EFFECTS OF MOLTING AND HYPOSALINITY STRESS ON THE EXPRESSION OF HIF-α, MOLTING, AND IMMUNE RESPONSE GENES IN JUVENILE *CANCER MAGISTER*

by

WYATT AUSTIN MILLER

A THESIS

Presented to the Department of Biology
and the Graduate School of the University of Oregon
in partial fulfillment of the requirements
for the degree of
Master of Science

March 2009

"Effects of Molting and Hyposalinity Stress on the Expression of HIF-α, Molting, and Immune Response Genes in Juvenile *Cancer magister*," a thesis prepared by Wyatt Austin Miller in partial fulfillment of the requirements for the Master of Science degree in the Department of Biology. This thesis has been approved and accepted by:

Nora B. Terwilliger, Chair of the Examining Committee

February 4, 2009
Date

Committee in Charge: Dr. Nora Terwilliger, Chair
Dr. Caren Braby
Dr. Alan L. Shanks

Accepted by:

Dean of the Graduate School

An Abstract of the Thesis of

Wyatt Austin Miller for the degree of Master of Science

in the Department of Biology to be taken March 2009

Title: EFFECTS OF MOLTING AND HYPOSALINITY STRESS ON THE EXPRESSION OF HIF-α, MOLTING, AND IMMUNE RESPONSE GENES IN JUVENILE *CANCER MAGISTER*

Approved: _____
Dr. Nora B. Terwilliger, Advisor

Molting and hyposalinity stress in crustaceans cause extensive morphological and metabolic changes. I hypothesize that in juvenile *Cancer magister*, which molt in nearshore and estuarine habitats, hypoxia inducible factor (HIF) participates in regulating target genes in response to molting and hyposalinity stress. This study investigated the mRNA expression of HIF-α, cryptocyanin 2, arthrodial membrane protein 6.0, anti-lipopolysaccharide factor (ALF), and β-actin in juvenile *C. magister* under normoxic conditions. One cohort of 1st instar juveniles was maintained across the entire molt cycle. Beginning at ecdysis and daily until 2nd instar, crabs were sampled for total RNA and analyzed by real-time quantitative PCR. A second cohort was exposed to 50% seawater for 3, 8, and 24 hours and then analyzed for mRNA expression.

All five genes showed molt stage-specific changes in mRNA expression during the molt cycle in normoxia, but the genes did not change expression due to hyposalinity stress.

CURRICULUM VITAE

NAME OF AUTHOR: Wyatt Austin Miller

PLACE OF BIRTH: Champaign, Illinois

DATE OF BIRTH: January 10, 1980

GRADUATE AND UNDERGRADUATE SCHOOLS ATTENDED:

University of Oregon, Eugene, Oregon
Illinois State University, Normal, Illinois

DEGREES AWARDED:

Master of Science in Biology, 2009, University of Oregon
Bachelor of Science in Biological Sciences, 2003, Illinois State University
Bachelor of Science in Theater, 2003, Illinois State University

AREAS OF SPECIAL INTEREST:

Marine Biology
Marine Molecular Physiology

PROFESSIONAL EXPERIENCE:

GK12 Fellow, National Science Foundation, Coos Bay, OR 2006-2008

CRUI Research Program for Undergraduates, National Science Foundation, Normal, IL 2000-2002

ACKNOWLEDGMENTS

I wish to express sincere appreciation to my advisor Dr. Nora Terwilliger for her encouragement and support. Also for your patient walkthrough of techniques and having the faith that eventually I'll get it. To Dr. Karen Guillemin, thank you and your staff for allowing a marine biology student into your molecular lab to take up your time and space. To Dr. David Towle, thank you for allowing me to use your space and resources, your help and knowledge on qPCR was greatly appreciated. In addition, special thanks are due to Brian Bingham, whose help with my understanding of statistics was invaluable. I also thank the faculty, staff, and graduate students of OIMB for making my time their wonderful. Thank you GK12, students, faculty, and staff of North Bay School I had a great time with you all. Finally, I would like to thank my family and friends for their continued support even when I am living across the country.

I dedicate this document to my parents and grandparents, for always encouraging me to follow my dreams.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER I

GENERAL INTRODUCTION

Cancer magister (Dana 1852), the Dungeness crab, is an important commercial species on the west coast of North America. Along the Oregon coast, the majority of larval *C. magister* metamorphose into megalopas offshore and migrate back to estuaries and near shore habitats from about mid April through June (Lough, 1976; Roegner et al., 2007). They settle and metamorphose into the first of many juvenile stages in preparation for adulthood. In crustaceans, molting is a necessary aspect of growth and development that involves extensive tissue remodeling, apoptosis, cell proliferation and growth (Skinner, 1985). The molt cycle consists of distinct stages, pre-molt, ecdysis, post-molt, and intermolt. Molting is a stressful and dangerous time for crustaceans. They shed their hard protective exoskeleton in order to grow larger. Molting requires precise timing of regulation of genes and proteins in order to synthesize and harden the new exoskeleton, as well as degrade and shed the old one. During molting the animal is most vulnerable to injury, which could lead to infection by pathogens and predation. Not only do *C. magister* juveniles have to face the challenges of the cyclical molt cycle, juveniles are also subject to other abiotic stresses like hypoxia, temperature change, and spatial salinity changes in the estuaries and near shore habitats of the Oregon coast.

Compared to megalopas and adult *C. magister*, 1st instar juveniles have the least osmoregulatory ability, and yet those that settle in the estuaries are exposed to the most extreme changes in temperature and salinity (Brown and Terwilliger, 1992).

Hypoxia inducible factor (HIF) is a highly conserved family of transcription factors found in many organisms and is known to regulate gene expression in response to hypoxia (Wenger, 2002) as well as other factors (Blouin et al., 2004; Dery et al., 2005; Hoogewijs et al., 2007). The active form of HIF is made up of two subunits, α and β. In normoxic conditions, HIF-α is rapidly degraded *via* the proteosome pathway, but under hypoxic conditions this subunit stabilizes and binds with HIF-β thus allowing for gene-specific targeting in the nucleus (Wang and Semenza, 1993). Recent evidence has shown HIF involvement in normoxia during specific stresses such as immunochallenge and temperature change (Blouin et al., 2004; Dery et al., 2005; Hoogewijs et al., 2007). HIF-α, a gene that has an important role during hypoxia, but is known to regulate genes during times of normoxia, could function in orchestrating some of the complex changes during the molt cycle and hyposalinity stress.

I investigated five genes that are involved in the molt cycle as well as possible candidates that may be involved in the response to hyposalinity stress. A cDNA microarray analysis of gene expression during the 1st instar juvenile molt cycle of *C. magister* has shown distinct patterns of up- and down-regulation at specific stages of the molt cycle (Phillips, 2007). Another microarray of 1st instar juveniles during hyposalinity stress showed gene specific up- and down-regulation in response to

hyposalinity stress (Phillips, 2007). I selected three of the genes identified from these microarrays, cryptocyanin 2 (Cc2), arthrodial membrane protein 6.0 (AMP6.0) and anti-lipopolysaccharide factor (ALF) for more detailed analysis. I investigated these genes as potential down-stream targets of HIF regulation during molting and hyposalinity stress. Cryptocyanin plays a role in forming the new exoskeleton (Terwilliger et al., 2005). AMP6.0 is a cuticle protein involved in the synthesis of uncalcified exoskeleton including arthrodial membrane (Wynn and Shafer, 2005). ALF is an anti-microbial protein that has a broad spectrum of anti-fungal and anti-bacterial properties (Somboonwiwat et al., 2005; de la Vega et al., 2008) and has been shown to up-regulate during hyposalinity stress in 1st instar juvenile *C. magister* (Phillips, 2007).

To more clearly understand the molting process and its regulation at the molecular level, I sampled *C. magister* crabs from their metamorphosis from megalopas into 1st instar juveniles through the molt cycle to 2nd instar (Chapter 2). I used real-time quantitative polymerase chain reaction (qPCR) to examine mRNA expression profiles of five genes during molting, HIF-α, Cc2, AMP 6.0, ALF, and β-actin (a potential qPCR housekeeping gene). I then examined the responses of *C. magister* HIF-α and target gene mRNA expression to hyposalinity stress (Chapter 3). Changes in salinity are key abiotic factors that affect *C. magister* along the Oregon coast. It was important to study the gene expression profiles of target genes during development.

This allowed me to delineate between gene expression that occurred during the molting process from those changes that occurred because of hyposalinity stress.

CHAPTER II

EFFECT OF MOLTING ON THE EXPRESSION OF HIF-α, CRYPTOCYANIN 2, ARTHRODIAL MEMBRANE PROTEIN 6.0, ANTI-LIPOPOLYSACCHARIDE FACTOR, AND β-ACTIN IN 1ST INSTAR JUVENILE *CANCER MAGISTER*

INTRODUCTION

In crustaceans, molting is a necessary aspect of growth and development that involves extensive tissue remodeling, apoptosis, cell proliferation and growth. The molt cycle consists of distinct stages, pre-molt, ecdysis, post-molt, and intermolt. Preparation for ecdysis begins during pre-molt when the epidermis separates from the old exoskeleton and secretion of the new exoskeleton is initiated. The molting process ends with the completion of the membranous layer of the new exoskeleton during post-molt (Skinner, 1985). Intermolt, the growth stage between each ecdysis when the epidermis is relatively quiescent, completes the cycle. The duration of the molt cycle increases with age: juvenile Dungeness crabs, *Cancer magister*, molt at least ten times during their first year, two to five times during their second year, and approximately once a year after that (Wainwright and Armstrong, 1993; Terwilliger et al., 2005).

Molting requires precise timing of regulation of genes and proteins in order to synthesize and harden the new exoskeleton, as well as degrade and shed the old one. The interaction of ecdysteroids and other hormones in regulating this process have been well documented (Chang et al., 1993; Willmer et al., 2005). I wondered whether the transcription factor, hypoxia inducible factor alpha (HIF-α), might also participate in orchestrating the molt process. HIF, a highly conserved family of transcription factors found in many organisms including mammals, teleosts, nematodes, *Drosophila*, *Daphnia*, and *C. magister*, is known to regulate gene expression in response to hypoxia. The active transcription factor, made up of two subunits, α and β, binds specifically to target genes in response to low partial pressure of oxygen. There are at least four isoforms of HIF-α, 1-4, in vertebrates (Law et al., 2006), but thus far, only one has been identified in crustaceans. In normoxic conditions, HIF-α is rapidly degraded *via* the proteosome pathway, but under hypoxic conditions this subunit stabilizes and binds with HIF-β thus allowing for gene specific targeting in the nucleus (Wang et al. 1993). In hypoxia-sensitive mammals, HIF-1α mRNA levels remain constant during hypoxia while the HIF-1α protein increases (Wenger et al., 1997; Hara et al., 1999; Soitamo et al., 2001), indicating that HIF is controlled at the protein level. However, recent studies on hypoxia-tolerant animals showed changes of HIF-α mRNA expression during hypoxia that resulted in increased HIF-α protein (Gorr et al., 2006; Law et al., 2006; Hoogewijs et al., 2007; Rahman et al., 2007). In specific circumstances in hypoxia-sensitive vertebrates, HIF-1α mRNA has also been

demonstrated to increase (Blouin et al., 2004; Dery et al., 2005). These increases in HIF mRNA and protein during normoxia in vertebrates in response to stressors that include inflammation, wound healing and thermal stress, suggest HIF may function to modulate multiple pathways of stress response in both hypoxia and normoxia (Blouin et al., 2004; Hoogewijs et al., 2007). HIF-α, a gene that has an important role during hypoxia, but is known to regulate genes during times of normoxia, could function in orchestrating some of the complex changes during the molt cycle. *Cancer magister* may activate the up-regulation of HIF-α mRNA in order to maintain homeostasis during molting stress. This study is unique in that a *C. magister* mRNA expression profile of HIF-α across the molt cycle has never been demonstrated.

Only recently have global changes in gene expression during molting received attention (Kuballa et al., 2007; Phillips, 2007; Shechter et al., 2007; Stillman et al., 2008). A cDNA microarray analysis of gene expression during the 1st instar juvenile molt cycle of *C. magister* identified many genes that exhibit distinct patterns of up- and down-regulation at specific stages of the molt cycle (Phillips, 2007). I selected two of the genes identified from this microarray, cryptocyanin 2 (Cc2) and arthrodial membrane protein 6.0 (AMP6.0) for real-time quantitative polymerase chain reaction (qPCR) analysis. These genes were used as a study control and qPCR was performed to verify the molt cycle microarray results. Cc2 and AMP6.0 may be potential down-stream targets of HIF regulation during molting. Cryptocyanin is a member of the hemocyanin family but unlike hemocyanin does not function in oxygen uptake.

Instead cryptocyanin plays a role in forming the new exoskeleton (Terwilliger et al., 1999; Terwilliger et al., 2005). In the global expression study, Cc2 mRNA increased expression during intermolt, decreased during late pre-molt, and remained low throughout post-molt and early intermolt (Phillips, 2007). AMP6.0 is a cuticle protein found only in uncalcified exoskeleton including the arthrodial membrane, and its mRNA expression has been shown to increase during pre-molt, stay constant through ecdysis, and decrease during post-molt (Wynn and Shafer, 2005; Phillips, 2007). These two genes both have critical roles in creating the new exoskeleton but the timing of their expression differs.

In the same microarray that showed molt stage-specific mRNA expression of Cc2 and AMP6.0, an abundance of pathogen defense genes increased expression during pre- and post-molt (Phillips, 2007). An anti-microbial protein, anti-lipopolysaccharide factor (ALF), has a broad spectrum of anti-fungal and anti-bacterial properties against filamentous fungi, Gram-positive, and Gram-negative bacteria (Somboonwiwat et al., 2005; de la Vega et al., 2008). My study examined changes in ALF expression as an indicator that genes in the innate immune response are activated during molting. It may be that ALF is one of those pathogen defense genes that increase expression as seen in the molt cycle microarray done by Phillips.

In addition to the regulatory gene HIF-α, microarray identified genes (Cc2 and AMP6.0), and ALF, I also looked at β-actin. β-actin, sometimes used in qPCR studies as a "housekeeping gene", is important for cell proliferation and structure, and it is a major component of epithelium and the membranous and endocuticular layers of the

exoskeleton (Mykles et al., 2000). I included β-actin in this study to determine whether it would serve as a good housekeeping gene for molting studies of *C. magister*.

To more clearly understand the molting process and its regulation at the molecular level, I sampled *C. magister* crabs from their metamorphosis from megalopas into 1st instar juveniles through the molt cycle to 2nd instar.
I used real-time quantitative polymerase chain reaction (qPCR) to examine mRNA expression profiles of five genes, HIF-α, Cc2, AMP 6.0, ALF, and β-actin, during molting.

MATERIALS AND METHODS

Animal Collection and Rearing

Cancer magister (Dana, 1852) megalopas were collected using a dip net from the docks of the Charleston, OR marina at the mouth of the Coos Bay estuary on May 5th, 2007. Megalopas were cultured at the Oregon Institute of Marine Biology in individual 16-oz flow-through containers in two aerated running seawater tables (13±1°C). Each afternoon, containers were cleaned, animals were fed *ad libitum* chopped *Mytilus sp.*, and the molt cycle of each individual was monitored. All megalopas metamorphosed into 1st instar juveniles within one to three days after

collection. The day that ecdysis occurred was considered day 0. Starting at day 0, five crabs per molt cycle day were rapidly frozen in liquid N_2 until the entire cohort reached 2nd instar ecdysis. The average duration of the 1st instar molt cycle was 16 ± 0.18 days. Samples were stored at –80° C until RNA purification.

Total RNA Isolation and cDNA Synthesis

Samples from each molt cycle day were randomly chosen for RNA isolation (n=3; Day 15, n=2). The entire frozen crab was quickly weighed, then homogenized in 3 ml denaturing solution. Total RNA was isolated using RNAse-free labware and RNAgents® Total RNA Isolation System (Promega). RNA quality and quantity were analyzed using a Nanodrop spectrophotometer (Thermo Scientific) and a DU-640 spectrophotometer (Beckman and Coulter). A few RNA samples with initially low 260/280 ratios were re-isolated using a RNAqueous-4PCR kit (Ambion) to ensure absence of genomic DNA or degradation products in all samples. For each sample an aliquot of 2 μg of total RNA was reverse transcribed using Superscript III First-Strand Synthesis (Invitrogen) with oligo-dT as primer. Samples of cDNA were stored at -20° C.

Quantification of Gene Expression

Non-degenerate primer pairs were designed to target specifically *C. magister* HIF-α (NCBI Accession # DQ535030), Cc2 (NCBI Accession # DQ230982)

(Terwilliger and Ryan, 2006), ALF, and AMP6.0 using Primer Premier software (Premier Biosoft). Degenerate primers based on three known arthropod sequences for β-actin were also used (Towle et al., 1997). The primers were synthesized by Integrated DNA Technologies (Coralville, IA, USA). Information on the primer name, nucleotide sequence, qPCR annealing temperature, and amplicon size can be found in Table 1. Analysis of mRNA expression was accomplished using target gene specific primers, SYBR GreenER Supermix (Stratagene), and an ABI Prism 7700 Sequence Detection System instrument at the University of Oregon (50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 sec and 60°C for 1 min). To assess product homogeneity, a thermal denaturation profile was generated following cDNA amplification. A single inflection point of the denaturation curve was observed for all targets indicating that a single product was present. A dilution series of cDNA from a day 7 intermolt animal was used to calculate relative expression values of each sample. The same day 7 cDNA dilution series was included in each qPCR run for all genes. All cDNA samples were diluted 1:10, run in triplicate, and the triplicate values were averaged. Expression values were normalized to total RNA content (2 μg) in the initial reverse transcription step. This method is an accepted way to normalize the data when the system does not have a validated housekeeping gene (Bustin et al., 2005). Statistical analyses using one-factor ANOVA with Scheffe posttests and quadratic regressions were accomplished using SPSS. Statistical significance threshold was set at $P<0.05$. In order to correct for high homogeneity of variance the data was $\log_{10}$ transformed when necessary.

Gene	Primer Name	Nucleotide sequence	QPCR annealing temperature (Celsius)	Amplicon Size (bp)
HIF alpha	NTCmaghifF20 NTCmaghifR20	5'-AACGAAGCAGAAACAGCGACAA-3' 5'-GGAGGATGGCAATGGTGAGC-3'	58	174
Cc2	NTCmagCc2F17 NTCmagCc2R17	5'-TACGCCACTATGCCACGACT-3' 5'-GCAAGGATGCGAATAACAGA-3'	60	101
ALF	NTCmagALFF15 NTCmagALFR15	5'-TTTGTCGCTGCTCCTCTTCG-3' 5'-ACCTCTGTGGTTCCGTCTTC-3'	60	120
AMP6.0	NTCmagAMP6F18 NTCmagAMP6R18	5'-CGTCGCCGATGAGAATGGTT-3' 5'-GTGGCAAGGAGTTCGAGTAGTGTT-3'	60	132
B-actin	BactinS BactinR	5'-GTCGGYGAYGARGCWCARAGCAA-3' 5'-GGRCARCGGAAWCGYTCATT-3'	63	613

Table 1. Nucleotide sequence, annealing temperature, and product size of degenerate and non-degenerate primers used for quantitative PCR. C. magister cDNAs taken from whole crabs during the 1st instar juvenile molt cycle.

RESULTS

Analysis of target gene expression during the 1st instar juvenile molt cycle of *C. magister* showed changes in expression of each gene based on days that correspond to specific stages of the molt cycle. Daily levels of mRNA expression were analyzed during post-molt, intermolt, and pre-molt stages, which were grouped by days 0-5, 6-10, and 11-16, respectively. Weights of 1st instar juveniles (avg. 0.15g ± 0.004) were more than double that of megalopas (avg. 0.06g ± .008) and remained constant across the molt cycle (Fig. 1), reflecting the gradual exchange of water weight for body mass within an instar that is typical of crustacean growth.

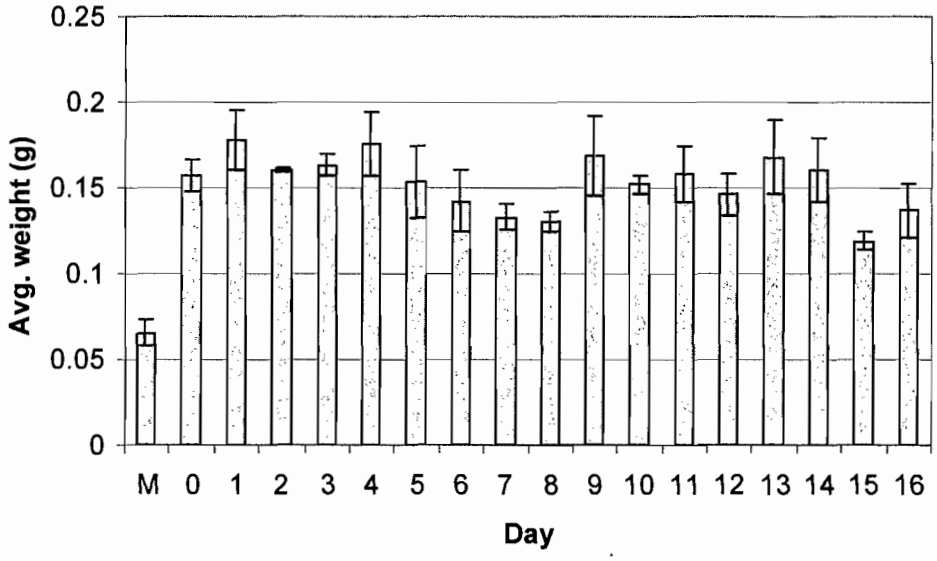

Fig. 1. Average weight of megalopas and 1st instar juveniles during the 1st instar molt cycle. Average duration of 1st instar molt cycle was 16 ± 0.18 days. M represents megalopas. Each value represents the mean ± S.E.M (n=3; Day 15 n=2).

Hypoxia Inducible Factor-α (HIF-α) Gene Expression

HIF-α mRNA expression differed between days and had high variability among individuals (Fig. 2A). There was a two-fold increase from day 0, the day of ecdysis, to day 1 followed by a sharp ten-fold decrease on day 2. On day 3 HIF-α mRNA expression rose and stayed high until day 6 when there was a two-fold decrease. This pattern continued throughout the molt cycle with another rise on days 8-9 and an increase again in late pre-molt. In a more general way, there is a trend towards an increased expression throughout post-molt and intermolt with decreased expression during early pre-molt.

Cryptocyanin 2 (Cc2) Gene Expression

The relative expression of Cc2 was very low during post-molt, days 0-5 (Fig. 2B). This expression of Cc2 was significantly different from both the high level of expression during the intermolt stage, days 6-10, ($F= 4.73$ $P<0.01$) and the decreased expression levels of the pre-molt stage, days 11-16 ($F=25.73$ $P<0.01$). The relative expression of Cc2 was three-fold higher during the intermolt stage than in post-molt. This expression slowly decreased from late intermolt into pre-molt. The relative expressions between these two stages of the molt cycle are not significantly different ($P=0.974$). There was a three-fold increase in Cc2 mRNA expression on day 16 right before ecdysis into the 2nd instar juvenile molt cycle. Except for this day 16 spike, the overall pattern of Cc2 mRNA expression is supported by previous analyses of cryptocyanin mRNA expression during the molt cycles of 1st and 5th instar juvenile *C. magister* (Terwilliger et al., 2005; Phillips, 2007).

Arthrodial Membrane Protein 6.0 (AMP6.0) Gene Expression

The relative expression of AMP6.0 mRNA was high in early post-molt and late pre-molt (Fig. 2C). The expression gradually decreased though post-molt until expression was almost nonexistent throughout intermolt and then gradually increased during pre-molt. This pattern fit a quadratic line ($f=169.45 - 37.01x + 2.19x^2$) ($F=22.094$ $P=0.001$). When comparing intermolt to post-molt and pre-molt animals, the difference in expression was significant ($F=32.120$ $P=0.001$).

Microarray data on AMP6.0 in *C. magister* during the 1st instar juvenile molt cycle showed a similar quadratic regression (Phillips, 2007).

Anti-lipopolysaccharide Factor (ALF) Gene Expression

Analysis of *C. magister* ALF gene expression during most of the 1st instar molt cycle showed very little change in expression. However, during day 4 there was a fifteen-fold increase in relative expression and on day 5, a ten-fold increase compared to every other day (Fig 2D). This unique spike in expression during day 4 was significantly different from every day (ANOVA Scheffe post-hoc $F=9.29$ $P=0.00$). Day 5 was almost significant from the other days ($P<0.06$). When comparing days 4 and 5 there was not a statistically significant difference ($P=0.71$).

β-actin Gene Expression

β-actin mRNA expression was high during early post-molt and late pre-molt and low during intermolt (Fig. 2E). This pattern is similar to AMP6.0 in that this data fits a quadratic line ($f = 2.3 - 0.49x + 0.3x^2$) ($F= 11.35$ $P =0.00$). Days 0-4 of post-molt and days 14-16 of late pre-molt were significantly different from the other days ($F= 3.5$ $P= 0.04$ and $F= 6.42$ $P= 0.03$, respectively).

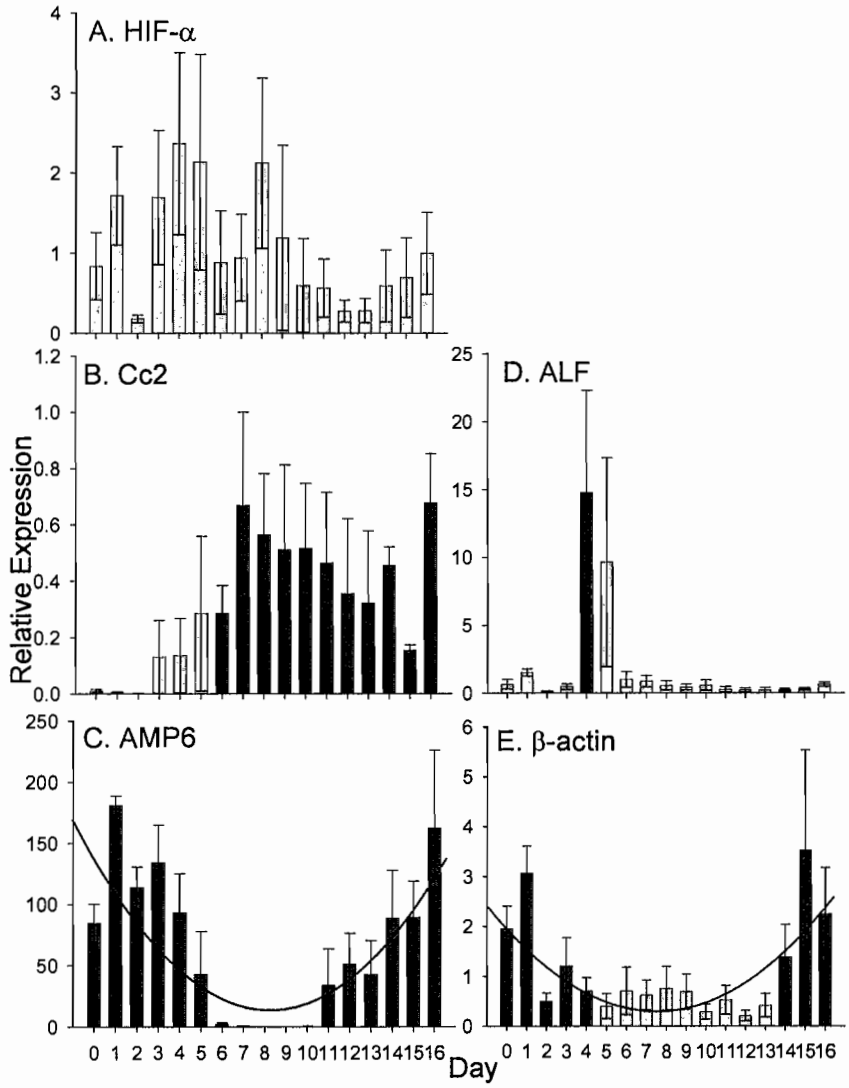

Fig. 2. Real-time quantitative PCR mRNA expression of five target mRNAs in *C. magister* 1st instar juveniles across the molt cycle: A) HIF-α, B) Cc2, C) AMP6.0, D) ALF, and E) β-actin. Day 0 is considered metamorphosis into the 1st juvenile molt cycle. Each value represents the mean ± S.E.M (n= 3; Day 15 n= 2). mRNA levels were normalized to total RNA (2 μg) in each sample. All dark shaded areas were statistically significant ($P<0.05$) from lighter shaded areas using ANOVA and Scheffe post-hoc analysis. Daily levels of mRNA expression were analyzed during post-molt, intermolt, and pre-molt stages, which were grouped by days 0-5, 6-10, and 11-16, respectively. B. Cc2 post-molt is significantly different from both the high level of expression during the intermolt stage ($F=14.734$ $P<0.001$) and the decreasing expression levels of the pre-molt stage ($F=25.734$ $P<0.001$). C. AMP6.0 line represents a quadratic regression (ANOVA) ($f = 169.45 - 37.01x + 2.19x^2$) ($F=22.094$ $P<0.001$). Intermolt was statistically significant from post-molt and pre-molt animals ($F=32.120$ $P<0.001$). D. ALF day 4 expression was significantly different from every day ($F=9.289$ $P=0.003$). E. β-actin line represents a quadratic regression (ANOVA) ($f = 2.3 - 0.492x + 0.3x^2$) ($F=11.35$ $P<0.001$). Days 0-4 of post-molt and days 14-16 of late pre-molt were significantly different from the other days ($F=3.5$ $P=0.042$ and $F=6.420$ $P=0.025$ respectively).

DISCUSSION

The five genes of juvenile *C. magister* analyzed in this study all showed changes in mRNA expression specific to molt stage when their expression levels were analyzed on a daily basis. Each gene is important in different physiological processes, ranging in function from regulation of other genes whose products are important in the cellular and tissue adaptation to stress (HIF-α), to direct involvement with the synthesis and deposition of new exoskeleton (Cc2, AMP6.0), defense against pathogens (ALF), and cellular proliferation (β-actin). Sampling each day from one ecdysis to the next provided the opportunity to observe closely when mRNA levels rise and fall in anticipation of product need.

The striking feature of the HIF-α mRNA profile of 1st instar juveniles is the extent of change from periods of low expression to peaks of high expression (Fig. 2A). In hypoxic activation of HIF-1α in hypoxia-sensitive mammals and rainbow trout, mRNA levels of HIF-1α are unaffected while posttranslational mechanisms allow HIF-1α protein to accumulate (Wenger et al., 1997; Hara et al., 1999; Soitamo et al., 2001). Alternatively, hypoxia-tolerant animals including mole rats, yak, Atlantic croaker, and grass carp express increased HIF-1α mRNA in response to hypoxia (Shams et al., 2004; Law et al., 2006; Wang et al., 2006; Rahman et al., 2007). In contrast to the above examples where HIF-α regulation of down-stream targets was triggered by hypoxia, HIF expression in normoxia has also been reported. In normoxic conditions

under certain stimuli, both HIF-α mRNA and HIF-α protein increase. Examples of this up-regulation of HIF activation in normoxia include immunostressed vertebrate macrophages and cold- acclimated Crucian carp (Blouin et al., 2004; Rissanen et al., 2006). My findings that HIF-α mRNA expression changes in *C. magister* under normoxic conditions support the theory that several HIF activation pathways, including but not restricted to environmental hypoxia, can occur in order to maintain tissue homeostasis or combat multiple stresses.

The activation of HIF-1α in vertebrate macrophages and smooth muscle cells by non-hypoxic stimuli including the inflammatory factor lipopolysaccharide, cytokines, and growth factors during environmental normoxia indicate a connection between HIF-1α, wound healing and immunochallenge (Page et al., 2002; Blouin et al., 2004; Dery et al., 2005). These findings have also suggested the use of an alternate pathway for HIF activation. The processes of tissue degradation and synthesis, controlled apoptosis, limb regeneration, and immunodefense that occur in molting are similar to the processes described above, where normoxic up-regulation of HIF-α occurs. These parallels led me to investigate HIF-α expression in the molting crab.

Another noteworthy feature is the periodicity of HIF-α mRNA expression that suggests HIF regulates certain genes for a restricted period of time and other genes later in the cycle as dictated by the requirements of molting. During pre-molt (days 11-16) the underlying epidermis separates from the old cuticle, the old cuticle is partially digested and reabsorbed, and new epi- and exocuticle are secreted (Skinner, 1985; Terwilliger et al., 2005). The increase in HIF-α mRNA expression just prior to this,

during late intermolt (days 8-10), may reflect HIF regulation of genes needed for the nearly simultaneous processes of apoptosis of the old exoskeleton and growth of the new exoskeleton. During post-molt (days 0-5) the new exoskeleton expands, deposition of the exocuticle continues, secretion of the endocuticle is initiated, and both sclerotization and calcification of the new exoskeleton occur. Again a prior increase of HIF-α mRNA expression from late pre-molt to early post-molt precedes the post-molt activities. Intermolt is a physiologically active time of cell proliferation and tissue growth. HIF may be involved in the down-regulation of post-molt-specific genes and up-regulation of intermolt-specific ones. Therefore the periodic increases in HIF-α mRNA expression across the molt-cycle may be in anticipation of HIF regulated product need. It may be that HIF-α is regulating Cc2, AMP6.0, ALF, and/or β-actin during the molt cycle. The up-regulation of HIF mRNA precedes up-regulation of each of these genes, suggesting possible downstream targets of HIF (Fig. 2).

Although the crabs were maintained in normoxic conditions, it may be that during the molt-cycle localized hypoxia occurred in specific tissues within the animal and triggered the peaks of HIF-α activation. Mammalian osteocytes have shown up-regulation of HIF-1α and HIF-2α mRNA expression, while under normoxic conditions (Gross et al., 2001). The up-regulation was postulated to be due to acute disuse and oxygen deprivation by the cells themselves and not a lack of oxygen in the environment (Gross et al., 2001). Hemocyanin levels in *C. magister* and other crabs decrease markedly during late premolt into early postmolt, lowering the oxygen carrying capacity of the hemolymph (Zuckerkandl, 1960; Terwilliger et al., 2005). In

addition, when the crab sheds its hard exoskeleton at ecdysis, it loses its ligamental connections between the heart and the dorsal carapace, presumably resulting in diminished heart stroke volume. Although the uptake of water at ecdysis (Mykles 1980; Magnum et al., 1985) aids in forming a temporary hydrostatic skeleton (Taylor and Kier, 2003), hemolymph circulation is probably diminished. These alterations in oxygen availability, in addition to the metabolic demands of molting, could cause localized areas of cellular hypoxia. Even in well-oxygenated seawater, internal hypoxia could be involved in the up-regulation of HIF-α activity.

Even though the HIF-α expression pattern is compelling, I do not have the replication necessary to pull out statistical significance. Increased replication might reduce the high variance and tighten the trends emerging in the current data. Another possibility is that crab HIF-α mRNA is an important component of the crab's metabolic control and is expressed constitutively, but is not inducible by environmental extremes or internal stress, as described for grass shrimp HIF-α (Li and Brouwer, 2007). Whether crab and/or shrimp HIF-α protein accumulates in response to stress remains to be demonstrated.

The up-regulation of Cc2 mRNA during the middle third of the molt cycle and a gradual decrease during pre-molt, reaching low levels after ecdysis during post-molt (days 0-5) are consistent with previous work on Cc mRNA in 1st and 5th instar juvenile *C. magister* and the crab *Portunus pelagicus* (Terwilliger et al., 2005; Phillips, 2007; Kuballa et al., 2007). Furthermore, cryptocyanin protein levels were seen to increase during pre-molt, and rapidly disappear following ecdysis as the molecule became

incorporated into the new exoskeleton and was taken up by reserve cells (Terwilliger et al., 2005). My data are consistent with the earlier experiments demonstrating that increased cryptocyanin mRNA precedes the high protein levels (Terwilliger et al., 2005; Phillips, 2007). The low levels of Cc2 mRNA during post-molt (day 0-5) in this study confirm earlier microarray analysis, qPCR, and immunoblot data (Terwilliger et al., 2005; Phillips, 2007). The new exoskeleton is formed in sequential layers throughout pre-molt and into post-molt. The mRNA up-regulation of Cc2 is synchronous with the synthesis of the new exoskeleton. The increase in Cc2 expression on day 16, although not statistically significant from intermolt or pre-molt days, is interesting considering that previous work showed a continual decrease of Cc mRNA during late pre-molt through ecdysis. The increase may be due to the variability in time to molt between animals, or perhaps Cc2 plays a previously unknown role right before ecdysis. My results support the role of cryptocyanin as a key protein, along with cuticular protein, in new exoskeleton formation.

Expression of arthrodial membrane protein 6.0 (AMP6.0) mRNA expression is up-regulated during pre- and post-molt and down-regulated during intermolt, confirming earlier microarray results (Phillips, 2007). The closest homologue of *C. magister* AMP6.0 that has been studied is *Callinectes sapidus* AMP6.0, which is also expressed in pre- and post-molt (Wynn and Shafer, 2005). The CsAMP6.0 protein has an important role in the deposition of the new exoskeleton during both pre- and post-molt (Wynn and Shafer, 2005), and *C. magister* AMP6.0 is thought to have a similar function. CsAMP6.0 is specifically incorporated into the flexible, permeable

membrane of the arthrodial joints, but is not found in the calcified cuticle of the hard exoskeleton (Wynn and Shafer, 2005). Other areas of uncalcified arthrodial type exoskeleton include the gill coverings and the branchial chambers. The timing of mRNA expression of *C. magister* AMP6.0 is consistent with the interval of the molt cycle when the animal is forming new arthrodial membrane.

In *C. magister*, anti-lipopolysaccharide factor (ALF) mRNA expression peaks during the transition from post-molt to intermolt. To defend themselves from the majority of invading pathogens, invertebrates make use of an innate immune system. This immune system includes pathogen recognition, phagocytosis, encapsulation, cytotoxicity, and melanization (Soderhall and Cerenius, 1992; Muta and Iwanaga, 1996). ALF binds to lipopolysaccharide on the cell walls of Gram-negative bacteria and inhibits the lipopolysaccharide-mediated coagulation cascade (Chaby, 2004). ALF's role in pathogen recognition and inhibition makes it an integral part of the innate immune system. In a microarray done on *C. magister* 1st instar juveniles, large clusters of pathogen defense genes had high transcript abundance during post-molt (Phillips, 2007). In *Homarus americanus* only one isoform of ALF, HamALF-1, was up-regulated in response to *Vibrio* infection (Beale et al., 2008). The other isoform, HamALF-2, like *C. magister* ALF, may be expressed during post-molt when the fragile, unscleratized exoskeleton is most vulnerable to injury and infection.

β-actin mRNA has high expression during pre-molt through post-molt but not in intermolt. In mammals actin is associated with cell growth (Bachvaroff et al., 1980). In the land crab, *Gecarcinus lateralis* and lobster, *Homarus americanus*, β-actin is one

of the major components of the epithelium, and the acellular membranous and endocuticular layers of the exoskeleton (Mykles et al., 2000). In *G. lateralis*, the synthesis of actin by the integument of the exoskeleton is stimulated during pre-molt (Stringfellow and Skinner, 1988; Varadaraj et al. 1997). My findings also support the need for β-actin synthesis in the production of the new exoskeleton. (Mykles et al., 2000). More analyses on β-actin's role in *C. magister* need to be performed, but the change in β-actin mRNA expression during molting make it a poor candidate for a housekeeping gene. The similar expression patterns of AMP6.0 and β-actin suggest a possible interrelationship among these genes in response to synthesizing the new exoskeleton.

All five genes showed changes in mRNA expression specific to stages within the molt cycle. HIF-α mRNA expression changed in normoxia during the molt cycle suggesting HIF may modulate multiple stress responses. The up-regulation of HIF-α mRNA precedes the expression of the genes in this study, supporting the idea that some of these genes may be downstream targets of HIF's response to the molt cycle. Even though this study did not specifically look at HIF and its direct downstream targets, the data warrant further investigation. Having described the gene expression during development, I can now look at mRNA expression of these genes during an abiotic stress.

BRIDGE TO CHAPTER III

Chapter II offers an introduction to the methods and techniques used to prepare whole juvenile *C. magister* total RNA for real-time qPCR analysis, an introduction essential for understanding how I obtained the data presented here and in the following chapter. By sampling on a daily basis I have a better understanding of the timing of product need for one regulatory, two exoskeleton, one immunodefense, and one cell structure and proliferation gene. Analysis of HIF-α mRNA expression has revealed the possibility of multiple gene regulation during the molt cycle. Patterns of mRNA expression of genes with more specific functions indicate the need for product during specific times of the molt cycle. These findings stimulate further investigation into alternative pathways for HIF regulation, and the functions of Cc2, AMP6.0, ALF, and β-actin as each relates to the molt cycle.

In the next chapter, I examine the responses of *C. magister* HIF-α and target gene mRNA expression to hyposalinity stress. Changes in salinity are key abiotic factors that affect *C. magister* along the Oregon coast. These changes occur seasonally and on a daily basis throughout the complex life history of *C. magister*. Chapter III builds on the data presented in Chapter II, and allows us to delineate between genes that are expressed as a function of the molt cycle versus those that are changing in response to hyposalinity.

CHAPTER III

EFFECTS OF HYPOSALINITY ON THE EXPRESSION OF HIF-α, CRYPTOCYANIN 2, ARTHRODIAL MEMBRANE PROTEIN 6.0, ANTI-LIPOPOLYSACCHARIDE FACTOR, AND β-ACTIN IN 1ST INSTAR JUVENILE *CANCER MAGISTER*

INTRODUCTION

Along the Oregon coast, Dungeness crab, *Cancer magister,* release embryos into the nearshore waters from December through March (Reed, 1969; Lough, 1976). The newly hatched larvae go through five zoeal stages while at sea, and they have been found as far as two hundred miles offshore (Lough, 1976). Zoeas metamorphose into actively swimming planktonic megalopas that reenter the coast and estuarine waters from Alaska to Baja California. The megalopas metamorphose into the first of multiple juvenile stages. The timing varies between latitudes, but the developmental process is the same. In Oregon, the majority of the young *C. magister* make the transition from pelagic to benthic lifestyle from mid April through June (Lough, 1976; Roegner et al., 2007) when seasonal rains decrease and the estuary becomes more oceanic. However, some megalopas occur as late as November off the Oregon coast (Roegner and Shanks,

2001). Successful recruitment of *C. magister* megalopas is strongly correlated with the timing of the spring transition (Shanks and Roegner, 2007). During this transition, the California Current system changes flow direction and carries megalopas back to settlement sites (Shanks and Roegner, 2007). Delays in the timing of this transition due to global climate change may influence *C. magister* juvenile populations dramatically (Tchernia, 1980; Shanks and Roegner, 2007).

The *C. magister* juveniles are subject to diurnal, seasonal and spatial salinity changes in the estuaries and nearshore habitats of the Oregon coast. Rainfall, evaporation, frequency and extent of tidal flooding, and topography all affect the salinity of *C. magister* habitat. In the Coos Bay estuary, as the tide recedes and freshwater input becomes more prominent, salinity levels can vary from ~33 ppt to ~14 ppt from summer to winter (South Slough Centralized Data Management Office, NOAA http://cdmo.baruch.sc.edu/). On the mudflats where large numbers of juvenile *C. magister* are found, summer temperatures can change from 10°C to 25°C when the tide has receeded and the mudflats are exposed. At the same time salinity drops from 32 ppt to 16 ppt as the freshwater lens passes down the mudflats (Brown, 1992; Brown and Terwilliger, 1992).

Changes in salinity affect growth, development, molting, behavior, aerobic metabolism, ion regulation, and the immune response in decapod crustaceans (Buchanan and Milleman, 1969; Brown and Bert, 1993; Sugarman et al., 1983; Brown and Terwilliger, 1999; Liu et al., 2006). Compared to megalopas and adult *C. magister*, 1st instar juveniles have the least osmoregulatory ability (Brown and

Terwilliger, 1992). For example, as salinity decreases, 1st instar juveniles do not show regulation of Ca^{2+}. Yet the 1st instars that settle in the estuary are exposed to the most extreme changes in salinity. Because of the salinity and temperature changes in the estuarine habitat, 1st instar juveniles may be near the limit of their respiratory and circulatory capacities (Brown and Terwilliger, 1999). Changes in environmental salinity cause many marine animals to allocate energy toward osmoregulation. As salinity decreases, hypoxia-tolerant marine animals begin to conserve energy by decreasing metabolism (Gorr et al., 2006). Another consequence of acute salinity change in crustaceans is an increased susceptibility to pathogens. In the shrimp *Fenneropenaeus chinensis*, acute hyposalinity change caused an increased susceptibility to white spot syndrome (WSS) ultimately leading to increased mortality (Liu et al., 2006). It is therefore important to understand the physiological mechanisms that allow *C. magister* juveniles to maintain functional homeostasis during times of hyposaline stress.

Hypoxia inducible factor, HIF, regulates target genes in response to low partial pressure of oxygen. Hypoxia, like salinity stress, can affect growth, development, metabolism, and the immune response. I wondered whether HIF, a highly conserved family of transcription factors found in yeast, mammals, teleosts, nematodes, arthropods such as *Drosophila*, *Daphnia*, and *C. magister,* and others, might regulate gene expression in response to salinity stress.

HIF is a heterodimeric protein composed of HIF-α and HIF-β subunits. In hypoxia-sensitive mammals, in normoxic conditions, HIF-1α (one of four HIF-α

isomers, 1-4, found in vertebrates) is rapidly degraded *via* the proteosome pathway, but under hypoxic conditions this subunit stabilizes and binds with HIF-1β thus allowing for gene specific targeting in the nucleus (Wang et al. 1993; Law et al., 2006). HIF-1α mRNA levels remain constant during hypoxia while the HIF-1α protein increases (Soitamo et al., 2001; Hara et al., 1999; Wenger et al., 1997), indicating that HIF is controlled at the protein level. However, recent studies on hypoxia-tolerant animals showed changes of HIF-α mRNA expression during hypoxia that resulted in increased HIF-α protein (Gorr et al., 2006; Law et al., 2006; Hoogewijs et al., 2007; Rahman et al., 2007). In specific circumstances in hypoxia-sensitive vertebrates, HIF-1α mRNA has also been demonstrated to increase (Blouin et al., 2004; Dery et al., 2005). In one case, under normoxic conditions, mice macrophages were exposed to an endotoxin, lipopolysaccharide (LPS). During immunochallenge HIF-1α mRNA expression increased in as little as 2 hours, reached maximal induction at 6 and 8 hours, and then decreased after 8 hours (Blouin et al., 2004). This finding suggests that the HIF-1 complex is involved in the immune response.

Changes in salinity affect metabolism and hemocyanin oxygen affinity in crustaceans (Brown and Terwilliger, 1992; Terwilliger and Brown, 1998), and changes in hemocyanin affinity directly affect an animal's ability to bind and transport oxygen. HIF may be up- or down-regulating genes during hyposalinity because of changes in metabolism and need for oxygen. My current study investigates the possibility of HIF involvement in the regulation of genes during hyposalinity stress under normoxic conditions in a weak osmoregulator, *C. magister*.

In a recent microarray study done on *C. magister* 1st instar juveniles, megalopas, and adults exposed to different salinities, several genes thought to not be directly involved in the response to hyposalinity showed unexpected changes in mRNA expression (Phillips, 2007). I selected three of these genes, cryptocyanin 2 (Cc2), antilipopolysaccharide factor (ALF), and arthrodial membrane protein 6.0 (AMP6.0), in addition to HIF-α and β-actin, for a time-course investigation of mRNA expression during hyposalinity stress in 1st instar *C. magister* juveniles.

MATERIALS AND METHODS

Animal Collection, Rearing, and Salinity Challenge

Cancer magister megalopas were collected using a dip net off the Charleston, OR marina docks at the mouth of the Coos Bay estuary on September 2, 2007. Megalopas were cultured at the Oregon Institute of Marine Biology in individual 16-oz flow-through containers in 2 aerated running seawater tables (15±1°C). Crabs were monitored for metamorphosis into 1st instar juveniles. On day 6 post-molt, four animals raised in running seawater were frozen for a time zero control. Starting on day 6 post-molt (intermolt stage), individuals were randomly assigned to filtered 100% (32 ppt) or 50% (16 ppt) seawater prepared from filtered seawater and distilled water. Each treatment consisted of a 38L aquarium filled with 12L of 50% or 100% SW, aerated, and placed in the running seawater table to maintain constant temperature (15±1°C). During the entire holding period, aquarium temperature and salinity were monitored

daily in the morning, afternoon, and evening. A refractometer (Reichert-Jung) was used to measure salinity. Every morning containers were cleaned and in the evening animals were fed *ad libitum* chopped *Mytilus sp.* Every other day, after feeding, 4 liters of water was replaced in each aquarium. Replacement water was prepared from filtered seawater as described above. Animals, in individual containers randomly assigned to one of four seawater tables, were then randomly assigned to 3 hrs, 8 hrs, or 24 hrs of treatment stress. One animal for each salinity treatment at each time point from each seawater table was frozen in liquid N_2 and stored at –80° C until RNA purification (24 animals total).

Total RNA Isolation and cDNA Synthesis

All samples were prepared for RNA isolation (n= 4) as in Chapter II. Briefly, the entire frozen crab was quickly weighed then homogenized in 3 ml denaturing solution. Total RNA was isolated using Rnase-free labware and a RNAgents® Total RNA Isolation System (Promega). RNA quality and quantity were analyzed using a Nanodrop spectrophotometer (Thermo Scientific) and a DU-640 spectrophotometer (Beckman and Coulter). A few samples were re-extracted using a RNAqueous-4PCR kit (Ambion) to insure absence of genomic DNA or degradation products in all the samples. For each sample an aliquot of 2 μg of total RNA was reverse transcribed using Superscript III First-Strand Synthesis (Invitrogen) with oligo-dT as primer. Samples of cDNA were stored at -80° C.

Species-specific Primer Design

Primers were designed as in Chapter II, Materials and Methods, Species-specific primer design.

Quantification of Gene Expression

Real-time quantitative PCR (qPCR) analysis was accomplished using target gene specific primers, SYBR GreenER Supermix (Stratagene), and an ABI Prism 7700 Sequence Detection System instrument at the University of Oregon. The thermal cycle was 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 sec and 60°C for 1 min. In order to assess product homogeneity, a thermal denaturation profile was generated following cDNA amplification. A standard curve dilution series from a day 6 juvenile in 50% SW for 24 hours was run and compared to samples to determine the relative expression of mRNA for each gene. All cDNA samples were diluted 1:10, run in triplicate, and the triplicate values were averaged. This method of adding the same amount of total RNA in each well is an accepted way to normalize the data when the system does not have a validated housekeeping gene (Bustin et al., 2005). Statistical analysis of time and salinity using repeated two-factor analysis of variance (ANOVAR) and Tukey posttests were accomplished using SAS. Time 0 was compared to controls using a one-factor ANOVA and Tukey posttests.

RESULTS

In all the real-time qPCR analyses the relative expression of mRNA was not due to differences in the placement of the aquariums (F=0.588, P=0.63).

Hypoxia Inducible Factor-α (HIF-α) Gene Expression

The qPCR analysis of HIF-α mRNA relative expression showed no significant differences between the salinity treatments, time, and the interaction of time and salinity (F=1.31 P=0.30, F=0.38 P=0.69, and F=0.93 P=0.42 respectively) (Fig. 3A).

Cryptocyanin 2 (Cc2) Gene Expression

The relative expression of Cc2 showed a 2-fold increase from 3 hrs to 24 hrs in both control and treatment groups (Fig. 3B) although not statistically significant (F=1.67 P=0.23). There was no significant difference in Cc2 mRNA relative expression between salinity treatment and control (F=0.12 P=0.74) nor was there an interaction of time and salinity (F=0.13 P=0.88).

Arthrodial Membrane Protein 6.0 (AMP6.0) Gene Expression

AMP6.0 mRNA relative expression decreased seven-fold from time 0 to 24 hours later (F=5.970 P=0.011) (Fig. 3C). This gradual decrease in expression is almost significant between 3 hours and 24 hours (F=3.47 P=0.06).

When analyzing the AMP6.0 mRNA relative expression of the salinity treatments and the interaction between time and salinity, there were no significant differences (F=0.01 P=0.93 and F=0.29 P=0.76, respectively).

Anti-lipopolysaccharide Factor (ALF) Gene Expression

The qPCR analysis of ALF mRNA relative expression showed no significant differences between the salinity treatments, time, and the interaction of time and salinity (F=1.26 P=0.31, F=1.38 P=0.29, and F=1.21 P=0.32, respectively) (Fig. 3D). Time and the interaction of time and salinity violated the assumption of sphericity. Therefore power was adjusted using the Greenhouse-Geisser estimator.

β-actin Gene Expression

β-actin mRNA relative expression had a significant difference between 3 hours and 24 hours (F=5.04 P=0.03). Time 0 would be significant from 24 hours if the threshold for P were set at P<0.1 (F=3.12 P=0.06). β-actin mRNA does appear to follow a trend of decreasing 2-fold over a 24-hour period (Fig. 3E). There was no significant difference in salinity treatments or the interaction between salinity and time.

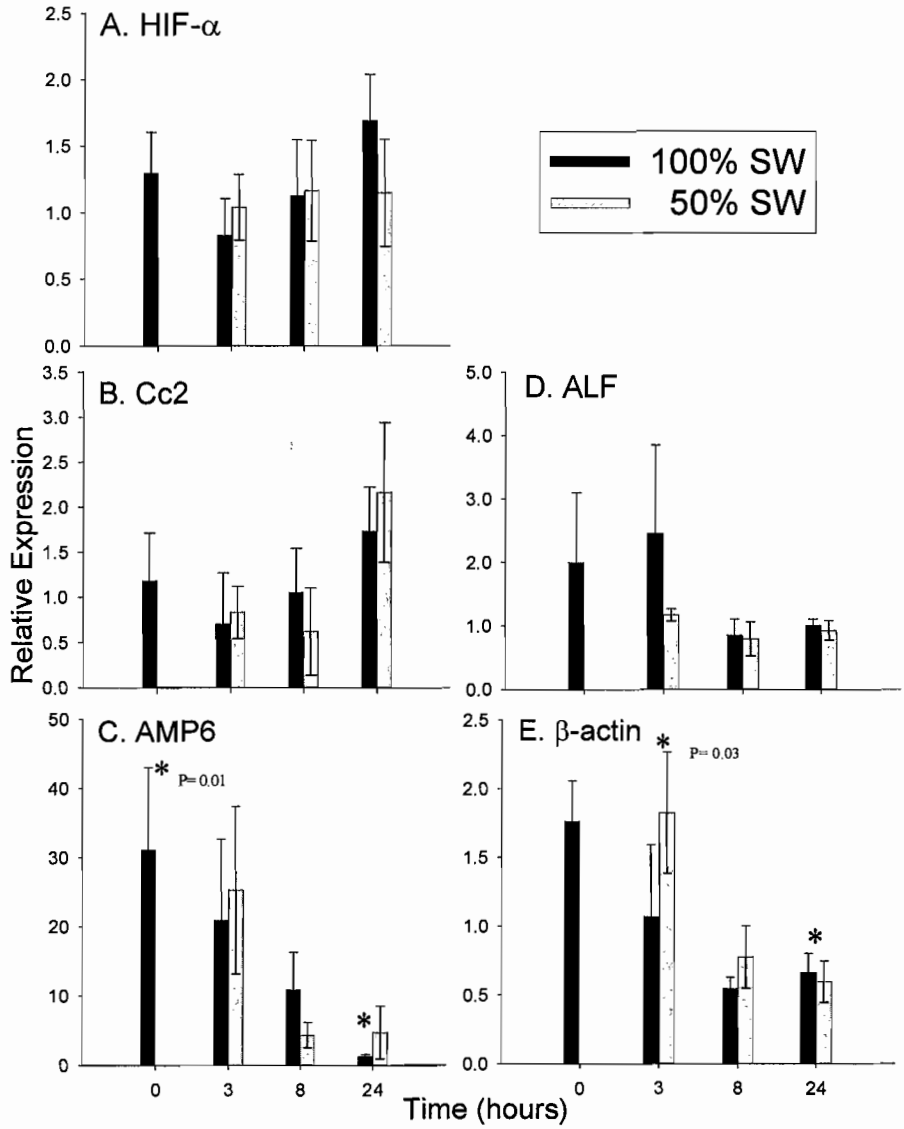

Fig. 3. Real-time quantitative PCR mRNA expression of five target mRNAs in C. magister 1st instar juveniles during hyposalinity (50% SW) stress: A) HIF-α, B) Cc2, C) AMP6.0, D) ALF, and E) β-actin. Dark bars represent 100% SW control and lighter bars represent 50% SW treatment. Time 0 are crabs before experiment started. All crabs started on day 6 intermolt stage. Each value represents the mean ± S.E.M (n= 4). Individuals were randomly assigned to 0, 3, 8 and 24 hours of hyposalinity stress, and mRNA levels were normalized to total RNA (2 μg) in each sample. Asterisks indicate statistically significant ($P<0.05$) using ANOVAR and Tukey post-hoc analysis. C. AMP6.0 time 0 is significantly different from control at 24 hours ($F=5.970$, $P=0.011$). E. β-actin mRNA relative expression is significantly different between 3 hours and 24 hours ($F=5.04$, $P=0.03$).

DISCUSSION

In a microarray analysis of gene expression in *C. magister* adult crabs, AMP6.0 was up-regulated after hyposalinity stress (50% SW for 8 hours) in gill tissue (Phillips, 2007). I investigated whether a similar increase in AMP6.0 mRNA occurs in an early developmental stage. In 1st instar juveniles, AMP6.0 mRNA expression decreased over 24 hours in both 50% SW and 100% SW (Fig.2D). The gradual decrease of expression over time is consistent with the down-regulation of AMP6.0 during intermolt (days 6-10) as seen in the molting study (Chapter II) and supports the hypothesis that AMP6.0 plays an integral role in the synthesis of the new exoskeleton. The juvenile crabs were stressed starting at day 6 to day 7 (intermolt) when little AMP6.0 mRNA is present (Chapter II). Hyposalinity did not increase mRNA expression. Based on my results, AMP6.0 clearly plays a specific role in the *C. magister* molt cycle (Chapter II), but it is not affected by hyposalinity stress. The up-regulation of AMP6.0 in the salinity stressed adult crab suggests AMP6.0 plays an additional role in adult *C. magister* (Phillips, 2007).

An alternative explanation could be that AMP6.0 mRNA is up-regulated in juvenile gill tissue as it is in the adult gill in response to hyposalinity stress, but analysis of whole crab mRNA is not as sensitive as using specific gill tissue. It is interesting to note the rapid decrease of AMP6.0 over 24 hours. In my molting study I sampled animals only every 24 hours, but the salinity results indicate that AMP6.0 mRNA expression can change in as little as 3 hours (Fig.3C).

During this study, the lack of any significant difference in HIF-α, Cc2, and ALF mRNA expression between controls and stressed individuals might be due to the high variability in mRNA expression among individuals. Repeating this experiment using more juveniles in order to increase power might result in a more definitive answer.

There does seem to be a suppression of HIF-α mRNA in 50% salinity when compared to the control at 24 hours. Perhaps activation of HIF-α mRNA in hyposalinity does not occur until 24 hours or more of stress. Sampling animals after a longer exposure time would be useful.

In adult *C. magister* hepatopancreas, Cc2 mRNA expression increased after 8 hours of hyposalinity stress according to microarray analysis (Phillips, 2007). This up-regulation of Cc2 was not seen in heart tissue (Phillips, 2007). As with AMP6.0 mRNA expression, it may be that using whole juvenile crab dilutes the results, and the current qPCR method using whole crab total RNA is not sensitive enough to see tissue-specific up-regulation of Cc2 due to hyposalinity stress. It could also be that there is a developmental difference in Cc2 expression and function between juveniles and adults.

In Phillips' study, ALF was up-regulated in 1st instar juveniles during 8 hours of hyposalinity stress, 50% SW (Phillips, 2007). My results do not show this change even after 24 hours, which is surprising. One possibility is seasonal variation between cohorts. The juveniles in this study were caught as megalopas in early September while Phillip's experiments used individuals caught in July.

There is a good possibility that late season megalopas in Coos Bay, OR are not local recruits but are recruits coming from Washington or British Columbia (Alan Shanks, personal communication). Genetic variation between Oregon and more northern populations might explain the differences in mRNA expression between cohorts. There is also the possibility that timing in the molt cycle plays a major role in up-regulation of ALF mRNA. The peak of ALF mRNA expression in the molt cycle occurs on days 4 and 5 (Chapter 2, Fig.2D). The crabs used in this study were day 6 crabs, post-ALF expression, and the crabs used in the Phillips' microarray study were younger than day 4-5, pre-ALF-expression. It may be that an increase in ALF expression due to hyposalinity stress occurs only during post-molt. It would be interesting to sample a cohort under hyposalinity stress across the entire molt cycle to see if this hypothesis is true.

β-actin mRNA showed a similar expression pattern as AMP6.0, a decrease of mRNA expression over time. β-actin did not respond to hyposalinity stress, but this pattern does support the molt cycle data in Chapter 2, where β-actin had little expression and decreased expression during intermolt (Fig.2E). β-actin would not be a good choice for a *C. magister* housekeeping gene when doing a timed study because of the rapid change in expression in less than 24 hours.

This time-course study examining the mRNA expression of HIF-α, Cc2, ALF, AMP6.0, and β-actin during 24 hours of hyposalinity stress did not show a change of expression in response to hyposalinity. There is a possibility that 50% salinity (16ppt) is not a sufficiently hyposaline stress in *C. magister* juveniles to stimulate mRNA

expression. It may be that regulation of mRNA does not occur until after 24 hours, and therefore longer stress times need to be performed. The changes in expression of AMP6.0 and β-actin mRNAs in both 50% and 100% salinity most likely reflect a molt cycle dependent change in expression from day 6 to day 7 crabs rather than because of hyposalinity stress. Genes have individual expression patterns during the molt cycle. It is imperative to take into consideration the stage of the molt cycle when doing gene expression studies in crustaceans.

Severe and prolonged salinity stresses can interfere with growth, ion and oxygen exchange, and require energy that would go to other key processes. HIF-α is an important transcription factor that during times of hypoxia regulates many genes. Cc2 and AMP6.0 are involved in building the new exoskeleton during the molt cycle, and ALF participates in the innate immune response. β-actin is part of the cytoskeleton and helps with cellular proliferation. Although their functions are very different, all showed stage-specific expression during the molt cycle (Chapter 2). However, their mRNA expression during hyposalinity still needs further investigation.

CHAPTER IV

CONCLUDING SUMMARY

The five genes of juvenile *C. magister* in the molting study all showed changes in mRNA expression specific to molt stage when their expression levels were analyzed on a daily basis. Hyposalinity did not seem to affect my target genes. The salinity study did show AMP6.0 and β-actin mRNA expressions decreasing over 24 hours. The gradual decrease of expression over time is consistent with my molting study and indicate a need for precise timing when doing experiments involving crustaceans and the molt cycle. There is a possibility of an interrelationship between AMP6.0 and β-actin because of their remarkably similar mRNA expression patterns during the molt cycle. Each gene is important in different physiological processes during the molt cycle so it would be interesting to investigate how they relate. HIF-α mRNA expression changed in normoxia during the molt cycle. This data supports the idea that HIF-α is responsible for regulating genes in response to multiple stressers not just hypoxia. The peaks of HIF-α mRNA expression precede the mRNA up-regulation of Cc2, AMP6.0, ALF, and β-actin, implicating these genes as possible HIF downstream targets during the molt cycle.

Cc2 and AMP6.0 seem to be directly involved with the synthesis and deposition of new exoskeleton because of the timing of mRNA expression during the synthesis of the new exoskeleton. ALF has a post-molt peak, which could indicate defense against pathogens. β-actin was up-regulated during pre- and post-molt indicating a need for the protein during the synthesis of the new exoskeleton. The changing levels of β-actin mRNA indicate that it would not be a good housekeeping gene. Sampling each day from one ecdysis to the next provided the opportunity to observe closely when mRNA levels rise and fall in anticipation of product need. Due to the rapid change of some genes and the unexpected little affect hyposalinity had on these genes, it would be interesting to test multiple times through-out the molt cycle under prolonged hyposalinity stress. When investigating the impact of an environmental stress it is important to consider the animal's natural development and note the developmental stage of test animals.

REFERENCES

Chapter I

Blouin, C.C., Page, E.L., Soucy, G.M. and Richard, D.E. (2004). Hypoxic gene activation by lipopolysaccharide in macrophages: implication of hypoxia-inducible factor 1α. *Blood* **103**, 1124-1130.

Brown, A.C. and Terwilliger, N.B. (1992). Developmental changes in ionic and osmotic regulation in the Dungeness crab, *Cancer magister*. *Biol. Bull.* **182**, 270-277.

de la Vega, E., Hall, M.R., Wilson, K.J., Reverter, A., Woods, R.G. and Degnan B.M. (2007). Stress-induced gene expression profiling in the black tiger shrimp *Penaeus monodon*. *Physiol. Genomics* **31**, 126-138.

Hoogewijs, D., Terwilliger, N.B., Webster, K.A., Powell-Coffman, J.A., Tokishita, S., Yamagata, H., Hankein, T., Burmester, T., Rytkonen, K.T., Nikinmaa, M., Abele, D., Heise, K., Lucassen, M., Fandrey, J., Maxwell, P.H., Pahlman, S. and Gorr, T.A. (2007). From critters to cancer: bridging trajectories between comparative and clinical research on oxygen sensing, HIF signaling, and adaptations towards hypoxia. *Integr. Comp. Biol.* **47**, 552-577.

Lough, R.G. (1976). Larval dynamics of the Dungeness crab, *Cancer magister*, off the central Oregon coast, 1970-71. *U.S. Natl. Mar. Fish. Serv. Fish. Bull* **74(2)**, 353-375.

Phillips, M.R. (2007). Functional genetic analysis of two non-model marine invertebrates: physiologically and environmentally induced changes in gene expression. Doctoral dissertation, University of Oregon.

Roegner, G.C., Armstrong, D.A., and Shanks, A.L. (2007). Wind and tidal influences on larval crab recruitment to an Oregon estuary. *MEPS.* **351**, 177-188.

Skinner, D. (1985). "Molting and Regeneration." *The Biology of Crustacea: Integument, Pigments, and Hormonal Processes*. New York: Academic Press Inc.

Somboonwiwat, K., Marcos, M., Tassanakajon, A., Klinbunga, S., Aumelas, A., Romestand, B., Gueguen, Y., Boze, H., Moulin, G. and Bachere, E. (2005). Recombinant expression and anti-microbial activity of anti-lipopolysaccharide factor (ALF) from the black tiger shrimp *Penaeus monodon*. *Dev. Comp. Immunol.* **29**, 841-851.

Terwilliger, N.B., Ryan, M.C., and Towle, D. (2005). Evolution of novel functions: cryptocyanin helps build new exoskeleton in *C. magister*. *J. Exp. Bio.* **208**, 2467-2474.

Wang, G.L. and Semenza, G.L. (1993). Characterization of hypoxia-inducible factor 1 and regulation of DNA binding activity by hypoxia. *J. Bio. Chem.* **268**, 21513-21518.

Wenger, R.H. (2002). Cellular adaptation to hypoxia: O_2-sensing protein hydroxylases, hypoxia-inducible transcription factors, and O_2-regulated gene expression. *FASEB J.* **16**, 1151-1162.

Wynn, A. and Shafer, T.H. (2005). Four differentially expressed cDNAs in *Callinectes sapidus* containing the Rebers-Riddiford consensus sequence. *Comp. Biol. Physiol. B.* **141**, 294-306.

Chapter II

Bachvaroff, R.J., Miller, F. and Rapaport, F.T. (1980). Appearance of cytoskeletal components on the surface of leukemia cells and of lymphocytes transformed by mitogens and Epstein-Barr virus. *Proc. Natl. Acad. Sci. USA* **77**, 4979-4983.

Beale, K.M., Towle, D.W., Jayasundara, N., Smith, C.M., Shields, J.D., Small H.J. and Greenwood, S.J. (2008). Anti-lipopolysaccharide factors in the American lobster *Homarus americanus*: Molecular characterization and transcriptional response to *Vibrio fluvialis* challenge. *Comp. Biochem. Physiol.* **3**, 263-269.

Blouin, C.C., Page, E.L., Soucy, G.M. and Richard, D.E. (2004). Hypoxic gene activation by lipopolysaccharide in macrophages: implication of hypoxia-inducible factor 1α. *Blood* **103**, 1124-1130.

Bustin, S.A., Benes, V., Nolan, T. and Pfaffl, M.W. (2005). Quantitative real-time RT-PCR – a perspective. *J. Mol. Endocrinol.* **34**, 597-601.

Chaby, R. (2004). Lipopolysaccharide-binding molecules: transporters, blockers and sensors. *Cell. Mol. Life Sci.* **61**, 1697-1713.

Chang, E.S., Bruce, M.J, and Tamone, S.L. (1993). Regulation of crustacean molting: a multi-hormonal system. *Amer. Zool.* **33**, 324-329.

de la Vega, E., Hall, M.R., Wilson, K.J., Reverter, A., Woods, R.G. and Degnan B.M. (2007). Stress-induced gene expression profiling in the black tiger shrimp *Penaeus monodon. Physiol. Genomics* **31**, 126-138.

Dery, M.C., Michaud, M.D. and Richard, D.E. (2004). Hypoxia-inducible factor 1: regulation by hypoxic and non-hypoxic activators. *Intern. J. Biochem. Cell Bio.* **37**, 535-540.

Gorr, T.A., Gassmann, M. and Wappner, P. (2006). Sensing and responding to hypoxia via HIF in model invertebrates. *J. Insect Physiol.* **52**, 349-364.

Gross, T.S., Akeno, N., Clemens, T.L., Komarova, S., Srinivasan, S., Weimer, D.A. and Mayorov, S. (2001). Physiological and genomic consequences of intermittent hypoxia selected contribution: Osteocytes upregulate HIF-1α in response to acute disuse and oxygen deprivation. *J. Appl. Physiol.* **90**, 2514-2519.

Hara, S., Kobayashi, C. and Imura, N. (1999). Molecular cloning of cDNAs encoding hypoxia-inducible factor (HIF)-1α and -2α of bovine arterial endothelial cells. *Biochim. Biophys. Acta* **1445**, 237-243.

Hoogewijs, D., Terwilliger, N.B., Webster, K.A., Powell-Coffman, J.A., Tokishita, S., Yamagata, H., Hankein, T., Burmester, T., Rytkonen, K.T., Nikinmaa, M., Abele, D., Heise, K., Lucassen, M., Fandrey, J., Maxwell, P.H., Pahlman, S. and Gorr, T.A. (2007). From critters to cancer: bridging trajectories between comparative and clinical research on oxygen sensing, HIF signaling, and adaptations towards hypoxia. *Int. Comp. Biol.* **47**, 552-577.

Kuballa, A.V., Merritt, D.J. and Elizur, A. (2007). Gene expression profiling of cuticular proteins across the moult cycle of the crab *Portunus pelagicus*. *BMC Biology* **5**, 45.

Law, S.H.W., Wu, R.S.S., Ng, P.K.S., Yu, R.M. and Kong, R.Y.C. (2006). Cloning and expression analysis of two distinct HIF-alpha isoforms – gcHIF-1alpha and gcHIF-4alpha - from the hypoxia-tolerant grass carp, *Ctenopharyngodon idellus. BMC Mol.Biol.* **7**, 15.

Li, T. and Brouwer, M. (2007). Hypoxia-inducible factor, gsHIF, of the grass shrimp *Palaemonetes pugio*: Molecular characterization and response to hypoxia. *Comp. Biochem. Physiol.* **147**, 11-19.

Mangum, C.P., McMahon, B.R., deFur, P.L. and Wheatly, M.G. (1985). Gas exchange, acid-base balance, and the oxygen supply to the tissues during a molt of the blue crab *Callinectes sapidus*. *J. Crust. Biol.* **5**, 188-206.

Muta, T. and Iwanaga, S. (1996). The role of hemolymph coagulation in innate immunity. *Current Opinion Immunology* **8**, 41-47.

Mykles, D.L. (1980). The mechanism of fluid absorption at ecdysis in the American lobster, *Homarus americanus*. *J. Exp. Biol.* **84**, 89-101.

Mykles, D.L., Haire, M.F., Skinner, D.M. (2000). Immunocytochemical localization of actin and tubulin in the integument of land crab (*Gecarcinus lateralis*) and lobster (*Homarus americanus*). *J. Exp. Zool.* **286**, 329-342.

Page, E.L., Robitaille, G.A., Pouyssegur, J. and Richard, D.E. (2002). Induction of hypoxia-inducible factor-1alpha by transcriptional and translational mechanisms. *J. Biol. Chem.* **277**, 48403-48409.

Phillips, M.R. (2007). Functional genetic analysis of two non-model marine invertebrates: physiologically and environmentally induced changes in gene expression. Doctoral dissertation, University of Oregon.

Rahman, M.S. and Thomas, P. (2007). Molecular cloning, characterization and expression of two hypoxia-inducible factor alpha subunits, HIF-1α and HIF-2α, in a hypoxia-tolerant marine teleost, Atlantic croaker (*Micropogonias undulates*). *Gene* **396**, 273-282.

Rissanen, E., Tranberg, H.K., Sollid, J., Nilsson, G.E. and Nikinmaa, M. (2006). Temperature regulates hypoxia-inducible factor-1 (HIF-1) in poikilothermic vertebrate, crucian carp (*Carassius carassius*). *J. Exp. Biol.* **209**, 994-1003.

Shams, I., Avivi, A. and Nevo, E. (2004). Hypoxic stress tolerance of the blind subterranean mole rat: Expression of erythropoietin and hypoxia-inducible factor 1α. *PNAS* **101**, 9698-9703.

Shechter, A., Tom, M., Yudkovski, Y., Weil, S., Chang, S.A., Chang, E.S., Chalifa-Caspi, V., Berman, A. and Sagi, A. (2007). Search for hepatopancreatic ecdysteroid- responsive genes during the crayfish molt cycle: from a single gene to multigenicity. *J. Exp. Biol.* **210**, 3525-3537.

Skinner, D. (1985). "Molting and Regeneration." *The Biology of Crustacea: Integument, Pigments, and Hormonal Processes*. New York: Academic Press Inc.

Soitamo, A.J., Rabergh, C.M., Gassmann, M., Sistonen, L. and Nikinman, M. (2001). Characterization of a Hypoxia-inducible Factor (HIF-1α) from Rainbow Trout. *J. Biol. Chem.* **276**, 19699-19705.

Soderhall, K. and Cerenius, L. (1992). Crustacean immunity. *Ann. Rev. Fish Dis.* **2**, 3-23.

Somboonwiwat, K., Marcos, M., Tassanakajon, A., Klinbunga, S., Aumelas, A., Romestand, B., Gueguen, Y., Boze, H., Moulin, G. and Bachere, E. (2005). Recombinant expression and anti-microbial activity of anti-lipopolysaccharide factor (ALF) from the black tiger shrimp *Penaeus monodon*. *Dev. Comp. Immunol.* **29**, 841-851.

Stillman, J.H., Colbourne, J.K., Lee, C.E., Patel, N.H., Phillips, M.R., Towle, D.W., Eads, B.D., Gelembulk, G.W., Henry, R.P., Johnson, E.A., Pfrender, M.E. and Terwilliger, N.B. (2008). Recent advances in crustacean genomics. *Int. Comp. Biol.* **48**, 852-868.

Stringfellow, L.A. and Skinner, D.M. (1988). Molt-cycle correlated patterns of synthesis of integumentary proteins in the land crab *Gecarcinus lateralis*. *Dev. Biol.* **128**, 97-110.

Taylor, J.R.A. and Kier, W.M. (2003). Switching skeletons: hydrostatic support in molting crabs. *Science* **301**, 209-210.

Terwilliger, N.B., Dangott, L.D. and Ryan,M.C. (1999). Cryptocyanin, a crustacean molting protein: evolutionary link with arthropod hemocyanins and insect hexamerins. *Proc. Natl. Acad. Sci. USA* **96**, 2013-2018.

Terwilliger, N.B., Ryan, M.C., and Towle, D. (2005). Evolution of novel functions: cryptocyanin helps build new exoskeleton in *C. magister*. *J. Exp. Bio.* **208,** 2467-2474.

Terwilliger, N.B. and Ryan, M.C. (2006). Functional and phylogenetic analyses of phenoloxidases from brachyuran (*Cancer magister*) and branchiopod (*Artemia franciscana, Triops longicaudatus*) crustaceans. *Biol. Bull.* **210**, 38-50.

Towle, D.W., Rushton, M.E., Heidysch, D., Magnani, J.J., Rose, M.J., Amstutz, A., Jordan, M.K., Shearer, D.W. and Wu, W.S. (1997). Sodium-proton antiporter in the euryhaline crab *Carcinus maenas*: Molecular cloning, expression and tissue distribution. *J. Exp. Biol.* **200**, 1003-1014.

Varadaraj, K., Kumari, S.S. and Skinner, D.M. (1997). Molecular characterization of four members of the α-tubulin gene family of the Bermuda land crab *Gecarcinus lateralis*. *J. Exp. Zool.* **278**, 63-77.

Wainwright, T. and Armstrong, D.A. (1993). Growth Patterns of the Dungeness Crab (*Cancer magister* Dana): Synthesis of Data and Comparison of Models. *J. Crust. Biol.* **13**, 36-50.

Wang, G.L. and Semenza, G.L. (1993). Characterization of hypoxia-inducible factor 1 and regulation of DNA binding activity by hypoxia. *J. Biol. Chem.* **268**, 21513-21518.

Wang, D.P., Li, H.G., Li, Y.J., Guo, S.C., Yang, J., Qi, D.L., Jin, C. and Zhao, X.Q. (2006). Hypoxia-inducible factor 1α cDNA cloning and its mRNA and protein tissue specific expression in domestic yak (*Bos grunniens*) from Qinghai-Tibetan plateau. *Biochem. Biophys. Res. Comm.* **348**, 310-319.

Wenger, R.H., Kvietikova, I., Rolfs, A., Gassmann, M. and Marti, H.H. (1997). Hypoxia-inducible factor-1 alpha is regulated at the post-mRNA level. *Kidney Int.* **51**, 560-563.

Willmer, P., Stone, G., and Johnston, I. (2005). *Environmental Physiology of Animals, 2nd Edition.* Oxford, UK: Blackwell Science Ltd.

Wynn, A. and Shafer, T.H. (2005). Four differentially expressed cDNAs in *Callinectes sapidus* containing the Rebers-Riddiford consensus sequence. *Comp. Biol. Physiol. B.* **141**, 294-306.

Zuckerkandl, E. (1960). Hemocyanine et cuivre chez un crustace decapode dans leurs rapports avec le cycle d'intermue. *Annales de l'Institit Oceanographique, Monaco* **38**, 1-122.

Chapter III

Blouin, C.C., Page, E.L., Soucy, G.M. and Richard, D.E. (2004). Hypoxic gene activation by lipopolysaccharide in macrophages: implication of hypoxia-inducible factor 1α. *Blood* **103**, 1124-1130.

Brown, A.C. (1992). Effects of salinity and temperature on the respiratory physiology of the Dungeness crab, *Cancer magister*. Doctoral dissertation. University of Oregon

Brown, A.C. and Terwilliger, N.B. (1992). Developmental changes in ionic and osmotic regulation in the Dungeness crab, *Cancer magister*. *Biol. Bull.* **182**, 270-277.

Brown, A.C. and Terwilliger, N.B. (1998). Ontogeny of hemocyanin function in the Dungeness crab *Cancer magister*: hemolymph modulation of hemocyanin oxygen-binding. *J. Exp. Biol.* **201**, 819-826.

Brown, A.C. and Terwilliger, N.B. (1999). Developmental changes in oxygen uptake in *Cancer magister* (Dana) in response to changes in salinity and temperature. *J. Exp. Marine Biol. Ecol.* **241**, 179-192.

Brown, S.D. and Bert, T.M. (1993). The effects of temperature and salinity on molting and survival of *Menippe adina* and *M. mercenaria* (Crustacea, Decapoda) postsettlement juveniles. *MEPS* **99**, 41-49.

Buchanan, D.W. Milleman, R.E. (1969). The prezoeal stage of the Dungeness crab, *Cancer magister* Dana. *Biol. Bull.* **137(2)**, 250-255.

Bustin, S.A., Benes, V., Nolan, T. and Pfaffl, M.W. (2005). Quantitative real-time RT-PCR – a perspective. *J. Mol. Endocrinol.* **34**, 597-601.

Dery, M.C., Michaud, M.D. and Richard, D.E. (2004). Hypoxia-inducible factor 1: regulation by hypoxic and non-hypoxic activatiors. *Intern. J. Biochem. Cell Bio.* **37**, 535-540.

Gorr, T.A., Gassmann, M. and Wappner, P. (2006). Sensing and responding to hypoxia via HIF in model invertebrates. *J. Insect Physiol.* **52**, 349-364.

Hara, S., Kobayashi, C. and Imura, N. (1999). Molecular cloning of cDNAs encoding hypoxia-inducible factor (HIF)-1α and -2α of bovine arterial endothelial cells. *Biochim. Biophys. Acta* **1445**, 237-243.

Hoogewijs, D., Terwilliger, N.B., Webster, K.A., Powell-Coffman, J.A., Tokishita, S., Yamagata, H., Hankein, T., Burmester, T., Rytkonen, K.T., Nikinmaa, M., Abele, D., Heise, K., Lucassen, M., Fandrey, J., Maxwell, P.H., Pahlman, S. and Gorr, T.A. (2007). From critters to cancer: bridging trajectories between comparative and clinical research on oxygen sensing, HIF signaling, and adaptations towards hypoxia. *Int. Comp. Biol.* **47**, 552-577.

Law, S.H.W., Wu, R.S.S., Ng, P.K.S., Yu, R.M. and Kong, R.Y.C. (2006). Cloning and expression analysis of two distinct HIF-alpha isoforms – gcHIF-1alpha and gcHIF-4alpha - from the hypoxia-tolerant grass carp, *Ctenopharyngodon idellus*. *BMC Mol. Biol.* **7**, 15.

Liu, B. Yu, Z. Song, X. Guan, Y. Jian, X. He, J. (2006). The effect of acute salinity change on white spot syndrome (WSS) outbreaks in *Fenneropenaeus chinensis*. *Aquaculture* **253,** 163-170.

Lough, R.G. (1976). Larval dynamics of the Dungeness crab, *Cancer magister*, off the central Oregon coast, 1970-71. *U.S. Natl. Mar. Fish. Serv. Fish. Bull* **74(2),** 353-375.

Phillips, M.R. (2007). Functional genetic analysis of two non-model marine invertebrates: physiologically and environmentally induced changes in gene expression. Doctoral dissertation, University of Oregon.

Rahman, M.S. and Thomas, P. (2007). Molecular cloning, characterization and expression of two hypoxia-inducible factor alpha subunits, HIF-1α and HIF-2α, in a hypoxia-tolerant marine teleost, Atlantic croaker (*Micropogonias undulates*). *Gene* **396**, 273-282.

Reed, P.N. (1969). Culture methods and effects of temperature and salinity on survival and growth of Dungeness crab *Cancer magister* larvae in the laboratory. *J. Fish. Res. Board Can.* **26(2)**, 389-397.

Roegner, G.C., Armstrong, D.A., and Shanks, A.L. (2007). Wind and tidal influences on larval crab recruitment to an Oregon estuary. *MEPS.* **351**, 177-188.

Roegner, G.C. and Shanks, A. (2001). Import of coastally-derived chlorophyll a to South Slough, Oregon. *Estuaries* **24**, 224-256.

Shanks, A.L. and Roegner, G.C. (2007). Recruitment limitation in Dungeness crab populations is driven by variation in atmospheric forcing. *Ecology* **88**, 1726-1737.

Soitamo, A.J., Rabergh, C.M., Gassmann, M., Sistonen, L. and Nikinman, M. (2001). Characterization of a Hypoxia-inducible Factor (HIF-1α) from Rainbow Trout. *J. Biol. Chem.* **276**, 19699-19705.

Tchernia, P. (1980). Descriptive regional oceanography. Pergamon Press, Oxford, UK.

Wang, G.L. and Semenza, G.L. (1993). Characterization of hypoxia-inducible factor 1 and regulation of DNA binding activity by hypoxia. *J. Biol. Chem.* **268**, 21513-21518.

Wenger, R.H., Kvietikova, I., Rolfs, A., Gassmann, M. and Marti, H.H. (1997). Hypoxia-inducible factor-1 alpha is regulated at the post-mRNA level. Kidney Int. **51**, 560-563.