4-1-2011

Activation Of AMP-Activated Protein Kinase As An Early Indicator For Stress In The Lobster, Homarus Americanus

Stephanie Podolski
University of New England

Activation of AMP-activated protein kinase as an early indicator for stress in the lobster, Homarus americanus

An Honors Thesis Presented to
The Faculty of the Department of Biology
University of New England

In partial fulfillment of the
Requirements for the Degree of
Bachelor of Science with Honors in
Medical Biology-Medical Sciences

by

Stephanie Podolski
Undergraduate Honors Medical Biology Student
University of New England
April 5, 2011

Thesis Examining Committee:

Markus Frederich, Ph.D, Thesis Advisor, (Department of Marine Sciences, CAS, UNE)
Geoffrey Ganter, Ph.D. (Department of Biology, CAS, UNE)
Stine Brown, Ph.D. (Department of Biology, CAS, UNE)

ACKNOWLEDGMENTS

For providing me this opportunity and for offering guidance, direction, and support, as well as being valued members of my thesis committee, I would like to thank the following:

Markus Frederich, Ph.D.

Geoffrey Ganter, Ph.D.

Christine Brown, Ph.D.

For collecting our model species, the American lobster, *Homarus americanus*. Thank you to the following:

Robert Bragdon

Robert Collard

James Sulikowski, Ph.D.

For laboratory assistance and for all of the support, guidance, and feedback throughout the course of my thesis preparation, I would like to thank the following:

Ellen Shores

Sabrina Klick

Casey Toombs

Jennifer Jost, Ph.D.

As well as my family and close friends

I would finally like to take a moment to thank again, **Dr. Markus Frederich** for his patience and dedication throughout my research experience. His enthusiasm and dynamic ability to teach and to provide unmatchable advice have made this experience an integral part of my education.

ABSTRACT

Variations in water temperature, salinity, pH, and oxygen concentration are stressors that marine invertebrates face on a daily basis. Each of these physiological stressors creates a large cellular demand for energy. In mammals, energy metabolism is regulated by the enzyme AMP-activated protein kinase (AMPK), which is highly conserved during evolution. This project was designed to test the hypothesis that AMPK is present and activated by temperature, hypoxia, and anoxia stress in the lobster, *Homarus americanus*.

Animals were exposed to a rapid and progressive increase in temperature (6ºC per hour) beginning at 14 ºC. We measured lactate concentrations and AMPK activity and heat shock protein 70 (HSP70) levels in 2ºC increments (14-32ºC) in heart, muscle, and liver tissue. Lactate concentration remained at low control levels between 14 and 28ºC and increased significantly (ANOVA, $p<0.05$) in heart, liver, and muscle between 28°C and 30°C. In the heart, liver, and muscle tissues HSP70 levels remained constant during the temperature exposure. AMPK activity significantly increased up to 2.2±1.2 at 30°C and 2.9±0.8 fold at 33º. In the liver AMPK activity remained constantly low, between 14 and 28ºC, but increased up to 2.7±0.4 at 30°C and 1.9±0.5 fold at 33º. In the muscle AMPK activity remained constantly low.

Secondly, lobsters were exposed for a 24 hour time period to the sub-lethal temperature of 28°C. The prolonged exposure to heat led to a significant increase in AMPK liver activity up to 2.1±0.1 fold between 0 and 24 hours. AMPK did not significantly increase in the heart or muscle tissues. HSP70 levels remained constant in heart, liver, and muscle tissues.

Lastly, to characterize the role of AMPK during hypoxia lobsters were exposed for 24 hours to a low oxygen concentration of 4 kPa. The same measurements as described above were performed at 0, 4 and 24 hours. We found up to a 6-fold increase in AMPK activity and a nearly

40 fold increase in AMPK mRNA expression of heart tissue after 24 h of hypoxia. In the liver nearly a 1000 fold increase was found in AMPK mRNA expression. HSP70 mRNA and protein expression remained unchanged.

The data show that in lobsters AMPK activation is an early indicator of stress when cellular energy levels are depleted, as indicated by the concurrence of AMPK activation and lactate accumulation. The traditionally used marker, HSP70, was a less reliable indicator for stress. Future comparison with other crustacean species will indicate whether AMPK activation during stress is a more potent mechanism than HSP70 for assessing stress levels in other populations of invertebrates.

INTRODUCTION

Climate change is a global phenomenon, however, from an individual organism stand point, any change in the environment is locally focused and therefore affects the physiological mechanisms and systems that allow these organisms to survive. Because there is such a large fluctuation and continuous increase in environmental parameters, such as air and water temperature on a global scale, it is important to understand how large scale signals are downscaled to the level of the organism and directly impact the organisms' physiological niche. This physiological niche can be described by optimal levels of performance at the cellular level. Stressors, such as water temperature based on year and season, body temperature, and gas concentrations within the organism's habitat, extend an organism's physiology beyond its niche level constraints and drive measureable physiological processes (Helmuth et al., 2010). It is vital to understand how the animals' physiology will be affected, particularly marine invertebrates, because of their inability to physiologically regulate body temperature and the frequent exposure to a wide range of water temperature fluctuations.

The cellular effects of stressful conditions, such as those created by climate change, can be quantified using physiological parameters. Physiologists often use heat shock proteins (HSP) as a marker for temperature or other types of stress because HSPs are found in nearly all living organisms. (e.g. Hoffmann et al., 2003, Tomanek, 2005). The rare exception remains Antarctic eelpouts which live in an exceptionally stable thermal environment (Hoffmann et al. 2000). Heat shock proteins (HSPs) act as chaperones and aid in the conformational stabilization of other proteins, preventing the proteins from aggregating and being rendered nonfunctional (Feige et al., 1996, Frydman, 2001). HSPs are controlled by a transcriptionally regulated increase in gene expression. In a non-stressed state the cells contain HSP bound to heat shock factors (HSF). Once cells are exposed to heat stress, HSP and HSF dissociate. HSP binds to denaturing proteins, while HSF translocates to the nucleus, activating transcription of the HSP gene, resulting in the production of HSP messenger RNA (mRNA) (Morimoto 1993). This mRNA is then translated into more HSPs that further bind to denatured proteins initially damaged by stress (Sorger 1991). When a sufficient amount of HSP is produced, HSP will reattach itself to HSF, stopping the cascade. With this mechanism, the heat shock response produces a measurable time lag between the actual stress and the detectable HSP response. As a result of this time lag, HSP often does not increase until after animals are returned to control conditions (Frederich et al. 2009). Furthermore, the heat shock response and production of heat shock proteins adds considerably to the cellular ATP demand because of the ATP consuming protein folding and denaturing pathways.

In order to identify a faster indicator of cellular stress than the standard HSP, this project targets the enzyme AMP-activated protein kinase, AMPK. AMPK is well described in mammalian systems, and, is involved in various diseases, such as diabetes, heart disease and

obesity (Hardie & Carling 1997). The key function of AMPK is to regulate the cellular ATP pool. Temperature stress and ATP pool regulation are tightly connected because temperature has a direct effect on metabolic rate, which affects the amount of ATP used (Hardie et al. 2006). However, very little is known about AMPK and the role it plays in regulating ATP synthesis and hydrolysis in invertebrates.

As described in mammalian systems, the AMPK protein is a heterotrimer that consists of three subunits; the alpha subunit is responsible for kinase activity, while the beta subunit connects the alpha and gamma subunits (Hardie & Carling 1997). The gamma subunit is the primary site where AMP binds, thus creating a conformational change in the AMPK structure. During this conformational change, the alpha subunit becomes exposed, increasing binding activity, and allowing for further AMPK activation (Hardie and Sakamoto 2006). The activation of AMPK is triggered by the binding of AMP and the activation through an upstream AMPK-kinase (AMPKK). AMP is an important indicator for metabolic rate and the respective breakdown of ATP, and is the first step of AMPK activation. This can be seen when ATP is hydrolyzed and converted to ADP and inorganic phosphate (P_i). Subsequently, the enzyme, adenylate kinase, converts two ADP molecules into ATP and AMP.

$$ATP \rightarrow ADP + Pi \qquad \text{(ATP hydrolysis)}$$
$$2ADP \rightarrow ATP + AMP \text{ (Adenylate kinase reaction)}$$

Therefore, AMP accumulates during enhanced ATP hydrolysis and is an ideal signal for increased energy demand. To meet this ATP demand, AMPK is consequently activated with increasing AMP concentrations (see figure 2). In addition to the allosteric activation of AMPK by AMP, AMPK is activated by phosphorylation of and upstream kinase, AMPKK, which is also activated by AMP. AMPKK phosphorylates AMPK at the threonine 172 site of the alpha subunit which leads to increased AMPK activity. Phosphorylation is the much stronger activator of

AMPK and therefore AMPK phosphorylation reflects the AMPK activity. Once AMPK is activated, it phosphorylates key enzymes of ATP consuming and producing pathways, which in turn maintains a constant ATP pool needed to maintain cellular activity.

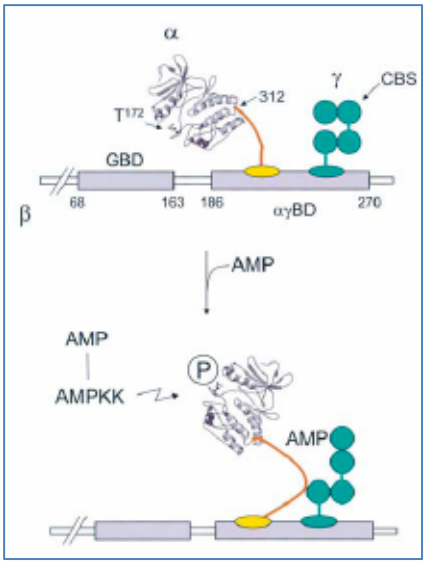

Figure 1: AMPK alpha, beta, and gamma subunits. AMP binds to the gamma subunit and also activates AMPKK which phosphorylates AMPK at the T172 binding site of the alpha subunit. Both mechanisms simultaneously cause a conformational change in the alpha and gamma subunit and activate AMPK. (from Rutter et al. 2000)

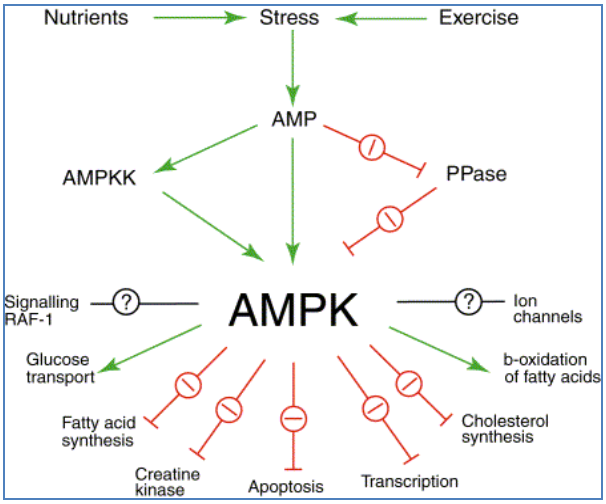

Figure 2: Stress, such as exercise and nutrient depletion, leads to the accumulation of AMP which activates AMPKK in order to phosphorylate AMPK. This cascade regulates ATP by either activating or inhibiting specific metabolic pathways in order to produce or conserve sufficient levels of ATP. (from Kemp et al. 1999)

Basic thermodynamics and enzyme function cause an increase of enzymatic activity with temperature. Since the cellular metabolism is just the sum of multiple enzyme reactions, a temperature increase will accelerate cellular metabolism and therefore, ATP use. This can be quantified as the Q_{10}. The ratio between two metabolic rates 10 degrees apart will be in the range of 2-3 (i.e. Q_{10} = 2-3) for most biological reactions. The doubling or tripling of cellular metabolism with a 10ºC increase requires an additional increase in the amount of ATP needed. Because of this, regulating ATP producing and ATP consuming pathways during thermal stress is critical.

Under non-stressful conditions, aerobic metabolism provides the fuel and energy needed to sustain and power any organism. In this metabolic pathway glycolysis yields pyruvate which is subsequently used in the Krebs cycle to form NADH and $FADH_2$. NADH is the electron donor for the oxidative phosphorylation used to form adenosine-triphosphate (ATP). At the end of the aerobic cycle, a theoretical maximum of thirty six molecules of ATP is yielded for each molecule of glucose used as the initial carbohydrate source. The alternative metabolism, which is an oxygen deficient process, is called anaerobic metabolism. During oxygen deficiency, the anaerobic metabolism process of glycolysis produces only two molecules of pyruvate for each molecule of glucose used. The produced pyruvate is then converted by the enzyme lactate dehydrogenase into lactic acid, which is the main anaerobic end product in mammals and crustaceans. Extreme temperatures can lead to a transition from aerobiosis to anaerobiosis, therefore reducing the amount of anaerobically produced ATP, eventually leading to death. This transition to anaerobiosis in the presence of sufficient oxygen is caused by a temperature-induced failure of oxygen delivery by ventilation or circulation. Subsequently the animal experiences a mismatch between oxygen demand and supply, resulting in anaerobiosis during heat stress.

Past research has shown the transition to anaerobiosis by heat stress in the heart tissue of the rock crab, *Cancer irroratus*, concomitant with AMPK activation, while HSP70 expression was delayed (Frederich et al. 2009). The activation of AMPK was actually shown to occur before the switch to anaerobiosis and to be a faster mechanism than elevated HSP70 expression during a fast progressive heat stress. Crabs were additionally exposed for 6 hours to the sub-lethal temperature of 26°C. As an additional adaptation to heat stress, extended exposure resulted in a consistent increase in AMPK mRNA levels. However, HSP70 mRNA and protein remained constant. The additional response in gene expression supported the conclusion that AMPK was an earlier marker for temperature stress than HSP70 in this crab species (Frederich et al. 2009).

That study raised the questions as to whether the observed early activation of AMPK during heat stress in the crab heart tissue is a general mechanism that can be applied to other crustacean species, like the lobster, and, whether the response seen was specific to heart tissue only. Therefore in this project we applied a similar approach and tested the temperature induced AMPK activation and the respective HSP70 expression, but focused on a different species, the commercially important lobster, *Homarus americanus*, and investigated the stress response in several different tissues. The similarities and differences between the lobster and the rock crab will reveal more about the mechanism of how fluctuations in temperature affects marine invertebrates, specifically the role of AMPK as a cellular marker. Furthermore, the switch to anaerobiosis at high temperatures raises the questions whether AMPK is activated by the increased temperature or the resulting hypoxia. To address this, we compared the temperature induced AMPK activation with the activation of AMPK cause by hypoxia only at control temperature.

The lobster is a poikilotherm, an animal unable to physiologically regulate its own body temperature, which inhabits marine environments where yearly water temperatures can vary over a 25°C range (0-25°C), depending on the winds, tides, and seasons (Camacho et al. 2006). Because of this thermal fluctuation, the lobsters' organ systems must physiologically adapt. The adaptation or reaction to temperature changes is well documented in the lobster and other crustaceans. The upper limit for cardiac function for lobsters that have been acclimated up to 20°C is just about 29°C, which is 5°C warmer than lobsters that were acclimated to 4°C and shows that lobsters have some plasticity based on acclimation (Camacho et al. 2006). The original thought that crustaceans were only active during a specific "energy range", was devised on maximizing energy output. However, thermal acclimatization in the environment between seasons or adaptation to a specific climate change event involves shifting of thermal windows for each organ system, such as the upper limit for cardiac function during heat stress (Pörtner 2009). The relationship between body temperature and different organ systems already shows that different unique responses occur for each organ system. However, there is not any information available about AMPK and the tissue specific response to temperature stress.

To investigate whether AMPK is activated in a tissue specific manner in the lobster during temperature stress, and whether the AMPK activation due to temperature-induced hypoxia is the same as in temperature-independent hypoxia, we tested the following hypotheses:

- AMPK is present and activated by temperature stress in the lobster, *Homarus americanus*.
- AMPK activation and expression occurs in a tissue specific manner due to the different energy demands of the respective tissues.
- AMPK is present and activated by hypoxia stress in the lobster, *Homarus americanus*.

MATERIALS AND METHODS

Animals: Lobsters (*Homarus americanus)* with an average carapace length 9.0±0.28 cm were obtained from a local fisherman in Winter Harbor, ME and Biddeford, ME. Animals were housed in the flow through sea water system in the Marine Science Center at 12°C for at least 24 hour before experimentation and were fed fish and squid, *ad libitum*.

Temperature loggers*:* To obtain more accurate temperature measurements in the habitat, the local fisherman equipped lobster traps with temperature data loggers that recorded the water temperature every thirty minutes over several days, providing information about the thermal history of the animals used.

Temperature Incubations: Animals (n=5 per temperature) were exposed to a fast and progressive increase in temperature, beginning at 14 ºC and increasing 6ºC per hour. The lobsters were sacrificed at 14, 16, 18, 20, 22, 24, 26, 28, 20, and 32 ºC. In a second experiment, lobsters were exposed to a long sub-lethal temperature of 26 ºC for a 24 hour time period. Lobsters were sacrificed at control temperatures (12 ºC), after 4 hours, and after 24 hours. For both short and long term temperature studies tissues were extracted from the liver, heart, brain, and tail muscle. Tissues used to measure lactate build up in lactate dehydrogenase assays and to measure AMPK activity and HSP70 protein levels were flash frozen with liquid nitrogen and stored at -80°C. The tissues used to measure AMPK gene expression were stored in RNAlater© solution (Ambion, Austin, TX) at 4°C.

Hypoxia/Anoxia Incubations: For hypoxia exposure, lobsters were incubated at 5% oxygen (4 kPa) for 24 hours, using a bubble stone connected to a nitrogen gas tank and a dissolved oxygen meter to ensure that the tank level remained stable. Lobsters were sacrificed after 0, 4, and 24 hours. For anoxia exposure, lobsters were incubated at 0.7% oxygen for two hours (0.3kPa) for 2 hours. Tissue for both hypoxia and anoxia experiments were extracted from the liver, heart, and tail muscle tissue at 0, 20, 40, 80, and 120 minutes. Tissues were used to measure lactate build up in lactate dehydrogenase assays and to measure AMPK activity and HSP70 protein levels were flash frozen with liquid nitrogen and stored at -80°C. The tissues were used to measure AMPK gene expression for hypoxia experiments were stored in RNAlater© solution (Ambion, Austin, TX) at 4°C.

Lactate: The lactate concentration in each tissue sample was measured to determine the onset of anaerobiosis, indicating severe temperature stress. For this assay, samples were homogenized under liquid nitrogen and protein was removed by acid precipitation. Lactate concentrations in the protein free supernatant were measured in a photometric test enzyme test (Bergmeyer 1985). The enzyme assay measures the buildup of NADH from the reaction lactate + NAD → pyruvate + NADH, catalyzed by lactate dehydrogenase. The lactate concentrations were normalized to the protein concentration in each sample that was measured using the Bradford method (Bradford 1976).

Heart Rate**:** Photoplethysmographs (IsITech, Bremerhaven, Germany), super-glued with dental wax to the carapace above the heart, were used to measure heart rate. Sensors were connected to a digital recording device (Power Lab, AD Instruments, Mountain View, CA).

Reaction Time: Lobsters were exposed to the above temperature, hypoxia, and anoxia conditions. Lobsters were flipped over on their backs and the amount of time it took for each lobster to right itself was recorded in seconds. In the same experiment, the percent of animals that stopped righting themselves was recorded. Animals were considered not respond if 5 minutes elapsed without a righting response.

***AMPK activity*:** AMPK activity was measured by western blots. For this method tissue samples were homogenized under liquid nitrogen and transferred to a buffer containing several phosphatase inhibitors, which were used to preserve the phosphorylation stage of the AMPK mechanism. After quantification of the protein concentration by the Bradford method, samples were diluted to equal amounts of protein concentrations and separated by SDS gel electrophoresis. The separated proteins were then transferred onto a nitrocellulose membrane. This membrane was blocked with 3% non-fat dry milk and then incubated with a primary anti-p172 AMPK antibody that was designed for mouse tissue, but cross reacts with lobster tissue as well. A secondary antibody with a linked horseradish peroxidase (HRP) enzyme was used to induce a chemiluminescent signal of the AMPK band. Membranes were scanned and the bands were analyzed using Image J. See Figure 3 below for an AMPK Western gel.

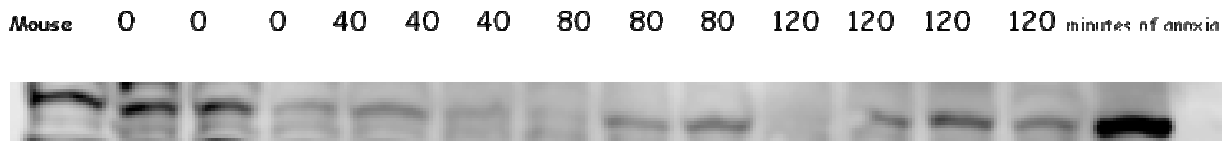

Figure 3: Representative AMPK western blot. The arrow and box indicates the row of bands that we use to analyze AMPK activation.

***HSP70 protein levels*:** HSP70 levels were measured using western blots and anti-HSP70 antibodies. For details of this method see above under AMPK activity. See Figure 4 below for a HSP70 Western gel.

Figure 4: HSP70 western gel. The arrow and box indicates the row of bands that we use to analyze HSP70 protein levels. The row used for AMPK and HSP70 analysis is the same in for both gels.

Sequencing, primer design and mRNA extraction: To design degenerate primers, we searched GenBank for AMPKγ protein sequences from various invertebrate and vertebrate species including the *Cancer irroratus* sequence (Frederich et. al 2009). Obtained sequences were aligned using the MultAlin tool (http://bioinfo.genotoul.fr/multalin/multalin.html) and from the alignment, conserved regions were used to design degenerate forward and reverse primers for PCR. For HSP70, we used primers designed for copepods have been proven to work with many crustacean species (Voznesensky et al. 2004). RNA was extracted and purified directly from the liver, heart, muscle of tail, and brain tissues, using the Promega Total RNA Isolation System, and then stored in RNAlater® solution. The RNA was then tested for purity, quality, and quantity by agarose gel electrophoresis and in a UV/VIS spectrophotometer at 260 and 280nm wavelength. RNA reverse transcription was used to synthesize a complementary DNA (cDNA) strand and was accomplished using the Invitrogen Super-Script First Strand Synthesis System. The cDNA was then subjected to PCR, amplifying the cDNA, with an annealing temperature of 45ºC for degenerate primers or 55ºC for specific primers. An agarose (8%) gel electrophoresis separated the cDNA. This gel was incubated in ethidium bromide solution ($C_{21}H_2OBrN_3$) for fifteen

minutes. When placed under ultraviolet light, we were able to determine which primers produced complete DNA bands. Bands were extracted from the agarose gel, and, the DNA was purified and sent for sequencing to the MDIBL sequencing facility in Salsbury Cove, ME. Sequences were analyzed with the 4Peaks program, and, BLAST nucleotide and protein searches compared the given sequence with known sequences. This verified that the purified DNA is actually the AMPK gene. Based on the defined sequence, we created lobster specific primers using the idtDNA.com primer design tool (See Table 1 below).

Table 1. Nucleotide sequence of *Cancer irroratus* primers used for amplification of AMPKγ, HSP70 and 18S

Target	Primer	Nucleotide Sequence	References
AMPKγ, degenerate	MF F2	5'-AAY ggN gTN MgN gCN gCN CCN YTN T-3'	This study
	MF R6	5'-ggR TCD ATN CAN ggN ARN CKR TgD AT-3'	
AMPKγ, specific	LobGF1	5'-AgA GgA CCA TCg gCT TgA AAC TTg-3'	This study
	LobGF2	5'-TTC AgC ATC TAC AAT ggg CAA ggC-3'	
18S, specific	18S F2	5'-gCC gCA CgA gAT TgA gCA ATA ACA-3'	Frederich et al. 2009
	18S R1	5'-ATT CTA gCC gCA CgA gAT TgA gCA-3'	
HSP70 , degenerate	Petra HSPF2	5'-gCN AA RAA YCA RgT NgC NAT gAA-3'	Voznesensky et al., 2004
	Petra HSPR2	5'-YTT YTC NgC RTC RTT NAC CAT-3'	
HSP70, specific	LobHSPF1	5'-gAg CgA CAT gAA ACA TTg gCC CTT-3'	This study
	LobHSPR1	5'-TAC CgA ggT ATg CTT CAg CCg TTT-3'	

Nucleotide code: g, guanine; C, cytosine; A, adenine; T, thymine; Y=T or C; R=A or g; D=A or g or T; N (any nucleotide) = A, g, C or T.

Quantifying gene expression: In order to determine the levels of gene expression, a Stratagene MX3005 Real-Time Quantitative PCR (QPCR) instruments was used. For QPCR a fluorescent dye (SYBR green) that binds to double stranded DNA is added to a PCR reaction and the fluorescence is detected after each PCR cycle. A standard dilution series of 1x, 10x, 100x, and 1000x was used to generate a standard curve for quantifying the amount of AMPKγ gene

expression. To ensure that the SYBR green signal is specific to AMPK we sequenced the PCR product and obtained an AMPK sequence. Additionally, after each QPCR run we performed a melting curve analysis in which the temperature is increased stepwise while monitoring the fluorescence signal. If the QPCR quantifies a single specific DNA product, sharp drop of the fluorescence signal at a specific temperature can be observed. Figure 5 shows the 1st derivation of the signal. The clean peak indicates that only one product was amplified. 18S was used as the house-keeping gene to normalize the AMPKγ signal.

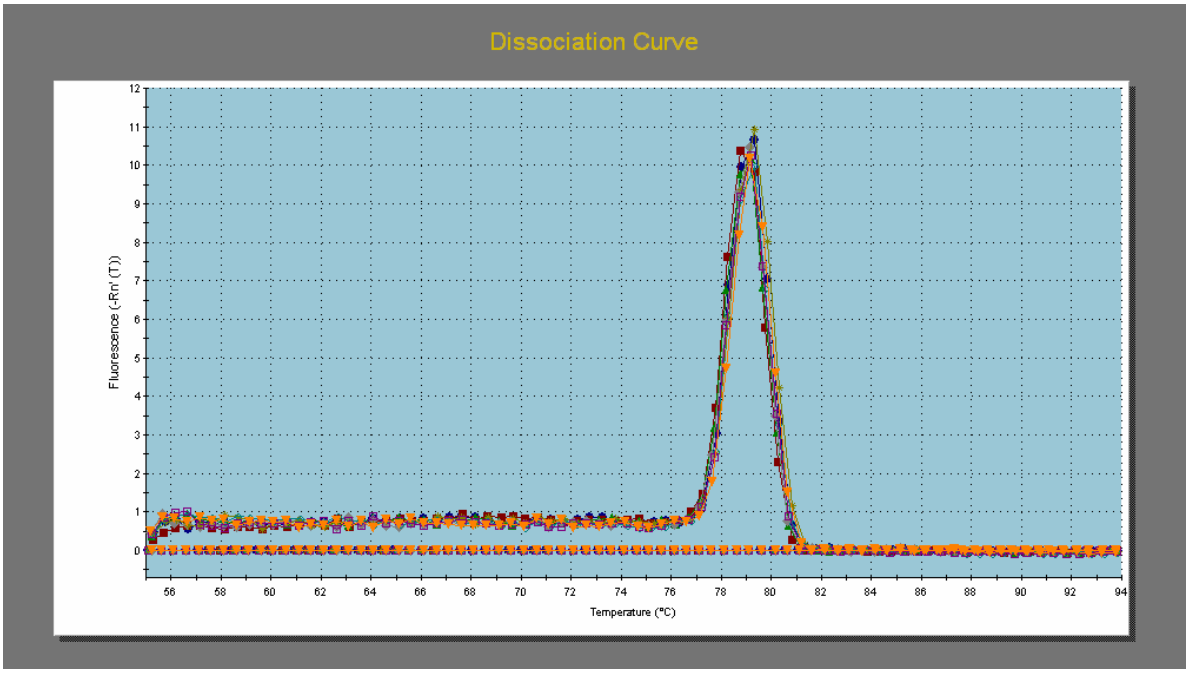

Figure 5: Melting curve representing the specificity of the AMPKγ QPCR curve above shows that only one product is amplified; the specific primers used for this quantative real time PCR were made specifically to target the AMPKγ gene. Bands obtained in regular PCR were sent for sequencing and confirmed this result.

Statistics: Data was tested with the GraphPad InStat software for significant differences using a standard t-test, ANOVA or repeated measures ANOVA depending on the single data set and a Tukey post hoc test. A $p<0.05$ was considered significant.

RESULTS

(Please Note: Immediate discussion of the respective data is often included in the results section due to the amount of data. A broader discussion is highlighted in the discussion section.)

Part 1 – AMPK Expression in Lobster, *Homarus americanus*

We tested the hypothesis that AMPK is present and activated by temperature stress in the lobster, *Homarus americanus*. Furthermore, we hypothesized that AMPK activation occurs in a tissue specific manner due to the different energy demands of the respective tissues.

To show that AMPK is expressed in lobsters we designed degenerate PCR primers based on conserved regions of the AMPK gene in other invertebrates for the alpha, beta and gamma subunit. We obtained a 235 base pair DNA sequence for the gamma subunit, which shows that AMPK is expressed in lobsters. Below are the partial DNA and respective amino acid sequences of the AMPK gamma subunit.

Lobster *Homarus americanus*

DNA sequence AMPKγ subunit

CACAGACTTCNTCCGCATTCTTCAGAATTTCTATAACTCACCCAATCGTAA
AATGGAAGAGCTAGAGGACCATCGGCTTGAAACTTGGCGCACCGTGTTGG
AGGATGAAGTACGGCCATTGATCAGCATTCGACCAGACGAGTCTCTGTAT
GTTGCAATACGATCTCTCATCCATCATAAAATTCNCCGTCTCCCTGTTATT
GATCCCGCCNCTGGCAATGTTCTGTATATTGTCACACACAAGCGCATTCT
CAAGTTCCTTTACTTATATATCAGTGAGCTGCCCAAGCCGTCCATCCTGC
AGAAGCCTCTAAGGGACCTGGAAATCGGCACGTATAAAAACATAGAAACA
GCAAGTCAGGATACGCTCATTATAGAAGCTCTTAACAAATTTGTGGAACAC
AGAATCTCTGCCTTGCCCATTGTAGATGCTGAAGGAAAACTGGTTGATATT
TATGCCAANTTCGACGTCATCAACCTCGCCGCCG

Deduced amino acid sequence

TDFXRILQNFYNSPNRKMEELEDHRLETWRTVLEDEVRPLISIRPDESLY

VAIRSLIHHKIXRLPVIDPAXGNVLYIVTHKRILKFLYLYISELPKPSIL

QKPLRDLEIGTYKNIETASQDTLIIEALNKFVEHRISALPIVDAEGKLVD

IYAXFDVINLAA

Figure 6: Obtained DNA sequence with translation to amino acids.

To show that AMPK is highly conserved during evolution we aligned the 235 base pair DNA sequence of the gamma subunit with the AMPK DNA sequences of the rock crab, *Cancer irroratus* (GenBank accession # NP_714966.1) and the mouse, *Mus musculus* (GenBank accession # ACL 13567.1). Many attempts were made to sequence the alpha and beta subunits of the AMPK gene, but the results were unsuccessful and the data were not crucial for this project.

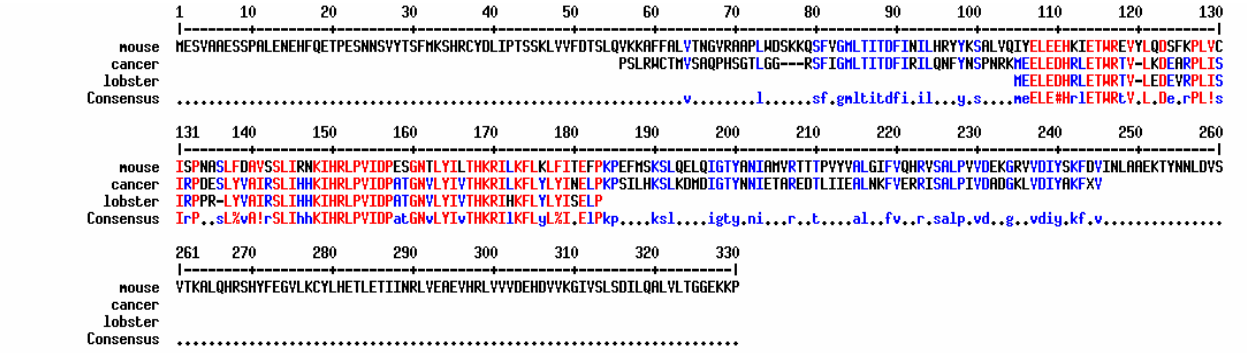

Figure 7:Alignment of the partial lobster AMPK gamma amino acid sequence with the respective sequence fragment from the rock crab, *Cancer irroratus* and the mouse. The high sequence conservation of AMPK is easily visible. (Partial sequence conservation shown in blue. Complete sequence conservation shown in red.)

We additionally tested the hypothesis that AMPK expression occurs in a tissue specific manner due to the different energy demands of the respective tissues. The figure below (Figure 8) shows tissue specific expression of the AMPK gene in the brain, heart, liver, and muscle tissues. AMPK mRNA expression was higher in tail and cardiac muscle and lower in liver and brain. The tail and cardiac muscle tissue were expected to have higher amounts of AMPK mRNA because they are the most active tissues in the lobster's natural environment. Liver and brain tissues are expected to be lower in AMPK mRNA expression because the energy supply to these tissues remains at a constantly lower rate. Tail muscle was 6 fold higher than brain tissue, 3 fold higher than liver tissue, and 2 fold higher than cardiac muscle tissue.

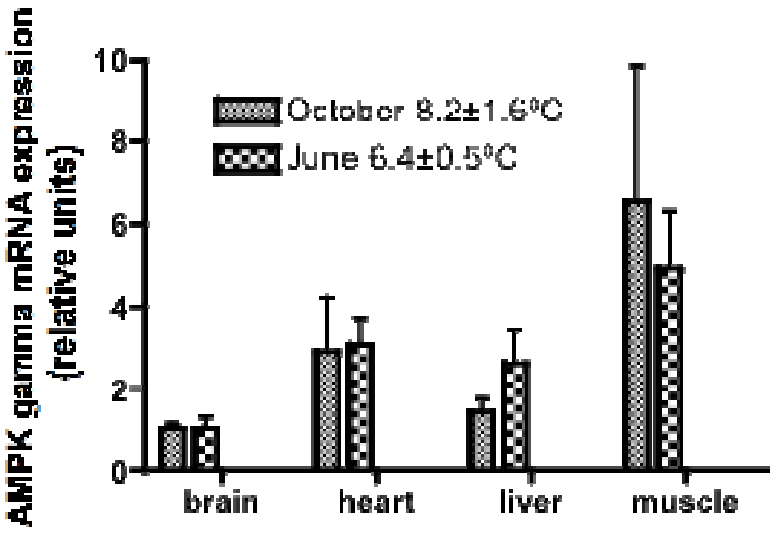

Figure 8: mRNA expression of the AMPK gene. AMPK gene expression for was much higher in tail and cardiac muscle tissues, and much lower in liver and brain tissues. (mean±SE, n=5 per data point)

This project was originally designed to examine the seasonality of temperature-induced activation of AMP-activated protein kinase (AMPK) in the lobster, *Homarus americanus,* along a temperature and latitudinal gradient beginning off the coast of Winter Harbor, Maine, continuing to Biddeford, Maine, and ending in Massachusetts Bay. In each of these three places, water temperature was measured in lobster traps and animals were collected for subsequent laboratory experiments. Available data bases were searched for temperature recordings to support the presence of a steep thermal gradient within this geographically limited range. The data from the GOMOOS data base (www.gomoos.org) over the 2008-2009 year range for the three buoys I01 - Eastern Maine Shelf, C02 - Casco Bay, and A01 - Massachusetts Bay, showed the highest spikes in water temperature for the summer months of August and September 2008 as 11.8ºC (I01 - Eastern Maine Shelf), 14.3ºC (C02 - Casco Bay) and 15.8ºC (A01 - Massachusetts Bay). Each of these buoys only measured water temperature at a depth 150 meters below the surface. However when attaching temperature loggers to traps in Biddeford and Winter Harbor,

Maine, we found no significant variation in temperature recordings between different seasons. AMPK expression was compared in lobsters caught in June and October, months that were reported to vary in temperature significantly in the GOMOOS database, and showed no difference in temperature (8.2 in June and 6.4 in October) as well as AMPK mRNA expression (Figure 8). With the unexpected problem of not being able to sample animals from different temperatures throughout the year, the original thermal gradient idea was abandoned and this thesis work explored AMPK activation in a tissue specific manner and in the presence of different forms of stress.

Part 2 – Fast Progressive Temperature Stress

Lobsters (n=5) were exposed to a rapid and progressive increase in temperature, beginning at 14 ºC and increasing at a rate of 6ºC per hour. Heart rate, measured between 14 and 33ºC, increased significantly (ANOVA, $p<0.05$) from 43.9±6.8 min^{-1} at 14ºC to 126.4±16.3 min^{-1} at 31ºC with a $Q_{10(14°C-30°C)}$ of 1.79 and a $p < 0.001$ (Figure 9).

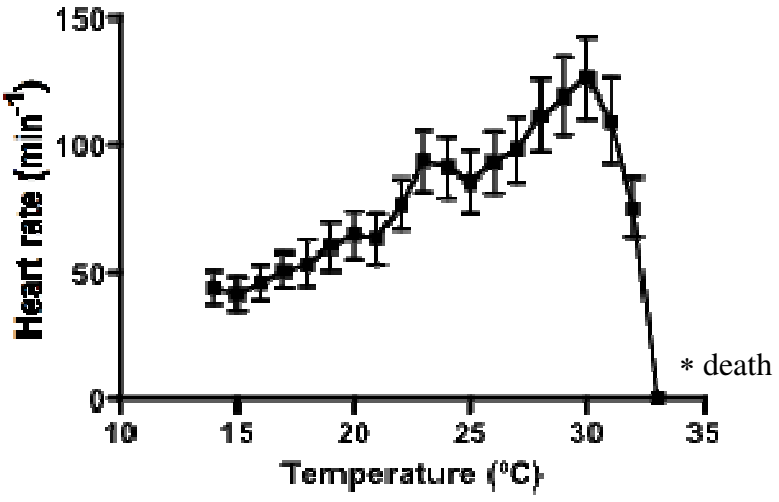

Figure 9: Heart rate for short term progressive temperature stress. Heart rate increased significantly (ANOVA, $p<0.05$) from 43.9±6.8 min^{-1} at 14ºC to 126.4±16.3 min^{-1} at 31ºC with a $Q_{10(14°C-30°C)}$ of 1.79 and a $p< 0.001$. 30°C was selected because it had the highest average heart rate. Death occurred at 33°C. (mean±SE, n=5 per data point)

During the same experimental parameters reaction time was recorded. Lobsters were flipped on their backs in two degree Celsius increments and the time it took for each animal to right itself was recorded. Figure 10A shows that at 28°C lobsters start to significantly slow down in righting themselves. A repeated measures ANOVA revealed a p-value = 0.073, which is not statistically significant. However, for the repeated measures ANOVA we needed to exclude the 30°C and 32°C data points because some of the lobsters stopped turning. Therefore, we did a one-way ANOVA, which shows a significant effect of temperature on reaction time, p-value = 0.0134. Despite this change in statistical analysis, the data clearly show the trend of animals slowing down during the heat stress. The percent of animals that stopped righting themselves as the temperature increase can be seen in figure 10B. Heart rate and reaction time experiments were performed to measure thermal stress at the whole animal level. At the organism level, it is clear that thermal stress is present, indicating further investigation of the physiological stress mechanisms at the tissue and cellular levels is merited.

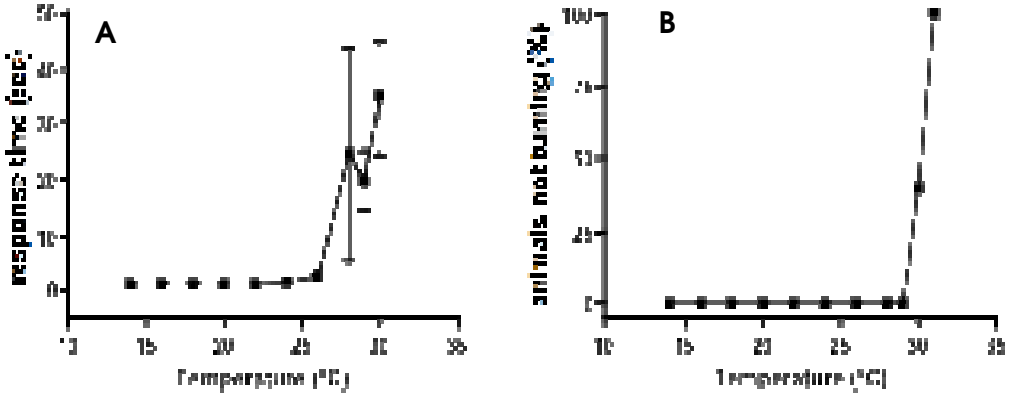

Figure 10: Reaction time decreased in the graph on the left indicating that lobster's started to slow in the righting response between 26° and 33°C. A regular ANOVA shows a significant effect of temperature on reaction time, p-value = 0.0134. The graph on the right shows the percent of animals that stopped responding; 30° and 32°C are the temperature points where animals were incapable of responding. (mean±SE, n=5 per data point)

Lactate concentration remained at low control levels between 14 and 28ºC (in nmol/g protein: 4.0±1.0, 3.5±0.9, 14.9 ±4.7, heart, liver, muscle, respectively) (Figure 11). Lactate levels increased significantly at 33°C (ANOVA, $p<0.05$) to 151.4±6.8 nmol/g protein in heart, 236.0±7.0 nmol/g protein in liver and 144.1±3.1 nmol/g protein in muscle. In the heart, liver, and muscle western blot analysis, HSP70 levels remained constant and were not significant during the temperature exposure (ANOVA, $p>0.05$). In the heart, AMPK activity increased linearly (ANOVA, $p<0.05$) up to 2.2±1.2 at 30 ºC 2.9±0.8 fold at 33ºC with a p-value = 0.0031. In the liver AMPK activity remained constantly low, between 14 and 28ºC, but increased significantly (ANOVA, $p<0.05$) up to 2.7±0.4 at 30ºC and 1.9±0.5 fold at 33ºC with a p-value = 0.0018. In the muscle AMPK activity remained constantly low and showed no significant increase (ANOVA, $p>0.05$).

The data show that lobsters, during severe heat stress, switch to anaerobic metabolism between 28 and 30ºC and that AMPK activation is an earlier indicator of heat stress and an adaptation for energy requirements when compared to the well established HSP70 response. The observed increases in AMPK activity, specifically in the lobster heart and liver tissues, confirm this hypothesis. The constantly low AMPK activity in the muscle may be explained by the heat-induced torpor. The inactivity of the muscle tissue at higher temperatures suggests that since decreased motor activity requires small amounts energy, AMPK activation does not occur. A key finding of this study part of the study is that tissue-specific AMPK activation patterns exist during heat stress. This result has not yet been reported for any other animal.

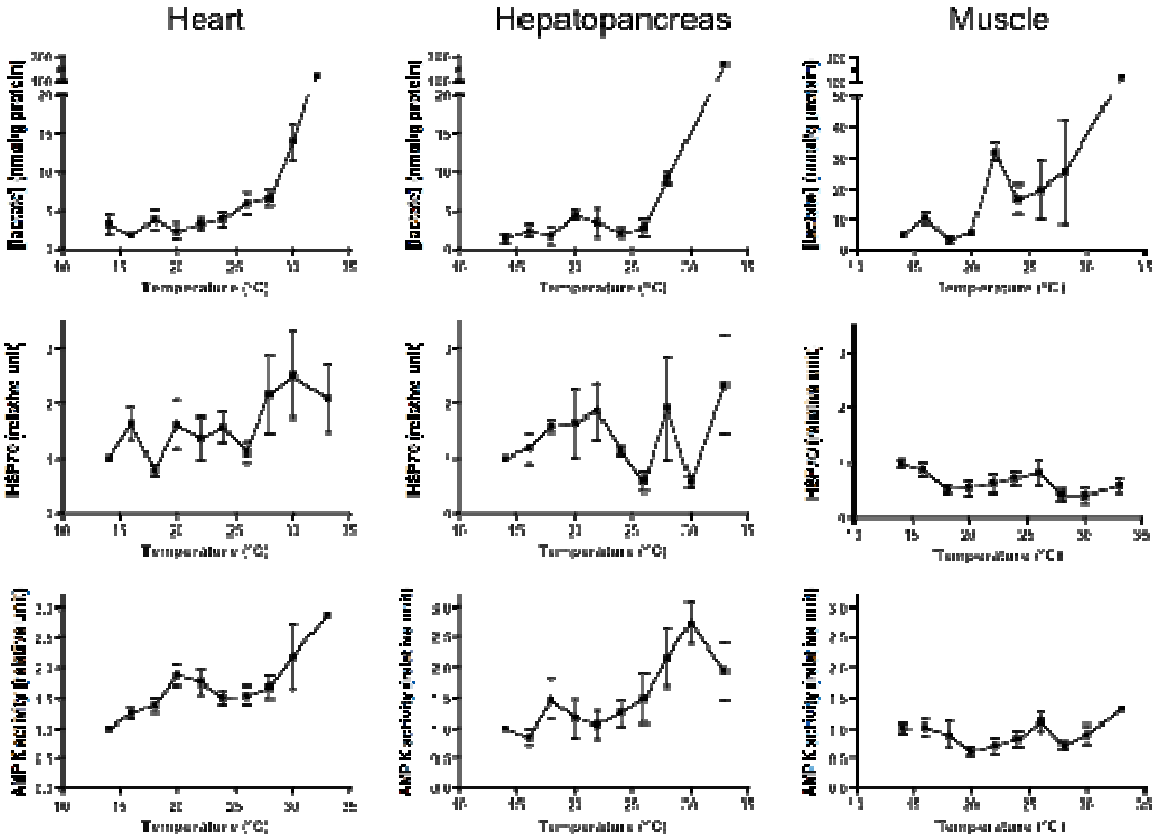

Figure 11: Lactate concentration, HSP70 protein levels, and AMPK activity in Lobster heart, hepatopancreas and claw muscle tissue (n=5 for all data). Lactate concentration 14 and 28ºC (in nmol/g protein: 4.0±1.0, 3.5±0.9, 14.9 ±4.7, heart, liver, muscle, respectively). Lactate levels at 33°C (ANOVA, $p<0.05$) to 151.4±6.8 nmol/g protein in heart, 236.0±7.0 nmol/g protein in liver and 144.1±3.1 nmol/g protein in muscle. HSP70 levels for all tissues remained constant and were not significant during the temperature exposure (ANOVA, $p>0.05$). AMPK activity increased linearly (ANOVA, $p<0.05$) up to 2.2±1.2 at 30 ºC 2.9±0.8 fold at 33ºC with a p-value = 0.0031. In the liver AMPK activity remained constantly low, between 14 and 28ºC, but increased significantly (ANOVA, $p<0.05$) up to 2.7±0.4 at 30ºC and 1.9±0.5 fold at 33ºC with a p-value = 0.0018. In the muscle AMPK activity remained constantly low and showed no significant increase (ANOVA, $p>0.05$). (mean±SE, n=5 per data point)

Part 3 – Sub-lethal Temperature Stress

Lobsters were exposed to 24 hour incubation at 28°C, a sub-lethal temperature that showed AMPK activation in the fast and progressive temperature study. The fast, progressive temperature increase described above results in an immediate response to thermal stress, shown by the fast activation of AMPK. The sub-lethal thermal stress study tests whether AMPK and HSP70 are affected differently during a period of extended thermal stress. The 28°C temperature

was chosen because it was previously identified as a temperature in which animals could survive without being overtaxed for energy on the cellular level. Heart rate, measured in two hour increments from 0 to 24 hours, increased significantly (ANOVA, $p<0.05$) from 26.9±0.7 min^{-1} at time point 0 to 101.7±0.7 min^{-1} at 6 hours with a p-value < 0.0001, and remained constant at 91.8±2.9 min^{-1} between 6 hours and 24 hours (Figure 12).

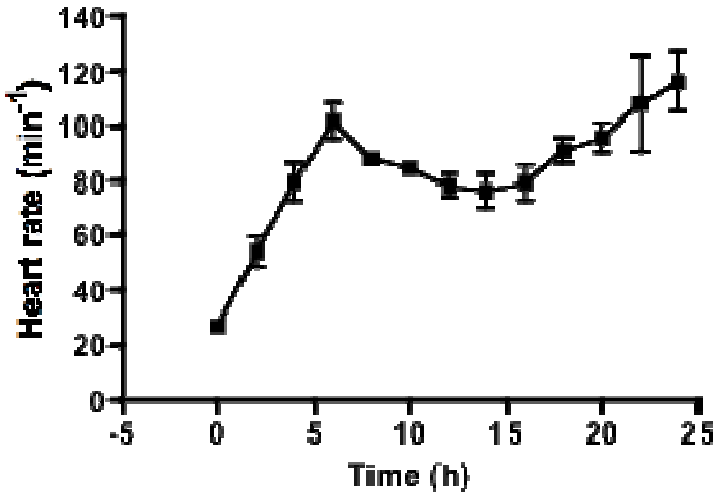

Figure 12. Heart rate data was collected for a sub-lethal temperature stress at a constant 28°C. Heart rate increased significantly (ANOVA, $p<0.05$) from 26.9±0.7 min^{-1} at time point 0 to 101.7±0.7 min^{-1} at 6 hours with a p-value < 0.0001, and remained constant at 91.8±2.9 min^{-1} value between 6 hours and 24 hours. (mean±SE, n=5 per data point)

During the same experiment, reaction time was recorded. Lobsters were flipped on their backs at the 0, 4, and 24 hour time points and the time it took for each animal to right itself was recorded. Figure 11 shows that from 6 to 10 hours lobsters start to significantly slow down in righting themselves before returning to normal righting rates around 22 hours. A repeated measures ANOVA showed significant increase at 6, 8, and 10 hours (ANOVA, $p<0.05$) and a p-value = 0.0001 (Figure 13).

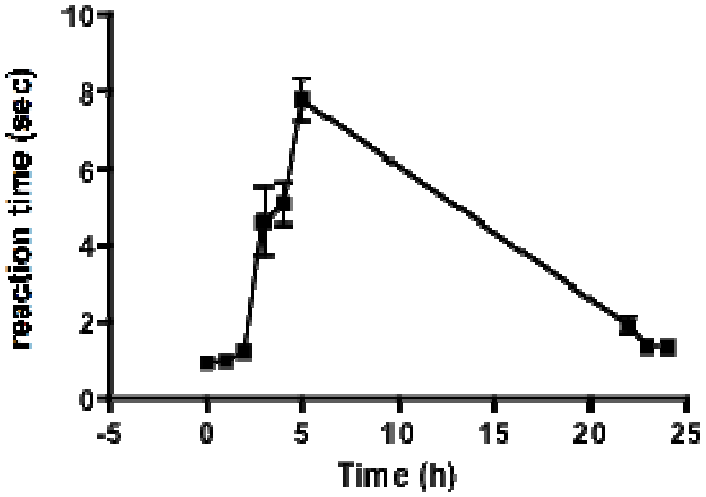

Figure 13. The reaction time graph shows that from 6 to 10 hours lobsters start to significantly slow down in righting themselves before returning to normal righting rates around 22 hours. When doing statistical analysis, a repeated measures ANOVA showed significant increase at 6, 8, and 10 hours (ANOVA, $p<0.05$) and a p-value = 0.0001. (mean±SE, n=5 per data point)

Lactate concentration remained at low control levels between 0 and 4 (in nmol/g protein: 3.2±0.6, 2.7±0.6, 7.1 ±2.3 in heart, liver, and muscle respectively) (Figure 14). Lactate increased between 0 and 24 hours significantly (ANOVA $p<0.05$) in the heart tissue from 3.2±0.6 to 27.4±7.3 nmol/g with a p value = 0.0019. Lactate increased between 0 and 4 hours significantly (ANOVA $p<0.05$) in the liver tissue from 2.7 ±0.6 to 3.0±1.5 nmol/g and between 0 and 24 hours from 2.7 ±0.6 to 20.7±5.2 nmol/g with a p value = 0.0016. Lactate increased between 0 and 24 hours significantly (ANOVA $p<0.05$) in the tail muscle tissue from 7.1 ±2.3 to 22.9±10.22 nmol/g with a p value = 0.010. In the heart, liver, and muscle western blot analysis, HSP70 levels remained constant and were not significant during the temperature exposure (ANOVA, $p>0.05$). AMPK activity remained constant and changes were not significant during the temperature exposure (ANOVA, $p>0.05$). In the liver, AMPK activity increased significantly (ANOVA, $p<0.05$) from 1.1±0.07 up to 1.3±0.2 between 0 and 4 hours and up to 2.1±0.1 between 0 and 24 hours with a p-value = 0.0038.

The data show that lobsters (Figure 14), during constant temperature stress, switch to anaerobic metabolism after four hours and activate AMPK as an earlier indicator of heart stress than the well established response given by HSP70 expression in the liver tissue only. The insignificant change in the AMPK activity of the heart can be directly correlated to the heart rate data; there were gradual increases followed by a constant heart rate for the majority of the experiment time. Since the lobsters were not experiencing a continuous increase in temperature, they may have been able to adapt to the constant temperature in the heart tissue specifically. Again, the continual increase in AMPK activity in the muscle may be explained by the heat-induced torpor. The data below are collected from five animals at each time point.

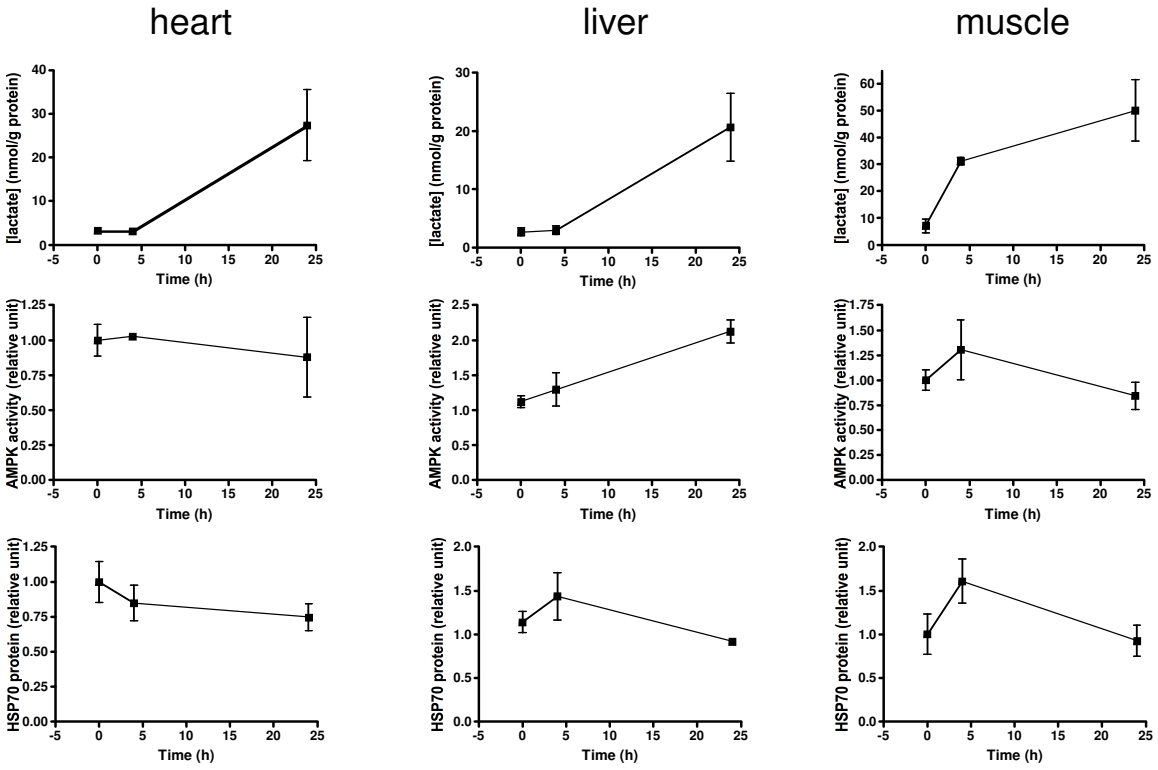

Figure 14. Lactate was at control levels between 0 and 4 (in nmol/g protein: 3.2±0.6, 2.7±0.6, 7.1 ±2.3 in heart, liver, and muscle respectively). Lactate increased between 0 and 24 hours significantly (ANOVA $p<0.05$) in the heart tissue from 3.2±0.6 to 27.4±7.3 nmol/g with a p value = 0.0019. Lactate increased between 0 and 4 hours significantly (ANOVA $p<0.05$) in the liver tissue from 2.7 ±0.6 to 3.0±1.5 nmol/g and between 0 and 24 hours from 2.7 ±0.6 to 20.7±5.2 nmol/g with a p value = 0.0016. Lactate increased between 0 and 24 hours significantly (ANOVA $p<0.05$) in the tail muscle tissue from 7.1 ±2.3 to 22.9±10.22 nmol/g with a p value = 0.010. In all

tissues, HSP70 levels remained constant and were not significant during (ANOVA, $p>0.05$). In the heart and muscle tissues, AMPK activity remained constant and was not significant (ANOVA, $p>0.05$). In the liver, AMPK activity increased significantly (ANOVA, $p<0.05$) from 1.1±0.07 up to 1.3±0.2 between 0 and 4 hours and up to 2.1±0.1 between 0 and 24 hours with a p-value = 0.0038. (mean±SE, n=5 per data point)

Part 4 – 24 Hour Hypoxia Stress

So far, the data clearly show that heat stress leads to anaerobiosis in the presence of adequate oxygen in the environment. Anaerobiosis in the tissues leads to diminished availability of ATP at the cellular level, which subsequently activates AMPK. In the next set of experiments, we tested whether we can elicit the same AMPK activation in a hypoxia environment at control temperatures. Therefore, we additionally tested the hypothesis that AMP-activated protein kinase (AMPK) is present and activated by hypoxia stress in the lobster, *Homarus americanus*. Lobsters were incubated at 5% oxygen (4 kPa) for 24 hours. We refrained from measuring the respective parameters in the muscle, as we saw no differences in AMPK activity in this tissue earlier and therefore wanted to focus on heart and liver. Heart rate (Figure 15) measured at two hour increments from 0 to 24 hours, did not increase significantly (ANOVA, $p>0.05$) during the 24 hour heart rate experiment. During the same experimental parameters reaction time was recorded with the same methods as the above temperature data sets. The righting response did not vary throughout the 24 hour time course.

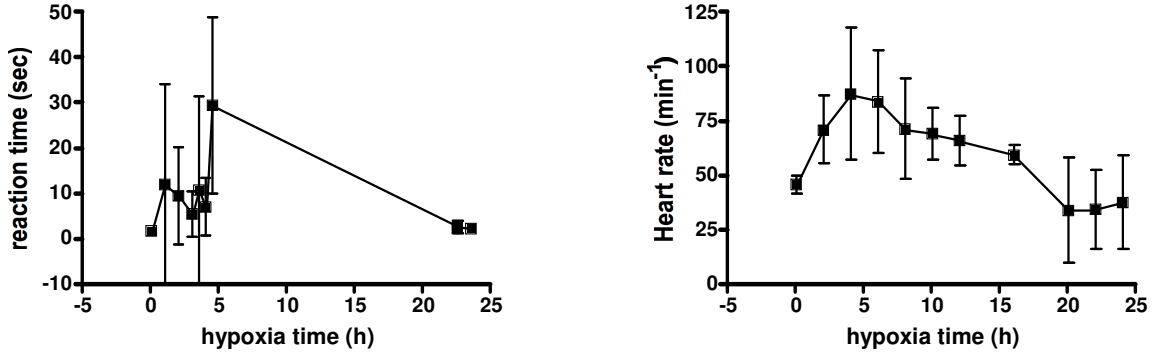

Figure 15: Reaction time, in the graph on the left, indicated that lobsters did not change in their ability to right themselves in the course of the experiment. The graph on the right shows the heart rate data collected for hypoxia stress. Heart rate did not significantly increase or decrease for the lobsters. (mean±SE, n=5 per data point)

In the liver tissue, lactate concentration remained at low control levels between 0 and 4 hours from 2.3±0.2 to 3.7±0.6 nmol/g protein. Lactate increased between 0 and 24 hours significantly (ANOVA $p<0.05$) in the heart tissue from 0.5±0.05 to 1.8±0.2 nmol/g and between 0 and 4 hours 0.5±0.05 to 1.7±0.1 from with a p value = 0.0001 (Figure 16). Lactate increased between 0 and 24 hours significantly (ANOVA $p<0.05$) in the liver tissue from 2.3±0.2 to 5.9±0.3 nmol/g and between 4 and 24 hours from 3.7±0.6 to 5.9±0.3 nmol/g with a p value = 0.0005. In the heart and liver western blot analysis, HSP70 levels remained constant and did not significantly change during the temperature exposure (ANOVA, $p>0.05$). In the heart, AMPK activity increased significantly (ANOVA, $p<0.05$) from 1.0±0.05 up to 6.1±1.1 between 0 and 24 hours and from 1.9±0.3 up to 6.1±1.1 between 4 and 24 hours with a p-value = 0.0021. In the liver, AMPK activity remained constant and was not significant during the temperature exposure.

The data show that lobsters (Figure 16), during constant hypoxia stress, switch to anaerobic metabolism after four hours and activate AMPK as an earlier indicator of hypoxia stress than the well established response given by HSP70 expression in the heart tissue only. The

insignificant change in the AMPK activity of the liver can be explained by the variability of the data. An increased sample size for this experiment may prove to decrease the amount of deviation.

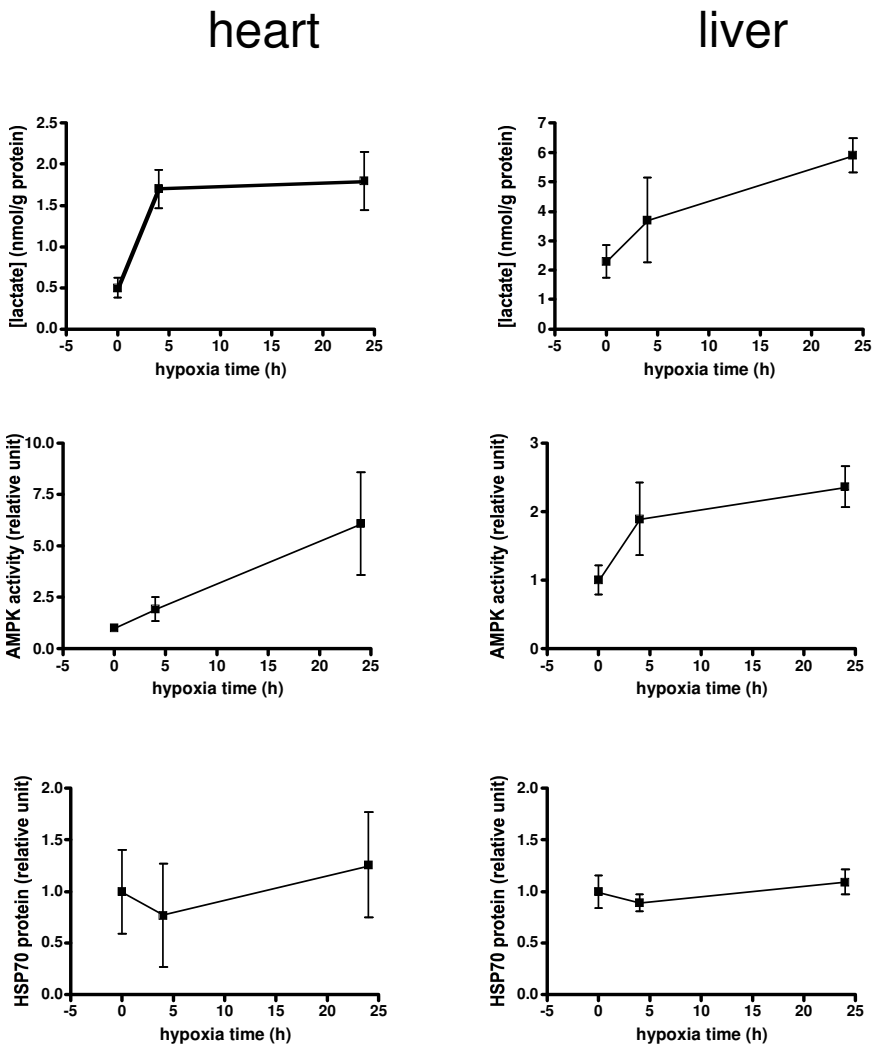

Figure 16: In the liver tissue, lactate concentration remained at low control levels between 0 and 4 hours from 2.3±0.2 to 3.7±0.6 nmol/g protein. Lactate increased between 0 and 24 hours significantly (ANOVA $p<0.05$) in the heart tissue from 0.5±0.05 to 1.8±0.2 nmol/g and between 0 and 4 hours 0.5±0.05 to 1.7±0.1 from with a p value = 0.0001. Lactate increased between 0 and 24 hours significantly (ANOVA $p<0.05$) in the liver tissue from 2.3±0.2 to 5.9±0.3 nmol/g and between 4 and 24 hours from 3.7±0.6 to 5.9±0.3 nmol/g with a p value = 0.0005. In the heart and liver western blot analysis, HSP70 levels were not significant during the temperature exposure (ANOVA, $p>0.05$). In the heart, AMPK activity increased significantly (ANOVA, $p<0.05$) from 1.0±0.05 up to 6.1±1.1 between 0 and 24 hours and from 1.9±0.3 up to 6.1±1.1 between 4 and 24 hours with a p-value = 0.0021. In the liver, AMPK activity remained constant and was not significant during the temperature exposure. (mean±SE, n=5 per data point)

AMPK and HSP70 mRNA gene expression:

As a fuel gauge, AMPK mRNA is expected to increase before animals surpass their temperature threshold and die. Since different tissues fulfill specific functions and have varying energy requirements, AMPK responses are expected to be much different within each tissue. The experiment below confirms this hypothesis in showing that in the heart AMPK mRNA expression resulted in a significant 20 fold increase during exposure to hypoxia (Figure 17). Significant increases were found between 0 and 24 hours significantly (ANOVA $p<0.05$) in the heart tissue from 2.2±0.6 to 40.1±8.7 and between 4 and 24 hours from 4.7±0.8 to 40.1±8.7 with a p value = 0.0017. The liver AMPK mRNA expression shows an approximate 1000 fold increase but was found to be statistically insignificant (ANOVA $p>0.05$) due to high variability of the data. Up-regulation of HSP70 mRNA in the heart tissue and liver tissue were found to be statistically insignificant (ANOVA $p>0.05$).

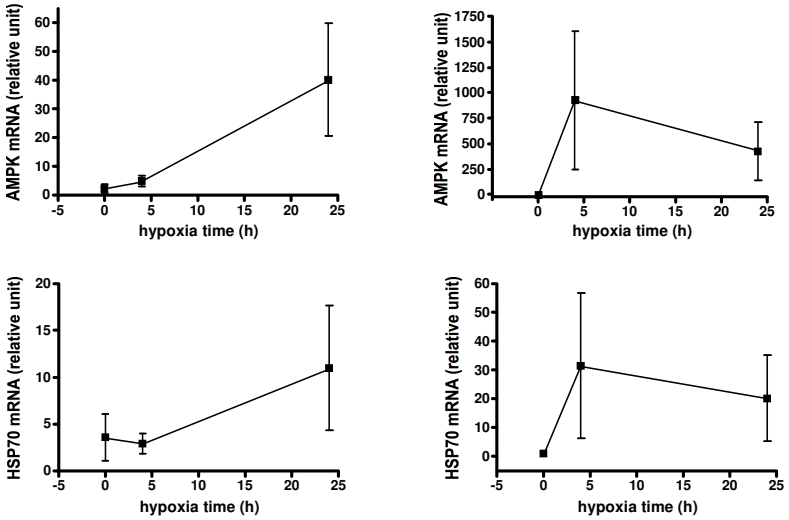

Figure 17: Heart and Liver AMPK mRNA levels (top graphs) show a 20 fold increase in AMPK expression in the heart tissue, while the liver AMPK mRNA expression resulted in about a 1000 fold increase. A 2 fold up-regulation of HSP70 mRNA was found in the heart tissue and a 30 fold up-regulation of HSP70 mRNA was found in the liver tissue. (mean±SE, n=5 per data point)

Part 5 – Anoxia Stress

In the hypoxia environment at control temperatures (Figure 18), AMPK activation and expression was only found to be significant in the heart tissue, showing that AMPK is an earlier indicator of hypoxia stress than the well established response given by HSP70. Because this activation was different than in the long and short term temperature experiments, we wanted to test oxygen deficiency on a more acute level. For anoxia exposure, lobsters were incubated at 0.7% oxygen (0.3 kPa) for 2 hours. In the heart, HSP70 levels remained constant and did not significantly change during the anoxia exposure (ANOVA, $p>0.05$). AMPK activity increased significantly (ANOVA, $p<0.05$) from 1.0±0.05 up to 2.6±0.1 between 0 and 120 minutes, from 1.3±0.1 up to 2.6±0.1 between 40 and 120 minutes, and from 1.4±0.3 up to 2.6±0.1 between 80 and 120 minutes with a $p < 0.0001$. The data confirm our earlier findings for temperature and hypoxia, showing that AMPK is an earlier indicator of stress than the well established response given by HSP70 expression in the heart tissue. Analysis of the lobster anoxia liver tissue is still in progress.

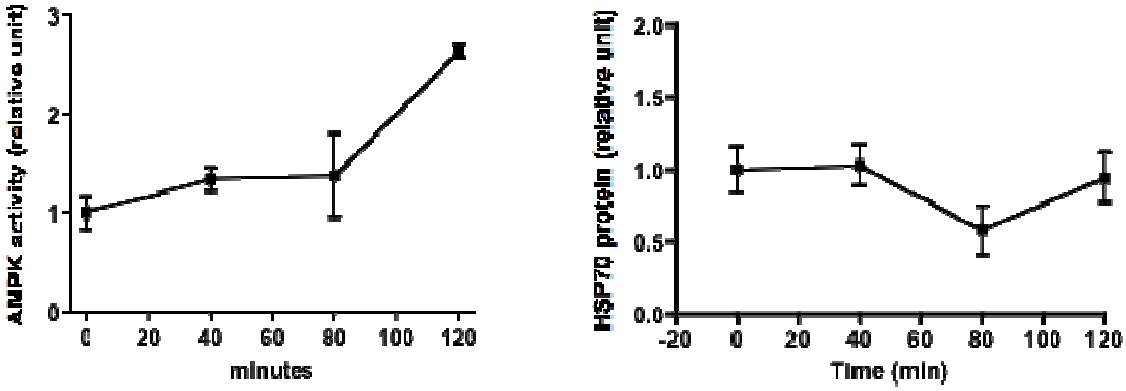

Figure 18: In the heart, HSP70 levels remained constant and did not significantly change during the anoxia exposure (ANOVA, $p>0.05$). AMPK activity increased significantly (ANOVA, $p<0.05$) from 1.0±0.05 up to 2.6±0.1 between 0 and 120 minutes, from 1.3±0.1 up to 2.6±0.1 between 40 and 120 minutes, and from 1.4±0.3 up to 2.6±0.1 between 80 and 120 minutes with a $p < 0.0001$. (mean±SE, n=5 per data point)

Part 6 – Comparison of AMPK activity during Temperature and Hypoxia on

To determine whether the activation of AMPK during the temperature-induced hypoxia is similar to the hypoxia induced by nitrogen bubbling, we correlated the lactate accumulation with the respective AMPK activity for each experiment. Assuming that lactate is a potential marker for the state of cellular energetics, lactate vs. AMPK activity was plotted in the graphs below (Figures 19). Curves were fitted with a hyperbolic function through the data to the equation y=aX/(b+X). The data show that in the heart and liver AMPK activity follow approximately the same trend during fast progressive and sub-lethal temperature stress. At the same time, AMPK activity increases at a faster rate during hypoxia exposure. This is most visible in the liver where the hypoxia induced AMPK activity reaches maximum at approximately 5 nmol lactate, while maximum AMPK activation is reached during temperature induced hypoxia at approximately 10 nmol lactate. Therefore, AMPK is activated at a faster rate in hypoxia. The respective correlation was not completed for muscle tissue because heat stress did not activate AMPK.

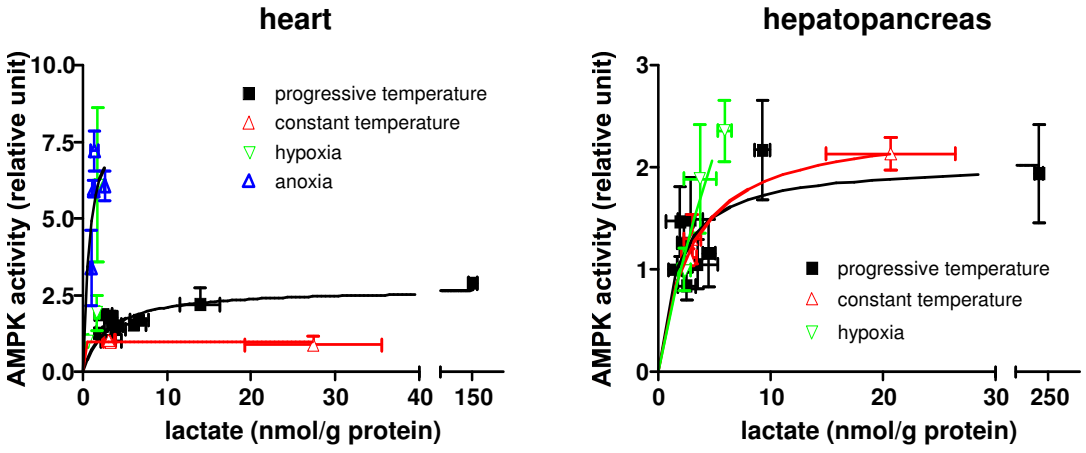

Figure 19: This figure compares increases in both, lactate and AMPK activity levels in the heart and liver tissue for progressive temperature, constant temperature, hypoxia, and anoxia stress. Curves were fitted with a hyperbolic function through the data, y=aX/(b+X) to show similarities or differences in trends. No valid fit could be found for hypoxia. The fast progressive temperature stress and the sub-lethal temperature stress followed a similar trend. At similar lactate concentrations AMPK activity increased at a faster rate during hypoxia and anoxia than during temperature-induced hypoxia.

DISCUSSION

This study investigates AMPK as a cellular marker for heat stress in the context of climate change. AMPK has been studied over the past years in the medical community especially in mammalian species in studies relating to obesity (Kola et al., 2008), heart failure (Hardie, 2008), and diabetes (Kola et al., 2008). In each of the published studies different types of metabolic stress are investigated and cellular ATP homeostasis is disrupted. AMPK's primary role in regulating cellular ATP quantities indicates that AMPK directly influences the physiological mechanisms of the diseases. A recent PubMed search revealed only 3 papers that focus on AMPK in crustacean species, including one on the rock crab, *Cancer irroratus* (Frederich et al. 2009) and the other two on the brine shrimp, *Atremia franciscana* (Zhu et al. 2007, Zhu et al. 2009). The limited knowledge about the AMPK response specifically in marine crustacean species is a principal reason why this study was developed. In order to characterize the role of AMPK as a cellular signal for temperature and hypoxia stress, long-term and short-term treatments were conducted with the lobster, *Homarus americanus*. Parameters described above, such as AMPK mRNA expression, AMPK activity, heart rate, and lactate accumulation, were used to observe and determine the role of AMPK during specified stressors. Once determined, the AMPK mechanism and response to stress was compared to that of HSP70.

Several specific results were briefly discussed in the results section; the following focuses on the broader importance of AMPK in the lobster and other marine invertebrates. AMPK was found to be present and expressed in a tissue specific manner in the lobster, *Homarus americanus*. Each tissue was found to show varying levels of AMPK expression, being higher in the tail and cardiac muscle tissues, and lower in the brain and liver. In the fast progressive temperature experiments, sub-lethal temperature exposure, hypoxia experiment, and anoxia experiment, AMPK was activated as an earlier indicator for stress than the well established

HSP70 expression. Each experimental data set differed in AMPK tissue activation, showing that the AMPK response is not the same for each environmental stressor. AMPK activation significantly increased in all of the above, while HSP70 levels remained at a constant level.

The sequencing of the AMPK gamma subunit in the lobster, *Homarus americanus,* had not been successfully completed prior to the beginning of this study. After the sequencing of the partial gamma AMPK 235 base pair sequence, it was aligned with the respective sequences of the rock crab, *Cancer irroratus*, the mouse, *Mus musculus.* High sequence conservation of the AMPK gamma sequence with other species of diverse phyla supports the assumption of AMPK as a general mechanism regulating cellular energy metabolism and shows that AMPK has been highly conserved throughout evolution. This conservation implies that the AMPK mechanism can be applied to many species and could predict the potential impacts of a specific stress on a species.

Unlike a similar study on the rock crab (Frederich et al., 2009), this lobster study focused on the tissue specific AMPK activity patterns, because all earlier work investigated heart tissue only. Using the lobster as the focus organism, this project identifies that the mechanism described in the rock crab does not have a universal trend that is present in other species as well. This project was the first study that investigates whether AMPK activation by heat and hypoxia stress is tissue specific. Since thermal tolerance can often be modified through acclimation or acclimatization, it was important to study AMPK activity and gene expression throughout multiple organ systems. This more detailed and specified understanding of the cellular and molecular processes relating to temperature and hypoxia stress indicated that there was a significant tissue specific difference in how each organ system responds to temperature and hypoxia stress individuals. The heart's primary role is to pump hemolymph throughout

crustaceans as a mechanism to keep the animals alive. Metabolically, as stress increases and transitions into moderate and lethal stress progress, the heart needs to apply mechanisms to provide a constant energy supply to itself in order to increase survivability. The liver is the largest and most active tissue because it regulates digestion, energy output, and energy storage. The liver's response to unique stressors could indicate how animals adapt to stress physiologically, by shunting energy to particular organ systems; a process similar to a human's sympathetic or parasympathetic response. Finally the tail muscle tissue is responsible for mobility and activity, particularly short bursts of activity during the escape response in the lobster. It is important to see how mobility and activity decrease as temperature and dissolved oxygen stress increase. This is the first advanced physiological understanding of the effects of how climate change and oxygen deficiency will affect energy regulation in an invertebrate species in a tissue specific manner.

The ecological law of tolerance devised by Shelford, described a range of tolerance to environmental or chemical factors, such as temperature, ischemia, or pH (Shelford, 1913). The physiological tolerance range is divided into sections based on animal activity, survivability, and physiological adaptability. The midpoint of these two scales is the optimum performance range, an ideal range where the animal's physiology can perform at its maximum level. When animals reach their upper and lower limits during exposure to an abiotic factor, their physiological processes worsen. A moderate amount of stress, a pejus range, is the physiological point where animals have limited aerobic activity. White the most severe stress range, a pessimum range, is considered to be lethal and extends beyond upper limits of survivability. The transition points between pejus and pessimum ranges can be labeled as critical temperatures, Tc. At Tc, marine invertebrates switch from aerobic to anaerobic metabolism despite sufficient levels of oxygen in

the environment and are indicated by the accumulation of a respective anaerobic end product. (first described by Zielinski and Pörtner 1996; for review see Pörtner 200l). While in the presence of a temperature above or below the upper or lower Tc, respectively, marine invertebrates experience a mismatch between the oxygen supply present and the cellular demand needed. The relationship between oxygen availability to tissues and the demand for oxygen is important to understand the oxygen limitation of thermal tolerance (Frederich and Pörtner 2000).

Shelford's law of tolerance and critical temperatures can theoretically account for the activation of AMPK through thermal stress with the transition into a pejus range. The transition points between optimum and pejus ranges can be labeled as pejus temperatures, Tp (Frederich and Pörtner 2000). Within an upper or lower pejus range, survival is possible but only if performance is limited. Specifically, AMPK activity is described as a cellular marker that indicates a *T*p (Frederich et al. 2009). At *T*p the animals leave their optimum range and enter a temperature range with a limited aerobic range for movement and physical activity. Under most circumstances the reduced amount of ATP during anaerobiosis is sufficient for an organism to survive for a short period of time, but is not enough for sustaining survival over an extended period of time due to cellular energy depletion and long-term lethal limits (Peck et al. 2002). The defined *T*p is reached occasionally during annual water temperature fluctuations and has higher biological significance than the earlier described critical temperatures at which the animals switch to anaerobic metabolism The later switch to anaerobic metabolism can be connected with the time lag period that HSP70 needs in order to be induced for expression.

The inability to adapt or remain physiologically stable has been directly connected to physiological system collapse in conjunction with an organism's lethal temperature. This process, also known as acute thermal death, has been directly connected to cardiac failure or

denaturing proteins (Helmuth et al., 2010). Protein and gene expression are crucial mechanisms in setting thermal tolerance limits in ectotherms (Somero, 2010). For example, increase or decrease in body temperature based on environmental stressors directly impacts gene expression, leading to the activation of heat shock protein response (Helmuth et al., 2010).

Using the data from this study and comparable data from a parallel study, we can describe the connection between Shelford's law and AMPK in three crustacean species, the green crab, *Carcinus maenas*, the rock crab, *Cancer irroratus*, and the lobster, *Homarus americanus* (Figure 20). The rock crab and the lobster both follow Shelford's law in that as temperature increases to a defined Tp for each species, the rock crab and lobster both transition in to a pejus range, indicated by the increase in AMPK activity. In order to increase chances of survival and conserve cellular energy, these animals decrease their activity. As temperature increases further to Tc, animals transition into a pessimum range and eventually die. In a parallel study, the green crab was found to not have a pejus range. This intertidal animal has an extended optimum range that transitions into a pessimum range when temperature reaches Tc. The increased adaptability of this animal and the daily exposure to a wide variety of temperature variations are believe to account for this variation in the law of tolerance (See appendix for a manuscript that describes this mechanism in more detail. The manuscript combines data from an earlier publication (Frederich et al. 2009), from this thesis, and additional data on the green crab).

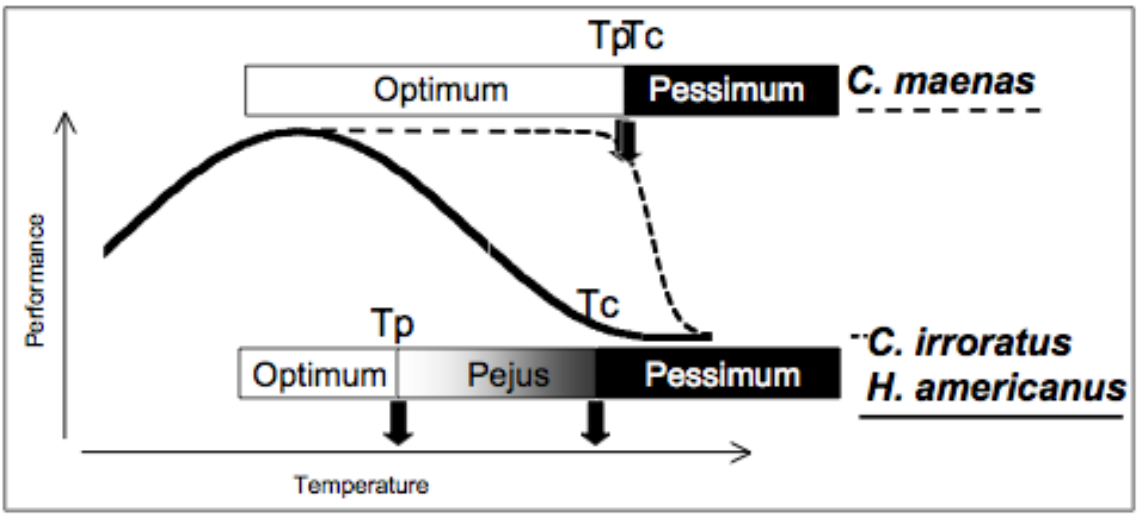

Figure 20: Shelford's law of tolerance (1913) with the more recently added thresholds, Tc and Tp. Tp is characterized by an increase in AMPK, Tc is characterized by the onset of anaerobic metabolism and increases in HSP70. Lobsters and rock crabs follow the sequence of optimum → pejus → pessimum range. Green crabs extend their optimum range and do not show AMPK activation by temperature stress. Green crabs transgress directly from an optimum to pessimum range, a potential adaptation to their highly instable habitat (see text and appendix for details).

In the lobster, *Homarus americanus*, the anaerobic end-product is lactate. The transition from aerobic to anaerobic metabolism, shown by the accumulation of lactate as an end product in heart, liver and muscle, indicates the presence of severe temperature stress (*T*c) within the lobster. During the switch to anaerobic metabolism, the concentration of lactate is expected to rapidly increase. This average stress threshold, also described as the pejus range, was seen between 28-30ºC with some variation among tissues. This range illustrates the upper limit for thermal tolerance within each specific tissue. Increased lactate concentration can be a direct marker for the upper thermal limit threshold of an organism and can be used to identify Tc. Additionally, lactate is a good tool when comparing the physiological processes of AMPK and HSP70 exposure to temperatures above the threshold leads to organ failure and eventually death.

AMPK activation plays a vital role in maintaining energy homeostasis on a cellular level. The AMPK mechanism acts as an evolutionary adaptation for regulating and providing for the

increased energy requirements during temperature stress. The observed increases in AMPK activity of the fast progressive temperature experiments, specifically in the lobster heart and liver tissues, confirm this hypothesis. The constant AMPK activity in the muscle may be explained by the heat-induced torpor. The inactivity of the muscle tissue at higher temperatures suggests that since very little energy is required, AMPK activation does not occur.

Similarly to AMPK, HSP70 is expected to increase in activation when animals are subjected to thermal stress. However, an increase in HSP70 was expected to occur much later than the increase of AMPK because of its mechanism of activation that requires gene transcription. Proteins denature due to the presence of heat and molecular chaperones assist in protein assembly by refolding denatured proteins (Hochachka & Somero, 2002) We found that the delay in detectable HSP level increases was so pronounced that when compared to AMPK activity, the increase was minimal or nonexistent. From the data gathered, HSP70 remains constant in the heart, liver, and muscle tissues of the lobster despite thermal stress. This confirms earlier studies completed with the rock crab, *Cancer irroratus* (Frederich et. al 2009), where it was shown that HSP70 does not increase in the heart during a fast, progressive temperature increase. Therefore, HSP70 is not a reliable indicator for acute thermal stress in crustaceans.

The 24 hour sub-lethal temperature experiment was designed to test the animal's physiological limits at a temperature within their upper pejus range, a study that has not been previously completed. A temperature of 26°C was selected for this study as it represented a temperature that was just below the complete metabolic switch from aerobic to anaerobic metabolism. The data show that lobsters switch to anaerobic metabolism after four hours at 30°C for each tissue. AMPK activation was found to significantly increase and was an earlier indicator of heat stress than the well established response given by HSP70 expression in the liver tissue

only. This tissue is thought to be the most active during this time point because it is contains many metabolic functions and physically consumes a large portion of the lobster's thorax. The increase of AMPK activity of the heart was found to be statistically insignificant. Since the lobsters were not having a continuous increase in temperature, they were able to adapt more readily to the constant temperature in the heart tissue specifically. The constant AMPK activity again in the muscle may be explained by the heat-induced torpor. To conclude, AMPK was found to be a faster and more reliable indicator for constant thermal stress in the liver tissue, while HSP70 remained unreliable and not significant. The activation in the liver tissue alone shows unique stressors have a greater impact on specific tissues depending on how the animals adapt to the stress.

When looking at the fast progressive temperature study and the sub-lethal temperature study, we see that the AMPK activation and increase is transient. In the fast progressive experiments, AMPK is activated in the heart and liver, two highly metabolic tissues. The lack of activity in the muscle tissue can be related to the reduced activity affects of Tp/Tc. In the sub-lethal temperature experiments, AMPK activation was not seen to be as significant in the heart tissue, mostly because animals were given enough time to adapt to the moderately stressful temperature of 28°C. The liver tissue had a significant increase in AMPK activity, again, because of its regulatory properties in energy storage and consumption during pejus range temperatures. HSP70 levels did not significantly increase either of the experiments. Many studies have discussed how HSP will elicit an increase but only after animals are returned to control conditions, allowing enough time for new HSPs to be transcribed (Tomanek et al. 2010 and Hoffmann et al. 2003).

Together, both temperature experiments show that AMPK is activated immediately during heat stress, but does not remain activated for an extended period of time, as shown in the long term temperature exposure. Initially, the AMPK mechanism may provide sufficient ATP, however, this will change once the cell reaches a new balanced state. Accumulation of HSP70 after the return to control conditions from an original heat stress seems to have a longer lasting impact on the cellular level. However, this up regulation occurs only after the return to control level from the initial stress. Therefore, our goal to identify a faster marker for cellular stress was achieved only for immediate stress. Using AMPK and HSP70 together could potentially help assess stress levels and the duration of the stress that an organism was exposed to. If AMPK activity increases, then the stress response happened immediately, and, if HSP70 is up-regulated, then stress was present but may have passed already. Before this methodology can be used, a standard must be determined.

Similarly to the sub-lethal temperature stress, hypoxia stress was measured over a 24 hour period under control temperatures and AMPK activity and HSP70 expression were compared at certain time increments. The data show that lobsters switch from aerobic to anaerobic metabolism after 4 hours. AMPK activation was found to significantly increase and was an earlier indicator of stress than the well established response given by HSP70 expression in the heart tissue only. The insignificant change in the AMPK activity of the liver can only be explained by statistical analysis. An increased sample size for this experiment may prove to decrease the amount of deviation.

In order to understand the impact that hypoxia stress has upon *Homarus americanus*, tissue analyses measuring AMPK activation and levels of mRNA expression was performed at varying time increments based upon physiological importance. The short term responses,

specific to daily oxygen concentration changes within the environment, represented the rapid biochemical process where AMPK activation is measured by the amount of phosphorylation of the gamma subunit. Long term responses are more specific to seasonal changes within the environment and can be experimentally measured using genetic processes where AMPK mRNA expression levels are quantified. AMPK results were compared to HSP70 protein and mRNA levels for the 24 hour response to hypoxia stress. Heart and Liver AMPK mRNA levels showed an extremely significant increase in AMPK expression in the heart and liver tissues. HSP70 mRNA levels were not significant, therefore confirming again that AMPK is a faster and more reliable mechanism for cellular stress response.

To test oxygen deficiency on a more acute level, lobsters were incubated at 0.7% oxygen (0.3 kPa) for 2 hours. In the heart western blot analysis, HSP70 levels remained constant and did not significantly change during the temperature exposure (ANOVA, $p>0.05$). In the heart, AMPK activity increased significantly against all data points up to 120 minutes. So far, the data show that lobsters, during acute anoxia stress, activate AMPK as an earlier indicator of anoxia stress than the well established response given by HSP70 expression in the heart tissue. Further analysis will help us to compare further hypoxia and acute anoxia AMPK activation with the transition to anaerobiosis during fast progressive and sub-lethal temperature stress.

Correlating lactate concentration and AMPK activity during both temperature experiments and the hypoxia incubation revealed a hyperbolic correlation for heart and liver tissue. Interestingly enough, the fitted curves for progressive temperature and for constant sub-lethal temperature exposure increase similarly. We expected that temperature stress would create a compromised energy metabolism with subsequent lactate accumulation. It was also anticipated that the increased ATP hydrolysis rate would lead to an increased AMP concentration, which

activates AMPK. Measuring AMP concentrations is difficult because most cellular AMP is bound to proteins. The AMP concentration needed is that of free AMP, which works to activate AMPK. Total AMP measurements often overestimate the amount of free AMP by an order of magnitude. The only way to reliably calculate free AMP concentrations is to use NMR spectroscopy (Frederich et al. 2005). Instead of measuring AMP, we use lactate as a crude indicator of the metabolic state. Although we are using lactate concentrations as a measure of metabolic transition, we are not suggesting that lactate directly activates AMPK.

It was most surprising to find that at matching lactate levels, AMPK activity increased at a much faster rate for nitrogen induced hypoxia than during temperature-induced hypoxia. This finding suggests that during hypoxia AMPK is activated faster than during temperature stress, signifying two separate mechanisms of AMPK activation. A similar study shows similar results for rat heart tissues (Frederich et al. 2005). In that study, nitrogen induced hypoxia and a chemical induced hypoxia caused by inhibition of the Citric acid cycle through inhibitors led to AMPK activation at different rates. As a result, a difference in the AMPK activating mechanism was proposed. The nature of the two different mechanisms remains indefinable but highlights the complexity of the involved regulatory pathways.

CONCLUSION

In conclusion, the effects of climate change, whether it's in dissolved oxygen concentration or water temperature fluctuations, can be seen to have a drastic impact on marine animals if water temperature highs increase and if dissolved oxygen levels decrease. To assess these impacts it would be helpful to have a cellular parameter that provides a snap shot of the stress level of an organism; AMPK has the potential to be that cellular marker. This is the first

study that investigates the effects of temperature and hypoxia stress on AMPK activity in any animal on a tissue specific level. A fast, progressive temperature increase shows three different rates of AMPK activation in the heart, liver and muscle tissues; the AMPK activation in the heart and muscle tissues is seen well before the heat shock response (HSP70) can be seen. The sub-lethal temperature increase again, showed a tissue specific activation of AMPK as an earlier indicator of temperature stress than HSP70 but only in the liver tissue. The increase in AMPK activity as temperature increases can be directly related to the survivability, responsiveness, and physical activity of the animal during temperature conditions in the environment. Similarly, the hypoxia response showed a tissue specific significant increase in AMPK activity for the heart tissue only but an increase in AMPK expression for both the heart and liver tissues. This activation of AMPK occurred again before the heat shock response. Furthermore, AMPK activation is transient and as a result independent of the stressor. Therefore, we conclude that AMPK is a better cellular marker for heat stress in the lobster, *Homarus americanus,* and occurs in a tissue specific manner. Rates of AMPK activation during temperature and hypoxia stress are different, indicating that there are potentially two mechanisms for activating AMPK. The details of this difference in activation remain to be elucidated.

References

Bergmeyer HU. (1985) Methods for Enzymatic Analysis (3rd ed.) Weinheim, Germany: VCH, vols 1-12.

Bradford MM. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry. 72: 248-254.

Camacho J, Qadri SA, Wang H, Worden MK. (2006) Temperature acclimation alters cardiac performance in the lobster *Homarus Americanus.* J Comp Physiol A. 192: 1327-1334.

Feige U, Morimoto RI, Yahara I, Polla BS. (1996) Stress Inducible Cellular Responses. Basel, Boston, Berlin: Birkhäuser Verlag

Frederich M, O'Rourke M, Furey N, Jost J. (2009) AMP-activated protein kinase (AMPK) in the rock crab, *Cancer irroratus*: an early indicator of temperature stress. J. Exp. Biol. 212, 722-730.

Frederich M, Pörtner HO. (2000) Oxygen limitation of thermal tolerance defined by cardiac and ventilator performance in spider crab, *Maja squinado*. Am J Physiol Regulatory Integrative Comp Physiology. 279: R1531-R1538.

Fredeich M, Zhang L, and Balschi JA. (2005) Hypoxia and AMP independently regulate AMP-activated protein kinase activity in heart. American Journal of Physiology: Heart and Circulatory Physiology. 288: H2412–H2421.

Frydman J. (2001) Folding of newly translated proteins in vivo: The role of molecular chaperones. Annu Rev Biochem 70: 603-649

Hardie, D. G. (2008). Role of AMP-activated protein kinase in the metabolic syndrome and in heart disease. *FEBS Lett.* 582, 81-89.

Hardie, D. G. and Carling, D. (1997). The AMP-activated protein kinase: fuel gauge of the mammalian cell? *Eur. J. Biochem*. 246, 259-273.

Hardie, D. G. and Sakamoto, K. (2006). AMPK: a key sensor of fuel and energy status in skeletal muscle. *Physiology* 21, 48-60.

Hardie, D. G., Salt, I. P., Hawley, S. A. and Davies, S. P. (1999). AMP-activated protein kinase: an ultrasensitive system for monitoring cellular energy charge. *Biochem. J.* 338, 717-722.

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

Helmuth B et al. (2010) Organismal climatology: analyzing environmental variability at scales relevant to physiological stress. J Exp. Biol. 213, 995-1003.

Hochachka PW, Somero GN. 2002. Biochemical adaptation, mechanism and process in physiological evolution. Oxford University Press, pp 466.

Hoffmann AA, Sørensen J, Loeschke V. (2003) Adaptation of *Drosophila* to temperature extremes: bringing together quantitative and molecular approaches. J Therm Biol 28: 175-216.

Kemp B, Mitchelhill KI, Stapleton D, Michell BJ, Chen Z, Witters LA. 1999. Dealing with energy demand: the AMP-activated protein kinase. TIBS 24: 22-24.

Kola, B., Grossman, A. B. and Korbonits, M. (2008). The role of AMP-activated protein kinase in obesity. *Front. Horm. Res.* 36, 198-211.

Morimoto RI (March 1993). "Cells in stress: transcriptional activation of heat shock genes". *Science (journal)* 259 (5100): 1409–10. PMID 8451637

Peck LS, Hardewig I, Pörtner HO. 2002. Metabolic demand, oxygen supply, and critical temperatures in the Antartic Bivalve *Laternula elliptica*. Physiol Biochem Zoology 75(2): 123-133.

Pörtner HO. 2001. Climate change and temperature-dependent biogeography: oxygen limitation of thermal tolerance in animals. Naturwissenschaften 88(4): 137-146.

Pörtner HO. 2009. Oxygen-and capacity-limitation of thermal tolerance: a matrix for integrating climate-related stressor effects in marine ecosystems. The Journal of Experimental Biology 213, 881-893.

Rutter GA, Tavare JM, Palmer DG. 2000. Regulation of mammalian gene expression by glucose. News Physiol Sci 15: 149-154.

Shelford VE. Animal Communities in Temperate America. Chicago, IL; Univ. of Chicago Press, 1913, p. 1-368.

Somero GN. (2010) The physiology of climate change: howpotentials for acclimatization and genetic adaptation will determine 'winners' and 'losers.' J Exp. Biol. 213, 912-920.

Sorger PK (May 1991). "Heat shock factor and the heat shock response". *Cell* **65** (3): 363–6. PMID 2018972

Tomanek L. (2005) Two-dimensional gel analysis of the heat-shock response in marine snails (genus *Tegula*): interspecific variation in protein expression and acclimation ability. J Exp. Biol. 208: 3133-3143.

Tomanek L. (2010) Variation in the heat shock response and its implication for predicting the effect of global climate change on species' biogeographical distribution ranges and metabolic costs. J Exp. Biol. 213: 971-979.

Valasek M, Repa J. (2005) The power of real-time PCR. J. Advances in Physiology Education. 29: 151-159.

Voznesensky, M., Lenz, P. H., Spanings-Pierrot, C. and Towle, D. W. (2004). Genomic approaches to detecting thermal stress in *Calanus finmarchicus* (Copepoda: Calanoida). *J. Exp. Mar. Biol. Ecol*. 311, 37-46.

Yeager, D. P. and Ultsch, G. R. (1989). Physiological regulation and conformation: A BASIC program for the determination of critical points. *Physiol. Zool*. 62, 888–907.

Zhu XJ, Dai JQ, Tan X, Zhao Y, Yang WJ. (2009). Activation of an AMP-activated protein kinase is involved in post-diapause development of Artemia franciscana encysted embryos. BMC Dev Biol. 16; 9:21.

Zhu XJ, Feng CZ, Dai ZM, Zhang RC, Yang WJ. (2007). AMPK alpha subunit gene characterization in Artemia and expression during development and in response to stress. Stress. 10(1): 53-63.

Zielinski S, and Pörtner HO. Energy metabolism and ATP free-energy change of the intertidal worm, Sipunculus nudus, below a critical temperature. J Comp Physiol [B] 166: 492–500, 1996.