THESIS

MOLECULAR REGULATION OF GROWTH AND MOLTING IN DECAPOD CRUSTACEANS

Submitted by

Megan Reese Mudron

Department of Biology

In partial fulfillment of the requirements

For the Degree of Master of Science

Colorado State University

Fort Collins, Colorado

Summer 2014

Master's Committee:

Advisor: Donald L. Mykles

Deborah M. Garrity
Norman P. Curthoys

ABSTRACT

MOLECULAR REGULATION OF GROWTH AND MOLTING

IN DECAPOD CRUSTACEANS

The green shore crab, *Carcinus maenas,* is a highly invasive species that inhabits coastal temperate zones worldwide. The reaction of *C. maenas* to acute temperature change was determined in six tissues (heart, gill, thoracic ganglion, eyestalk ganglion, Y-organ, and claw muscle) using genetic markers for temperature-induced metabolic stress, including HSP70, AMPKγ, mTOR, and Rheb. Animals were exposed to temperatures between 5° and 30°C for 1 or 2 h. mRNA levels in six tissues were quantified by quantitative RT-PCR (qPCR). The results indicate that *C. maenas* tolerated a wide temperature range, requiring 2-h exposures at 5 ˚C and 30 ˚C to affect tissue-specific changes in gene expression. *Cm-HSP70* expression was robustly increased at 30 °C in all tissues.

Ecdysteroids produced from the molting gland (Y-organ or YO) induce molting in decapod crustaceans. Reduction in molt-inhibiting hormone (MIH) activates the YO and animals enter premolt. At mid-premolt, YOs transition to the committed state, during which ecdysteroid production increases further. In blackback land crab (*Gecarcinus lateralis*), a tropical decapod species, SB1431542, an inhibitor of Activin receptors, decreases hemolymph ecdysteroid titers in premolt animals, suggesting that an Activin-like transforming-growth factor (TGF-β) is produced by the activated YO and drives the transition of the YO to the committed state. Myostatin (Gl-Mstn) is an Activin-like factor that is highly expressed in skeletal muscle. Rapamycin lowers hemolymph ecdysteroid titers by inhibiting mTOR, which controls global

translation of mRNA into protein. Endpoint RT-PCR established that *Gl-Mstn* was expressed in the YO, not just muscle tissue. YOs were harvested from intact (intermolt) animals and from animals at 1, 3, 5, 7, and 14 days post-ESA. Quantitative PCR was used to quantify the effects of molt induction by eyestalk ablation (ESA) on gene expression. Expression of mTOR components peaked at 3 days post-ESA, which is consistent with the increased activity required for activation of the YO. *Gl-Mstn* expression also peaked at 3 days post-ESA, which is before the transition to the committed state at 7 days post-ESA. These results indicate that mTOR components are involved in activation of the YO, and Mstn is involved in transitioning the YO to the committed state.

ACKNOWLEDGEMENTS

I would like to thank Dr. Donald Mykles, my thesis advisor and mentor, for all of his guidance and support during my time at Colorado State University. Dr. Mykles challenged me to think as a scientist, not just a student, and encouraged my career interests. I would also like to thank Drs. Debbie Garrity and Norm Curthoys for their time and assistance in preparing and editing my research and thesis.

My time in the lab would not have been the same without the people working alongside of me. I am deeply grateful for Natalie Pitts, my dissecting companion and confidante, as well as Kathy Cosenza, Dr. Sukkrit Nimitkul, Dr. Astrid Wittman, and Dr. Sunetra Das for all of their support and assistance with my research. I also would like to acknowledge numerous undergraduates for their help with homogenizing tissues, preparing RNA samples, and crab care: Kent Schnacke, for helping me design and complete the green crab experiments; Tara Pianko and Rachel McGee for completing RNA extractions for several tissues; Talia Head and Hannah Jesberger for assisting with sample preparation, and countless others that helped as well for crab care. I thank Hector Horta and Dr. Sukkrit Nimitkul for collecting *G. lateralis* and *C. maenas,* respectively. The research was supported by a grant to DLM from the National Science Foundation (IOS-1257732), and the *C. maenas* experiments were supported by The Journal of Experimental Biology Travel Fellowship. Of course, I must thank Molly Zellar for her positive attitude and moral support and love of coffee.

Lastly, but perhaps most importantly, I would like to thank my boyfriend, Brett Hines, and my parents, Margaret and David Mudron, for their perpetual belief in me.

TABLE OF CONTENTS

CHAPTER ONE: INTRODUCTION

Endocrine control of growth and reproduction in arthropods

Arthropods possess an exoskeleton, which must be molted periodically in order to grow. Beginning from a larval stage and progressing through a series of juvenile and adult stages, metamorphosis events occur that significantly change the structure of the organism. Many insects have at least one larval, pupa, and adult stage (Truman and Riddiford, 1999), whereas decapod crustaceans have at least one larval stage (nauplius, zoea, and megalopa), and an adult stage (Chang et al. 2001).

In insects, prothoracicotropic hormone (PTTH), a neuropeptide released from the brain, stimulates production of ecdysteroids from the prothoracic gland (molting gland)(Hopkins, 2012). Ecdysteroids, including 20-hydroxyecdysone (20E), are major hormonal regulators that are synthesized from cholesterol. 20E binds to and stabilizes the dimerized ecdysteroid receptor, comprised of the ecdysone receptor (EcR) and retinoid X receptor (RXR)(Mykles, 2011). PTTH induces phosphorylation of p70 S6 kinase via the mTOR pathway, which can be experimentally blocked by rapamycin (Song and Gilbert, 1994, 1997). Molting frequency is determined by a critical weight, which varies depending on species (Callier and Nijhout, 2013), and PTTH level, which varies depending on the age of the organism (Covi et al. 2012). Juvenile hormone regulates 20E, and helps specify if the insect will molt into a subsequent larval stage or into a pupal stage (Marchal et al. 2010).

In crustaceans, ecdysteroids are produced from the Y-organ (YO) in response to decreased molt-inhibiting hormone (MIH) release. Active ecdysteroids, which include 20E and ponasterone A, are synthesized by the Halloween genes (Mykles, 2011). As above, these ecdysteroids bind to EcR and RXR to induce molting processes. MIH is a neurosecretory

hormone produced in the eyestalk in the X-organ/sinus gland complex and is released in a pulsatile manner during intermolt. When MIH levels decrease in response to environmental cues, the YO becomes activated and the animal enters premolt (Chang and Mykles, 2011). The activated stage is concurrent with the up-regulation of mTOR signaling in the YO, which leads to increased protein and ecdysteroid synthesis (Fig. 1). It is hypothesized that a TGF-β factor is involved in transitioning the animal into the committed stage through Activin/Smad signaling, which further activates the mTOR pathway (Fig. 2). The molt cycle can be manipulated experimentally by injection of rapamycin or SB431542. Rapamycin blocks the activation of premolt, whereas SB431542 blocks the Activin receptor and thus delays the commitment stage (Fig. 3).

A primary player in protein synthesis during premolt, mechanistic Target of Rapamycin (mTOR) is a serine/threonine protein kinase that is highly conserved. mTOR combines with Rheb (Ras homolog enriched in brain), Raptor, and GβL (also known as LST8) to form mTOR Complex-1 (mTORC1)(Ma and Blenis, 2009). mTORC1 is a downstream target of the Akt/PI_3 kinase insulin-signaling pathway, which is crucial for cell growth, development, molting, and reproduction (Rommel et al. 2001). mTORC1 is activated when Rheb-GDP is converted to Rheb-GTP, which phosphorylates ribosomal S6 kinase (S6K) and 4E-binding protein 1 (4EBP1) to increase mRNA translation (Holz et al. 2005; Inoki et al. 2012). mTORC1 activity can be inhibited by Rheb-GAP, also known as TSC1/TSC2 (tuberous sclerosis complex 1 and 2), or FK506 binding protein (FKBP12) (Figs. 1 and 2).

The molt cycle can be manipulated in crustaceans, particularly *G. lateralis*. Eyestalk ablation (ESA) removes the source of MIH, thus effectively allowing the YO to become activated and the animal can enter premolt; this method allows molting to be induced and

proceed more rapidly (e.g. Costlow, 1963; McCarthy and Skinner, 1977). ESA provides a predictable time frame in which the animal will molt, allowing researchers to be able to harvest tissues at 3, 7, and 14 days, which are approximate to the animal's activation, commitment, and repressed states. Alternatively, multiple leg autonomy (MLA) can be performed, in which five or more walking legs are autotomized (voluntarily let go). This is triggered by a putative Limb autotomy factor – anecdysis (LAF_{an}) (Skinner, 1985). This method represents a more natural mode of molt induction by mimicking the effects of a predator (Skinner and Graham, 1972). *C. maenas* is refractory to both ESA and MLA, which appears to be due to its secondary sources of MIH that are present in thoracic ganglia and brain tissues (Abuhagr et al. 2014a). This MIH production appears to be sufficient to keep the YO in a suppressed state, thereby preventing the animal from inducing molting.

A brief overview of the crustacean molt cycle

When MIH levels decrease in response to environmental cues, the molt cycle is initiated and the animal enters early premolt (D_0). At this point, the YO has become activated. Prior to mid-premolt (D_{1-2}), a putative transforming growth factor β (TGF-β) is upregulated, which makes the animal committed to molting. Before the commitment stage, the animal can pause the molt cycle and decrease ecdysteroid synthesis. This can be performed experimentally by removal of a primary limb bud (LB) regenerate. Limb buds are grown to replace lost walking legs or claws during premolt, which allows the animals to molt with a full set of legs. Once the animal has reached the committed stage, it must proceed through the molt cycle, even if LBs are lost after this point. At the committed stage, the animal also has a low sensitivity to MIH. Hemolymph ecdysteroid levels continue to rise until late premolt (D_{3-4}), and drop precipitously

immediately prior to ecdysis (E) (Skinner, 1985; Mykles, 2011). Basal ecdysteroid levels return during the postmolt stage. Ecdysteroid levels can be measured via a competitive ELISA, as described previously (Abuhagr et al. 2014b). Hemolymph ecdysteroid titers allow for an approximate measure of molt stage. An overview of ecdysteroid level changes during the molt cycle is presented (Fig. 4).

Environmental regulation of molting and growth

A large body of literature exists on the environmental effects on molting and growth in comparison to the relatively small amount that is understood on the endocrinology of molting. Photoperiod (darkness), crowding, and temperature are factors that regulate the inhibition of MIH that precedes premolt in *Gecarcinus lateralis*. These are evolutionarily suitable habitats for the animal because they reduce the animal's likelihood of survival when they are especially vulnerable with their soft shell (Bliss, 1964; Hoang et al. 2003). Crustacean larvae have even been recommended as an indicator of water quality, as their existence indicates a relatively healthy environment (Gannon and Stemberger, 1978).

In contrast, negative factors can be stressful for an animal and can prevent the onset of molting and therefore growth. Temperature is one of the most well studied factors that influence growth, and is also one of the main considerations when projecting future population expansions (Compton, 2010). Other stressors include hypoxia, salinity, and pollution. Pollution can have a wide range of effects, but can alter neurotransmitter function and therefore behavior or release of particular hormones, as well as reduce the rate of limb regeneration (Rodriguez et al. 1998; Weis et al. 1992). In addition, the health and immune response of crustaceans becomes limited as stress increases (Moullac and Haffner, 2000). Calcification of chitinous exoskeletons, an

essential component of the crustacean body structure (Vigh and Dendinger, 1982), is reduced in water with low pH, which occurs at an increasing rate as a result of global climate change (Whiteley, 2011). One of the goals of this thesis is to investigate the tolerance of crustaceans in response to stress.

Choice of study organisms

Carcinus maenas, the green shore crab, and *Gecarcinus lateralis,* the blackback land crab, were the species chosen for these studies. Both species have been widely studied in the field of crustacean endocrinology, and ample information is also available regarding their environment (see above).

C. maenas is a highly invasive species, originating from Western Europe and Northern Africa but now inhabiting environments in temperate zones worldwide. Several well-studied populations exist- the native population in Europe, the invasive population on the East Coast of North America, and the invasive population on the West Coast 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). The West Coast population has the least genetic diversity, since it was only established around 1990 from a single introduction event; however, it has also been the most successful in populating new habitat (Darling et al. 2008). A model of success, this crab can withstand extreme temperatures, salinity, hypoxia, and pH in comparison to closely related species, which has led researchers to conclude that this species will be easily able to expand its current habitat (Jost et al. 2012; Klassen and Locke, 2007). It is suggested that temperature may be the most important indicator of where the species will be able to expand (Compton et al. 2010). *C. maenas* is considered an "ecosystem engineer" in that it modifies any

new environment it inhabits through consumption of and competition with native fauna (Klassen and Locke, 2007). Due to the invasiveness of this species, it is readily available for use in our studies. Six tissues were collected during the study, and the external and internal structures of the animal are provided (Fig. 5).

G. lateralis is a tropical species that is native to the Caribbean region, including Florida and Texas in the United States, the Bahamas, Bermuda, and the Atlantic side of Central America (Bliss et al. 1978). This species is mostly nocturnal, and usually spends the daytime in their underground sand burrows, although there have been exceptions noted in certain areas. This allows them to remain at a consistent temperature, and prevents dessication (Bliss, 1964). This animal will remain hydrated by collecting moisture from damp sand or dew (Bliss, 1979). During twilight and nighttime, the land crab will forage omnivorously. In their habitat, there is a rainy summer season during which this species molts, and a dry winter season, although the temperature does not vary much (Bliss, 1964). The molt cycle is easily manipulated by eyestalk ablation (ESA) or multiple leg autonomy (MLA). Using these techniques, we have learned quite a bit about the molt cycle of this species (Chang and Mykles, 2011), especially the molecular changes that occur in the YO (Fig. 6). We are able to provide care for these animals at Colorado State University without a flow-through seawater system as they live on land. In the near future, we hope to complete the genome and Y-organ transcriptome sequencing of this species, which will provide further insight into changes that occur during molting.

In this thesis, an increased understanding of the gene expression that occurs during stressful episodes in *C. maenas* and during the molt cycle in *G. lateralis* is presented. In Chapter 2, we determine that *C. maenas* does experience heat stress after exposure to acute temperature changes, but our data demonstrate that this heat stress alters only HSP70 expression, not

expression of AMPKγ or mTOR components. In Chapter 3, we replicate a previous experiment that shows the changes in mTOR expression over the molt cycle, and further demonstrate that Mstn, a TGF-β factor in the Activin family, is at least partially responsible for transitioning the animal into the committed stage of molting. These results provide further insight into the mechanisms of molting and growth in decapod crustaceans.

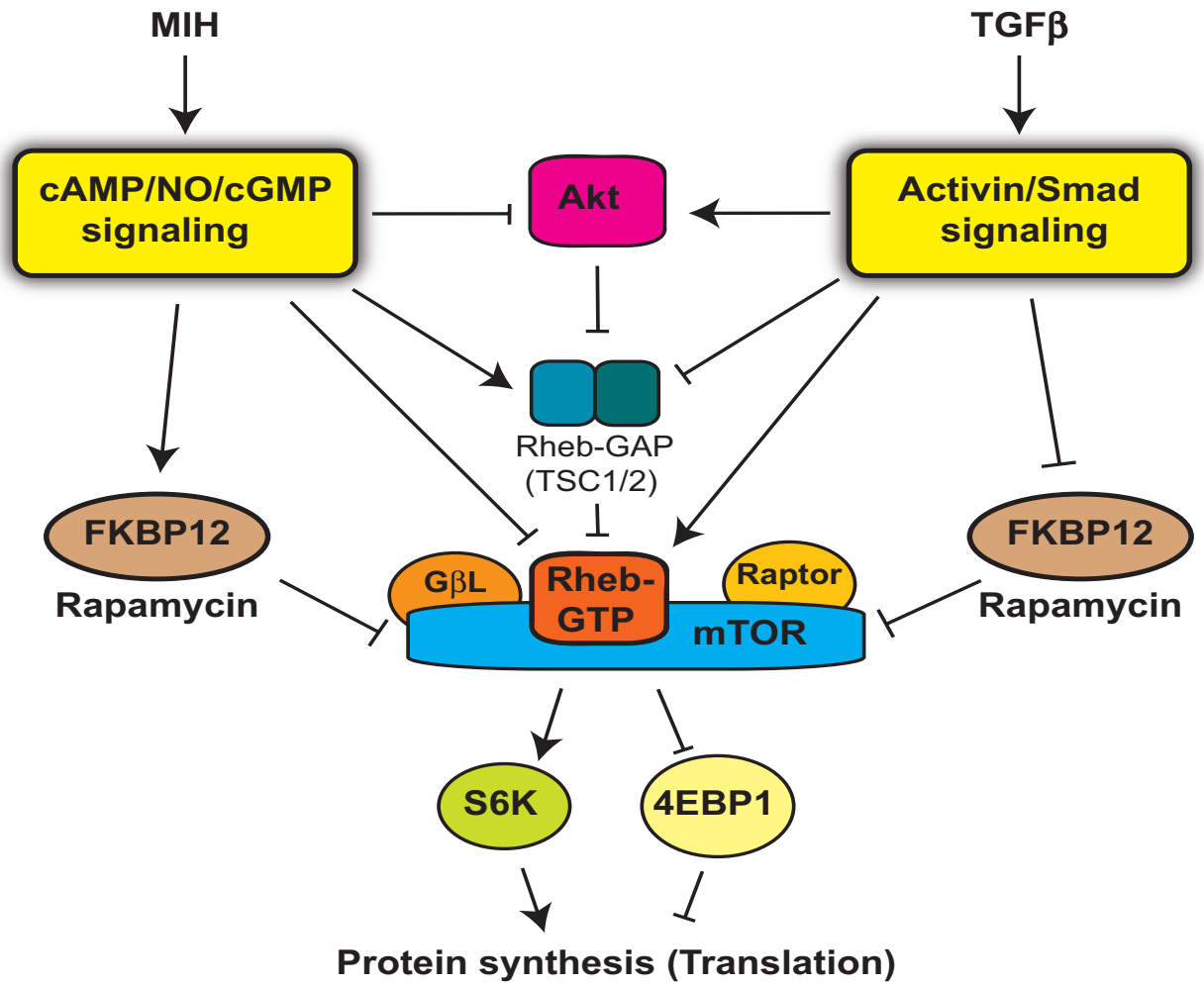

Fig 1.1. Proposed regulation of the mTOR pathway. MIH inhibits molting through repression of mTOR, which is responsible for protein synthesis and sustained ecdysteroid synthesis. Mstn, a TGFβ-like protein, stimulates mTOR. The mTORC1 complex, consisting of mTOR, Rheb-GTP, and Raptor, are primary regulators of protein synthesis and mediate mRNA translation into protein.

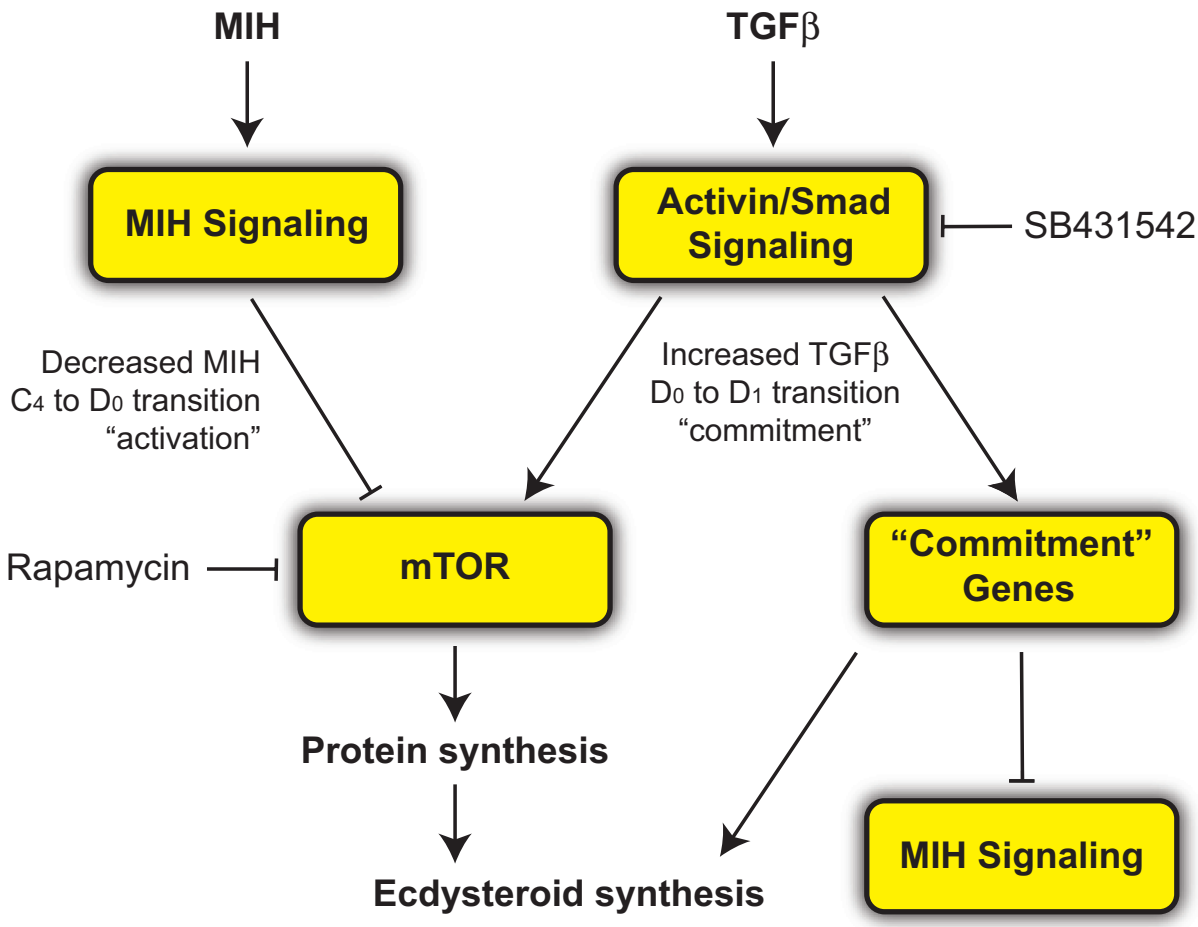

Fig 1.2. Proposed Signaling Pathway of Gl-Mstn, a TGF-β factor, in the YO. MIH inhibits molting through repression of the mTOR pathway, which is responsible for protein synthesis and growth. Activin and Smad signaling activate the mTOR pathway to stimulate molting, perhaps by triggering the animal to commit to molting and enter late premolt.

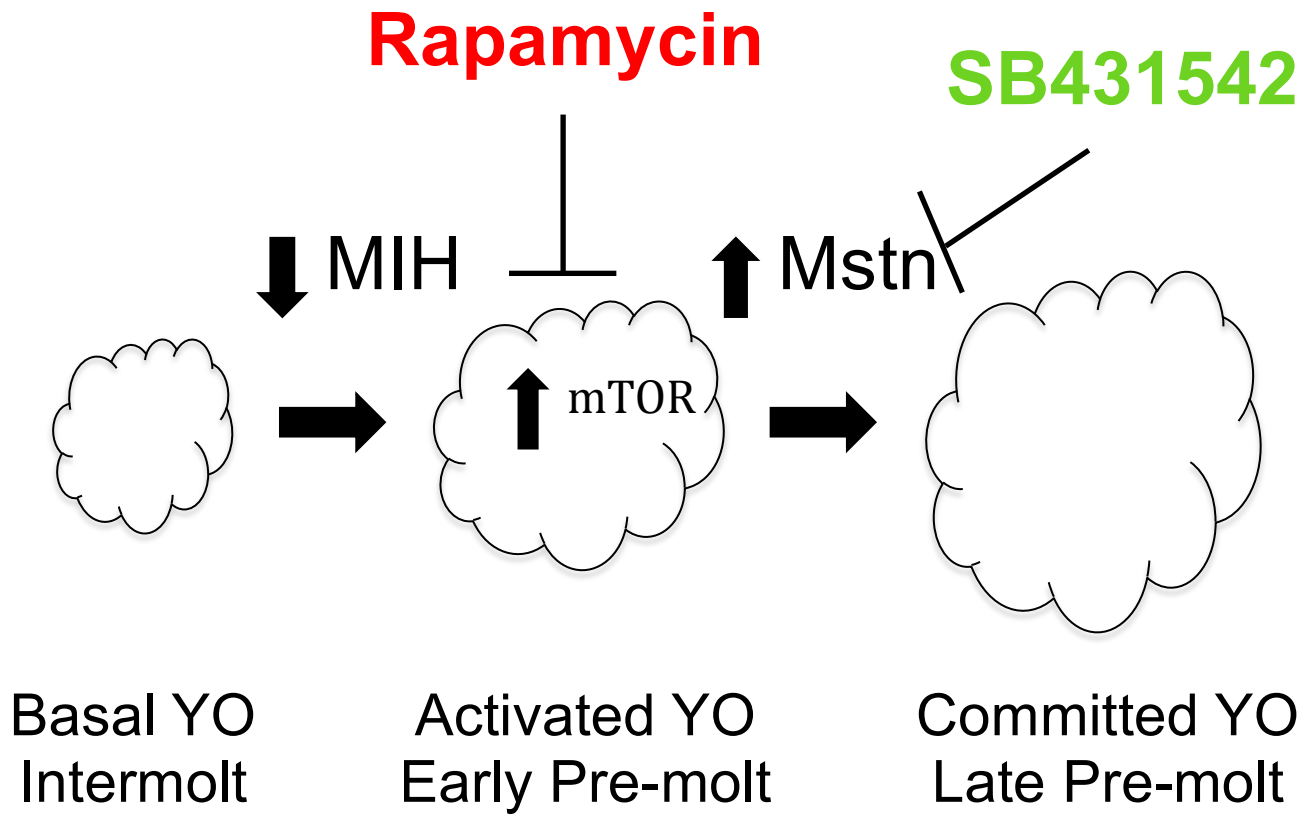

Fig 1.3. Proposed effects of molt cycle manipulation on crustacean molting. A decrease in MIH activates the YO and the animal enters premolt. The activated YO expresses Mstn, which induces the transition of the YO to the committed state in mid premolt. Rapamycin inhibits YO activation, while SB431542 prevents commitment by inhibiting Activin/Smad signaling.

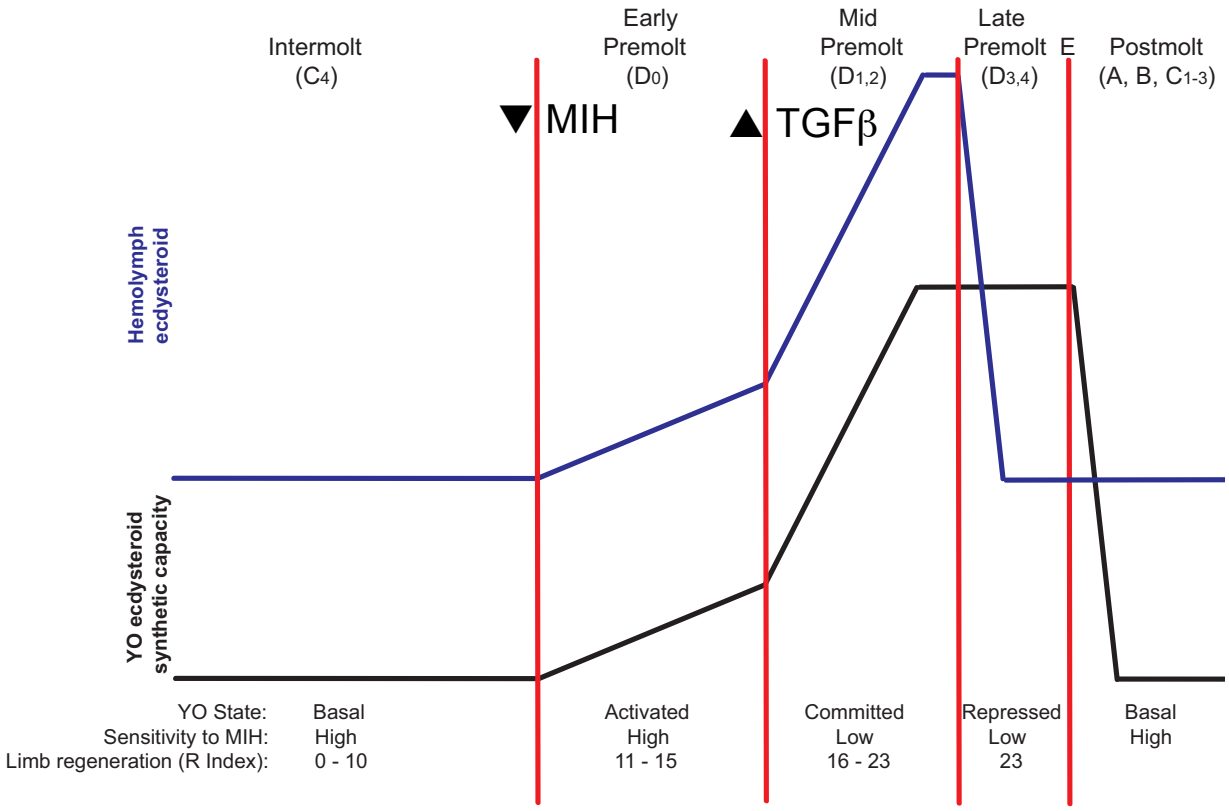

Fig 1.4. Ecdysteroid level changes during the molt cycle. Ecdysteroid levels are low during the basal state (intermolt), but begin to rise once the MIH signal is turned off. This activates the pathway and cues the animal to begin molting. The animal becomes committed to molt once a TGF-β signal is received. Ecdysteroid levels continue to rise, reaching a peak during late premolt. Shortly after, the crab will molt and return to intermolt.

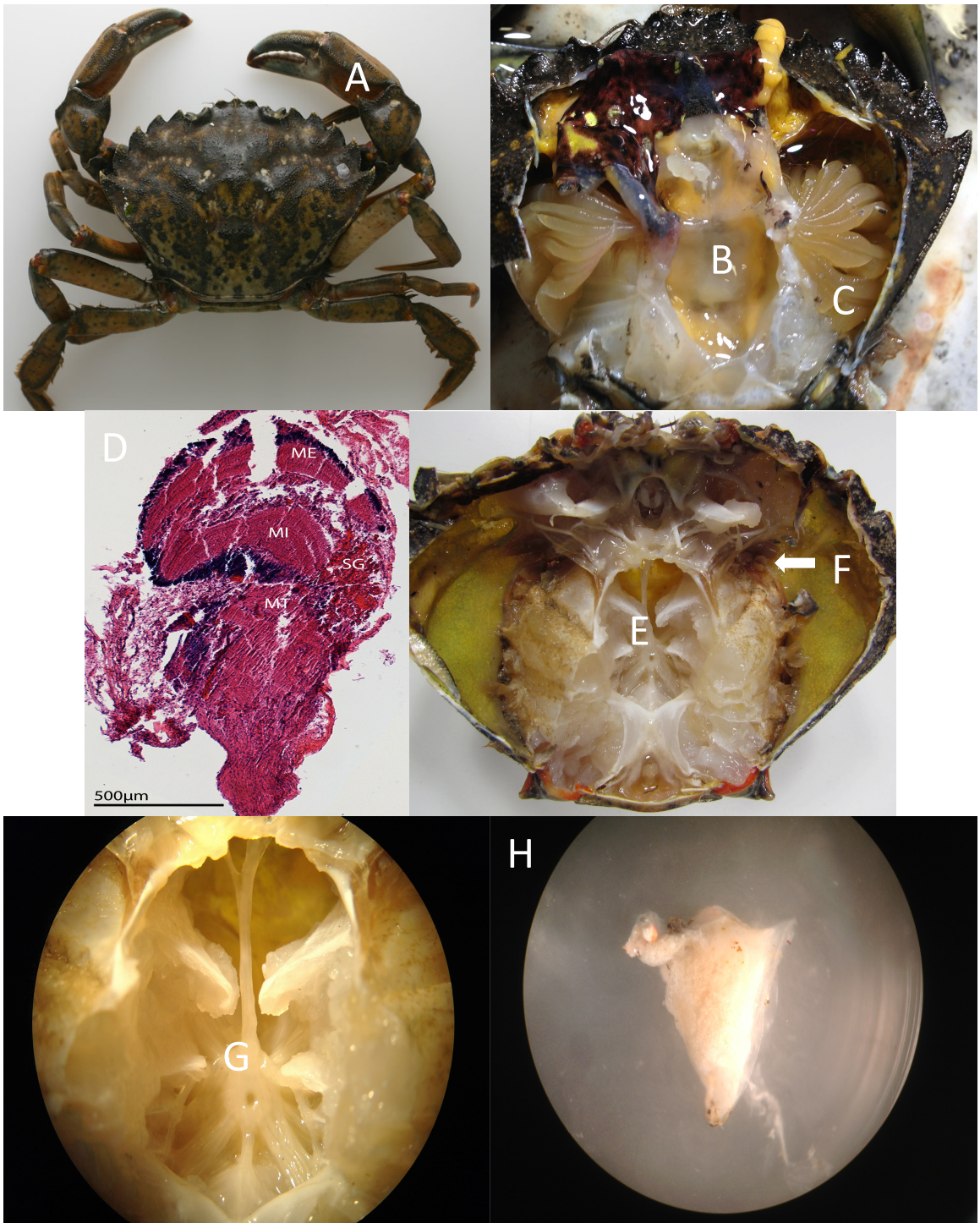

Fig 1.5. External and internal structure of *C. maenas*, depicting the tissues that were dissected for the acute temperature stress experiment. (A) Claw muscle; (B) Heart; (C) Posterior right gill; (D) Eyestalk ganglia (dissected from animal; located in the eyestalk of the animal on the anterior side, and stained with hematoxylin and eosin (H&E)); Interior of animal, which depicts dorsal organs removed with the thoracic ganglia (E) intact and also indicates location of Y-organ (F);

(G) Thoracic ganglia; (H) Y-Organ (dissected from animal; located in the cephalothorax region, anterior to the branchial chamber). Abbreviations on eyestalk ganglia- ME, medulla externa; MI, medulla interna; MT, medulla terminalis. Photo credit to Talia Head and Natalie Pitts (Colorado State University) for photo of eyestalk ganglia. Photo credit to Sukkrit Nimitkul (Bodega Marine Laboratory) for photos of thoracic ganglia and Y-organ.

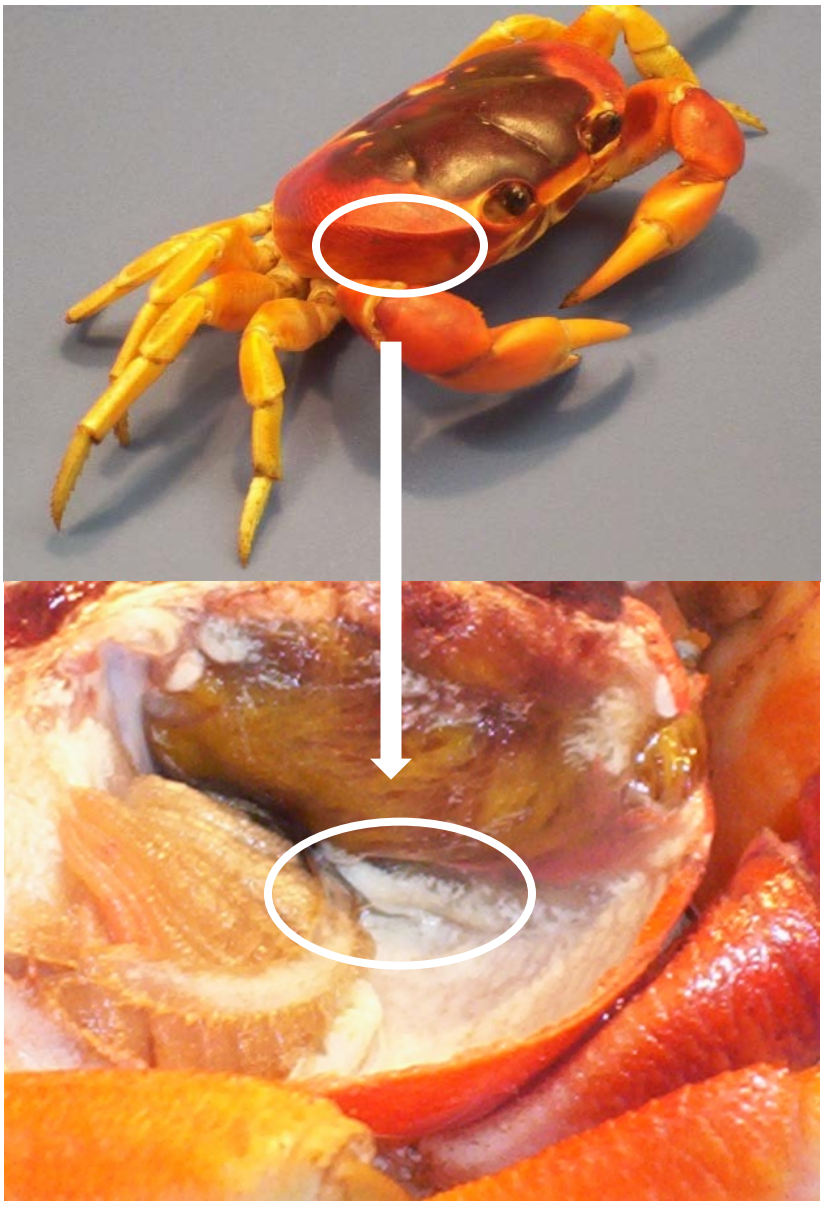

Fig 1.6. The blackback land crab's, *Gecarcinus lateralis,* molting gland (Y-Organ; YO). The paired YO is located in either side of the cephalothorax region, anterior to the branchial chamber and beneath the hepatopancreas.

REFERENCES

Abuhagr, A., 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., vol. 217, pp. 796-808: J Exp Biol.

Abuhagr, A., 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*., vol. 168, pp. 25-39: Comp Biochem Phys A.

Bliss, D. E. (1979). From sea to tree: saga of a land crab, vol. 19, pp. 385-410: Int Comp Biol.

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

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

Callier, V. and Nijhout, H. F. (2013). Body size determination in insects: a review and synthesis of size- and brain-dependent and independent mechanisms, vol. 88, pp. 944-954: Biol Rev.

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*, vol. 30, pp. 1809-1820: J Biogeog.

Chang, E. S., Chang, S. A. and Mulder, E. P. (2001). Hormones in the lives of crustaceans: an overview, vol. 41, pp. 1090-1097: Amer. Zool.

Chang, E. S. and Mykles, D. L. (2011). Regulation of crustacean molting: a review and our perspectives, vol. 172, pp. 323-330: Gen Comp Endocr.

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*), vol. 16, pp. 243-255: Divers and Dist.

Costlow, J. D. (1963). The effect of eyestalk extirpation on metamorphosis of megalops of the blue crab, *Callinectes sapidus*, vol. 3, pp. 120-130: Gen Comp Endocr.

Covi, J. A., Chang, E. S. and Mykles, D. L. (2012). Neuropeptide signaling mechanisms in crustacean and insect molting glands, vol. 56, pp. 33-49: Invert Reprod Dev.

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*, vol. 17, pp. 4992-5007: Mol Ecol.

Gannon, J. E. and Stemberger, R. S. (1978). Zooplankton (especially crustaceans and rotifers) as indicators of water quality, vol. 97, pp. 16-35: T Am Microsc Soc.

Hoang, T., Barchiesis, M., Lee, S. Y., Keenan, C. P. and Marsden, G. E. (2003). Influences of light intensity and photoperiod on molting and growth of *Penaeus merguiensis* cultured under laboratory conditions, vol. 216, pp. 343-354: Aquaculture.

Holz, M. K., Ballif, B. A., Gygi, S. P. and Blenis, J. (2005). mTOR and S6K1 mediate assembly of the translation preinitiation complex through dynamic protein interchange and ordered phosphorylation events, vol. 123, pp. 569-580: Cell.

Hopkins, P. M. (2012). The eyes have it: A brief history of crustacean neuroendocrinology, vol. 175, pp. 357-366: Gen Comp Endocr.

Inoki, K., Kim, J. and Guan, K. (2012). AMPK and mTOR in cellular energy homeostasis and drug targets, vol. 52, pp. 381-400: Annu Rev Pharmacolog.

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, vol. 444, pp. 463-474: Mar Ecol Prog Ser.

Klassen, G. and Locke, A. (2007). A biological synopsis of the European green crab, *Carcinus maenas*. , pp. 75: Can Rep Fish Aquat Sci.

Ma, X. M. and Blenis, J. (2009). Molecular mechanisms of mTOR-mediated translational control, vol. 10, pp. 307-318: Mol Cell Biol.

Marchal, E., Vandersmissen, H. P., Basisco, L., Van de Velde, S., Verlinden, H., Iga, M., Van Wielendaele, P., Huybrechts, R., Simonet, G., Smagghe, G. et al. (2010). Control of ecdysteroidogenesis in prothoracic glands of insects: a review, vol. 31, pp. 506-519: Peptides.

McCarthy, J. F. and Skinner, D. M. (1977). Proecdysial changes in serum ecdysone titers, gastrolith formation, and limb regeneration following molt induction by limb bud autonomy and/or eyestalk removal in the land crab, *Gecarcinus lateralis*, vol. 33, pp. 278-292: Gen Comp Endocr.

Moullac, G. and Haffner, P. (2000). Environmental factors affecting immune response in Crustacea, vol. 191, pp. 121-132: Aquaculture.

Mykles, D. L. (2011). Ecdysteroid metabolism in crustaceans, vol. 127, pp. 196-203: J Steroid Biochem.

Rodriguez, E. M., Medesani, D. A. and Fingerman, M. (2007). Endocrine disruption in crustaceans due to pollutants: a review, vol. 146, pp. 661-671: Comp Biochem Phys A.

Rommel, C., Bodine, S. C., Clarke, B. A., Rossman, R., Nunez, L., Stitt, T. N., Yancopoulos, G. D. and Glass, D. J. (2001). Mediation of IGF-1-induced skeletal myotube hypertrophy by PI(3)K/Akt/mTOR and PI(3)K/Akt/GSK3 pathways, vol. 3, pp. 1009-1013: Nature Cell Biol.

Skinner, D. M. (1985). Interacting factors in the control of the crustacean molt cycle, vol. 25, pp. 275-284: Amer Zool.

Skinner, D. M. and Graham, D. E. (1972). Loss of limbs as a stimulus to ecdysis in Brachyura (true crabs), vol. 143, pp. 222-233: Biol Bull.

Song, Q. and Gilbert, L. I. (1994). S6 phosphorylation results from prothoracicotropic hormone stimulation of insect prothoracic glands: a role for S6 kinase, vol. 15, pp. 332-338: Dev Genetics.

Song, Q. and Gilbert, L. I. (1997). Molecular cloning, developmental expression, and phosphoryation of ribosomal protein S6 in the endocrine gland responsible for insect molting, vol. 272, pp. 4429-4435: J Biol Chem.

Truman, J. W. and Riddifod, L. M. (1999). The origins of insect metamorphosis, vol. 401, pp. 447-452: Nature.

Vigh, D. A. and Dendinger, J. E. (1982). Temporal relationships of post molt deposition of calcium, magnesium, chitin, and protein in the cuticle of the atlantic blue crab, *Callinectes sapidus*, vol. 72, pp. 365-369: Comp Biochem Phys A.

Weis, J. S., Cristini, A. and Roa, K. R. (1992). Effects of pollutants on molting and regeneration in Crustacea, vol. 32, pp. 495-500: Amer Zool.

Whiteley, N. M. (2011). Physiological and ecological responses of crustaceans to ocean acidification, vol. 430, pp. 257-271: Mar Ecol Prog Ser.

CHAPTER TWO

EFFECTS OF ACUTE TEMPERATURE SHIFTS ON TISSUE EXPRESSION OF mTOR SIGNALING AND STRESS RESPONSE GENES IN THE GREEN SHORE CRAB, *CARCINUS MAENAS*

Summary

The green shore crab, *Carcinus maenas,* is a highly invasive species that inhabits coastal temperate zones worldwide. The reaction of *C. maenas* to acute temperature change was determined in six tissues (heart, gill, thoracic ganglion, eyestalk ganglion, Y-organ, and claw muscle) using genetic markers for stress proteins. The HSP70 chaperone gene was used as a proxy for the upper limit of the pejus temperature range. AMPK expression and activity has been suggested as an early indicator of stress in crustaceans. AMPKγ, mTOR, and Rheb genes were selected as markers for temperature-induced metabolic stress. The 3' end of a partial sequence for *AMPKγ* was extended by 3' RACE. The hypothesis predicts that an increase in temperature up-regulates *AMPKγ* and *HSP70* expression. However, the response would likely vary between tissues, as tissues with higher metabolic activity may be more sensitive to temperature than tissues with lower metabolic activity. Animals were exposed to temperatures between 5° and 30°C for 1 or 2 h. *Cm-HSP70*, *Cm-AMPKγ*, *Cm-mTOR*, and *Cm-Rheb* mRNA levels in six tissues were quantified by quantitative RT-PCR (qPCR). *Cm-Elongation Factor-2* (*Cm-EF2*), a constitutively expressed gene required for translation, was also quantified. The possible involvement of eyestalk neuropeptides was assessed by quantifying: (1) *Cm-MIH* and *Cm-CHH* mRNA levels in eyestalk ganglia and (2) *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-h exposures at 5 ˚C and 30 ˚C to affect tissue-specific changes in gene expression. *Cm-HSP70* expression was robustly increased at 30 °C in all tissues.

Introduction

The green shore crab, *Carcinus maenas*, is a highly invasive species in temperate coastal regions worldwide. The species is considered an "ecosystem engineer", in that it modifies any new environment it inhabits through consumption of and competition with native fauna (Klassen and Locke, 2007). 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). 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 of the species to tolerate wide ranges in temperatures, salinity, oxygen levels, and pH (Jost et al., 2012; Klassen and Locke, 2007). Temperature is an important determinant in the geographic distribution of a species (Compton et al., 2010). Predictions on climate change indicate that the ocean is warming, and will continue to do so (Stocker et al., 2014, in IPCC report). Thus, if *C. maenas* continues to expand its current range into warming waters, it will likely continue to be ecologically competitive, which may impact the biodiversity of coastal communities (Worm et al., 2006).

C. maenas, an intertidal species, tolerates short term temperature stress and can acclimate to a wide range of temperatures. In comparison to *C. maenas*, other decapod crustaceans like

Cancer irroratus and *Homarus americanus* have a lower tolerance for environmental stress. Multiple stress proteins have been shown to increase in various invertebrates after exposure to acute and/or prolonged environmental stressors (Frederich et al. 2009; Guévélou et al. 2013, Jost et al. 2012; Podolski, 2011). *C. maenas* was superior at righting itself and regulating its heart rate at higher temperatures, and switched to anaerobic metabolism at relatively high temperatures (measured by lactate accumulation in the heart)(Jost et al. 2012). Animals acclimated to extreme temperatures tend to tolerate acute changes in temperature more readily than those not acclimated (Tepolt and Somero, 2014), which gives *C. maenas* an even greater advantage. *C. maenas* may be more tolerant than closely related species because it does not seem to exhibit a pejus range, but rather enters into a pessimum range at temperatures between 34-37°C (Jost et al. 2012). The term pejus range, or "turning worse", coined by Frederich and Pörtner (2000), refers to the point where an animal's performance begins to decline. Once the animal reaches a critical point (the pessimum range), they switch into anaerobic metabolism and performance drops precipitously; at this point they are unlikely to recover (Shelford, 1931). This coping mechanism is often found in species that experience wide environmental conditions, especially temperature; this includes species located in the intertidal zone (Jost et al. 2012).

Heat shock proteins (HSPs) display a classic up-regulation in response to elevated temperatures (Kregel, 2002). This ubiquitous class of protein, which is highly conserved and among the most abundant superfamily present in all organisms (Jung et al. 2013), acts as a molecular chaperone, binding to existing functioning proteins and preventing them from unfolding, misfolding, or aggregating (Morimoto, 1998). In a previous study, lobsters subjected to acute thermal stress had increased *HSP70* and *HSP90* expression after 1-h of heat shock when transferred from ambient water conditions (13 °C)(Spees et al. 2002). Adenosine

monophosphate-activated protein kinase (AMPK) is commonly used as a stress indicator in crustaceans and other species (Zhu et al. 2007; Frederich et al. 2009; Han et al. 2013). AMPK activity did not increase in *C. maenas* after temperature stress; AMPK is thought to be an early indicator of stress in crustaceans (Jost et al. 2012). Gene expression of heat shock proteins, protein deacetylases SIRT1, cytochrome c oxidase, and AMPK, among others, were up-regulated during episodes of stress in various tissues in lobsters, shrimp, and other invertebrates (Chang et al., 1999; Han et al., 2013; Podolski, 2011; Zhao et al., 2009; Zhou et al., 2007).

AMPK is a heterotrimer that is regulated by AMP:ATP levels in the cell (Hardie et al., 2006). Higher AMP concentrations result from ATP hydrolysis to provide cellular energy. The α subunit contains the catalytic site; phosphorylation of Thr172 in mammalian AMPK results in activation of the kinase domain (Hardie et al., 2006). The β, or regulatory, subunit interacts with the kinase domain at the C-terminus of the α subunit (Xiao et al. 2011), and allows the α and γ subunits to come in contact with each other (Inoki et al. 2012). The β subunit is also involved in AMPK cellular localization and may play a critical role in sensing cellular energy (Inoki et al., 2012). The γ subunit, another regulatory subunit, contains cystathionine β-synthase (CBS), or Bateman, domains that preferentially bind AMP (Kemp, 2004; Xiao et al. 2011). AMPK inhibits mechanistic Target of Rapamycin (mTOR)-dependent protein synthesis through Tuberous Sclerosis Complex (TSC) and Rheb (for Ras homolog enriched in brain). mTOR is a serine/threonine protein kinase that associates with Rheb and other proteins to form mTOR Complex-1 (mTORC1; Laplante and Sabatini, 2012). Rheb is a GTP-binding protein that regulates mTORC1 activity. mTORC1 is activated by Rheb-GTP. TSC, also designated Rheb GTPase-activating protein (Rheb-GAP), promotes the hydrolysis of GTP to GDP, which inactivates Rheb (Laplante and Sabatini, 2012). mTORC1 stimulates protein synthesis by

phosphorylating ribosomal S6 kinase (S6K) and 4E-binding protein 1 (4EBP1) (Inoki et al., 2012). Thus, activation of AMPK by low energy or stress conditions stimulates TSC/Rheb-GAP activity, which inhibits mTORC1 by promoting the hydrolysis of Rheb-GTP to Rheb-GDP in mTORC1 (Xu et al., 2012). While AMPK and mTOR are regulated by phosphorylation, Rheb protein level is correlated with mRNA level and activity. Over-expression of Rheb in *Drosophila* cells increases protein synthesis and growth (Hall et al., 2007; Patel et al., 2003). Moreover, the 3'-untranslated region (UTR) of decapod crustacean Rheb transcripts contains a K-box motif, which suggests that Rheb mRNAs are negatively regulated by microRNA (MacLea et al., 2012).

The mTOR signaling pathway plays a critical role in regulating molting and tissue growth in decapod crustaceans. Orthologs of mTOR, S6K, and Akt, a protein kinase that inhibits TSC/Rheb-GAP), have been characterized in *C. maenas* and *Gecarcinus lateralis* and Rheb orthologs have been characterized in *C. maenas*, *G. lateralis*, *Scylla paramamosain*, and *H. americanus* (Abuhagr et al., 2014; MacLea et al., 2012). Increased *Gl-Rheb* expression is correlated with an increase in protein synthesis in claw closer skeletal muscle during the premolt period (Covi et al., 2010; MacLea et al., 2012). 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 (Covi et al., 2012; Mykles, 2011). Rapamycin, an mTOR inhibitor, inhibits ecdysteroid secretion by the YO *in vitro* in *C. maenas* and *G. lateralis* and *Gl-mTOR*, *Gl-Akt*, and *Gl-EF2* are up regulated in the *G. lateralis* YO during premolt (Abuhagr et al., 2014). The YO is negatively regulated by neuropeptides synthesized and secreted by the X-organ/sinus gland (XO/SG) complex in the eyestalk ganglia: molt-inhibiting hormone (MIH) and crustacean hyperglycemic hormone (CHH) (Chang and Mykles, 2011; Hopkins, 2012; Katayama et al., 2013; Webster et al., 2012). Both MIH and CHH inhibit YO ecdysteroidogenesis *in vitro*

(Lee et al., 2007; Zarubin et al., 2009). MIH is primarily responsible controlling the molt cycle; a decrease in MIH release from the XO/SG complex activates the YO and the animal enters premolt (Chang and Mykles, 2011; Covi et al., 2012). In addition to regulating carbohydrate metabolism and ion and water transport, CHH may inhibit molting in response to stress (Chang et al., 1999; Chang, 2005; Chung et al., 2010; Lee et al., 2014; Webster et al. 2012). These data suggest that the mTOR signaling pathway in the YO is inhibited by MIH and CHH, thus controlling the ecdysteroid synthetic activity and capacity in response to environmental conditions.

The effects of *C. maenas* to acute temperature change were determined using genetic markers for stress proteins. 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-induced metabolic stress. The 3' end of a partial sequence for AMPKγ in the GenBank database (JN134159) was extended by 3' RACE. As the three proteins regulate global translation of mRNAs, it is hypothesized that their expression may be affected by temperature stress. The hypothesis predicts that an increase in temperature up regulates *AMPKγ* and down regulates *mTOR* and *Rheb*, which would repress translation and allow cellular energy to be redirected for repair. However, the response would likely vary between tissues, as tissues with higher metabolic activity (e.g., eyestalk ganglia, thoracic ganglion, and heart) may be more sensitive to temperature than tissues with lower metabolic activity (e.g., claw skeletal muscle, gill, and YO). Animals were exposed to temperatures between 5° and 30°C for 1 or 2 h. *Cm-HSP70*, *Cm-AMPKγ*, *Cm-mTOR*, and *Cm-Rheb* mRNA levels in six tissues were quantified by quantitative RT-PCR (qPCR). *Cm-Elongation Factor-2* (*Cm-EF2*), a constitutively expressed gene required for translation, was also quantified. The possible involvement of eyestalk

neuropeptides was assessed by quantifying: (1) *Cm-MIH* and *Cm-CHH* mRNA levels in eyestalk ganglia and (2) *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-h exposures at 5 ˚C and 30 ˚C to effect tissue-specific changes in gene expression.

Materials and Methods

Animals and experimental treatments

Adult male and non-ovigerous female *Carcinus maenas* were collected from Bodega Harbor, Bodega Bay, CA, and maintained in a flow-through seawater system at Bodega Marine Laboratory (BML) at ambient temperature (~15 °C) and an approximately 12 h:12 h light:dark cycle. Animals were fed squid twice a week.

For the first set of experiments, animals were exposed to 5, 10, 20, 25, or 30 °C for 1 h. A second set of experiments exposed intact animals to either 5 or 30 °C for 2 h. A third set of experiments exposed eyestalk-ablated (ESA) animals to either 5 or 30 °C for 1 h. Exposure time was implemented based on previous research indicating that crustaceans respond to heat shock in as little as one hour after exposure (Spees, 2002). Intact animals that were kept at ambient temperature (~15 °C) served as controls. Seawater was equilibrated at each temperature for 24 h in an environmental chamber and oxygenated with an air supply. Molt stage was determined by hemolymph edcysteroid concentration. Only intermolt animals (hemolymph ecdysteroid titers <30 pg/μl) were used in the experiments.

Hemolymph samples (100 μl) were taken before and after treatment and combined with 300 μl methanol. Ecdysteroids were quantified by a competitive enzyme-linked immunoassay

(ELISA) as described (Abuhagr et al. 2014). Presence of limb-bud regenerates was noted, as well as color morph (red or green), carapace width, and mass. Males were larger than females in both mass and carapace width (males: 98.6 ± 4.3 g and 73.9 ± 1.0 mm, n = 60; females: 59.6 ± 2.0 g and 64.4 ± 0.7 mm, n = 48)(see Table 2). Mass was correlated with carapace width (R2 = 0.9195 for males and 0.9227 for females). Intact male and female animals used for the first and second set of experiments; only males were used in the third set of experiments. Red color morphs were the predominant phenotype (red:green ratio = ~5:1). Phenotypes were distributed proportionally into the different temperature treatments.

RNA isolation and cDNA synthesis

Heart, gill (right posterior), thoracic ganglia (TG), Y-organ (molting gland; YO), eyestalk ganglia (ESG), and claw muscle (CM) were harvested immediately after temperature treatment. Tissues were placed in at least 10 volumes RNA Later (Qiagen, Valencia, CA, USA), stored at 4 °C for 1 day to 2 weeks, and shipped to Colorado State University on ice. Tissues were stored at -20 °C for 2-3 months before processing.

RNA was isolated using TRIzol and chloroform/phenol extraction as described (Covi, et al. 2010). YOs and ESG were homogenized by hand in 1 ml TRIzol Reagent (Ambion/Life Technologies, Grand Island, NY, USA) for 5 min at room temperature. All other tissues were homogenized using a TissueLyser II (Qiagen) for 2 min at a frequency of 30/s and mixed with 1 ml TRIzol Reagent. After phenol-chloroform extraction, RNA pellets were rehydrated in 22 μl RNase/DNase/protease free water and received DNase I treatment according to manufacturer's instructions (Thermo Scientific, Grand Island, NY, USA). Ten units (0.75 μl) Ribolock (Thermo Scientific) were included in the DNase I treatment to prevent RNA degradation. A second

phenol-chloroform extraction used 24:1 chloroform:isoamyl alcohol, and RNA was precipitated by adding 0.5 volume sodium acetate (3 M, pH 5.2) to 1.5 volumes isopropanol. Pellets were rehydrated in 22 μl RNase/DNase/protease free water, and the RNA concentration was determined using a Nanodrop 1000 (Thermo Scientific). One μg total RNA was reverse-transcribed to cDNA using Quanta qScript cDNA SuperMix (Quanta Biosciences, Gaithersburg, MD, USA), according to the manufacturer's instructions. cDNA was stored at -20 °C.

Sequencing cDNA encoding Cm-AMPKγ

3'-RACE was used to extend a partial sequence for Cm-AMPKγ (Accession no. JN134159). All primers were synthesized by IDT (Coralville, IA, USA) and diluted to 10 μM. Nested 3' RACE was used to extend the 3' end of Cm-AMPKγ using YO and CM template cDNA according to First-Choice RLM-RACE kit manufacturer's instructions (Applied Biosystems, Austin, TX, USA). RACE reactions contained 1 μl template cDNA, 25 μl 2X PCR Master Mix (Thermo Scientific, Carlsbad, CA, USA), 2 μl forward gene-specific outer primer (10 μM), 2 μl 3' RACE outer primer, and 20 μl nuclease-free water. PCR conditions were: denaturation at 94 °C for 3 min, 35 cycles (denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 2 min), and final extension at 72 °C for 4 min. PCR was repeated using nested gene-specific forward inner primer and the 3' RACE inner primer and 1 μl first-round PCR reaction as template. PCR products were separated by 1.2% agarose gel electrophoresis, stained with ethidium bromide, and purified using the Qiaex II Gel Extraction Kit (Qiagen). The DNA was sequenced, using the inner primers, by Davis Sequencing (Davis, CA, USA). Sequences were aligned with orthologs in the NCBI database using ClustalX2 and Gene Doc. Bateman domains

of Cm-AMPKγ were located using the NCBI Conserved Domain Search. The sequence was deposited in GenBank (Accession no. KJ652006).

Tissue expression of EF2, AMPKγ, and HSP70

End-point PCR was used to determine the tissue expression of Cm-EF2 (GU808334.1), Cm-AMPKγ (KJ652006), and Cm-HSP70 (AM116767.1). Reactions contained 1 μl each forward and reverse gene-specific primers (Table 1), 5 μl 2X PCR Master Mix (Thermo Scientific, Carlsbad, CA, USA), 2 μl nuclease-free water, and 1 μl cDNA template. PCR conditions were initial denaturation at 95 °C for 3 min, 35 cycles (95 °C for 30 s, 62 °C for 30 s, and 72 °C for 30 s), and final extension at 72 °C for 7 min. PCR products were separated by 1% agarose gel electrophoresis, stained with ethidium bromide, and imaged under ultraviolet light.

Quantitative PCR (qPCR) was performed using a Lightcycler 480 Thermocycler (Roche Applied Science, Indianapolis, IN, USA). Reactions contained 0.5 μl each forward and reverse gene-specific primers (Table 1), 5 μl SyBr Green (Roche), 3 μl nuclease-free water, and 1 μl cDNA template. qPCR conditions were denaturation at 95 °C for 3 min, 45 cycles (95 °C for 30 s, 62 °C for 30 s, and 72 °C for 20 s), and final extension at 72 °C for 7 min. Concentrations were quantified by comparing to a standard curve for each gene. The data are presented as mean copy number/μg total equivalent RNA ± 1 S.E.M. (n).

Statistical analysis

Copy numbers were log transformed and outliers were removed using a Nalimov test (Wittman et al., 2012). Statistical significance ($p < 0.05$) between temperatures according to gene and tissue were evaluated using Tukey or Dunn's one-way ANOVA (Sigma Plot 12.0, Systat

Software, San Jose, CA USA). All experimental temperatures were compared to the intact animals harvested from the ambient water temperature (~15 °C). Data are presented as mean ± 1 S.E.M. (n).

Results

Sequencing and tissue expression of Cm-AMPKγ

A 540-bp partial sequence of Cm-AMPKγ in the GenBank database (JN134159) was used to extend the 3' sequence by 3' RACE. The RACE product (2165 bp) contained the 3' end of the open reading frame (ORF) and the 3' untranslated region (UTR). Several attempts to extend the 5' sequence by 5' RACE failed. The consensus sequence (KJ652006) was 1265 bp in length and encoded most of the ORF (Fig. 1). The deduced amino acid sequence encoded four Bateman domains, which are characteristic of the γ subunit of AMPK (Fig. 1). A multiple alignment of the Cm-AMPKγ amino acid sequence with sequences from two other decapod crustaceans (*Cancer irroratus* and *Homarus americanus*), *Daphnia*, 3 insect species, and human revealed a high sequence identity (Fig. 2).

Endpoint RT-PCR was used to determine the tissue distribution of *Cm-AMPKγ*, *Cm-EF2*, and *Cm-HSP70* mRNAs. The three genes were expressed in all 12 tissues at apparently high levels (Fig. 3). The PCR products were consistent with predicted sizes: 247 bp for *Cm-AMPKγ*, 160 bp for *Cm-HSP70*, and 278 bp for *Cm-EF2*. The identities of the PCR products were verified by direct sequencing.

Effects of acute temperature change

C. maenas has 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° 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 effect on hemolymph ecdysteroid titers (Fig. 4A).

qPCR was used to determine the mRNA levels of *Cm-EF2*, *Cm-AMPKγ*, *Cm-HSP70*, *Cm-mTOR*, and *Cm-Rheb* in six tissues. The first set of experiments determined the effects of an acute 1-h exposure of temperatures between 5˚C and 30˚C in animals acclimated to 15˚C. The analysis focused on comparing differences between control (~15°C) and experimental temperatures. Significant differences between the means at other temperatures for genes and tissues are reported in Table 3. There was no significant effect of temperature on *Cm-EF2*, a constitutively gene involved in translation, in five of the six tissues (Fig. 4B). Temperature also had little or no effect on *Cm-AMPKγ* expression (Fig. 4C). *Cm-AMPKγ* mRNA levels were not significantly different from control levels in claw muscle, eyestalk ganglia, and heart. The only significant differences were at 25˚C in gill (11-fold higher) and thoracic ganglion (15.2-fold lower), and at 30˚C in Y-organ (2-fold higher) (Fig. 4C, asterisks). By contrast, *Cm-HSP70* expression was induced at 30˚C in five of the six tissues (Fig. 4D, asterisks). *Cm-HSP70* mRNA levels were increased 119-fold at 30˚C in heart, 72-fold in thoracic ganglion, 1590-fold in eyestalk ganglia, 726-fold in Y-organ, and 29-fold in claw muscle. The expression of *Cm-mTOR* and *Cm-Rheb* was generally refractory to temperature shifts (Fig. 4E, F). *Cm-mTOR* mRNA levels were two to three orders of magnitude higher in heart, thoracic ganglion, and claw muscle

than in gill, eyestalk ganglia, and Y-organ (Fig. 4E). Only two means were significantly different from control: at 25˚C, *Cm-mTOR* mRNA levels were 1.9-fold higher in claw muscle and 22.9-fold lower in thoracic ganglion (Fig. 4E, asterisks). For *Cm-Rheb*, mRNA levels were 11-fold lower in thoracic ganglion and 232-fold higher in gill at 25˚C and 2.2-fold higher in claw muscle at 30˚C (Fig. 4F, asterisks). In summary, shifts to lower temperatures had no effect on the expression of the five genes. Gene expression was more variable in gill than in the other tissues. Any significant changes in gene expression occurred at higher temperatures, but there were no apparent trends in expression with respect to tissue or gene, except for *Cm-HSP70*. The only consistent affect of temperature was the large increase in *Cm-HSP70* mRNA levels at 30˚C.

The second experiment determined the effects of a 2-h exposure to 5 ˚C and 30 ˚C of animals acclimated to 15 ˚C. In general, the 2-h treatment had similar effects on gene expression as the 1-h treatment, except that there were more significant differences between the means in all tissues (Fig. 5). Temperature had little effect on *Cm-EF2* and *Cm-AMPKγ* expression in the six tissues. There was no significant effect of temperature on *Cm-EF2* mRNA levels in gill, eyestalk ganglia, and claw muscle (Fig. 5A). In heart, *Cm-EF2* mRNA level was 3.4-fold lower at 30˚C compared to the control (Fig. 5A, bracket). In thoracic ganglion, *Cm-EF2* mRNA level was 5.2-fold lower at 5˚C. In Y-organ, *Cm-EF2* mRNA levels were 2.3-fold and 1.8-fold higher at 5 ˚C than at 15 ˚C and 30 ˚C, respectively (Fig. 5A, brackets). *Cm-AMPKγ* expression in heart, gill, eyestalk ganglia, and claw muscle was not affected by temperature (Fig. 5B). In thoracic ganglion, *Cm-AMPKγ* mRNA level at 5 ˚C was 6-fold lower than that at 15 ˚C (Fig. 5B, bracket). In Y-organ, *Cm-AMPKγ* mRNA levels were ~2-fold lower at 15 ˚C than at 5 ˚C and 30 ˚C (Fig. 5B, brackets). *Cm-HSP70* was significantly up-regulated at 30 ˚C in all tissues; the mRNA levels at 5˚C and 15 ˚C were not significantly different (Fig. 5C, brackets). The magnitude increase

from 15 ˚C to 30 ˚C varied between tissues: 48-fold in heart; 34-fold in gill; 45-fold in thoracic ganglion; 975-fold in eyestalk ganglia; 75-fold in Y-organ; and 24-fold in claw muscle. There were significant changes in *Cm-mTOR* expression in heart, thoracic ganglion, eyestalk ganglia, and Y-organ, with higher mRNA levels at 15 ˚C in heart and thoracic ganglion and lower mRNA levels at 15 ˚C in eyestalk ganglia and Y-organ (Fig. 5D, brackets). Compared to the control (15 ˚C), *Cm-Rheb* mRNA levels were increased ~14-fold in gill at 30 ˚C, decreased 5.6-fold in thoracic ganglion at 5 ˚C, and increased 2.4-fold in Y-organ at 5 ˚C (Fig. 5E, brackets). Temperature had no effect on Cm-Rheb mRNA levels in heart, eyestalk ganglia, and claw muscle (Fig. 5E). Significant differences between the means at other temperatures for genes and tissues are reported in Table 4. In summary, Y-organ and thoracic ganglion were more sensitive to temperature, as indicated by significant changes in the expression of all five genes at 5 ˚C and/or 30 ˚C. All six tissues showed a large induction of *Cm-HSP70* at 30 ˚C. A 2-h exposure at 5 ˚C altered expression of *Cm-EF2*, *Cm-AMPKγ*, *Cm-mTOR*, and *Cm-Rheb* in certain tissues, suggesting that a longer treatment period was necessary to affect mRNA levels in thoracic ganglion, Y-organ, and eyestalk ganglia.

The third experiment determined the effects of eyestalk ablation on temperature-dependent gene expression. Animals were acclimated to 15 ˚C, eyestalk-ablated, and transferred to 5 ˚C or 30 ˚C for 1 h. There was no effect of temperature on *Cm-EF2* and *Cm-mTOR* expression, except for Y-organ at 5 ˚C and 30 ˚C (Fig. 6A & D, brackets). *Cm-AMPKγ* mRNA levels were increased 2.3-fold in heart at 30 ˚C, decreased 7-fold in thoracic ganglion at 5 ˚C, and increased 9.6-fold in Y-organ, relative to control levels (Fig. 6B, brackets). Exposure to 30 ˚C increased *Cm-HSP70* expression 137-fold in heart, 21,800-fold in Y-organ, and 82-fold in claw muscle (Fig. 6C). There was no significant effect on *Cm-HSP70* mRNA levels in gill and thoracic

ganglion. There was little or no effect of temperature on the expression of mTOR signaling genes. In Y-organ, *Cm-mTOR* and *Cm-Rheb* mRNA levels were increased ~6-fold and ~4-fold at 5 ˚C and 30 ˚C, respectively (Fig. 6 D & E, brackets). In thoracic ganglion, *Cm-Rheb* mRNA level was ~3-fold higher than the levels at 5 ˚C and 30 ˚C (Fig. 6E, brackets). Significant differences between the means at other temperatures for genes and tissues are reported in Table 5. In summary, temperature had limited effects on the expression of *Cm-EF2*, *Cm-AMPKγ*, *Cm-mTOR*, and *Cm-Rheb* in eyestalk-ablated animals. Exposure to 30 ˚C resulted in a large increase in Cm-HSP70 mRNA levels in heart, Y-organ, and claw muscle, but not in gill and thoracic ganglion.

As CHH is secreted into the hemolymph in response to stress, the effects of temperature on the expression of *Cm-CHH* and *Cm-MIH* in the eyestalk ganglia was quantified in animals from 1-h and 2-h treatments. *Cm-CHH* mRNA levels were higher than *Cm-MIH* levels at all temperatures (Fig. 7). There was no effect of temperature on the expression of either gene (Fig. 7).

Discussion

C. maenas did not experience heat stress until a 1-h exposure at 30 °C. While there are significant differences in *Cm-EF2*, *Cm-AMPKγ*, *Cm-mTOR*, and *Cm-Rheb* mRNA levels in intact animals at elevated temperatures, there is no general tissue response to temperature stress (Fig. 4). Moreover, the changes in expression were relatively modest, even at the extremes of the temperature range. By contrast, *Cm-HSP70* mRNA levels were significantly increased at 30 °C in all six tissues after 1-h (Fig. 4D) and 2-h (Fig. 5C). This temperature approaches the pessimum temperature, which suggests that *C. maenas* can tolerate short-term exposures to 25 ˚C

without the induction of chaperones to stabilize intracellular proteins. HSP70 is regulated by inducible transcription in response to environmental stress (Morimoto, 1998). The significant differences in *Cm-EF2, Cm-AMPKγ, Cm-mTOR*, and *Cm-Rheb* expression are likely due to a response mechanism that is stimulated prior to the classic heat shock response, which may or may not be related to a heat stress response. Rather, these genes are involved in the regulation of translation (EF2) and resource allocation (AMPK, mTOR, and Rheb), which respond to any changes in energy levels. This causes ATP levels to be conserved, and limits protein synthesis. As these significant differences in the means are not correlated to a specific temperature or tissue, they could be involved in the general maintenance of cellular metabolism rather than displaying a heat shock response. The exception to this is *Cm-Rheb* expression in gill, where expression increased in response to higher temperatures in intact animals (Figs. 4F and 5D). This may be an early response that indicates increased protein synthetic capacity with increased metabolism. However, this trend in *Cm-Rheb* expression cannot be generalized among all tissues. Perhaps gill tissue showed a response due to its function in gas exchange and ion transport. *Cm-mTOR* expression was generally stable in most tissues in reponse to temperature stress; it showed between 1- and 3-fold changes in the tissues, except for a ~36-fold decrease in thoracic ganglion at 25 °C compared to the control (Table 3). Like AMPK, mTOR is regulated by phosphorylation and is altered primarily at the protein level to allow rapid response to environmental conditions. Therefore, mRNA levels may not accurately reflect mTOR activity, especially after such a short exposure time. Rheb-GTP binding allows the mTORC1 to become active; Rheb activity is decreased by low energy levels (i.e., stress) through the AMPK/TSC1/TSC2 pathway (Avruch et al. 2006). This pathway seems to suggest that *Cm-Rheb* expression would decrease with temperature stress, so that mTOR activity and protein synthesis

are reduced as the AMP:ATP ratio increases. However, at least in gill, we see the opposite response. Perhaps an increased *Cm-Rheb* mRNA compensates for lower mTORC1 activity at low cellular energy supplies. Since Rheb is an upstream of mTOR signaling, it may respond first to temperature strress. Another possibility is that increased expression of *Cm-Rheb* will lead to increased protein synthesis and cell growth through the mTOR pathway as a result of increased temperature and metabolism. It has been established that crustacean metabolism increases with temperature up to a critical point; in *C. maenas*, the critical point is suggested to be between 32-34°C, a temperature that was not tested in this experiment (Jost et al. 2012). The data are consistent with physiological studies. *C. maenas* tissues can tolerate 1- and 2-hour exposures to temperatures between 5 ˚C and 30 °C, as there is no consistent effect on the expression of metabolic genes (*Cm-AMPKγ*, *Cm-mTOR*, and *Cm-Rheb*) in the 6 tissues and temperature.

Contrary to our hypothesis, *Cm-AMPKγ* mRNA expression was not increased at high temperature. Rather, while expression was variable between tissue types, there was generally very little variation between temperatures at 1- and 2-h exposure times as in mTOR genes. This may be due in part to the fact that AMPK is regulated primarily at the protein level through phosphorylation on the α subunit, instead of at the mRNA level. Therefore, the animal does not have to expend additional energy through the process of synthesizing mRNA, but can rather change activation state quickly through phosphorylation. The general trend of *Cm-AMPKγ* down-regulation at 5 °C compared to higher temperatures may simply suggest a depression of metabolism at lower temperatures, which is common to ectotherms (Leffler, 1972). ESA and intact animals exposed to temperatures for 1 h reacted similarly, although there was a less robust response in *Cm-HSP70* expression in ESA animals (Fig. 6). It makes sense than ESA animals act as the intact ones in their response to stress, considering the recent evidence that suggests *C.*

maenas is refractory to molting after ESA, since there are additional MIH release points in the thoracic ganglia and brain that prevent the animal from entering premolt (Abuhagr et al. 2014b). For these reasons, we conclude that *Cm-AMPKγ* expression is not sufficient for measuring stress in *C. maenas* under the tested parameters.

AMPKγ expression and activity has been shown to change in other decapod species in response to stress. In *C. irroratus*, both *HSP70* and *AMPKγ* expression increase after 1 h exposure to 26 °C (compared to the ambient temperature of 12-15 °C) (Frederich et al., 2009). A similar pattern was observed in *H. americanus* (Podolski, 2011), where *AMPKγ* mRNA levels increased up to 1000-fold in the hepatopancreas and 20-fold in the heart after hypoxia via emersion for 24 hours. *HSP70* expression was reduced, with a 30-fold change in the hepatopancreas. AMPK activity responded similarly to mRNA expression, and increased linearly in lobster heart and hepatopancreas with temperature; however, HSP70 activity did not change (Podolski, 2011). Previous studies were not able to determine a temperature where either HSP70 or AMPK activity changed in *C. maenas* (Jost et al. 2012), or where HSP70 activity changed in *C. irroratus* (Frederich et al. 2009). We observed that *Cm-AMPKγ* expression changed little in most tissues but *Cm-HSP70* expression was robustly increased at 30 °C. This suggests that *Cm-HSP70* is an indicator of early response to temperature stress in *C. maenas*.

Studies suggest that, unlike decapod species that live in a narrower range and enter anaerobic metabolism around 28-30 °C, *C. maenas* does not have a pejus range but instead transitions directly into its pessimum range at 32-34 °C (Jost et al. 2012). The highest temperature that was tested in this study was 30 ˚C for 2 h. Perhaps the animals were not exposed to temperatures high enough or long enough to effect an increase in *Cm-AMPKγ* mRNA levels. In *C. irroratus,* it required 4-6 h for *AMPKα* and *HSP70* expression to increase after

exposure to high temperatures (Frederich et al. 2009). *C. maenas*, compared to these subtidal species, is an invasive species that is known to tolerate a large range of temperatures and salinities. Since this species is more tolerant of habitat variability, it may have alternative mechanisms to regulate stress at the molecular level.

Cm-CHH and *Cm-MIH* mRNA levels in the ESG were not affected by temperature (Fig. 7). CHH has previously been used as an indicator of stress at the mRNA level in *G. lateralis* (Lee et al. 2007). Increased CHH levels indicate an animal experiences hyperglycemia (an increase in glycogen mobilization); this response allows an animal to restore ATP levels (Webster, 2012). Hemolymph CHH has also been observed to increase after exposure to thermal stress, among other stresses (temperatures 10-15°C higher than the acclimated temperature) in *H. americanus* (Chang et al. 1998). The absence of effect of temperature on *Cm-CHH* and *Cm-MIH* expression indicates, at least at the transcriptional level, that short exposures to high temperature do not elicit a stress response mediated by eyestalk neuropeptides.

MIH is a hormone unique to crustaceans that prevents the initiation of molting. The MIH signal is received by a receptor on the YO, and if absent, signals the YO to increase ecdysteroidogenesis by way of mTOR signaling (Chang and Mykles, 2011). It is interesting to note that even though *Cm-MIH* expression in the ESG and hemolymph ecdysteroid titers are not affected by temperature shifts, expression of *Cm-mTOR, Cm-Rheb, Cm-AMPKγ,* and *Cm-EF2* in the YO are affected by temperature extremes. This suggests that the YO is sensitive to temperature, which may affect molting and growth.

These results have implications on the geographic expansion of *C. maenas*. Currently, the species is found in temperate coastal waters worldwide (Tepolt and Somero, 2014). With projected climate change, it is thought that this temperate zone will expand toward the poles

(Stocker et al. 2014, in IPCC report). Theoretically, this means that *C. maenas* would be able to expand its current range into new areas. Not only would this species be able to survive in these new locations, but it would be able to grow and reproduce at comparatively higher rates in warming waters than endemic species, in addition to the colder waters it already inhabits.

In conclusion, *C. maenas* was highly tolerant to acute temperature changes. The green shore crab is highly invasive; a portion of its success is likely driven by its thermal flexibility and tolerance to changes at the molecular level. Invasive species generally show a limited response to stress as compared to related species that live within a narrower habitat range. *C. maenas* showed a limited response to heat shock at the gene expression level. Contrary to our hypothesis, while *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. *Cm-HSP70* did exhibit the classic heat shock response, showing increased expression at 25 °C in some tissues, and at 30 °C in all tissues. Our results are surprising in that we discovered little 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. Perhaps HSP70, in its role as a chaperone protein, is sufficient to compensate for the temperature stress so that other genes do not have to become overexpressed. 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. *C. maenas* tissues can tolerate a wide range of temperatures without altering mRNA levels of metabolic genes. It appears the up regulation of *Cm-HSP70* allows *C. maenas* to cope with short-term exposures to temperatures approaching its pessimus range.

Table 2.1. Oligonucleotide primer sequences used in sequencing and expression analysis of *C. maenas* genes. Reverse primers are written as reverse and complement. Annealing temperature was 62 °C for each PCR primer set, and 60 °C for 3' RACE. Abbreviations: EF2, elongation factor-2; AMPK, AMP-activated protein kinase; HSP70, heat shock protein 70; mTOR, mechanistic Target of Rapamycin; Rheb, Ras homolog enriched in brain; F, forward; R, reverse.

Primer	Sequence (5' → 3')	Use	Product size (bp)
Cm-AMPKg F1	AGCCGTCAATCCTACACAAGC	3' RACE	-
Cm-AMPKg F2	GGCAAACTGGTGGACATCTATGC	3' RACE	-
Cm-EF2 F1	CCATCAAGAGCTCCGACAATG	PCR	278
Cm-EF2 R1	CATTTCGGCACGGTACTTCTG	PCR	
Cm-AMPKg F1	TGGAGGACCATCGCTTGGAGACT	PCR	247
Cm-AMPKg R1	GGCTTGTGTAGGATTGACGGCT	PCR	
Cm-HSP70 F1	GACAACAGAATGGTGAATCACTTCGCTC	PCR	160
Cm-HSP70 R1	GAGAGTCGATCTCTATGCTGGCCTG	PCR	
Cm-mTOR F2	CATCCCTCAAACCTCATGCT	PCR	319
Cm-mTOR R2	CACCCACCACAGAACGCTTT	PCR	
Cm-Rheb F1	ATGGGCAAAGTCACAGTTCC	PCR	281
Cm-Rheb R1	GTCAGGAAGATGGTGGCAAT	PCR	

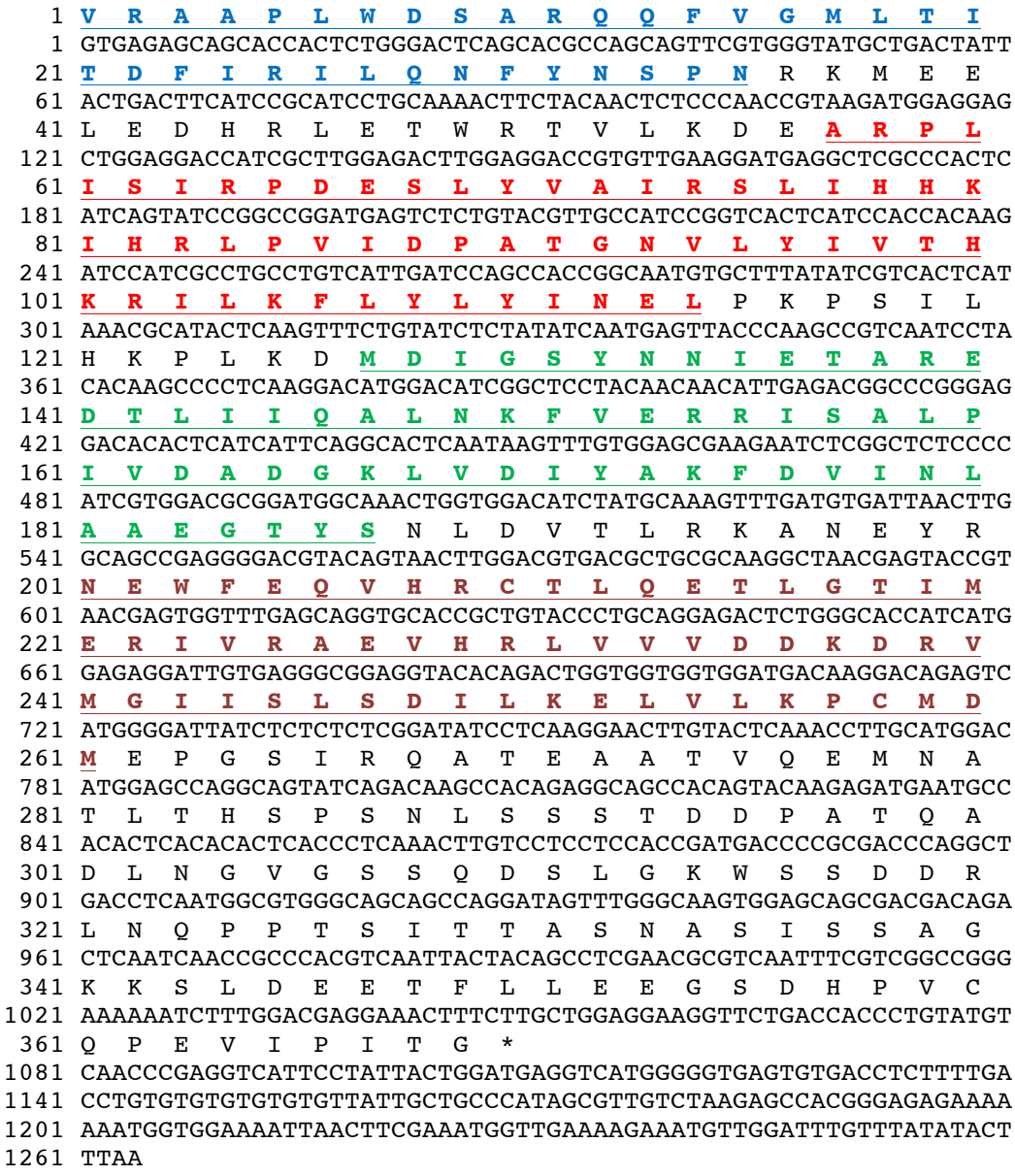

Fig 2.1. Nucleotide and deduced amino acid sequences of cDNA encoding *Cm-AMPKγ* (KJ652006). ORF is incomplete at the 5' end but does contain the stop codon (*) at the 3' end and partial 3' UTR. The translated sequence contained four Bateman (ATP/AMP binding) domains: blue, CBS 1; red, CBS 2; green, CBS 3; orange, CBS 4.

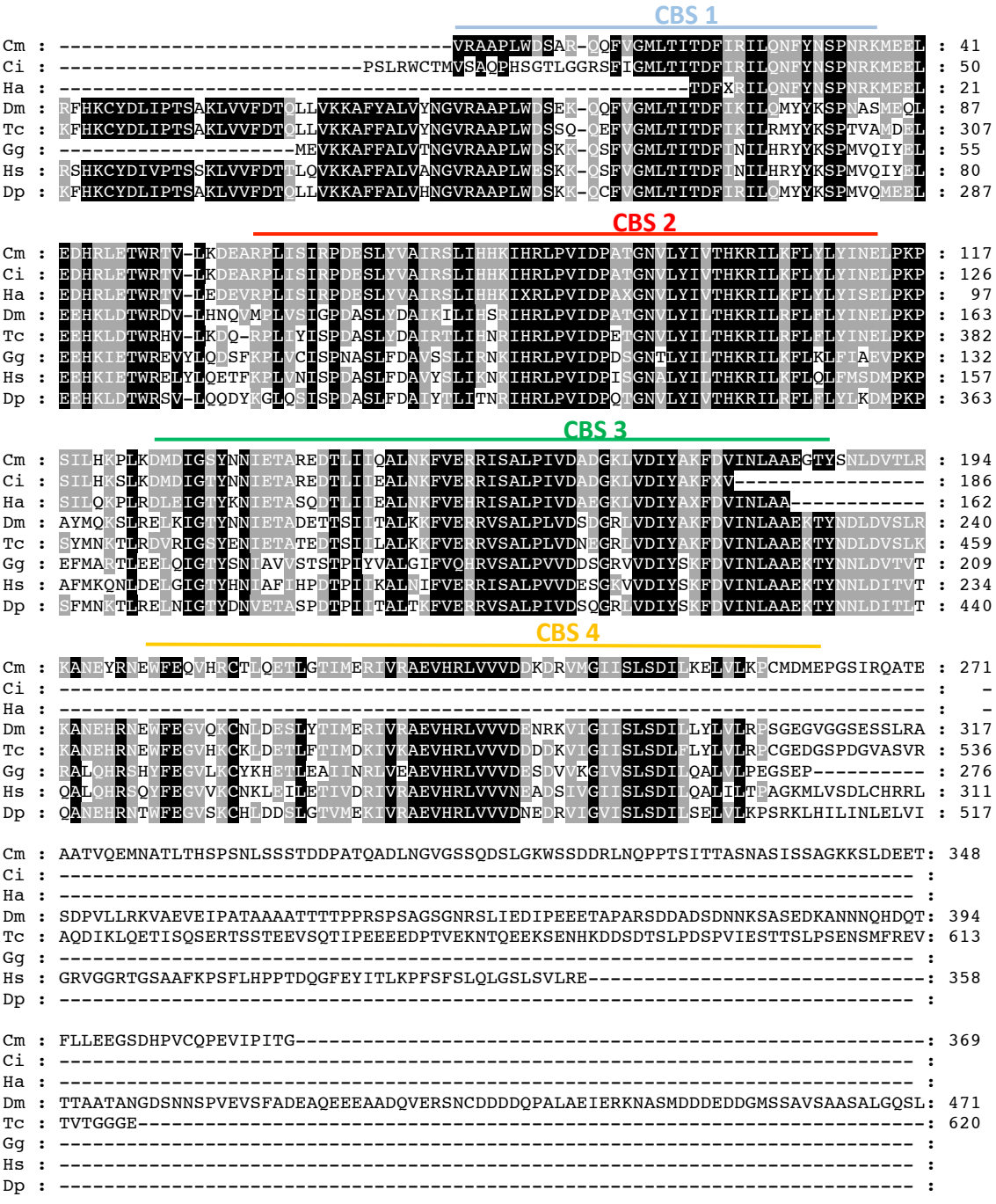
CBS 1
CBS 2
CBS 3
CBS 4

Fig 2.2. Multiple alignment of deduced amino acid sequences of the AMPKγ in *C. maenas* and orthologs from other arthropod species and human. Abbreviations: Cm, *C. maenas* (KJ652006); Ci, *Cancer irroratus* (ACL13567.1); Ha, *Homarus americanus* (AEO22037.1); Dm, *Drosophila melanogaster* (AAC95305.1); Tc, *Tribolium castaneum* (EFA04370.1); Gg, *Gallus gallus* (NP_001029999.1); Hs, *Homo sapiens* (XP_005250066.1); Dp, *Daphnia pulex* (EFX89994.1). Black shading indicates complete amino acid sequence identity/similarity in all species; gray shading indicates identity/similarity (80%) in most species. The colored solid lines above the sequences indicate the four Bateman domains for ATP/AMP binding: blue, CBS 1; red, CBS 2; green, CBS 3; orange, CBS 4.

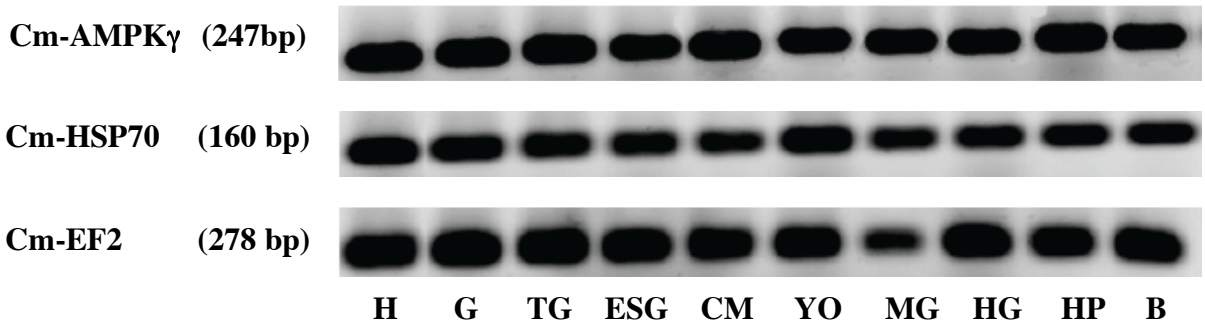

Fig 2.3. Tissue expression of *Cm-EF2, Cm-AMPKγ,* and *Cm-HSP70* using end-point PCR. PCR products after 35 cycles were resolved by agarose gel electrophoresis and are shown as a reversed ethidium bromide-stained image. Expected sizes of each product are indicated on the left. The three genes were expressed in all tissues. Abbreviations, from left to right: H, heart; G, gill; TG, thoracic ganglion; ESG, eyestalk ganglion; CM, claw muscle; YO, Y-organ; MG, mid-gut; HG, hind-gut; HP, hepatopancreas; B, brain.

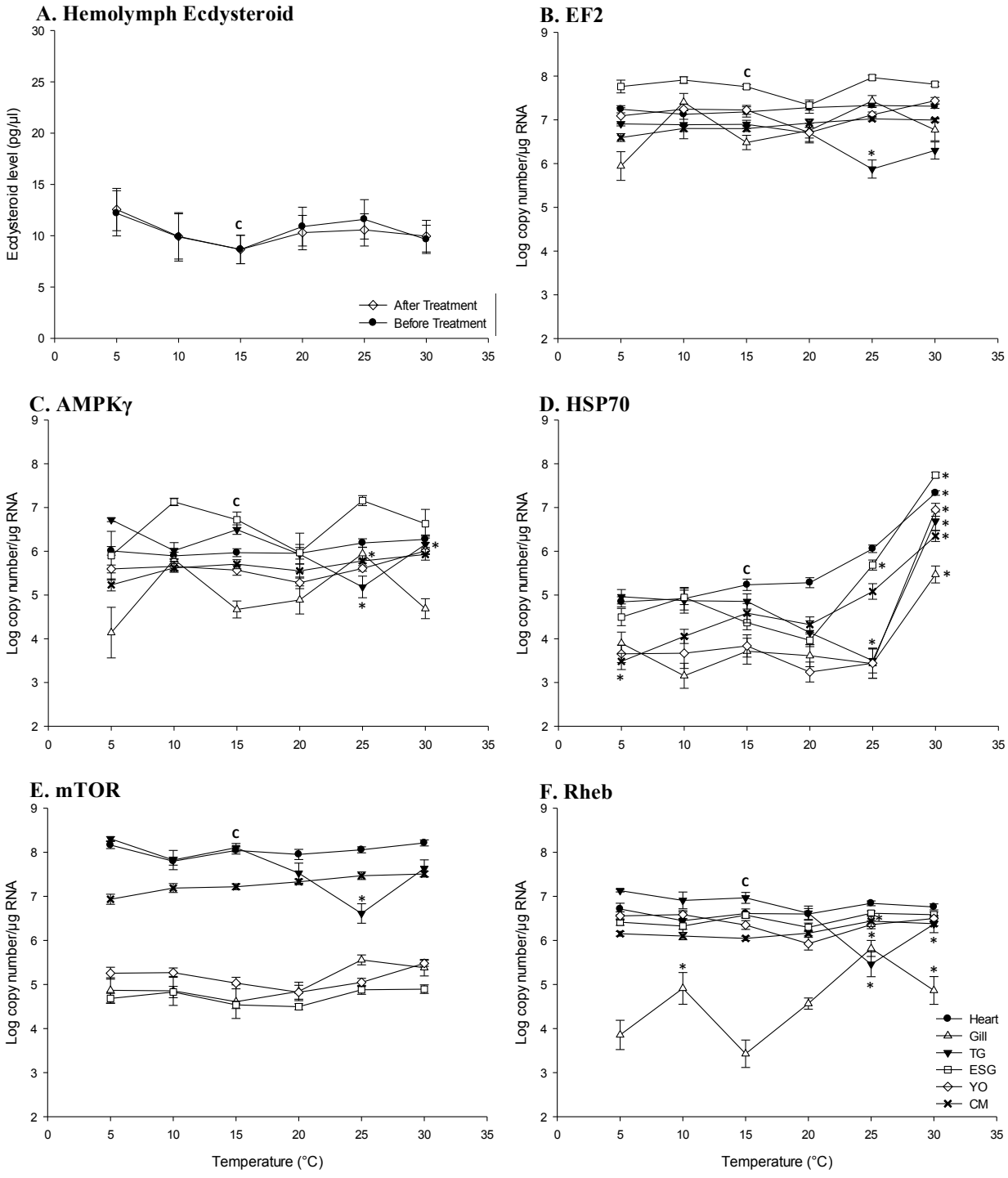
A. Hemolymph Ecdysteroid
Ecdysteroid level (pg/µl)
After Treatment
Before Treatment
B. EF2
Log copy number/µg RNA
C. AMPKγ
Log copy number/µg RNA
D. HSP70
Log copy number/µg RNA
E. mTOR
Log copy number/µg RNA
Temperature (°C)
F. Rheb
Log copy number/µg RNA
Heart
Gill
TG
ESG
YO
CM
Temperature (°C)

Fig 2.4. Effects of 1 h temperature treatment on hemolymph ecdysteroid levels and mRNA levels in *C. maenas* tissues. Animals were transferred from 15 ˚C to an experimental temperature for 1 h. Hemolymph ecdysteroid levels (A) before and after treatment were quantified by ELISA. *Cm-EF2* (B), *Cm-AMPKγ* (C), *Cm-HSP70* (D), *Cm-mTOR* (E), and *Cm-Rheb* (F) mRNA levels were quantified by qPCR. Data are presented as mean ± SEM (n for each subset is indicated in appendix). Asterisks (*) indicate a mean of an experimental temperature that was significantly different from the control (c) temperature ($p < 0.05$). Abbreviations: CM, claw muscle; ESG, eyestalk ganglia; TG, thoracic ganglion; and YO, Y-organ.

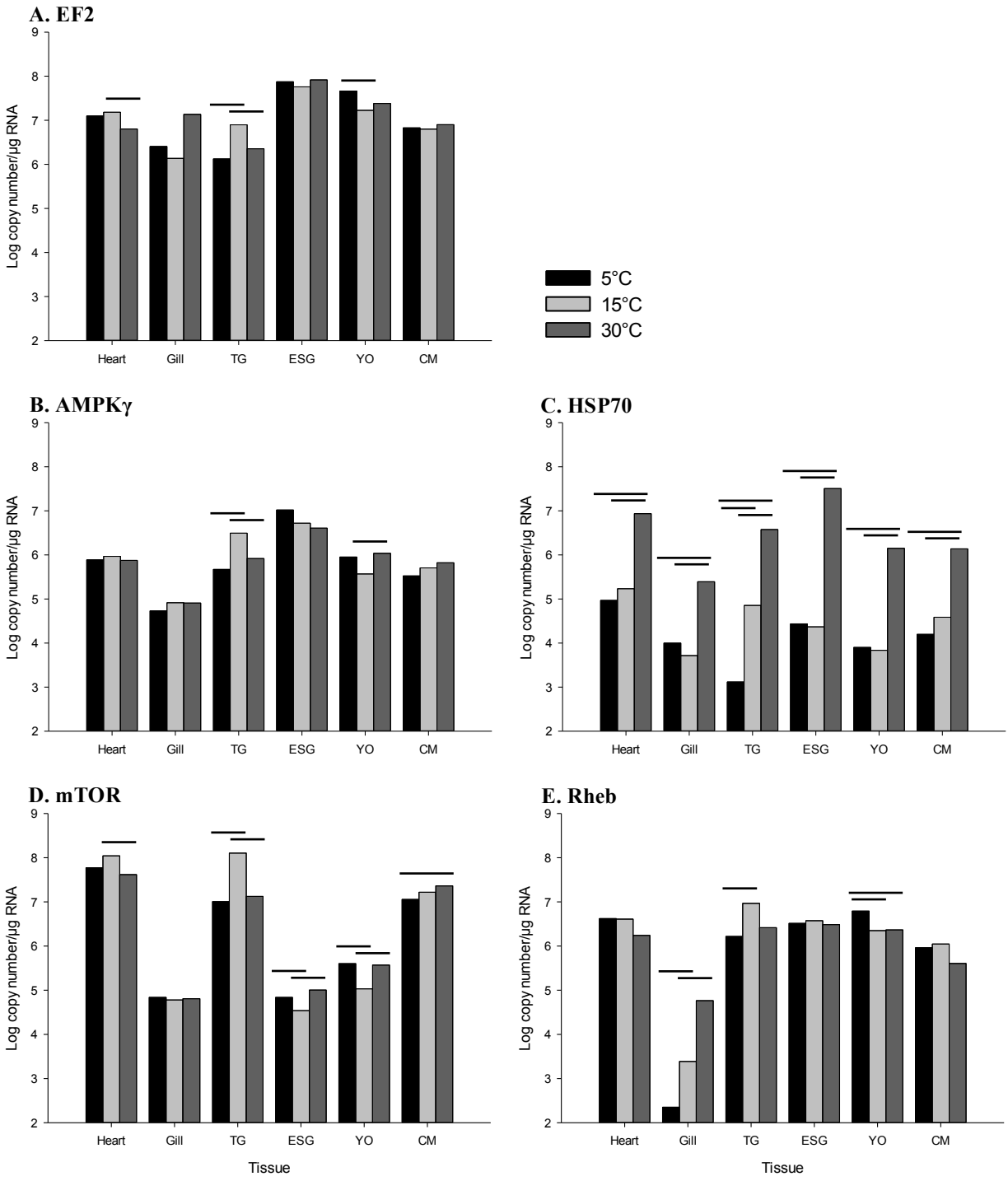
A. EF2
B. AMPKγ
C. HSP70
D. mTOR
E. Rheb
Log copy number/µg RNA
Heart
Gill
TG
ESG
YO
CM
Tissue
5°C
15°C
30°C

Fig 2.5. Effects of 2 h temperature treatment on mRNA levels in *C. maenas* tissues. Animals were transferred from 15 ˚C to an experimental temperature for 2 h. *Cm-EF2* (A), *Cm-AMPKγ* (B), *Cm-HSP70* (C), *Cm-mTOR* (D), and *Cm-Rheb* (E) mRNA levels were quantified by qPCR. Data are presented as mean ± SEM (n for each subset is indicated in appendix). Brackets indicate significance between temperatures ($p < 0.05$). Abbreviations: CM, claw muscle; ESG, eyestalk ganglia; TG, thoracic ganglion; and YO, Y-organ.

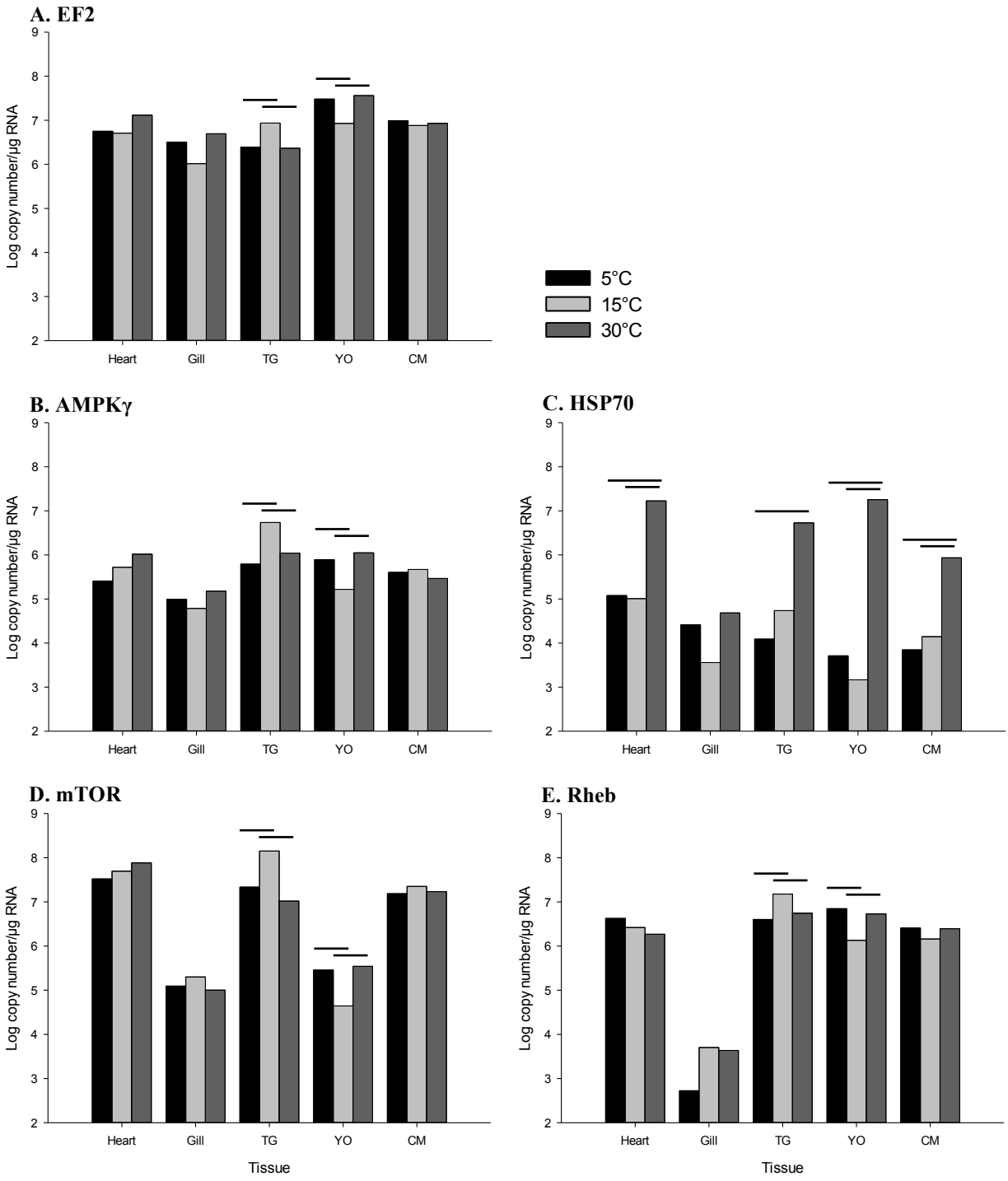
A. EF2
B. AMPKγ
C. HSP70
D. mTOR
E. Rheb
Log copy number/µg RNA
Heart
Gill
TG
YO
CM
Tissue
5°C
15°C
30°C

Fig 2.6. Effects of 1 h temperature treatment on mRNA levels ES-ablated *C. maenas* tissues. (heart, gill, TG, YO, and CM)**.** (A) Hemolymph ecdysteroid levels were quantified by ELISA. *Cm-EF2* (B), *Cm-AMPKγ* (C), *Cm-HSP70* (D), *Cm-mTOR* (E), and *Cm-Rheb* (F) mRNA expression at 5, 15 (control), and 30°C were quantified by qPCR. Data are presented as mean ± SEM (n for each subset is indicated in appendix). Brackets indicate significance between temperatures ($p < 0.05$). Abbreviations: CM, claw muscle; TG, thoracic ganglion; and YO, Y-organ.

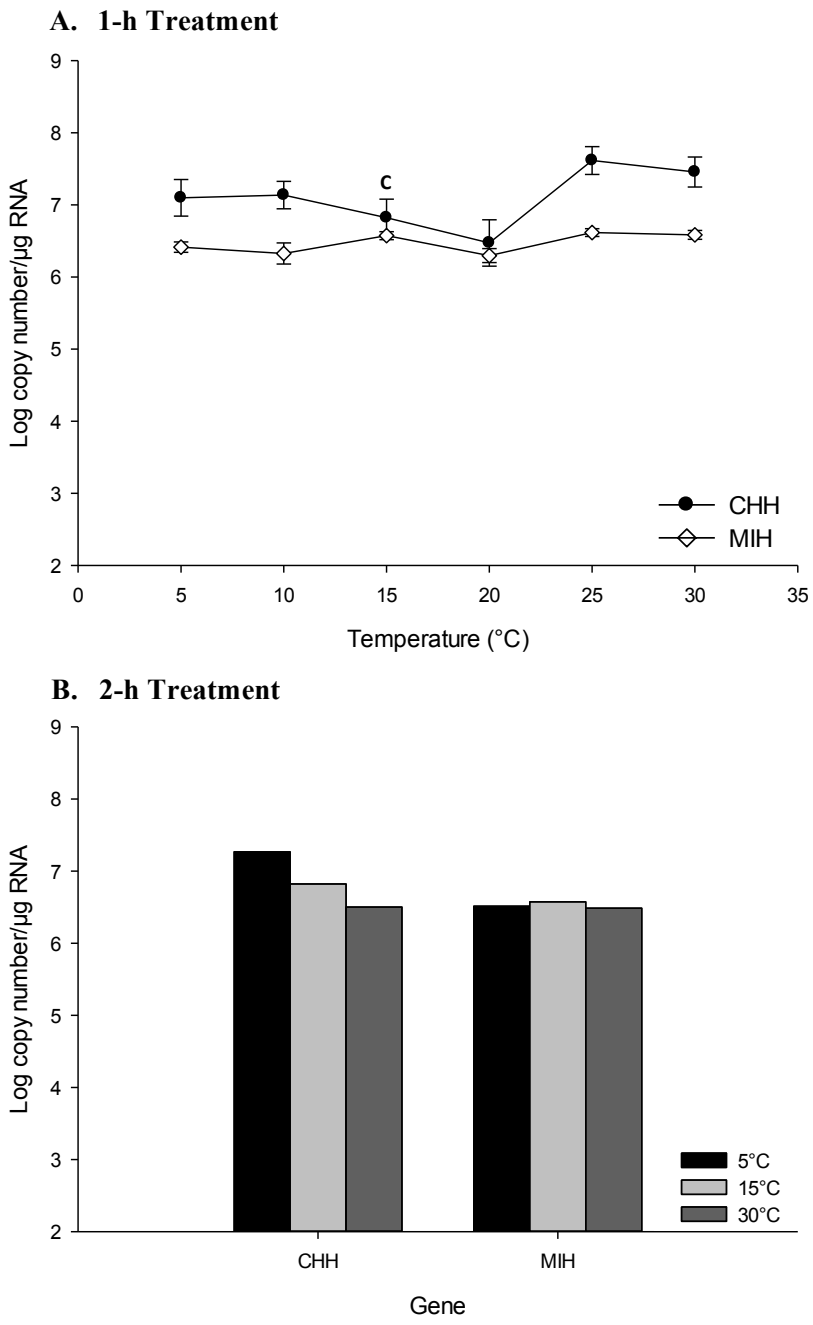

Fig 2.7. Effects of temperature on CHH and MIH mRNA levels after 1-h treatment (A) and 2-h treatment (B) in eyestalk ganglia. Data are presented as mean ± SEM (n for each subset is indicated in appendix). **C** indicates the control temperature point (15°C). There were no significant differences between temperatures.

Table 2.2. Mass (g) and carapace width (mm) for *C. maenas* used in the three treatments. Data are presented as mean ± S.E.M.

Treatment	Sex	Mass (g)	Carapace width (mm)	Sample Size
Intact, one hour immersion	M	92.6 ± 4.3	72.6 ± 1.2	36
	F	60.7 ± 2.5	64.9 ± 0.9	36
Intact, two hour immersion	M	83.0 ± 5.0	70.1 ± 1.5	12
	F	56.5 ± 5.8	62.9 ± 2.1	12
ESA, one hour immersion	M	135.2 ± 10.1	81.7 ± 1.9	12

Table 2.3. ANOVA analysis of mean mRNA levels after acute temperature treatment of *C. maenas* for 1-h. The fold differences are given for the genes that showed significant changes ($P < 0.05$) between the indicated temperatures, where the reference temperature is the first temperature in the pair. Tissues: heart, gill, thoracic ganglion (TG), eyestalk ganglia (ESG), Y-organ (YO), and claw muscle (CM). Data are presented in Fig. 4.

Gene	Tissue	Temperature Comparison	Fold Change
EF2	Gill	5°C vs. 25°C	5.6
		5°C vs. 10°C	2.1
	TG	5°C vs. 25°C	-7.3
		15°C vs. 25°C	-5.2
	ESG	10°C vs. 20°C	-3.4
		20°C vs. 25°C	3.8
	YO	20°C vs. 30°C	4.7
	CM	5°C vs. 20°C	2.2
		5°C vs. 25°C	2.2
		5°C vs. 30°C	2.2
AMPKγ	Gill	5°C vs. 25°C	8.6
		15°C vs. 25°C	11.1
		5°C vs. 25°C	-23.2
	TG	15°C vs. 25°C	-15.2
		15°C vs. 30°C	2.1
	YO	20°C vs. 30°C	4.9
	CM	5°C vs. 30°C	4.6
HSP70	Heart	5°C vs. 30°C	345
		10°C vs. 30°C	347
		15°C vs. 30°C	119
		20°C vs. 30°C	121
		5°C vs. 25°C	21
		10°C vs. 25°C	21
	Gill	5°C vs. 30°C	22
		10°C vs. 30°C	151
		15°C vs. 30°C	34
		20°C vs. 30°C	36
		25°C vs. 30°C	87
	TG	5°C vs. 30°C	58
		10°C vs. 30°C	55
		15°C vs. 30°C	72
		20°C vs. 30°C	182
		25°C vs. 30°C	2657
		10°C vs. 25°C	-49
		15°C vs. 25°C	-67

Table 2.3 (continued).

	ESG	5°C vs. 30°C	571
		10°C vs. 30°C	651
		15°C vs. 30°C	1590
		20°C vs. 30°C	4886
		25°C vs. 30°C	109
		5°C vs. 25°C	5
		10°C vs. 25°C	6
		15°C vs. 25°C	15
		20°C vs. 25°C	45
		10°C vs. 20°C	8
	YO	5°C vs. 30°C	1646
		10°C vs. 30°C	1156
		15°C vs. 30°C	726
		20°C vs. 30°C	2441
		25°C vs. 30°C	1484
	CM	5°C vs. 30°C	307
		10°C vs. 30°C	151
		15°C vs. 30°C	29
		20°C vs. 30°C	76
		25°C vs. 30°C	9
		5°C vs. 25°C	35
		10°C vs. 25°C	17
		20°C vs. 25°C	9
		5°C vs. 15°C	11
		5°C vs. 20°C	4
mTOR	Heart	10°C vs. 30°C	3.2
	TG	5°C vs. 25°C	-35.5
		15°C vs. 25°C	-22.9
	YO	20°C vs. 30°C	4.6
	CM	5°C vs. 20°C	1.9
		5°C vs. 25°C	3
		5°C vs. 30°C	2.5

Table 2.3 (continued).

Rheb	Gill	10°C vs. 15°C	14.8
		5°C vs. 25°C	57.2
		15°C vs. 25°C	232
		20°C vs. 25°C	26
		15°C vs. 30°C	44.6
	TG	5°C vs. 25°C	-16.5
		10°C vs. 25°C	-15
		15°C vs. 25°C	-11
	YO	5°C vs. 20°C	-3.8
		10°C vs. 20°C	-3.5
	CM	15°C vs. 25°C	2.5
		15°C vs. 30°C	2.2

Table 2.4. ANOVA analysis of mean mRNA levels after acute temperature treatment of *C. maenas* for 2-h. The fold differences are given for the genes that showed significant changes ($P < 0.05$) between the indicated temperatures, where the reference temperature is the first temperature in the pair. Tissues: heart, gill, thoracic ganglion (TG), eyestalk ganglia (ESG), Y-organ (YO), and claw muscle (CM). Data are presented in Fig. 5.

Gene	Tissue	Temperature Comparison	Fold Change
EF2	Heart	15°C vs. 30°C	-3.4
	TG	5°C vs. 15°C	5.2
		15°C vs. 30°C	-2.5
	YO	5°C vs. 15°C	-2.3
AMPKγ	TG	5°C vs. 15°C	6
		15°C vs. 30°C	-3.3
	YO	15°C vs. 30°C	2
HSP70	Heart	5°C vs. 30°C	76
		15°C vs. 30°C	48
	Gill	5°C vs. 30°C	41
		15°C vs. 30°C	34
	TG	5°C vs. 30°C	1603
		5°C vs. 15°C	35.9
		15°C vs. 30°C	45
	ESG	5°C vs. 30°C	1072
		15°C vs. 30°C	975
	YO	5°C vs. 30°C	152
		15°C vs. 30°C	75
	CM	5°C vs. 30°C	76
		15°C vs. 30°C	24
mTOR	Heart	15°C vs. 30°C	-2.5
	TG	5°C vs. 15°C	11.8
		15°C vs. 30°C	-8.7
	ESG	5°C vs. 15°C	-2.7
		15°C vs. 30°C	3.5
	YO	5°C vs. 15°C	-2.9
		15°C vs. 30°C	1.4
	CM	5°C vs. 30°C	2
Rheb	Gill	5°C vs. 30°C	38.7
		15°C vs. 30°C	13.9
	TG	5°C vs. 15°C	5.6
	YO	5°C vs. 15°C	-2.4
		5°C vs. 30°C	-2.3

Table 2.5. ANOVA analysis of mean mRNA levels after acute temperature treatment of eyestalk-ablated *C. maenas* for 1 h. The fold differences are given for the genes that showed significant changes ($P < 0.05$) between the indicated temperatures, where the reference temperature is the first temperature in the pair. Tissues: heart, gill, thoracic ganglion (TG), eyestalk ganglia (ESG), Y-organ (YO), and claw muscle (CM). Data are presented in Fig. 6.

Gene	Tissue	Temperature Comparison	Fold Change
EF2	TG	5°C vs. 15°C	2.8
		15°C vs. 30°C	-2.9
	YO	5°C vs. 15°C	-3.9
		15°C vs. 30°C	3.9
AMPKγ	TG	5°C vs. 15°C	7
		15°C vs. 30°C	-3.6
	YO	5°C vs. 15°C	-4.3
		15°C vs. 30°C	9.6
HSP70	Heart	5°C vs. 30°C	30
		15°C vs. 30°C	137
	TG	5°C vs. 30°C	635.8
	YO	5°C vs. 30°C	2120
		15°C vs. 30°C	21800
	CM	5°C vs. 30°C	144
		15°C vs. 30°C	82
mTOR	TG	5°C vs. 15°C	7
		15°C vs. 30°C	-6.3
	YO	5°C vs. 15°C	-5.9
		15°C vs. 30°C	2.4
Rheb	TG	5°C vs. 15°C	3.2
		15°C vs. 30°C	-2.9
	YO	5°C vs. 15°C	-3.2
		15°C vs. 30°C	3.8

REFERENCES

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

Abuhagr, A., Maclea, K. S., Chang, E. S. and Mykles, D. L. (2014). 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*., vol. 168, pp. 25-39: Comp Biochem Phys A.

Appelhans, Y., Thomsen, J., Pansch, C., Melzner, F. and Wahl, M. (2012). Sour times: seawater acidification effects on growth, feeding behavior, and acid-base status of *Asteria rubens* and *Carcinus maenas*., vol. 459, pp. 85-97: Mar Ecol Prog Ser.

Avruch, J., Hara, K., Lin, Y., Liu, M., Long, X., Ortiz-Vega, S. and Yonezawa, K. (2006). Insulin and amino-acid regulation of mTOR signaling and kinase activity through the Rheb GTP-ase, vol. 25, pp. 6361-6372: Oncogene.

Baker, M. R., Gobush, K. S. and Vynne, C. H. (2013). Review of factors influencing stress hormones in fish and wildlife, vol. 21, pp. 309-318: J Nat Conserv.

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

Burgos, S.A., Kim, J.J.M., Dai, M., Cant, J.P. (2013). Energy depletion of bovine mammary epithelial cells activates AMPK and suppresses protein synthesis through inhibition of mTORC1 signaling. In *Horm Metab Res*, vol. 45, pp. 183-189.

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*, vol. 30, pp. 1809-1820: J Biogeogr.

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

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, vol. 39, pp. 487-495: Integr Comp Biol.

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, vol. 111, pp. 359-366: Gen Comp Endocr.

Chang, E. S. and Mykles, D. L. (2011). Regulation of crustacean molting: a review and our perspectives, vol. 172, pp. 323-330: Gen Comp Endocr.

Chang, E. S. and O'Connor, J. D. (1977). *In vitro* secretion and hydroxylation of alpha-ecdysone as a function of the crustacean molt cycle, vol. 36, pp. 151-160: Gen Comp Endocr.

Chung, J. S., Zmora, N., Katayama, H. and Tsutsui, N. (2010). Crustacean hyperglycemic hormone (CHH) neuropeptides family: functions, titer, and binding to target tissues, vol. 166, pp. 447-454: Gen Comp Endocr.

Clark, T. D., Sandblom, E. and Jutfelt, F. (2013). Aerobic scope measurements of fishes in an era of climate change: respirometry, relevance and recommendations, vol. 216, pp. 2771-2782: J Exp Biol.

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*), vol. 16, pp. 243-255: Diversity and Distributions.

Cookie, I. M. and Sullivan, R. E. (1982). The Biology of Crustacea- Ch 6, Hormones and Neurosecretion. New York, NY: Academic Press, Inc.

Covi, J. A., Bader, B. D., Chang, E. S. and Mykles, D. L. (2010). Molt cycle regulation of protein synthesis in skeletal muscle of the blackback land crab, *Gecarcinus lateralis,* and the differential expression of a myostatin-like factor during atrophy indicated by molting or unweighting, vol. 213, pp. 172-183: J Exp Biol.

Covi, J. A., Chang, E. S. and Mykles, D. L. (2012). Neuropeptide signaling mechanisms in crustacean and insect molting glands, vol. 56, pp. 33-49: Invertebr Repr Dev.

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*, vol. 17, pp. 4992-5007: Mol Ecol.

Das, A. K., Yang, Q.-Y., Fu, X., Liang, J.-F., Duarte, M. S., Zhu, M.-J., Trobridge, G. D. and Du, M. (2012). AMP-activated protein kinase stimulates myostatin expression in C2C12 cells. *Biochem Biophys Res Comm* **427**, 36-40.

Das, S. and Durica, D. S. (2013). Ecdysteroid receptor signaling disruption obstructs blastema cell proliferation during limb regeneration in the fiddler crab, *Uca pugilator*, vol. 365, pp. 249-259: Mol Cell Endocr.

Feder, M. E. and Hofmann, G. E. (1999). Heat-shock proteins, molecular chaperones, and the stress response, vol. 61, pp. 243-282: Annu Rev Phys.

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*., vol. 212, pp. 722-730.: J Exp Biol.

Frederich, M. and Pörtner, H. O. (2000). Oxygen limitation of thermal tolerance defined by cardiac and ventilatory performance in spider crab, *Maja squinado*, vol. 279, pp. 1531-1538: Am J Physiol.

Gorr, T. A., Wichmann, D., Hu, T., Hermes-Lima, M., Welker, A. F., Terwilliger, N., Wren, J. R., Viney, M., Morris, S., Nilsson, G. E. et al. (2010). Hypoxia tolerance in animals: Biology and application, vol. 83, pp. 733-752: Physiol Biochem Zool.

Guévélou E., Huvet, A., Sussarellu, R., Milan, M., Guo, X., Li, L., Zhang, G., Quillien, V., Daniel, J.-Y., Quere, C., Boudry, P., Corporeau, C. (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*, vol. 183, pp. 597-611: J Comp Phys B.

Hall, D. J., Grewal, S. S., De La Cruz, A. F. A. and Edgar, B. A. (2007). Rheb-TOR signaling promotes protein synthesis, but not glucose or amino acid import, in *Drosophila*, vol. 5: BMC Biol.

Han, G., Zhang, S., Marshall, D. J., Ke, C. and Dong, Y. (2013). Metabolic energy sensors (AMPK and SIRT1), protein carbonization and cardiac failure as biomarkers of thermal stress in an intertidal limpet: linking energetic allocation with environmental temperature during aerial emersion, vol. 216, pp. 3273-3282: J Exp Biol.

Hardie, D. G. (2012). AMP-activated/SNF1 protein kinases: conserved guardians of cellular energy, vol. 8, pp. 774-785: Nature.

Hardie, D. G., Hawley, S. A. and Scott, J. W. (2006). AMP-activated protein kinase-development of the energy sensor concept, vol. 574, pp. 7-15: J Physiol.

Havird, J. C., Santos, S. R. and Henry, R. P. (2014). Osmoregulation in the Hawaiian anchialine shrimp Halocaridina rubra (Crustacea: Atyidae): expression of ion transporters, mitochondria-rich cell proliferation, and hemolymph osmolality during salinity transfers. *The Journal of Experimental Biology*.

Hay, N. and Sonenberg, N. (2004). Upstream and downstream of mTOR, vol. 18, pp. 1926-1945: Genes Dev.

Hensold, J. O., Hunt, C. R., Calderwood, S. K., Housman, D. E. and Kingston, R. E. (1990). DNA binding of heat shock factor to the heat shock element in insufficient for transcriptional activation in murine erythroleukemia cells, vol. 41, pp. 1600-1608: Mol Cell Biol.

Hopkins, P. M. (2012). The eyes have it: A brief history of crustacean neuroendocrinology, vol. 175, pp. 357-366: Gen Comp Endocr.

Hopkins, P. M., Bliss, D. E., Sheehan, S. W. and Boyer, J. R. (1979). Limb growth-controlling factors in the crab *Gecarcinus lateralis* with special reference to the limb growth-inhibiting factor, vol. 39, pp. 192-207: Gen Comp Endocr.

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, vol. 444, pp. 463-474: Mar Ecol Prog Ser.

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, vol. 4, pp. 1-34: Aquaculture.

Katayama, H., Ohira, T. and Nagasawa, H. (2013). Crustacean peptide hormones: structure, gene expression, and function, vol. 6, pp. 49-90: Aqua-BioSci Monogr.

Kemp, B. E. (2004). Bateman domains and adenosine derivatives form a binding contract, vol. 113, pp. 182-184: J Clin Invest.

Kiang, J. G. and Tsokos, G. C. (1998). Heat shock protein 70 kDa: Molecular biology, biochemistry, and physiology, vol. 80, pp. 183-201: Pharm Therap.

Klassen, G. and Locke, A. (2007). A biological synopsis of the European green crab, *Carcinus maenas*. , pp. 75: Can Manuscr Rep Fish Aquat Sci.

Kregel, K. C. (2002). Heat shock proteins: modifying factors in physiological stress responses and acquired thermotolerance, vol. 92, pp. 2177-2186: J Appl Phys.

Lafferty, K. D. and Kuris, A. M. (1999). How environmental stress affects the impacts of parasites, vol. 44, pp. 925-931: Limnol Oceanogr.

Laplante, M. and Sabatini, D. M. (2012). mTOR signaling in growth control and disease, vol. 149, pp. 274-293: Cell.

Layalle, S., Arquier, N. and Leopold, P. (2008). The TOR pathway couples nutrition and developmental timing in *Drosophila*, vol. 15, pp. 568-577: Dev Cell.

Lee, C. Y., Tsai, K. W., Tsai, W. S., Jiang, J. Y. and Chen, Y. J. (2014). Crustacean hyperglycemic hormone: structural variants, physiological function, and cellular mechanism of action, vol. 22, pp. 75-81: J Mar Sci Technol Taiwan.

Lee, K. J., Doran, R. M. and Mykles, D. L. (2007a). Crustacean hyperglycemic hormone from the tropical land crab, *Gecarcinus lateralis*: Cloning, isoforms, and tissue expression., vol. 154, pp. 174-183: Gen Comp Endocr.

Lee, K. J., Kim, H. W., Gomez, A. M., Chang, E. S., Covi, J. A. and Mykles, D. L. (2007b). Molt-inhibiting hormone from the tropical land crab, *Gecarcinus lateralis:* Cloning, tissue

expression, and expression of biologically active recombinant peptide in yeast, vol. 150, pp. 505-513: Gen Comp Endocr.

Leffler, C. W. (1972). Some effects of temperature on the growth and metabolic rate of juvenile blue crabs, *Callinectes sapidus,* in the laboratory, vol. 14, pp. 104-110: Mar Biol.

Lindquist, S. and Craig, E. A. (1988). The heat-shock proteins, vol. 22, pp. 631-677: Annu Rev Genet.

Long, W. C., Swiney, K. M., Harris, C., Page, H. N. and Foy, R. J. (2013). Effects of ocean acidification on juvenile Red King Crab (*Paralithodes camtschaticus*) and Tanner Crab (*Chionoecetes bairdi*) growth, condition, calcification, and survival., vol. 8, pp. 1-10: PLoS One.

Ma, X. M. and Blenis, J. (2009). Molecular mechanisms of mTOR-mediated translational control, vol. 10, pp. 307-318: Mol Cel Biol.

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 in skeletal muscle, vol. 215, pp. 590-604: J Exp Biol.

Mallouk, Y., Vayssier-Taussat, M., Bonventre, J. V. and Polla, B. S. (1999). Heat shock protein 70 and ATP as partners in cell homeostasis, vol. 4, pp. 463-537: Int J Mol Med.

Mattson, M. P. and Spaziani, E. (1985). Stress reduces hemolymph ecdysteroid levels in the crab: Mediation by the eyestalks, vol. 234, pp. 319-323: J Exp Biol.

McCarthy, J. F. and Skinner, D. M. (1977). Proecdysial changes in serum ecdysone titers, gastrolith formation, and limb regeneration following molt induction by limb bud autonomy and/or eyestalk removal in the land crab, *Gecarcinus lateralis*, vol. 33, pp. 278-292: Gen Comp Endocr.

McDonald, A. A., Chang, E. S. and Mykles, D. L. (2011). Cloning of a nitric oxide synthase from green shore crab, *Carcinus 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*, vol. 158, pp. 150-162: Comp Biochem Phys A.

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*, vol. 37, pp. 570-578: J Therm Biol.

Morimoto, R. I. (1998). Regulation of the heat shock transcriptional response: cross talk between a family of heat shock factors, molecular chaperones, and negative regulators, vol. 12, pp. 3788-3796: Gene Dev.

Mykles, D. L. (2011). Ecdysteroid metabolism in crustaceans, vol. 127, pp. 196-203: J Steroid

Biochem.

Patel, P. H., Thapar, N., Guo, L., Martinez, M., Maris, J., Gau, C. L., Lengyel, J. A. and Tamanoi, F. (2003). *Drosophila* Rheb GTPase is required for cell cycle progression and cell growth, vol. 116, pp. 3601-3610: J Cell Sci.

Podolski, S. (2011). Activation of AMP-activated protein kinase as an early indicator for stress in the lobster, Homarus americanus. *Theses* **Paper 1**.

Qian, Z., Liu, X., Wang, L., Wang, X., Xiang, J. and Wang, P. (2012). Gene expression profiles for four heat shock proteins in response to different acute stresses in shrimp, *Litopenaeus vannamei*, vol. 156, pp. 211-220: Comp Biochem Phys C.

Quackenbush, L. S. (1986). Crustacean endocrinology, a review, vol. 43, pp. 2271-2282: Can J Fish Aquat Sci.

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 mono don*, vol. 34, pp. 1082-1089: Dev Comp Immunol.

Schreck, C. B., Contreras-Sanchez, W. and Fitzpatrick, M. S. (2001). Effects of stress on fish reproduction, gamete quality, and progeny, vol. 197, pp. 3-24: Aquaculture.

Shelford, V. E. (1931). Some concepts of bioecology, vol. 12, pp. 455-467: Ecology.

Sibly, R. M. and Atkinson, D. (1994). How rearing temperature affects optimal size in ectotherms, vol. 8, pp. 486-493: Funct Ecol.

Snyder, M. J. and Mulder, E. P. (2001). Environmental endocrine disruption in decapod crustacean larvae: hormone titers, cytochrome P450, and stress protein responses to heptachlor exposure, vol. 55, pp. 177-190: Aquat Toxicol.

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., vol. 7, pp. 97-106: Cell Stress Chaperon.

Stentiford, G. D., Chang, E. S., Chang, S. A. and Neil, D. M. (2001). Carbohydrate dynamics and the crustacean hyperglycemic hormone (CHH): Effects of parasitic infection in Norway Lobsters (*Nephrops norvegicus)*, vol. 121, pp. 13-22: Gen Comp Endocr.

Stocker, T. F., Qin, D., Plattner, G.-K., Tignor, M., Allen, S. K., Böschung, J., Nauels, A., Xia, Y., Bex, V. and Midgley, P. M. (2014). *Climate Change 2013: The Physical Science Basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change*. Cambridge, United Kingdom and New York,

NY, USA.: Cambridge University Press.

Stoner, A. W., Ottmar, M. L. and Coperman, L. A. (2010). Temperature effects on the molting, growth, and lipid composition of newly-settled red king crab, vol. 393, pp. 138-147: J Exp Mar Biol Ecol.

Suzuki, T., Bridges, D., Nakasa, D., Skiniotis, G., Morrison, S. J., Lin, J. D., Saltiel, A. R. and Inoki, K. (2013). Inhibition of AMPK Catabolic Action by GSK3, vol. 50, pp. 407-419: Mol Cell.

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*., vol. 217, pp. 1129-1138: J Exp Biol.

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, vol. 213, pp. 3559-3574: J Exp Biol.

Towle, D. W., Henry, R. P. and Terwilliger, N. B. (2011). Micro-array detected changes in gene expression in gill of green crabs (*Carcinus maenas*) upon dilution of environmental salinity, vol. 6, pp. 115-125: Comp Biochem Phys D.

Van Weerd, J. H. and Komen, J. (1998). The effects of chronic stress on growth in fish: a critical appraisal., vol. 120, pp. 107-112: Comp Biochem Phys A.

Webster, S. G., Keller, R. and Dircksten, H. (2012). The CHH-superfamily of multifunctional peptide hormones controlling crustacean metabolism, osmoregulation, moulting, and reproduction., vol. 175, pp. 217-233: Gen Comp Endocr.

Wittman, A. C., Pörtner, H. O. and Sartoris, F. J. (2012). A role for oxygen delivery and extracellular magnesium in limiting cold tolerance of the Sub-Antarctic Stone Crab *Paralomis granulosa*, vol. 85, pp. 285-298: Physiol Biochem Zool.

Xiao, B., Sanders, M. J., Underwood, E., Heath, R., Mayer, F. V., Carmena, D., Jing, C., Walker, P. A., Eccleston, J. F., Haire, L. F. et al. (2011). Structure of mammalian AMPK and its regulation by ADP. *Nature* **472**, 230-233.

Xiao-Jing, Z., Feng, C.-Z., Dai, Z.-M., Zhang, R.-C. and Yang, W.-J. (2007). AMPK alpha subunit gene characterization in Artemia and expression during development and in response to stress. *Stress* **10**, 53-63.

Xu, J., Ji, J. and Yan, X. H. (2012). Cross-talk between AMPK and mTOR in regulating energy balance, vol. 52, pp. 373-381: Crit Rev Food Sci Nutr.

Zarubin, T. P., Chang, E. S. and Mykles, D. L. (2009). Expression of recombinant eyestalk crustacean hyperglycemic hormone from the tropical land crab, *Gecarcinus lateralis,* that

inhibits Y-organ ecdysteroidogenesis *in vitro*., vol. 36, pp. 1231-1237: Mol Biol Rep.

Zhao, L., Pridgeon, J. W., Becnel, J. J., Clark, G. G. and Linthicum, K. J. (2009). Identification of genes differentially expressed during heat shock treatment in *Aedes aegypti*, vol. 46, pp. 490-495: J Med Entomol.

Zhou, J., Wang, L., Xin, Y., Wang, W., He, W., Wang, A. and Liu, Y. (2010). Effect of temperature on antioxidant enzyme gene expression and stress protein response in white shrimp, *Litopenaeus vannamei* vol. 35, pp. 284-289: J Therm Biol.

Zilka, A., Garalapati, S., Dahan, E., Yoalsky, V. and Shapira, M. (2001). 3' Processing and mRNA stability control transcript abundance, and translation is directed by a determinant in the 3' untranslated region, vol. 276, pp. 47922-47929: J Biol Chem.

CHAPTER THREE

MYOSTATIN AND mTOR EXPRESSION IN THE Y-ORGAN AFTER MOLT CYCLE MANIPULATION IN THE BLACKBACK LAND CRAB, *GECARCINUS LATERALIS*

Summary

Ecdysteroids produced from the molting gland (Y-organ or YO) induce molting in decapod crustaceans. Reduction in molt-inhibiting hormone (MIH) activates the YO and animals enter premolt. At mid-premolt, YOs transition to the committed state, during which ecdysteroid production increases further. In blackback land crab (*Gecarcinus lateralis*), SB1431542, an inhibitor of Activin receptors, decreases hemolymph ecdysteroid titers in premolt animals, suggesting that an Activin-like transforming-growth factor (TGF-β) is produced by the activated YO and drives the transition of the YO to the committed state. Myostatin (Gl-Mstn) is an Activin-like factor that is highly expressed in skeletal muscle. Rapamycin lowers hemolymph ecdysteroid titers by inhibiting mTOR, which controls global translation of mRNA into protein. As *Gl-Mstn* is expressed in tissues in addition to muscle, the effects of molting on *Gl-Mstn* expression in the YO were determined, and compared to prior results of *Gl-mTOR*, *Gl-Rheb*, *Gl-Akt*, and *Gl-S6K* expression in the YO. Endpoint RT-PCR established that *Gl-Mstn* was expressed in the YO. Quantitative PCR was used to quantify the effects of molt induction by eyestalk ablation (ESA) on gene expression. YOs were harvested from intact (intermolt) animals and from animals at 1, 3, 5, 7, and 14 days post-ESA. Expression of mTOR components peaked at 3 days post-ESA, which is consistent with the increased activity required for activation of the YO. *Gl-Mstn* expression also peaked at 3 days post-ESA, which is before the transition to the

committed state at 7 days post-ESA. Expression of ecdysteroid receptor (*Gl-EcR* and *Gl-RXR*) did not change. Expression of elongation factor-2 (*Gl-EF2*) increased at 7 days post-ESA, indicating an increase in protein synthetic capacity coincides with YO commitment to molt. The SB431542 compound seemed to affect the gene expression in the YO between 7 and 14 days post-ESA, as hemolymph levels were lower in experimental animals. Rapamycin affected the gene expression as early as 1 day post-ESA, as there were significant differences in hemolymph concentration and expression of *Gl-Mstn* was delayed at 3 days post-ESA. These results indicate that mTOR components are involved in activation of the YO, and Mstn is involved in transitioning the YO to the committed state.

Introduction

Decapod crustaceans must molt in order to grow. These animals molt periodically at predictable intervals, which are based on environmental conditions, such as temperature and photoperiod. The Y-organ (YO) is the crustacean molting gland that secretes ecdysteroids (molting hormones), particularly ecdysone (Mykles, 2011). Molt-inhibiting hormone (MIH) is an inhibitory peptide that suppresses ecdysteroidogenesis in the YO during intermolt (Webster and Keller, 1986). The rate of ecdysteroidogenesis increases in response to a decrease in MIH secretion, which occurs prior to onset of the premolt period. MIH is secreted primarily from the X-organ/sinus gland complex located in the eyestalk ganglia, although secondary locations have recently been identified in other nervous tissues in *Carcinus maenas* as well (Abuhagr et al. 2014a; Huang et al. 2014). The YO is homologous to the insect prothoracic gland, which secretes ecdysteroids in response to a signal from the prothoracicotropic hormone (PTTH). Contrary to the crustacean YO, which is negatively regulated by MIH, PTTH stimulates molting in insects (Chang, 1993).

When MIH levels decrease in response to environmental cues, molting is initiated and the animal enters premolt (Stage D_0). At this point, the YO has become activated (Chang and Mykles, 2011). Prior to mid-premolt (D_{1-2}), a putative transforming growth factor β (TGF-β) is up regulated, which commits the animal to proceed through premolt and molt. Before commitment, the animal can pause the molt cycle and decrease ecdysteroid synthesis (Skinner, 1985). This can be performed experimentally by removal of a primary limb bud (LB) regenerate. Limb buds are grown to replace lost walking legs or chelae during premolt, which allows the animals to molt with a full set of appendages (Yu et al. 2002). Once the animal has reached the committed state, it must proceed through the molt cycle, even if LBs are lost after this point. At the committed state, the animal also has a low sensitivity to MIH. Hemolymph ecdysteroid levels continue to rise until late premolt (D_{3-4}), and drop precipitously immediately prior to ecdysis (E) (Skinner, 1985; Mykles, 2011). Basal ecdysteroid levels return post molt.

The molt cycle can be manipulated in crustaceans, particularly *Gecarcinus lateralis*, the blackback land crab. Eyestalk ablation (ESA) removes the source of MIH, thus effectively allowing the YO to become activated and the animal can enter premolt; this method allows molting to be induced and proceed rapidly (e.g. Costlow, 1963; McCarthy and Skinner, 1977). ESA provides a predictable time frame in which the animal will molt, allowing researchers to be able to harvest tissues at 3, 7, and 14 days which correspond to the YO activated, committed, and repressed states. Alternatively, multiple leg autonomy (MLA) can be performed, where five or more walking legs can be autotomized, which triggers a reduction in MIH. This method represents a more natural mode of molt induction by mimicking the effects of a predator or injury (Skinner and Graham, 1972).

During premolt, global protein synthesis in the YO is increased. A primary player in protein synthesis, mechanistic Target of Rapamycin (mTOR) is a serine/threonine protein kinase that is highly conserved. mTOR is a major regulator of protein synthesis in the insect and crustacean molting gland (Covi et al., 2010; Abuhagr et al., 2014b; Layalle et al., 2008). mTOR combines with Rheb (Ras homolog enriched in brain), Raptor, and GβL (also known as LST8) to form mTOR Complex-1 (mTORC1)(Ma and Blenis, 2009). This pathway has been shown to be involved in many essential functions, such cell growth and survival, as well as cancer (Hay and Sonenberg, 2004), and is driven by nutrient-rich cellular conditions (Proud, 2009). mTORC1 is a downstream target of the Akt/PI_3 kinase insulin-signaling pathway, which is crucial for growth, development, molting, and reproduction (Rommel et al., 2001). mTORC1 is activated when Rheb exchanges GTP for GDP. mTORC1 phosphorylates ribosomal S6 kinase (S6K) and 4E-binding protein 1 (4EBP1), which stimulates global translation of mRNA to protein (Holz et al., 2005; Inoki et al. 2012). mTORC1 activity can be inhibited by Rheb-GAP (GTPase-activating protein; also known as TSC1/TSC2 or tuberous sclerosis complex 1 and 2). Reduced protein synthesis indicates a reduction of growth at the cellular level. Elongation factor-2 (EF2), like mTOR, is involved in translation (Ryazanov et al., 1988). mTOR, Rheb, Akt, S6K, and EF2 are expressed in all crustacean tissues, including the YO (Abuhagr et al., 2014b; MacLea et al., 2012). In *G. lateralis*, *Gl-mTOR*, *Gl-Akt*, and *Gl-EF2* are up regulated in the YO during premolt and rapamycin inhibits YO ecdysteroidogenesis *in vitro* (Abuhagr et al., 2014b), which suggests that YO activation requires mTORC1-dependent protein synthesis.

Myostatin (Mstn) is a member of the TGF-β superfamily, particularly the Activin signaling group. This superfamily consists of cytokines that regulate genes through transcriptional activation or repression through Smad signaling (Weiss and Attisano, 2013). In mammals, Mstn

is expressed in skeletal muscle and is a negative regulator of muscle growth (Lee and McPherron, 2001). In mammalian skeletal muscle, Mstn inhibits mTORC1-dependent protein synthesis (Otto and Patel, 2010). In crustaceans, a Mstn-like factor is involved in the atrophy of the claw closer muscle during premolt, functioning as a repressor of protein synthesis. The reduction in muscle size allows the appendage to be pulled through the basi-ischial joint during ecdysis (Covi et al., 2008, 2010). However, unlike mammalian Mstn, the crustacean Mstn-like gene is expressed in other tissues (Covi et al., 2008), suggesting that it functions as a general regulator of cellular protein synthesis and growth. Previous work hypothesized that a TGF-β factor, specifically one that binds to an Activin receptor, may be involved in the transition from the activated to the committed state at mid-premolt. This would result in mTOR activation and continuous expression, up-regulation of enzymes that are involved in ecdysteroidogenesis, and down-regulation of MIH signaling (Abuhagr et al., 2014b). In insects, molting is stimulated by the prothoracic gland (PG), which is the physiological equivalent of the crustacean YO as it secrets ecdysone in response to PTTH. Ecdysone secretion in the PG is dependent on mTOR signaling to allow the larva to transition to pupa in *Drosophila*. RNAi knockdown of the Type I receptor, Type II receptor, Co-Smad, or R-Smad prevents Activin signaling and a peak in ecdysteroids that trigger metamorphosis in *Drosophila*. The same study showed that up regulation of Activin signaling results in early metamorphosis (Gibbens, 2011). We hypothesize that Mstn is the TGF-β factor that is up regulated in the activated YO and is responsible for transitioning the YO to the committed state.

Molt induction by MLA up regulates *Gl-mTOR*, *Gl-Akt*, and *Gl-EF2* in the YO (Abuhagr et al., 2014a). In this study, the effects of molt induction by ESA on YO gene expression were determined. mRNA levels of mTOR signaling genes (*Gl-mTOR*, *Gl-Akt*, *Gl-S6K*, and *Gl-Rheb*),

ecdysteroid receptor (*Gl-EcR* and *Gl-RXR*), *Gl-Mstn*, and *Gl-EF2* were quantified by quantitative polymerase chain reaction (qPCR). In addition, the effects of SB431542, an inhibitor of Activin receptor signaling, and rapamycin, an mTORC1 inhibitor, on YO gene expression were determined. The data suggest that the Mstn-like gene is the TGF-β factor responsible for the transition of the YO from the activated to committed state.

Materials and Methods

Animals and experimental treatments

Adult male *Gecarcinus lateralis* were collected from the Dominican Republic and maintained at Colorado State University, CO. Animals were acclimated for one month after arrival in late May and maintained at ~27 °C and 75-90% humidity with a 12 h:12 h light:dark cycle. Intermolt individuals were kept in communal plastic cages containing aspen bedding moistened with 5 ppt Instant Ocean (Aquarium Systems, Mentor, OH). Crabs were fed twice weekly with lettuce, carrots, and raisins (Covi et al. 2010). These animals usually molt once a year after the rainy season commences, which occurs prior to their shipment to Colorado.

Molting was induced by eyestalk ablation (ESA), which eliminated the source of MIH (Skinner, 1985). The YO is immediately activated and ecdysteroid titers increase by 1 day post-ESA (Lee et al. 2007). Animals progress through premolt (~3 weeks), but usually do not complete ecdysis (Covi et al. 2010). Molt stage was determined by the growth of the limb bud (LB). The regenerate index (R index), is the percentage of the LB length compared to its body size, and is calculated by the equation: (regenerate length x 100)/carapace width. The third right walking leg was autotomized immediately after arrival and a basal regenerate formed (R = 8-10). During premolt, the regenerate grows in response to ecdysteroids and reaches an R index of 23-

25 prior to ecdysis (Hopkins, 2001; Skinner and Graham, 1972; Yu et al. 2002). Molt stage was confirmed by hemolymph ecdysteroid titers and integumentary structure (presence or absence of the membranous layer and setal development in the maxillae; Moriyasu and Mallet, 1986). Hemolymph ecdysteroids were quantified by competitive ELISA as described (Abuhagr et al. 2014b).

The effects of SB431542 (Selleck Chem, Houston, TX, USA) and rapamycin (Selleck Chem) on YO gene expression were determined by *in vivo* experiments. Intact and ESA crabs were injected with either compound (10 μM final concentration) or vehicle (DMSO, ~1% final concentration) at Day 0. The amount to inject assumed that the hemolymph volume is 30% of the wet weight in grams, using the equation: mass (g) x 0.3 μl = volume of a 10 mM stock solution. Hemolymph samples (100 μl) were taken prior to injection and at time of dissection and combined with 300 μl methanol. YOs were harvested at 0, 1, 3, 5, 7, and 14 days post-ESA.

Gene mRNA levels in G. lateralis *tissues*

RNA was isolated using TRIzol and chloroform/phenol extraction as described (Covi et al., 2010). YOs were placed immediately into 1 ml TRIzol Reagent (Ambion/Life Technologies, Grand Island, NY, USA) in an RNase/DNase/protein free tube and homogenized by hand for 7 minutes. All other tissues represented in Fig. 1 were homogenized using a Tissue Lyser II (Qiagen) for 2 min at a frequency of 30/s in 1 ml TRIzol Reagent. Homogenates were centrifuged at 16,000g for 15 min to remove cellular debris. The supernatant fraction was subjected to phenol-chloroform extraction, and RNA pellets were dissolved in 22 μl RNase/DNase/protease free water. Each sample received DNase I treatment according to manufacturer's instructions (Thermo Scientific, Grand Island, NY, USA). Ten units (0.75 μl) of

Ribolock (Thermo Scientific) were included in the DNase I treatment to prevent RNA degradation. A second phenol-chloroform extraction used 24:1 chloroform isoamyl alcohol, and RNA was precipitated by adding 0.5 volume 3 M sodium acetate (pH 5.2) to 1.5 volumes isopropanol. Pellets were dissolved in 22 μl RNase/DNase/protease free water, and the RNA concentration was determined using a Nanodrop 1000 (Thermo Scientific). One μg total RNA was reverse-transcribed to cDNA using Quanta qScript cDNA SuperMix (Quanta Biosciences, Gaithersburg, MD, USA), according to the manufacturer's instructions. cDNA samples were stored at -20°C.

End-point PCR was used to determine the tissue expression of *Cm-EF2* and *Cm-Mstn*. All primers were synthesized using IDT (Coralville, IA, USA) and diluted to 10 µM. PCR conditions were as follows: 1 μl each of forward and reverse gene-specific primers (Table 1), 5 μl of 2X PCR Master Mix (Thermo Scientific, Carlsbad, CA, USA), 2 μl nuclease-free water, and 1 μl cDNA template were combined. After denaturation at 95 °C for 3 min, 35 PCR cycles were repeated at 95 °C for 30 sec, 62 °C for 30 sec, and 72 °C for 30 sec, with a final extension time of 7 min at 72 °C. Single band products were separated with 1.2% agarose gel electrophoresis, stained with ethidium bromide, and imaged under ultraviolet (UV) light.

Quantitative PCR (qPCR) was performed using a Lightcycler 480 Thermocycler (Roche Applied Science, Indianapolis, IN, USA). qPCR conditions were as follows: 0.5 μl each of forward and reverse gene-specific primers (Table 1), 5 μl SyBr Green (Roche), 3 μl nuclease-free water, and 1 μl cDNA template were combined. After denaturation at 95 °C for 3 min, 45 PCR cycles were repeated at 95 °C for 30 sec, 62 °C for 30 sec, and 72 °C for 20 sec, with a final extension time of 7 min at 72 °C. Concentration of each gene was quantified by comparing to an

established standard curve. The data are presented as mean copy number/μg total RNA ± 1 S.E.M.

Statistical analysis

Outliers were identified using a Nalimov test on the sum of copy numbers from each individual prior to calculation of means and SEM. Mean copy number was calculated and log transformed. Statistical significance ($p<0.05$) between time points were evaluated using a one-way ANOVA and significance between experimental and control at a given time point was determined using a student's t-test (Sigma Plot 12.0, Systat Software, San Jose, CA USA). All ESA time points were compared to the intact animals harvested at Day 0. Fold changes discussed in the text refer to the differences in mean raw expression per gene.

Results

Tissue expression of Mstn

Gl-Mstn and *Gl-EF2* were constitutively expressed in all tissues (Fig. 1). *Gl-Mstn* mRNA levels appeared to be highest in muscle, midgut, hindgut, and hepatopancreas, and at lower levels in heart, YO, eyestalk ganglia, and thoracic ganglion. The lowest expression was in the testis.

Effects of SB431542 on hemolymph ecdysteroid titers and YO gene expression in eyestalk-ablated animals

Animals were ES-ablated and received a single injection of either SB431542 dissolved in DMSO or DMSO alone at Day 0. Intact animals served as the Day 0 control. SB431542 had no effect on hemolymph ecdysteroid titers. Hemolymph ecdysteroid titers in ES-ablated animals increased after 1-day post-ESA and peaked at 14 days post-ESA (Fig. 2A). There were no

significant differences between the two groups, which is contrary to a previous report, in which SB431542 reduced hemolymph ecdysteroid after 7 days post-ESA (Abuhagr, 2012).

Gl-EF2 expression did not change significantly between control and experimental treatments or between time points (Fig. 3A). By contrast, *Gl-Mstn* mRNA level was significantly increased in the control treatment at 3 days post-ESA compared to 1 day post-ESA ($p<0.05$) with a 2.6 fold-change (Fig. 3B). *Gl-EcR* significantly increased between 5 and 7 days post-ESA in the vehicle ($p<0.05$), and there were significant differences between experimental and control at 1 day ($p=0.0256$), 7 days ($p=0.034$), and 14 days ($p=0.048$) post-ESA (Fig. 3C). *Gl-RXR* expression was unchanged, and there were no significant differences between treatments (Fig. 3D). *Gl-mTOR* expression was significantly higher at 7 days post-ESA than at 5 days and 14 days post-ESA in control samples ($p<0.05$)(Fig. 3E). *Gl-Rheb* was significantly increased at 7 days post-ESA compared to 0 day in the vehicle samples ($p<0.05$), and there were significant differences between experimental and control treatments at 1 ($p=0.0236$) and 14 days post-ESA ($p=0.0222$)(Fig. 3F). *Gl-S6K* had significantly increased expression at 3 days compared to 5 days post-ESA in both treatment groups ($p<0.05$), and there were significant changes in gene expression at 7 days ($p=0.0459$) and 14 days post-ESA ($p=0.034$) between experimental and control (Fig. 3G). *Gl-Akt* showed the same general pattern as *Gl-mTOR*, *Gl-Rheb*, and *Gl-S6K*, but there was only a significant difference between experimental and control at 14 days post-ESA ($p=0.039$)(Fig. 3H). In most genes, except RXR, there was an increase in expression at 3 days in both experimental and control, a subsequent decrease at 5 days, and an increase in control expression at 7 days and an increase in experimental expression at 14 days post-ESA. SB431542 had an effect toward the end of the experiment.

The effects of rapamycin on hemolymph ecdysteroid titers and YO gene expression in eyestalk-ablated animals

Intermolt intact and ES-ablated animals were injected with rapamycin dissolved in DMSO or DMSO alone at Day 0. Hemolymph ecdysteroid titers increased in the control animals to 49.5 ± 7.0 pg/μl, and rapamycin inhibited the increase. There were significant differences between rapamycin and the vehicle at 1 and 3 days ($p<0.01$)(Fig. 2B).

There was a significant increase in *Gl-EF2* expression at 3 and 14 days post-ESA compared to 1 day post-ESA in control samples, with a 3.9 and 4.7-fold change, respectively ($p<0.05$)(Table 3). There were also significant increases in gene expression in control compared to the rapamycin injection at 3 and 14 days ($p=0.0099$; Fig 4A). *Gl-Mstn* expression peaked at 3 days post-ESA in control samples, which was significantly higher than experimental samples ($p=0.009$). *Gl-Mstn* expression in the experimental samples peaked later and to a lesser degree at 7 days post-ESA (Fig. 4B). *Gl-EcR* expression was variable; the only significant change occurred between treatments at 3 days post-ESA ($p=0.013$; Fig. 4C). *Gl-RXR* expression was unchanged, and there was no difference between the control and experimental treatments (Fig. 4D). All mTOR signaling genes components showed a similar trend: expression in the control samples peaked at 3 days post-ESA, subsequently dropped at 5 days post-ESA, and slowly increased to peak again at 14 days post-ESA. In the experimental treatment, gene expression was generally delayed compared to the control (*Gl-mTOR* and *Gl-Akt*) or did not peak at all (*Gl-Rheb* and *Gl-S6K*). Significant differences ($p<0.01$) were observed at 3 days in all four genes (Fig. 4E-H; Table 3).

Discussion

Previous experiments with *G. lateralis* have shown that mTOR activity contributes to the increase global protein synthesis that occurs during the premolt period in preparation for ecdysis (Abuhagr et al. 2014b). This increase in protein synthesis, demonstrated by expression of mTOR and its components, peaks around 3 days post-ESA (Fig. 3 and 4; Abuhagr, 2012). While this is consistent with the hypothesis that mTOR is required for activation of the YO, we also wanted to determine how the molting gland becomes committed to molting prior at mid-premolt. We hypothesized that the activated YO produces a TGF-β-like factor that drives the transition of the YO to the committed state (Chang and Mykles, 2011).

The response to ESA was not as robust as in previous experiments. Typically, hemolymph ecdysteroid titers increase by 1 day post-ESA and exceed 100 pg/μl by 7 days post-ESA (Covi et al., 2010; McDonald et al., 2011; MacLea et al., 2012). By contrast, the mean ecdysteroid titers never exceeded 46 pg/μl after 14 days post-ESA (Fig 2). Moreover, in most of the animals, ESA did not stimulate growth of the basal LB. This suggests that many of the animals were not in intermolt (stage C_4) at the time of ESA, even though the animals were acclimated 1 month before treatment. In the field, *G. lateralis* molt soon after the onset of the rainy season in early spring. Animals are collected in late May and shipped to Colorado. Soon after arrival, the third walking leg is autotomized and animals are acclimated for 1 month, during which time a basal regenerate forms. In previous years, the 1-month interval was sufficient for animals to reach stage C_4 (Covi et al., 2010; MacLea et al., 2012). The basal regenerate remains small (R = 8-10) until it is stimulated to grow by ESA. The YO of postmolt animals is not as responsive to ESA, as the YO is in a repressed state during postmolt (stages A, B, and C_{1-3}; Nakatsuji and Sonobe, 2004); this is indicated by very low hemolymph ecdysteroid titers (Covi et al., 2010). At 3 days post-ESA and

later intervals, the hemolymph ecdysteroid titers of the SB431542-injected animals were lower than the control animals, but the differences in the means were not significant (Fig. 2A). By contrast, rapamycin significantly reduced ecdysteroid titers at 1 and 3 days post-ESA (Fig. 2B). In addition, low concentrations of rapamycin (< 1 μM) inhibit YO ecdysteroid secretion *in vitro* (Abuhagr et al., 2014). These data indicate that YO activation and ecdysteroidogenesis requires mTORC1, but not an Activin TGF-β-like factor.

The expression of mTOR signaling genes (*Gl-mTOR* and *Gl-Akt*) and *Gl-EF2* in the YO is up regulated in animals induced to molt by multiple leg autonomy (Abuhagr et al., 2014b). In the SB431542 experiment, ESA had modest effects on mTOR signaling gene expression and there was no significant differences between the means of experimental and control treatment animals at any time point (Fig. 3). In the rapamycin experiment, transcript levels of *Gl-EF2*, *Gl-Mstn*, *Gl-mTOR*, *Gl-Rheb*, *Gl-S6K*, and *Gl-Akt* were significantly increased at 3 days post-ESA in control animals (Fig. 4A, B, E-H). This suggests that YO hypertrophy requires the up regulation of translation (EF2) and mTOR pathway (mTOR, Rheb, S6K, and Akt) genes (Fig. 1 in Chapter 1). Rapamycin blocked or delayed the effects of ESA on *Gl-mTOR*, *Gl-Rheb*, *Gl-S6K*, and *Gl-Ak*t expression compared to the control (Fig. 4E-H), suggesting that mTOR-dependent protein synthesis is required for activation of the YO (Chapter 1, Fig. 1).

The upregulation of *Gl-Mstn* in control animals at 3 days post-ESA (Fig. 3B and 4B) is consistent with the hypothesis that *Gl-Mstn* is the Activin-like factor that induces the transition of the YO from the activated to committed states (Chapter 1, Fig. 4). *Gl-Mstn* is upregulated in the activated YO prior to entering the committed state, which occurs approximately 7 days post-ESA when they transition from stage D_0 to stage D_1 (Covi et al., 2010). *Gl-Mstn* expression was delayed by rapamycin treatment, reaching a maximum at 5 days post-ESA (Fig. 4B). However,

SB431542 had no effect on *Gl-Mstn* expression, suggesting that the YO activation does not involve Activin/Smad signaling (Fig. 3B). Since SB431542 prevents Mstn from binding to its receptor, Mstn transcription would not be affected initially, although the pathway would be affected downstream. There was little to no effect of ESA on *Gl-EcR* or *Gl-RXR* mRNA levels in either the SB431542 or rapamycin treatment (Figs. 3C-D and 4C-D), which suggests that a change in ecdysteroid receptor expression is not required for YO activation.

Our findings support previous findings that *Gl-mTOR* and mTOR components allow activation of the molt cycle. Additionally, we suggest that *Gl-Mstn* is at least partially responsible for the transition from activated to committed stages in the YO. Although these animals appeared to be refractory to eyestalk ablation as they were likely still in postmolt, SB431542 and especially rapamycin blocked or delayed the effects of molting in *G. lateralis* by suppressing gene expression changes necessary during premolt. Further research will investigate the effects of molt cycle manipulation on gene expression in intermolt animals and will focus on the effects of Myostatin signaling.

Table 3.1. Oligonucleotide primer sequences used in PCR expression analysis of *G. lateralis* genes. Reverse primers are written as reverse and complement. Annealing temperature was 62°C for each PCR primer set. Abbreviations: EF2, elongation factor-2; Mstn, myostatin; EcR, ecdysteroid receptor; RXR, retinoid X receptor; mTOR, mechanistic target of Rapamycin; Rheb, Ras homolog enriched in brain; Akt, protein kinase B; S6K, p70 S6 kinase; F, forward; R, reverse.

Primer	Sequence (5' → 3')	Product size (bp)
Gl-EF2 F1	TTCTATGCCTTTGGCCGTGTCTTCTC	227
Gl-EF2 R1	ATGGTGCCCGTCTTAACCA	
Gl-Mstn F1	GCTGTCGCCGATGAAGATGT	118
Gl-Mstn R1	GGCTGGGGACCTCAATCCCGT	
Gl-EcR F1	AAGAATGCCGTGTACCAGTGTAAATATG	566
Gl-EcR R1	GAGGTCATGATGCTTCGAGCAGC	
Gl-RXR F1	CTCAGGCAAGCACTATGGCGT	164
Gl-RXR R1	CTGCCGCTACCAGAAGTGCTTGA	
Gl-mTOR F2	AGAAGATCCTGCTGAACATCGAG	159
Gl-mTOR R2	AGGAGGGACTCTTGAAACCACAG	
Gl-Rheb F1	TTTGTGGACAGCTATGATCCC	119
Gl-Rheb R1	AAGATGCTATACTCATCCTGACC	
Gl-Akt F2	AACTCAAGTACTCCAGCGATGATG	156
Gl-Akt R1	GGTTGCTACTCTTTTCACGACAGA	
Gl-S6K F1	GGACATGTGAAGCTCACAGACTTT	239
Gl-S6K R1	TTCCCCTTCAGGATCTTCTCTATG	

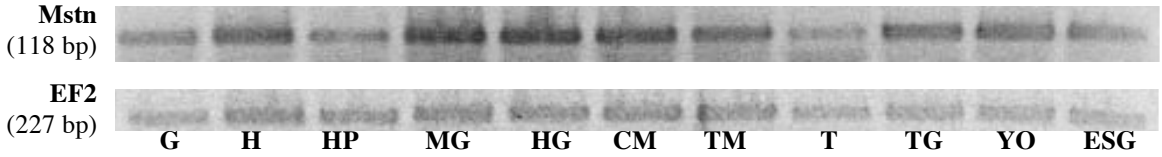

Fig 3.1. Expression of Mstn and EF2 in multiple tissues of *G. lateralis* after end-point PCR. Expression of Mstn is highest in muscle and gut tissues, but is also highly expressed in heart, TG, and YO. Lowest expression of Mstn is in testes. Abbreviations: G, gill; H, heart; HP, heptatopancreas; MG, midgut; HG, hindgut; CM, claw muscle; TM, thoracic muscle; T, testes; TG, thoracic ganglia; YO, Y-organ; ESG, eyestalk ganglia.

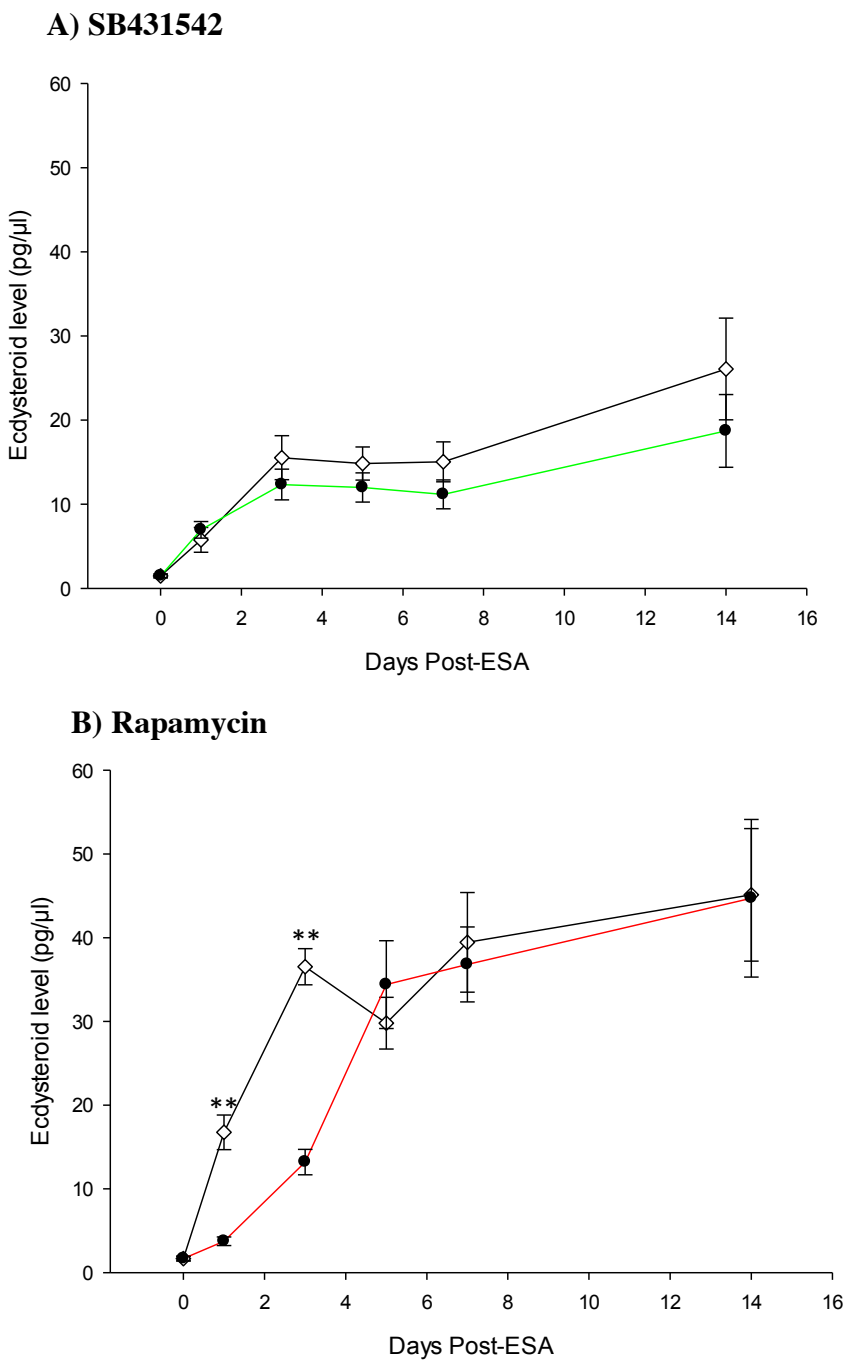

Fig 3.2. Hemolymph ecdysteroid titers of *G. lateralis* after ESA and injection with SB431542 (A) or rapamycin (B). Colored lines indicate experimental treatment, black lines indicate control treatment with DMSO. ** indicate significance where $p<0.01$.

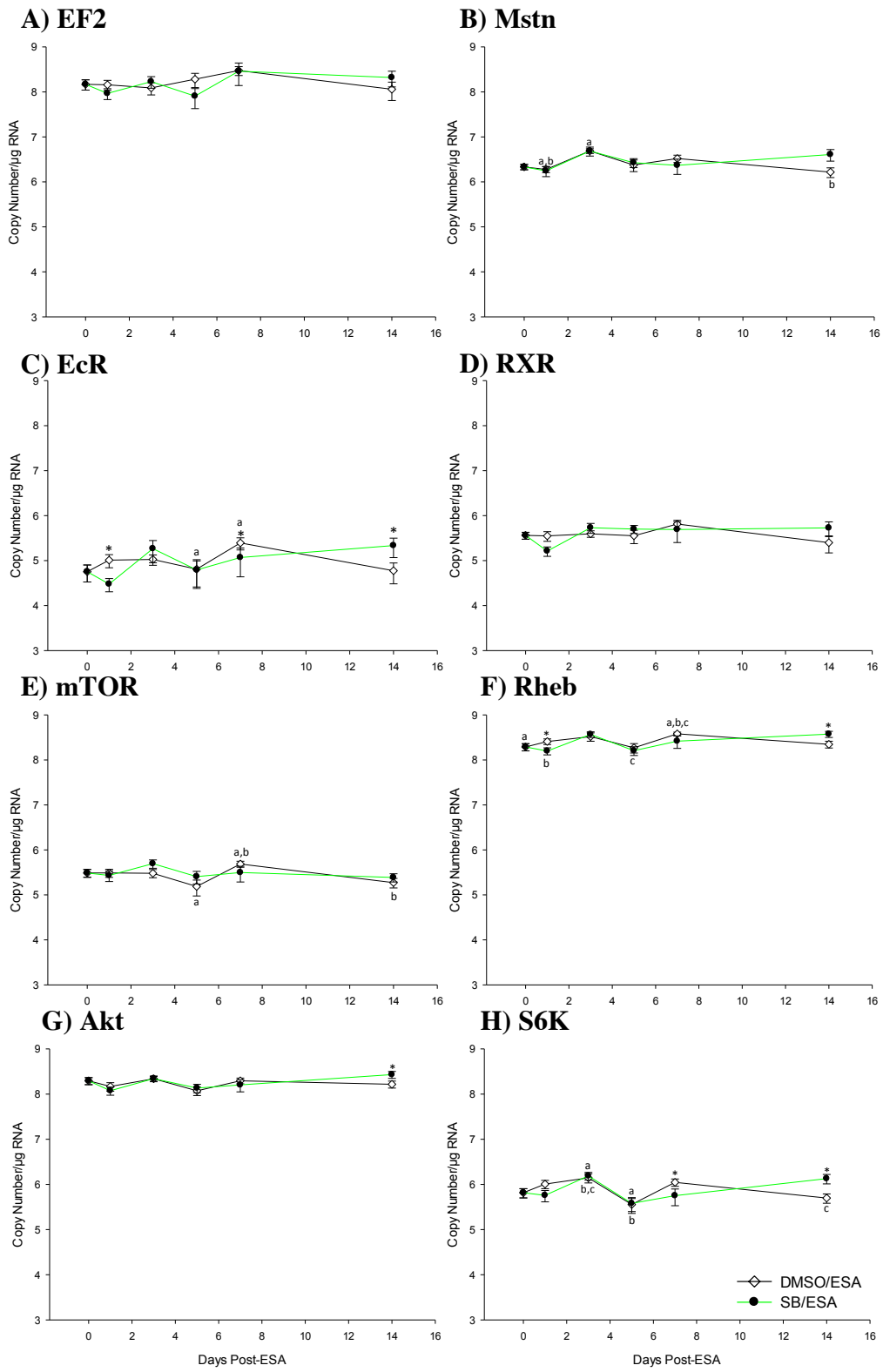
A) EF2
B) Mstn
C) EcR
D) RXR
E) mTOR
F) Rheb
G) Akt
H) S6K
Copy Number/µg RNA
Days Post-ESA
DMSO/ESA
SB/ESA

Fig 3.3. Effects of Activin receptor antagonist SB431542 on *G. lateralis* YO gene expression of EF2 (A), Mstn components (B-D), and mTOR components (E-H). Intact and ES-ablated animals were injected with a single dose of SB431542 in DMSO (10 μM) or 1% DMSO vehicle at Day 0. Data are presented as mean ± SEM (sample size for each treatment indicated in appendix). * indicate significance between treatments at the same time point. Letters indicate significance within the same treatment at varying time points. Abbreviations: EF2, elongation factor-2; Mstn, myostatin; EcR, ecdysteroid receptor; RXR, retinoid X receptor; mTOR, mechanistic target of Rapamycin; Rheb, Ras homolog enriched in brain; Akt, protein kinase B; S6K, p70 S6 kinase.

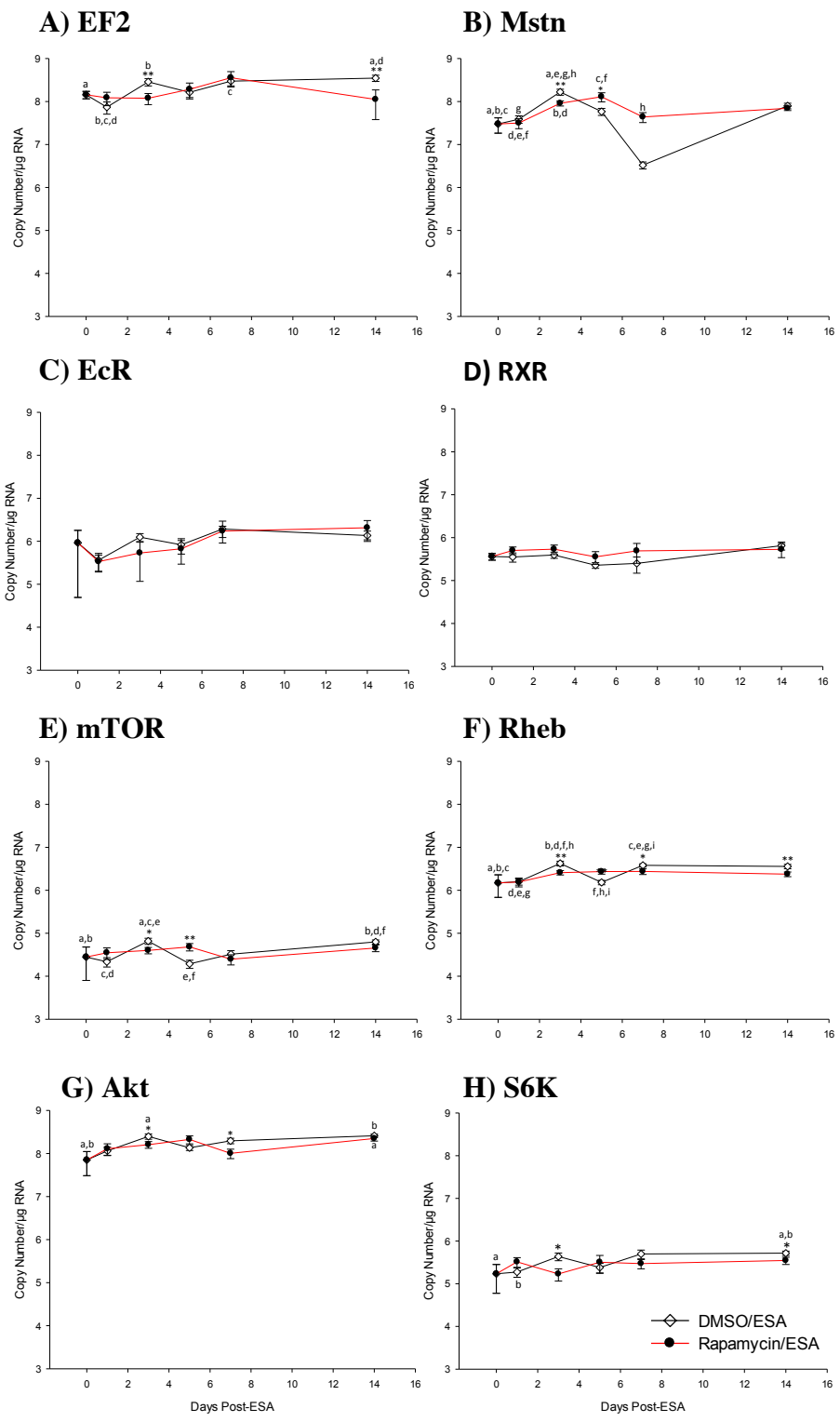
A) EF2
B) Mstn
C) EcR
D) RXR
E) mTOR
F) Rheb
G) Akt
H) S6K
Copy Number/µg RNA
Days Post-ESA
DMSO/ESA
Rapamycin/ESA

Fig 3.4. Effects of rapamycin on *G. lateralis* YO gene expression of EF2 (A), Mstn components (B-D), and mTOR components (E-H). Intact and ES-ablated animals were injected with a single dose of rapamycin in DMSO (10 μM) or 1% DMSO vehicle at Day 0. Data are presented as mean ± SEM (sample size for each treatment indicated in appendix). * indicate significance between treatments at the same time point (* means $p<0.05$; ** means $p<0.01$). Letters indicate significance within the same treatment at varying time points. Abbreviations: EF2, elongation factor-2; Mstn, myostatin; EcR, ecdysteroid receptor; RXR, retinoid X receptor; mTOR, mechanistic target of Rapamycin; Rheb, Ras homolog enriched in brain; Akt, protein kinase B; S6K, p70 S6 kinase.

Table 3.2. Fold changes in mRNA expression after SB431542 injection in *G. lateralis* YO that showed significant changes at the indicated time points. Abbreviations: Mstn, myostatin; EcR, ecdysteroid receptor; mTOR, mechanistic target of Rapamycin; Rheb, Ras homolog enriched in brain; S6K, p70 S6 kinase.

Gene	Control or Experimental?	Time Points Compared	Fold Change
Mstn	Control	1 Day vs 3 Day	2.6
	Control	1 Day vs 14 Day	-2.3
EcR	Control	5 Day vs 7 Day	3.8
mTOR	Control	5 Day vs 7 Day	3.2
	Control	7 Day vs 14 Day	-2.6
Rheb	Control	0 Day vs 7 Day	2.0
	Between both	1 Day (SB) vs 7 Day (C)	2.4
	Between both	5 Day (SB) vs 7 Day (C)	2.4
S6K	Experimental	3 Day vs 5 Day	-4.1
	Control	3 Day vs 5 Day	-3.9
	Control	3 Day vs 14 Day	-2.8

Table 3.3. Fold changes in mRNA expression after rapamycin injection in *G. lateralis* YO that showed significant changes at the indicated time points. Abbreviations: EF2, elongation factor-2; Mstn, myostatin; EcR, ecdysteroid receptor; RXR, retinoid X receptor; mTOR, mechanistic target of Rapamycin; Rheb, Ras homolog enriched in brain; Akt, protein kinase B; S6K, p70 S6 kinase.

Gene	Control or Experimental?	Time Points Compared	Fold Change
EF2	Control	0 Day vs 14 Day	2.4
	Control	1 Day vs 3 Day	3.9
	Control	1 Day vs 7 Day	4.8
	Control	1 Day vs 14 Day	4.7
Mstn	Control	0 Day vs 3 Day	5.5
	Experimental	0 Day vs 3 Day	3.0
	Experimental	0 Day vs 5 Day	4.3
	Experimental	1 Day vs 3 Day	2.9
	Between both	1 Day (R) vs 3 Day (C)	5.3
	Experimental	1 Day vs 5 Day	4.1
	Control	1 Day vs 3 Day	4.3
	Between both	3 Day (C) vs 7 Day (R)	2.1
mTOR	Control	0 Day vs 3 Day	2.4
	Control	0 Day vs 14 Day	2.3
	Control	1 Day vs 3 Day	3.0
	Control	1 Day vs 14 Day	2.3
	Control	3 Day vs 5 Day	-3.4
	Control	5 Day vs 14 Day	3.2
Rheb	Control	0 Day vs 3 Day	2.8
	Control	0 Day vs 7 Day	1.8
	Control	1 Day vs 3 Day	2.7
	Control	1 Day vs 7 Day	1.7
	Control	3 Day vs 5 Day	-2.8
	Control	5 Day vs 7 Day	1.8
Akt	Control	0 Day vs 3 Day	3.2
	Control	0 Day vs 14 Day	3.3
S6K	Control	0 Day vs 14 Day	3.1
	Control	1 Day vs 14 Day	2.8

REFERENCES

Abuhagr, A. (2012). Role of mechanistic Target of Rapamycin (mTOR) and TGF-B signaling in the crustacean Y-organ during the molt cycle. In *Thesis*, pp. 52-67. Fort Collins, CO: Colorado State University.

Abuhagr, A., 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., vol. 217, pp. 796-808: J Exp Biol.

Abuhagr, A., 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*., vol. 168, pp. 25-39: Comp Biochem Phys A.

Chang, E. S. (1993). Comparative endocrinology of molting and reproduction: insects and crustaceans, vol. 1993, pp. 161-180: Annu Rev Entomol.

Chang, E. S. and Mykles, D. L. (2011). Regulation of crustacean molting: a review and our perspectives, vol. 172, pp. 323-330: Gen Comp Endocr.

Costlow, J. D. (1963). The effect of eyestalk extirpation on metamorphosis of megalops of the blue crab, *Callinectes sapidus*, vol. 3, pp. 120-130: Gen Comp Endocr.

Covi, J. A., Bader, B. D., Chang, E. S. and Mykles, D. L. (2010). Molt cycle regulation of protein synthesis in skeletal muscle of the blackback land crab, *Gecarcinus lateralis,* and the differential expression of a myostatin-like factor during atrophy indicated by molting or unweighting, vol. 213, pp. 172-183: J Exp Biol.

Covi, J. A., Kim, H. and Mykles, D. L. (2008). Expression of alternatively spliced transcripts for a myostatin-like protein in the blackback land crab, *Gecarcinus lateralis*, vol. 150, pp. 423-430: Comp Biochem Phys A.

Gibbens, Y. Y., Warren, J. T., Gilbert, L. I. and O'Connor, M. B. (2011). Neuroendocrine regulation of *Drosophila* metamorphosis requires TGF-B/Activin signaling, vol. 138, pp. 2693-2703: Development.

Hay, N. and Sonenberg, N. (2004). Upstream and downstream of mTOR, vol. 18, pp. 1926-1945: Genes and Development.

Holz, M. K., Ballif, B. A., Gygi, S. P. and Blenis, J. (2005). mTOR and S6K1 mediate assembly of the translation preinitiation complex through dynamic protein interchange and ordered phosphorylation events, vol. 123, pp. 569-580: Cell.

Hopkins, P. M. (2012). The eyes have it: A brief history of crustacean neuroendocrinology, vol. 175, pp. 357-366: Gen Comp Endocr.

Huang, H., Fu, C., Chen, X., Gong, J., Huang, X. and Ye, H. (2014). Molt-inhibiting hormone (MIH) gene from the green mud crab *Scylla paramamosain* and its expression during the molting and ovarian cycle, vol. in preparation: Aqua Res.

Inoki, K., Kim, J. and Guan, K. (2012). AMPK and mTOR in cellular energy homeostasis and drug targets, vol. 52, pp. 381-400: Annu Rev Pharmacolog.

Layalle, S., Arquier, N. and Leopold, P. (2008). The TOR pathway couples nutrition and development timing in *Drosophila*, vol. 15, pp. 568-577: Dev Cell.

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, vol. 150, pp. 505-513: Gen Comp Endocr.

Lee, S. and McPherron, A. C. (2001). Regulation of myostatin activity and muscle growth, vol. 98, pp. 9306-9311: PNAS.

Ma, X. M. and Blenis, J. (2009). Molecular mechanisms of mTOR-mediated translational control, vol. 10, pp. 307-318: Mol Cel Biol.

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 in skeletal muscle, vol. 215, pp. 590-604: J Exp Biol.

McCarthy, J. F. and Skinner, D. M. (1977). Proecdysial changes in serum ecdysone titers, gastrolith formation, and limb regeneration following molt induction by limb bud autonomy and/or eyestalk removal in the land crab, *Gecarcinus lateralis*, vol. 33, pp. 278-292: Gen Comp Endocr.

McDonald, A. A., Chang, E. S. and Mykles, D. L. (2011). Cloning of a nitric oxide synthase from green shore crab, *Carcinus 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*, vol. 158, pp. 150-162: Comp Biochem Phys A.

Mykles, D. L. (2011). Ecdysteroid metabolism in crustaceans, vol. 127, pp. 196-203: J Steroid Biochem.

Nakatsuki, T. and Sonobe, H. (2004). Regulation of ecdysteroid secretion from the Y-organ by molt-inhibiting hormone in the American crayfish, *Procambarus clarkii*, vol. 135, pp. 358-364: Gen Comp Endocr.

Otto, A. and Patel, K. (2010). Signaling and the control of skeletal muscle size, vol. 361, pp. 3059-3066: Exp Cell Res.

Proud, C. G. (2009). mTORC1 signalling and mRNA translation, vol. 37, pp. 227-231: Biochem Soc Trans.

Rommel, C., Bodine, S. C., Clarke, B. A., Rossman, R., Nunez, L., Stitt, T. N., Yancopoulos, G. D. and Glass, D. J. (2001). Mediation of IGF-1-induced skeletal myotube hypertrophy by PI(3)K/Akt/mTOR and PI(3)K/Akt/GSK3 pathways, vol. 3, pp. 1009-1013: Nature Cell Biol.

Ryazanov, A. G., Shestakova, E. A. and Natapov, P. G. (1988). Phosphorylation of elongation factor 2 by EF-2 kinase affects rate of translation, vol. 334, pp. 170-173: Nature.

Skinner, D. M. (1985). Interacting factors in the control of the crustacean molt cycle, vol. 25, pp. 275-284: Amer Zool.

Skinner, D. M. and Graham, D. E. (1972). Loss of limbs as a stimulus to ecdysis in Brachyura (true crabs), vol. 143, pp. 222-233: Biol Bull.

Webster, S. G. and Keller, R. (1986). Purification, characterisation and amino acid composition of the putative moult-inhibiting hormone (MIH) of *Carcinus maenas* (Crustacea, Decapoda), vol. 156, pp. 617-624: J Comp Phys.

Weiss, A. and Attisano, L. (2013). The TGFbeta superfamily signaling pathway, vol. 2, pp. 47-63: WIREs Dev Biol.

Yu, X., Chang, E. S. and Mykles, D. L. (2002). Characterization of limb autonomy factor-proecdysis (LAF-pro), isolated from limb regenerates, that suspends molting in the land crab *Gecarcinus laterals*, vol. 202, pp. 204-212: Biol Bull.

CHAPTER FOUR

DIRECTION OF FUTURE RESEARCH

Temperature stress in Carcinus maenas

I examined how gene expression varied in response to acute temperature changes in six tissues from *Carcinus maenas*. In order to further understand how this species reacts to stress, I recommend the following studies. Firstly, AMPK activity and total protein, HSP70 total protein and inducible protein, and mTOR activity and total protein should be assessed using the same parameters as our experiment. This would allow a correlation between transcript and protein to be made, and would determine at what level these genes are regulated.

I also recommend expanding this study to include additional crustacean species to complement the studies already completed on *C. maenas, Cancer irroratus,* and *Homarus americanus. Gecarcinus lateralis*, the blackback land crab, is a species whose molt cycle is well understood. This species is tropical and lives near the equator, where temperatures are stable; therefore, it does not experience temperature fluctuations. I would expect that this species would exhibit gene changes in response to relatively small changes in temperature, in contrast to *C. maenas*. *Metacarcinus magister*, the Dungeness crab, inhabits the same habitat as *C. maenas* in California during their juvenile stage, before they migrate to the open ocean. Since they are experiencing the same temperature fluctuations as *C. maenas*, it would be useful to also compare this species over a range of temperature changes.

During this study, I noticed that *HSP70* expression was robustly increased by 25° or 30°C during an acute temperature shift, depending on tissue type. I hypothesize that HSP70 may be

able to be used as a marker of acclimation- if *HSP70* expression leveled off after an initial increase, it may indicate that the animal has become acclimated to its surroundings.

Finally, I recommend that additional stress studies in *C. maenas* should be completed using AMPK, HSP70, and mTOR components as stress markers. This would give a completed picture of the molecular changes that occur during stress, which may affect molting, growth, and therefore range expansion of this invasive species.

Myostatin *and* mTOR *expression in* Gecarcinus lateralis *Y-organ*

In this experiment, I wanted to determine the effects of molt cycle inhibitors, rapamycin and SB431542, on gene expression during various stages in the molt cycle. I did not see the expected effects since the animals did not respond to eyestalk ablation, suggesting that they may still have been in post-molt from the previous molting season. If this experiment were to be repeated, I recommend that it should begin at a later date than in our experiment to ensure the animals are in intermolt.

An additional experiment could involve injecting follistatin to inhibit myostain activity at different points during the molt cycle. Alternatively, myostatin itself could be injected to determine whether the committed stage could be mimicked; this could use limb-bud autonomy to determine whether the animal continue or suspend molting.