82.

Rodríguez-Fuentes, G., Murúa-Castillo, M., Díaz, F., Rosas, C., Caamal-Monsreal, C., Sánchez, A., Paschke, K. and Pascual, C. (2017). Ecophysiological biomarkers defining the thermal biology of the Caribbean lobster *Panulirus argus*. *Ecological Indicators* **78**, 192-204.

Roman, J. and Palumbi, S. R. (2004). A global invader at home: population structure of the green crab, *Carcinus maenas*, in Europe. *Mol Ecol* **13**, 2891-8.

Ruderman, N. B., Xu, X. J., Nelson, L., Cacicedo, J. M., Saha, A. K., Lan, F. and Ido, Y. (2010). AMPK and SIRT1: a long-standing partnership? *Am J Physiol Endocrinol Metab* **298**, E751-60.

Rungrassamee, W., Leelatanawit, R., Jiravanichpaisal, P., Klinbunga, S. and Karoonuthaisiri, N. (2010). Expression and distribution of three heat shock protein genes under

heat shock stress and under exposure to *Vibrio harveyi* in *Penaeus monodon*. *Dev Comp Immunol* **34**, 1082-9.

Sanli, T., Steinberg, G. R., Singh, G. and Tsakiridis, T. (2014). AMP-activated protein kinase (AMPK) beyond metabolism: a novel genomic stress sensor participating in the DNA damage response pathway. *Cancer Biol Ther* **15**, 156-69.

Santiago-Sotelo, P. and Ramirez-Prado, J. H. (2012). prfectBLAST: a platform-independent portable front end for the command terminal BLAST+ stand-alone suite. *Biotechniques* **53**, 299-300.

Santiago, J. and Sturgill, T. (2001). Identification of the S6 kinase activity stimulated in quiescent brine shrimp embryos upon entry to preemergence development as p70 ribosomal protein S6 kinase: isolation of *Artemia franciscana* p70S6k cDNA. *Biochem Cell Biol* **79**, 141 - 52.

Saxton, R. A. and Sabatini, D. M. (2017). mTOR Signaling in growth, metabolism, and disease. *Cell* **168**, 960-976.

Schlesinger, M. J. (1990). Heat shock proteins. *J Biol Chem* **265**, 12111-4.

Schumpert, C. A., Anderson, C., Dudycha, J. L. and Patel, R. C. (2016). Involvement of *Daphnia pulicaria* Sir2 in regulating stress response and lifespan. *Aging (Albany NY)* **8**, 402-17.

Semenza, G. L. (1998). Hypoxia-inducible factor 1: master regulator of O2 homeostasis. *Curr Opin Genet Dev* **8**, 588-94.

Semenza, G. L. (2001a). HIF-1 and mechanisms of hypoxia sensing. *Curr Opin Cell Biol* **13**, 167-71.

Semenza, G. L. (2001b). HIF-1, O2, and the 3 PHDs. *Cell* **107**, 1-3.

Semenza, G. L. (2001c). HIF-1, O2, and the 3 PHDs: How Animal Cells Signal Hypoxia to the Nucleus. *Cell* **107**, 1-3.

Semmouri, I., Asselman, J., Van Nieuwerburgh, F., Deforce, D., Janssen, C. R. and De Schamphelaere, K. A. C. (2019). The transcriptome of the marine calanoid copepod *Temora longicornis* under heat stress and recovery. *Mar Environ Res* **143**, 10-23.

Sharp, F. R. and Bernaudin, M. (2004). HIF1 and oxygen sensing in the brain. *Nat Rev Neurosci* **5**, 437-48.

Sheng, B., Liu, J. and Li, G. H. (2012). Metformin preconditioning protects Daphnia pulex from lethal hypoxic insult involving AMPK, HIF and mTOR signaling. *Comp Biochem Physiol B Biochem Mol Biol* **163**, 51-8.

Simonik, E. and Henry, R. P. (2014). Physiological responses to emersion in the intertidal green crab, *Carcinus maenas* (L.). *Marine and Freshwater Behaviour and Physiology* **47**, 101-115.

Skinner, D. M. (1962). The structure and metabolism of a crustacean integumentary tissue during a molt cycle. *The Biological Bulletin* **123**, 635-647.

Skinner, D. M. (1965). Amino acid incorporation into protein during the molt cycle of the land crab, *Gecarcinus lateralis*. *J Exp Zool* **160**, 225-33.

Skinner, D. M. (1985). Molting and Regeneration. In *The Biology of Crustacea*, vol. 9: *Integument, Pigments and Hormonal Processes*. (ed. D. E. Bliss and L. H. Mantel), pp. 43-146. San Diego: Academic Press.

Skinner, D. M. and Graham, D. E. (1972). Loss of limbs as a stimulus to ecdysis in brachyura (true crabs). *The Biological Bulletin* **143**, 222-233.

Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H.,

Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J. and Klenk, D. C. (1985). Measurement of protein using bicinchoninic acid. *Analytical Biochemistry* **150**, 76-85.

Snyder, M. J. and Chang, E. S. (1991). Ecdysteroids in relation to the molt cycle of the American lobster, *Homarus americanus* I. Hemolymph Titers and Metabolites. *General and Comparative Endocrinology* **81**, 133-145.

Snyder, M. J. and Rossi, S. (2007). Stress protein (HSP70 family) expression in intertidal benthic organisms: the example of *Anthopleura elegantissima* (Cnidaria: Anthozoa). *Scientia Marina* **68**, 155-162.

Somero, G. N. (2002). Thermal physiology and vertical zonation of intertidal animals: optima, limits, and costs of living. *Integr Comp Biol* **42**, 780-9.

Sonanez-Organis, J. G., Peregrino-Uriarte, A. B., Gomez-Jimenez, S., Lopez-Zavala, A., Forman, H. J. and Yepiz-Plascencia, G. (2009). Molecular characterization of hypoxia inducible factor-1 (HIF-1) from the white shrimp *Litopenaeus vannamei* and tissue-specific expression under hypoxia. *Comp Biochem Physiol C Toxicol Pharmacol* **150**, 395-405.

Soñanez-Organis, J. G., Racotta, I. S. and Yepiz-Plascencia, G. (2010). Silencing of the hypoxia inducible factor 1 -HIF-1- obliterates the effects of hypoxia on glucose and lactate concentrations in a tissue-specific manner in the shrimp *Litopenaeus vannamei*. *Journal of Experimental Marine Biology and Ecology* **393**, 51-58.

Sonna, L. A., Fujita, J., Gaffin, S. L. and Lilly, C. M. (2002). Invited review: effects of heat and cold stress on mammalian gene expression. *J Appl Physiol (1985)* **92**, 1725-42.

Sørensen, J. G., Kristensen, T. N. and Loeschcke, V. (2003). The evolutionary and ecological role of heat shock proteins. *Ecology Letters* **6**, 1025-1037.

Sorte, C. J., Ibanez, I., Blumenthal, D. M., Molinari, N. A., Miller, L. P., Grosholz,

E. D., Diez, J. M., D'Antonio, C. M., Olden, J. D., Jones, S. J. et al. (2013). Poised to prosper? A cross-system comparison of climate change effects on native and non-native species performance. *Ecol Lett* **16**, 261-70.

Soumoff, C. and O'Connor, J. D. (1982). Repression of Y-organ secretory activity by molt inhibiting hormone in the crab *Pachygrapsus crassipes*. *Gen Comp Endocrinol* **48**, 432-9.

Soumoff, C. and Skinner, D. M. (1983). Ecdysteroid titers during the molt cycle of the blue crab resemble those of other crustacea. *The Biological Bulletin* **165**, 321-329.

Spees, J. L., Chang, S. A., Snyder, M. J. and Chang, E. S. (2002). Thermal acclimation and stress in the American lobster, *Homarus americanus*: equivalent temperature shifts elicit unique gene expression patterns for molecular chaperones and polyubiquitin. *Cell Stress Chaperones* **7**, 97-106.

Spindler, K.-D. and Anger, K. (1986). Ecdysteroid levels during the larval development of the spider crab *Hyas araneus*. *General and Comparative Endocrinology* **64**, 122-128.

Stapleton, D., Woollatt, E., Mitchelhill, K. I., Nicholl, J. K., Fernandez, C. S., Michell, B. J., Witters, L. A., Power, D. A., Sutherland, G. R. and Kemp, B. E. (1997). AMP-activated protein kinase isoenzyme family: subunit structure and chromosomal location. *FEBS Lett* **409**, 452-6.

Stevens, M., Lown, A. E. and Wood, L. E. (2014). Camouflage and individual variation in shore crabs (*Carcinus maenas*) from different habitats. *PLoS One* **9**, e115586.

Stillman, J. and Somero, G. (1996). Adaptation to temperature stress and aerial exposure in congeneric species of intertidal porcelain crabs (genus *Petrolisthes*): correlation of physiology, biochemistry and morphology with vertical distribution. *J Exp Biol* **199**, 1845-55.

Sugumar, V., Vijayalakshmi, G. and Saranya, K. (2013). Molt cycle related changes

and effect of short term starvation on the biochemical constituents of the blue swimmer crab *Portunus pelagicus*. *Saudi J Biol Sci* **20**, 93-103.

Sun, S., Xuan, F., Fu, H., Ge, X., Zhu, J., Qiao, H., Jin, S. and Zhang, W. (2016). Molecular characterization and mRNA expression of hypoxia inducible factor-1 and cognate inhibiting factor in *Macrobrachium nipponense* in response to hypoxia. *Comp Biochem Physiol B Biochem Mol Biol* **196-197**, 48-56.

Sun, S., Xuan, F., Fu, H., Zhu, J., Ge, X. and Gu, Z. (2015). Transcriptomic and histological analysis of hepatopancreas, muscle and gill tissues of oriental river prawn (*Macrobrachium nipponense*) in response to chronic hypoxia. *BMC Genomics* **16**, 491.

Suwansa-Ard, S., Thongbuakaew, T., Wang, T., Zhao, M., Elizur, A., Hanna, P. J., Sretarugsa, P., Cummins, S. F. and Sobhon, P. (2015). In silico Neuropeptidome of Female Macrobrachium rosenbergii Based on Transcriptome and Peptide Mining of Eyestalk, Central Nervous System and Ovary. *PLoS One* **10**, e0123848.

Tagmount, A., Wang, M., Lindquist, E., Tanaka, Y., Teranishi, K. S., Sunagawa, S., Wong, M. and Stillman, J. H. (2010). The porcelain crab transcriptome and PCAD, the porcelain crab microarray and sequence database. *PLoS One* **5**, e9327.

Tamone, S. L., Adams, M. M. and Dutton, J. M. (2005). Effect of eyestalk-ablation on circulating ecdysteroids in hemolymph of snow crabs, *Chionoecetes opilio*: physiological evidence for a terminal molt. *Integr Comp Biol* **45**, 166-71.

Tan, C. Y. and Hagen, T. (2013). Post-translational regulation of mTOR complex 1 in hypoxia and reoxygenation. *Cell Signal* **25**, 1235-44.

Tarrant, A. M., Baumgartner, M. F., Hansen, B. H., Altin, D., Nordtug, T. and Olsen, A. J. (2014). Transcriptional profiling of reproductive development, lipid storage and

molting throughout the last juvenile stage of the marine copepod *Calanus finmarchicus*. *Front Zool* **11**, 91.

Taylor, A. C. and Davies, P. S. (1981). Respiration in the Land Crab, Gecarcinus-Lateralis. *Journal of Experimental Biology* **93**, 197-208.

Taylor, A. C. and Davies, P. S. (1982). Aquatic respiration in the land crab, Gecarcinus lateralis (Fréminville). *Comparative Biochemistry and Physiology Part A: Physiology* **72**, 683-688.

Taylor, A. C. and Spicer, J. I. (1987). Metabolic responses of the prawns *Palaemon elegans* and *P. serratus* (Crustacea: Decapoda) to acute hypoxia and anoxia. *Marine Biology* **95**, 521-530.

Taylor, B. L. and Zhulin, I. B. (1999). PAS domains: internal sensors of oxygen, redox potential, and light. *Microbiol Mol Biol Rev* **63**, 479-506.

Taylor, E. W., Butler, P. J. and Al-Wassia, A. (1977). Some responses of the shore crab, *Carcinus maenas* (L.) to progressive hypoxia at different acclimation temperatures and salinities. *J. Comp. Physiol.* **122**, 391-402.

Taylor, E. W., Butler, P. J. and Sherlock, P. J. (1973). The respiratory and cardiovascular changes associated with the emersion response of *Carcinus maenas* (L.) during environmental hypoxia, at three different temperatures. *Journal of Comparative Physiology* **86**, 95-115.

Techa, S., Alvarez, J. V. and Sook Chung, J. (2015). Changes in ecdysteroid levels and expression patterns of ecdysteroid-responsive factors and neuropeptide hormones during the embryogenesis of the blue crab, *Callinectes sapidus*. *Gen Comp Endocrinol* **214**, 157-66.

Techa, S. and Chung, J. S. (2013). Ecdysone and retinoid-X receptors of the blue crab,

Callinectes sapidus: cloning and their expression patterns in eyestalks and Y-organs during the molt cycle. *Gene* **527**, 139-53.

Techa, S. and Chung, J. S. (2015). Ecdysteroids regulate the levels of molt-inhibiting hormone (MIH) expression in the blue crab, *Callinectes sapidus*. *PLoS One* **10**, e0117278.

Tee, A. R., Blenis, J. and Proud, C. G. (2005). Analysis of mTOR signaling by the small G-proteins, Rheb and RhebL1. *FEBS Lett* **579**, 4763-8.

Teigen, L. E., Orczewska, J. I., McLaughlin, J. and O'Brien, K. M. (2015). Cold acclimation increases levels of some heat shock protein and sirtuin isoforms in threespine stickleback. *Comp Biochem Physiol A Mol Integr Physiol* **188**, 139-47.

Tepolt, C. K. and Palumbi, S. R. (2015). Transcriptome sequencing reveals both neutral and adaptive genome dynamics in a marine invader. *Mol Ecol* **24**, 4145-58.

Tepolt, C. K. and Somero, G. N. (2014). Master of all trades: thermal acclimation and adaptation of cardiac function in a broadly distributed marine invasive species, the European green crab, *Carcinus maenas*. *J Exp Biol* **217**, 1129-38.

Teranishi, K. S. and Stillman, J. H. (2007). A cDNA microarray analysis of the response to heat stress in hepatopancreas tissue of the porcelain crab *Petrolisthes cinctipes*. *Comp Biochem Physiol Part D Genomics Proteomics* **2**, 53-62.

Thomas, L. W. and Ashcroft, M. (2019). Exploring the molecular interface between hypoxia-inducible factor signalling and mitochondria. *Cell Mol Life Sci* **76**, 1759-1777.

Thomton, J. D., Tamone, S. L. and Atkinson, S. (2006). Circulating ecdysteroid concentrations in Alaskan dungeness crab (*Cancer magister*). *Journal of Crustacean Biology* **26**, 176-181.

Thorat, L. and Nath, B. B. (2015). Tolerance to desiccation stress in *Chironomus*

Ramosus through plasticity in homeostatic control. *European Journal of Environmental Sciences* **5**, 86-91.

Tian, Z., Lin, G. and Jiao, C. (2019). Identification of a S6 kinase transcript in the Chinese mitten crab *Eriocheir sinensis* and its molting-related expression in muscle tissues. *Fisheries Science* **85**, 737-746.

Tomanek, L. (2002). The heat-shock response: its variation, regulation and ecological importance in intertidal gastropods (genus *Tegula*). *Integr Comp Biol* **42**, 797-807.

Tomanek, L. (2015). Proteomic responses to environmentally induced oxidative stress. *J Exp Biol* **218**, 1867-79.

Tomanek, L. and Zuzow, M. J. (2010). The proteomic response of the mussel congeners *Mytilus galloprovincialis* and *M. trossulus* to acute heat stress: implications for thermal tolerance limits and metabolic costs of thermal stress. *J Exp Biol* **213**, 3559-74.

Torres, P. and González-Pisani, X. (2016). Primer registro del cangrejo verde, *Carcinus maenas* (Linnaeus, 1758), en Golfo Nuevo, Argentina: un nuevo límite norte de distribución en costas patagónicas. *Ecol. austral* **26**, 134-137.

Truchot, J. P. (1990). Respiratory and ionic regulation in invertebrates exposed to both water and air. *Annu Rev Physiol* **52**, 61-76.

Tsai, K.-W., Chang, S.-J., Wu, H.-J., Shih, H.-Y., Chen, C.-H. and Lee, C.-Y. (2008). Molecular cloning and differential expression pattern of two structural variants of the crustacean hyperglycemic hormone family from the mud crab *Scylla olivacea*. *Gen Comp Endocrinol* **159**, 16-25.

Tsai, Y. C., Greco, T. M. and Cristea, I. M. (2014). Sirtuin 7 plays a role in ribosome biogenesis and protein synthesis. *Mol Cell Proteomics* **13**, 73-83.

Tyagi, R., Shahani, N., Gorgen, L., Ferretti, M., Pryor, W., Chen, P. Y., Swarnkar, S., Worley, P. F., Karbstein, K., Snyder, S. H. et al. (2015). Rheb inhibits protein synthesis by activating the PERK-eIF2alpha signaling cascade. *Cell Rep* **10**, 684-693.

Vasquez, M. C., Beam, M., Blackwell, S., Zuzow, M. J. and Tomanek, L. (2017). Sirtuins regulate proteomic responses near thermal tolerance limits in the blue mussels *Mytilus galloprovincialis* and *Mytilus trossulus*. *J Exp Biol* **220**, 4515-4534.

Velazque-Amado, R. M., Escamilla-Chimal, E. G. and Fanjul-Moles, M. L. (2012). Daily light-dark cycles influence hypoxia-inducible factor 1 and heat shock protein levels in the pacemakers of crayfish. *Photochem Photobiol* **88**, 81-9.

Ventura-Lopez, C., Gomez-Anduro, G., Arcos, F. G., Llera-Herrera, R., Racotta, I. S. and Ibarra, A. M. (2016). A novel CHH gene from the Pacific white shrimp *Litopenaeus vannamei* was characterized and found highly expressed in gut and less in eyestalk and other extra-eyestalk tissues. *Gene* **582**, 148-60.

Verberk, W., Leuven, R., van der Velde, G. and Gabel, F. (2018). Thermal limits in native and alien freshwater peracarid Crustacea: The role of habitat use and oxygen limitation. *Funct Ecol* **32**, 926-936.

Verbruggen, B., Bickley, L. K., Santos, E. M., Tyler, C. R., Stentiford, G. D., Bateman, K. S. and van Aerle, R. (2015). De novo assembly of the *Carcinus maenas* transcriptome and characterization of innate immune system pathways. *BMC Genomics* **16**, 458.

Vermeer, G. K. (1987). Effects of air exposure on desiccation rate, hemolymph chemistry, and escape behavior of the spiny lobster, *Panulirus argus*. *Fishery Bulletin* **85**, 45-51.

Vernberg, F. J. and Tashian, R. E. (1959). Studies on the physiological variation between tropical and temperate zone fiddler crabs of the genus *Uca* I. Thermal death limits.

Ecology **40**, 589-593.

Vinagre, A. S. and Chung, J. S. (2016). Effects of starvation on energy metabolism and crustacean hyperglycemic hormone (CHH) of the Atlantic ghost crab *Ocypode quadrata* (Fabricius, 1787). *Marine Biology* **163**.

Vinuesa, J. (2007). Molt and reproduction of the European green crab *Carcinus maenas* (Decapoda: Portunidae) in Patagonia, Argentina. *Revista de Biología Tropical* **55**.

von Prahl, H. and Manjarrés, G. (1984). Cangrejos Gecarcinidos (Crustacea; Gecarcinidae) de Colombia. *Caldasia* **XIV**, 149-168.

Wang, T., Yu, Q., Chen, J., Deng, B., Qian, L. and Le, Y. (2010). PP2A mediated AMPK inhibition promotes HSP70 expression in heat shock response. *PLoS One* **5**.

Watts, A. J., Lewis, C., Goodhead, R. M., Beckett, S. J., Moger, J., Tyler, C. R. and Galloway, T. S. (2014). Uptake and retention of microplastics by the shore crab *Carcinus maenas*. *Environ Sci Technol* **48**, 8823-30.

Webster, S. (1996). Measurement of crustacean hyperglycaemic hormone levels in the edible crab *Cancer pagurus* during emersion stress. *J Exp Biol* **199**, 1579-85.

Webster, S. G. (1997). High-affinity binding of putative moult-inhibiting hormone (MIH) and crustacean hyperglycaemic hormone (CHH) to membrane-bound receptors on the Y-organ of the shore crab *Carcinus maenas*. *Proceedings of the Royal Society of London. Series B: Biological Sciences* **251**, 53-59.

Webster, S. G., Keller, R. and Dircksen, H. (2012). The CHH-superfamily of multifunctional peptide hormones controlling crustacean metabolism, osmoregulation, moulting, and reproduction. *Gen Comp Endocrinol* **175**, 217-33.

Westerheide, S. D., Anckar, J., Stevens, S. M., Jr., Sistonen, L. and Morimoto, R. I.

(2009). Stress-inducible regulation of heat shock factor 1 by the deacetylase SIRT1. *Science* **323**, 1063-6.

Whiteley, N. M., Robertson, R. F., Meagor, J., El Haj, A. J. and Taylor, E. W. (2001). Protein synthesis and specific dynamic action in crustaceans: effects of temperature. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology* **128**, 593-604.

Whiteley, N. M., Taylor, E. W. and El Haj, A. J. (1997). Seasonal and latitudinal adaptation to temperature in crustaceans. *Journal of Thermal Biology* **22**, 419-427.

Wilkens, J. L. and Milton, F. (1965). Heat tolerance and temperature relationships of the fiddler crab, *Uca pugilator*, with reference to body coloration. *Biological Bulletin* **128**, 133-141.

Willows, R., Navaratnam, N., Lima, A., Read, J. and Carling, D. (2017). Effect of different gamma-subunit isoforms on the regulation of AMPK. *Biochem J* **474**, 1741-1754.

Wittmann, A. C., Benrabaa, S. A. M., Lopez-Ceron, D. A., Chang, E. S. and Mykles, D. L. (2018). Effects of temperature on survival, moulting, and expression of neuropeptide and mTOR signalling genes in juvenile Dungeness crab (*Metacarcinus magister*). *J Exp Biol* **221**.

Wong, K. A. and Lodish, H. F. (2006). A revised model for AMP-activated protein kinase structure: The alpha-subunit binds to both the beta- and gamma-subunits although there is no direct binding between the beta- and gamma-subunits. *J Biol Chem* **281**, 36434-42.

Worm, B., Barbier, E. B., Beaumont, N., Duffy, J. E., Folke, C., Halpern, B. S., Jackson, J. B., Lotze, H. K., Micheli, F., Palumbi, S. R. et al. (2006). Impacts of biodiversity loss on ocean ecosystem services. *Science* **314**, 787-90.

Xiao, B., Sanders, M. J., Carmena, D., Bright, N. J., Haire, L. F., Underwood, E.,

Patel, B. R., Heath, R. B., Walker, P. A., Hallen, S. et al. (2013). Structural basis of AMPK regulation by small molecule activators. *Nat Commun* **4**, 3017.

Xie, X., Zhu, D., Yang, J., Qiu, X., Cui, X. and Tang, J. (2014). Molecular cloning of two structure variants of crustacean hyperglycemic hormone (CHH) from the swimming crab (*Portunus trituberculatus*), and their gene expression during molting and ovarian development. *Zoolog Sci* **31**, 802-9.

Xu, D., Sun, L., Liu, S., Zhang, L., Ru, X., Zhao, Y. and Yang, H. (2014). Molecular cloning of heat shock protein 10 (Hsp10) and 60 (Hsp60) cDNAs and their expression analysis under thermal stress in the sea cucumber *Apostichopus japonicus*. *Comp Biochem Physiol B Biochem Mol Biol* **171**, 49-57.

Xu, J., Ji, J. and Yan, X. H. (2012). Cross-talk between AMPK and mTOR in regulating energy balance. *Crit Rev Food Sci Nutr* **52**, 373-81.

Yang, X. Q., Zhang, Y. L., Wang, X. Q., Dong, H., Gao, P. and Jia, L. Y. (2016). Characterization of multiple Heat-Shock Protein transcripts from *Cydia pomonella*: their response to extreme temperature and insecticide exposure. *J Agric Food Chem* **64**, 4288-98.

Yang, Y., Ye, H., Huang, H., Li, S., Zeng, X., Gong, J. and Huang, X. (2013). Characterization and expression of SpHsp60 in hemocytes after challenge to bacterial, osmotic and thermal stress from the mud crab *Scylla paramamosain*. *Fish Shellfish Immunol* **35**, 1185-91.

Yi, J., Wu, H., Liu, J., Lai, X., Guo, J., Li, D. and Zhang, G. (2018). Molecular characterization and expression of six heat shock protein genes in relation to development and temperature in *Trichogramma chilonis*. *PLoS One* **13**, e0203904.

Yoon, H., Shin, S. H., Shin, D. H., Chun, Y. S. and Park, J. W. (2014). Differential roles of Sirt1 in HIF-1a and HIF-2a mediated hypoxic responses. *Biochem Biophys Res Commun*

444, 36-43.

Yorde, D. E., Sasse, E. A., Wang, T. Y., Hussa, R. O. and Garancis, J. C. (1976). Competitive enzyme-linked immunoassay with use of soluble enzyme/antibody immune complexes for labeling. I. Measurement of human choriogonadotropin. *Clin Chem* **22**, 1372-7.

Yoshihara, T., Naito, H., Kakigi, R., Ichinoseki-Sekine, N., Ogura, Y., Sugiura, T. and Katamoto, S. (2013). Heat stress activates the Akt/mTOR signalling pathway in rat skeletal muscle. *Acta Physiol (Oxf)* **207**, 416-26.

Yu, X., Chang, E. S. and Mykles, D. L. (2002). Characterization of limb autotomy factor-proecdysis (LAF(pro)), isolated from limb regenerates, that suspends molting in the land crab *Gecarcinus lateralis*. *Biol Bull* **202**, 204-12.

Yuan, Y., Sun, P., Jin, M., Wang, X. and Zhou, Q. (2019). Regulation of Dietary Lipid Sources on Tissue Lipid Classes and Mitochondrial Energy Metabolism of Juvenile Swimming Crab, Portunus trituberculatus. *Front Physiol* **10**, 454.

Zagorska, A. and Dulak, J. (2004). HIF-1: the knowns and unknowns of hypoxia sensing. *Acta Biochim Pol* **51**, 563-85.

Zhang, D., Guo, X., Wang, F. and Dong, S. (2016). Effects of periodical salinity fluctuation on the growth, molting, energy homeostasis and molting-related gene expression of *Litopenaeus vannamei*. *Journal of Ocean University of China* **15**, 911-917.

Zhang, K., Ezemaduka, A. N., Wang, Z., Hu, H., Shi, X., Liu, C., Lu, X., Fu, X., Chang, Z. and Yin, C. C. (2015). A novel mechanism for small heat shock proteins to function as molecular chaperones. *Sci Rep* **5**, 8811.

Zheng, J., Cao, J., Mao, Y., Su, Y. and Wang, J. (2019). Comparative transcriptome analysis provides comprehensive insights into the heat stress response of Marsupenaeus

japonicus. *Aquaculture* **502**, 338-346.

Zheng, J., Chen, H. Y., Choi, C. Y., Roer, R. D. and Watson, R. D. (2010). Molecular cloning of a putative crustacean hyperglycemic hormone (CHH) isoform from extra-eyestalk tissue of the blue crab (*Callinectes sapidus*), and determination of temporal and spatial patterns of CHH gene expression. *Gen Comp Endocrinol* **169**, 174-81.

Zheng, J., Li, L., Dong, H., Mao, Y., Su, Y. and Wang, J. (2018). Molecular cloning of heat shock protein 60 from *Marsupenaeus japonicus* and its expression profiles at early developmental stages and response to heat stress. *Aquaculture Research* **49**, 301-312.

CHAPTER FIVE: SUMMARY AND FUTURE RESEARCH

SUMMARY

Molting or ecdysis is the act of molting the integument, a behavior all crustaceans exhibit and involves biochemical, morphological, physiological and behavioral changes. Neuropeptides formed by neurosecretory cells in the X-organ are accumulated and released from the sinus gland in the eyestalk (XO/SG). This complex secretes the molt-inhibiting hormone (MIH) and suppresses production of ecdysteroids by a pair of molting glands or Y-organs (YO) located in the anterior region of the body. The mechanistic target of rapamycin (mTOR) is a highly conserved protein kinase controlling cell growth and regulating ecdysteroidogenesis in YO. In the blackback land crab, *Gecarcinus lateralis*, molting can be induced by multiple leg autotomy of 5 or more walking legs (MLA) and eyestalk ablation (ESA). During the molt cycle, the YO transitions through four molt states: under control of MIH an intermolt stage (C4), following decreased MIH expression and early (D0), mid (D1,2), and late premolt (D3,4) stages.

I isolated, identified and characterized orthologs of genes of stress response *AMPkα*-, *β*-, and *γ*-subunits; *SIRT1*; *SIRT7*; *HSP60*; *HSP70*; and *HIFα*- and *β*-subunits, in intermolt, early premolt, mid premolt, late premolt, and postmolt stages of the molt cycle of *G. lateralis* and green crab *Carcinus maenas*. These genes shared structural properties among arthropod species and exhibited unique profiles of expression in YOs, claw muscle, gill, heart, and thoracic ganglion. Gene expression was generally higher in YO and heart and lower in claw muscles of both species, but with no specific tissue distribution.

I used Quantitative PCR and Western Blot to determine the effect of acute thermal stress on the blackback land crab *G. lateralis*, and its impact on YO ecdysteroidogenesis and mRNA

levels of the stress response genes *AMPk*, *SIRT1*, *SIRT7*, *HSP60*, *HSP70*, and *HIF*. Ecdysteroid levels in MLA and ESA crabs were affected by molt stage and temperature. *AMPk*, *SIRT1*, *SIRT7*, *HSP60* and *HIF* mRNA levels of MLA animals were mostly affected by temperature. Gene expression mostly varied at late premolt and postmolt stages with lower mRNA levels at 27°C, and lower concentration at 35°C at the same molt stage at 27 and 32°C. *SIRT1*, *HSP60*, *HSP70* and *HIF* were moderately affected by ESA but not by temperature in ESA animals. Higher activated protein at 35 °C may result of stored protein being activated as immediate response, while crabs increase transcription and translation of genes of stress response after heat shock stress. Lower and increasing mRNA levels in premolt may reduce the metabolic cost at higher temperatures. mRNA levels were invariable in ESA and unrelated to higher ecdysteroid titers at 35°C. Although protein expression was not determined in ESA animals, protein synthesis may have a fixed rate at optimal temperatures of 27°C more ATP consumption and lower protein synthesis at 35°C. Acute temperature exposure for 1 hour might not represent sufficient time to observe a robust response and more pronounced evidence of alterations in mRNA levels and protein expression in *G. lateralis*.

In our previous study, HSP70, AMPKγ, mTOR, and Rheb genes were used as biomarkers to determine the effects of acute temperature change and emersion in non-desiccated-desiccated conditions in Y-organ, heart, gill, claw muscle, thoracic ganglion, eyestalk ganglion and brain of MLA and ESA *C. maenas*. Different to *G. lateralis*, *C. maenas* tolerated a wide temperature range and showed a rapid response with tissue-specific changes in gene expression after 1 hour at high temperatures. *C. maenas* tolerates emersion stress under non-desiccated conditions, but desiccation affected heart and nervous tissues, showing reduced expression of genes after 4 and 6 hours of emersion. Complementary information to mRNA data and protein expression of some

of these biomarkers will explain the response the thermal stress and the physiology of emersion in green crabs.

The study of mRNA expression, total and activated forms of AMPK, SIRT, HSP and mTOR should be addressed using similar parameters of temperature range and humidity levels. With about 85 land crab species existing in the tropical and temperate regions, future research should be addressed to determine possible patterns of gene expression and ecdysteroids in the molt cycle of crabs under environmental and internal stress in a comparative approach. In the same way, to study the positive and negative impact of environmental stress on invasive species, other invasive species are appropriate model organisms that explain the level of accommodation and acclimatization these foreign organisms undergo. Additional variables other than gene and protein expression such as physiological hypoxia, hypercapnia, lactate concentration in hemolymph, ATP or NAD^+ consumption, AMP and ADP production, can help to describe environmental effect on molting and physiology of crustacean species.