4-1-2006

Temperature Tolerance In Crustaceans: Critical Temperatures And The Heart

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Temperature Tolerance in Crustaceans: Critical Temperatures and the Heart

An Honors Thesis

Presented to
The faculty of the Biological Sciences Department
University of New England

In fulfillment of the
requirements for the Degree of
Bachelor of Science
with Honors

By

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Biddeford, Maine
April 3rd 2006

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Acknowledgments:

Markus Frederich, Ph.D. – For all the time, help, advice and patience he has shown me.

Michaela O'Rouke – For helping to set up and run the AMPK and HSP westerns.

Elizabeth Mitchelle – For helping care for the crabs and for running some photometric tests.

Dylan Perry – For helping to collect animals and in getting the whole animal data.

Gregg Zogg – For sharing the lab and helping me get through the thesis process.

A. Christine Brown, Ph.D. – For being a member of my committee and for feedback.

David Koester, Ph.D. - For being a member of my committee and for feedback.

Geoffrey Ganter, Ph.D. - For being a member of my committee and for feedback.

Proposal Review Committee – For all the great feedback and ideas in my proposal.

The UNE Community – To all those who allowed me to use lab space, borrow equipment, liquid nitrogen etc….

My family and friends – For all the support they gave me through out the process.

Abstract

At certain upper and lower threshold temperatures (critical temperatures (Tc) crustaceans switch to anaerobic metabolism despite sufficient oxygen availability in the environment. I tested the hypothesis that failure of the heart at critical temperatures leads to insufficient oxygen delivery and subsequent anaerobiosis in peripheral tissues.

I exposed rock crabs, *Cancer irroratus*, as whole animals, and their buffer-perfused semi-isolated hearts to a progressive temperature increase, while monitoring heart rate and lactate accumulation. The whole animals heart rate increased with temperature following a Q_{10} of 2.8. An abrupt decline in heart rate occurred at 25℃ and lactate accumulation occurred between 25℃ and 30℃. The semi-isolated hearts followed a Q_{10} of only 1.2 during temperature increase. A second set of semi-isolated hearts were perfused and paced at a heart rate comparable to the whole animals at each temperature. The lactate concentration in the paced semi-isolated hearts did not significantly increase. AMPK activity and HSP70 levels were measured to investigate cellular changes occurring at Tc. Preliminary data suggests they are not good indicating parameters of Tc for isolated hearts.

I conclude that the whole animal's Tc is 5℃ lower than that of the heart muscle. Therefore, the onset of anaerobiosis in the peripheral tissues is not due to failure of the heart muscle. The processes within the whole animal that lead to anaerobiosis are set by other organ systems.

Introduction:

This study investigates the mechanism responsible for setting the upper threshold of temperature tolerance in the rock crab, *Cancer irroratus.* I compared the upper temperature threshold of the animal to the temperature threshold of semi-isolated hearts to determine whether the circulatory system is responsible for initiating the temperature induced onset of anaerobiosis in peripheral tissues.

Cancer irroratus, the rock crab

The rock crab, *Cancer irroratus*, is a decapod crustacean, closely related to the commercially used Jonah crab, *Cancer borealis*. Rock crabs are larger crabs ranging in size from 50 – 140 mm (Wong and Barbeau 2005, Stehlik *et al.* 2004, Gendron 2001). They can be found under rocks, buried in the mud, or in small crevasses along the shore line and within the subtidal zone of the Atlantic Ocean. They are found as far north has Labrador, Canada and as far south as South Carolina, USA. Rock crabs are scavengers who eat a variety of things, including: pieces of fish, algae, polychaetes, mussels, gastropods, and various other crustaceans (Gendron 2001). They live mainly in the subtidal zone, which begins at the lowest tide line, below the intertidal zone, and is always submerged. The areas of the subtidal zone vary in depth. It reaches as deep as the bottom of the sea floor where current from the waves can still be felt. However this wave current is much smaller than in areas that are affected by the tides. On a day to day basis there is very little temperature or salinity change,

providing a consistent environment for sea animals. However, these animals do have to be able to adapt to seasonal temperature changes. The winter temperatures are usually around 6℃, and in the summer it gets up to 21.5℃ (Wong and Barbeau 2005, Stehlik *et al.* 2004).

Cancer irroratus is not often used as a model organism in scientific studies. As of March 30, 2006 only 24 papers were found on PubMed and only 12 in Academic Search Premiere using the keyword "*Cancer irroratus*". The current published papers investigated topics such as food habits (Ristvey and Rebach 1999), behavioral interactions with other crustaceans (Gendron *et al.* 2001), and the effects of harmful pollution in the water on metabolism (Chou *et al.* 2002, Tucker and Matte 1980); but none looked at anaerobic metabolism or temperature tolerance. I chose these animals because, they are large, easily accessible, inexpensive to catch and care for, and they provided a good model organism to test the hypothesis of the current project.

Circulatory System

Rock crabs, like all decapod crustaceans, have a single chambered heart that is attached to the dorsal carapace of the crab. The heart is suspended by a series of ligaments within the pericardial cavity, which totally surrounds the heart. The pericardial cavity acts as a second chamber of the heart because the returning hemolymph collects here prior to reentering the actual heart chamber. In mammalian hearts this function is fulfilled by the atria. Prior research has shown that with an increase in heart rate there is a decrease in the cardiac

output because hemolymph does not move fast enough through the pericardial cavity and into the heart chamber before the next contraction (McMahon and Burnett 1990, Cooke 1988, Wilkens and Kuramoto 1998, Saver *et al.* 1998). Even with these limiting factors of a single chamber, the heart is still capable of pumping with enough force to account for the complete circulation of hemolymph throughout the entire body (McMahon and Burnett 1990). Past studies have found the following average cardiac outputs and stroke volumes for several decapods under normal conditions.

Table 1: Cardiac output and stroke volume for three decapod crustaceans.

Species	Cardiac Output ($mL \cdot kg^{-1} \cdot min^{-1}$)	Stroke Volume ($mL \cdot kg^{-1}$)	Reference
Cancer magister	159 $\pm$ 56	1.63 $\pm$ .58	McMahon and Burnett 1990
Carcinus maenas	118	1.3	Taylor and Butler 1978
Cancer productus	103 – 275	1.9 – 2.8	McMahon and Wilkens 1977

The rate of the heart is controlled by the central nervous system in three ways: 1.) by direct innervation of the heart; 2.) through pacemaker cells located under the heart, which are nerve cells as oppose to muscle cells as in humans; and 3.) by interaction with neurohormonal agents. While the myocardium is innervated, the heart is more closely regulated by the cardiac ganglion located between the heart and dorsal carapace. The cardiac ganglion consists of 9 neurogenic pacemaker cells clustered into three nerves, two posterior excitatory nerves and one anterior inhibitory nerve (figure 1). The cardiac ganglion neurons innervate muscle fibers in the heart and respond to stretch (McMahon and

Burnett 1990). Several studies have reported a direct correlation between the cardiac ganglion bursts and the observed heart rate (Saver *et al.* 1998, Cooke 1988). The excitatory or inhibitory state of the cardiac ganglion of the crab is influenced by circulating neurohormonal agents, i.e. peptide hormones, such as FMRFamide-related peptide ($F_1 + F_2$), and proctolin (PR). These hormones increase calcium uptake into the cells. The cardiac ganglion is also influenced by central excitatory or inhibitory discharge from pericardial organs, and by auto regulation of the amount of stretch applied to the walls of the heart which affect the dendritic trees of the pacemaker cells (Cooke 1988).

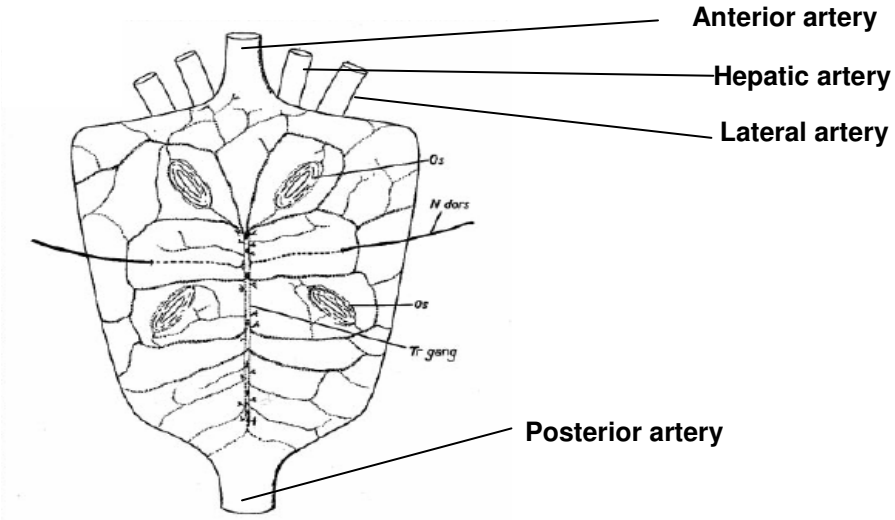


Figure 1: Nervous system in the dorsal wall of the heart of a lobster. Identified in this figure is the ganglionic trunk and its nerve-cells (*Tr gang*), the dorsal nerve piercing the heart-wall (*N dors)* and the ostium (*Os).* In the crab the posterior artery does not extend directly from the heart but it is connected to the sternal artery (see Figure 2). (modified after Cooke 1988).

Crabs have a complex open circulatory system. They have arteries that divide to form small tubules similar to human capillaries which help direct the initial flow (McMahon and Burnett 1990). Specifically there are two hepatic, two lateral, one anterior and one posterior artery (Maynard 1960)(Figure 2). The

difference between the arteries in crabs and those in humans is that as oppose to smooth muscle in the walls they have elastic fibers, which have regulatory capabilities like arteries in vertebrates. The control of these arteries comes from excitatory and inhibitory nerve fibers that innervate the elastic walls and cause either constriction (excitatory) or dilation (inhibitory) (McMahon and Burnett 1990). The hemolymph that is ejected from the heart is pumped through the arteries, into the tissues and then into the interstitial space. From there it moves into the lacunae, which are analogous to our veins. However, they are just open spaces between the tissues without a separate epithelial lining. These lacunae direct the hemolymph into the pericardial cavity and the hemolymph then enters the heart chamber through dorsal valves known as ostia, when the pressure in the pericardium cavity exceeds the pressure in the heart chamber (McMahon and Burnett 1990).

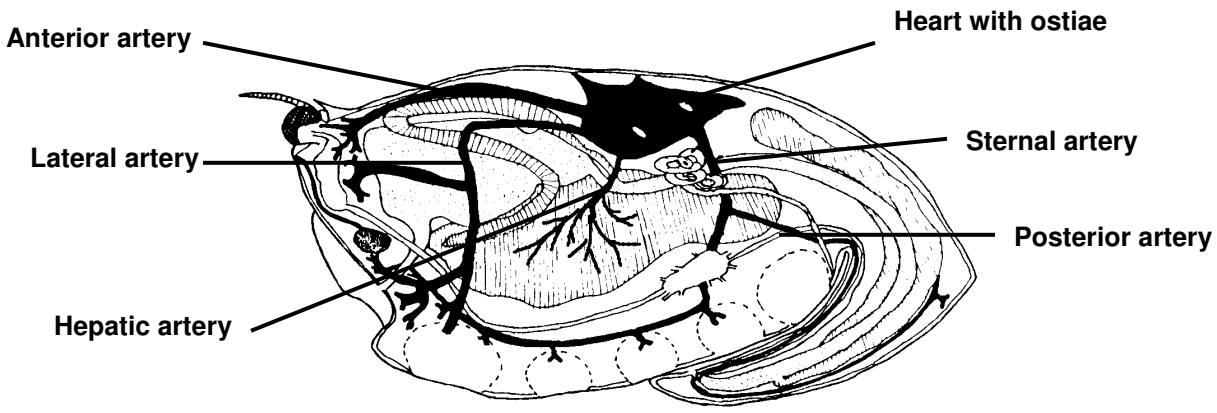


Figure 2: An internal view of the open circulatory system in decapod crustaceans (modified after McMahon and Burnett 1990).

Temperature

Crustaceans, and other invertebrates, are ectothermic which means that their body temperature changes in response to their environment. Therefore, the processes in their body are directly affected by the temperature of their environment. In contrast, humans are endothermic and maintain a constant body temperature such that their internal processes are not directly affected by environmental temperature changes. Temperature induced changes in ectotherms affect all biochemical and physiological processes. In addition, behavioral changes have been observed in some crustaceans, like the lobster, due to temperature variations (Crossins *et al.* 1998). Not only do the rates of these processes rise and fall with temperature, but these changes usually double with a temperature increase of 10°C. The doubling or tripling of metabolic rates and/or other processes in 10°C intervals is described as the Q_{10} relationship. For example, if at 10°C the rate of a biological process is 100, then by 20°C the rate will increase to 200 and by 30°C it will increase to 400. This temperature-rate relationship has been observed for many different biochemical and physiological processes in many different animals (Figure 3). The Q_{10} relationship for oxygen consumption as a measure of metabolic rate in various species was described by Krogh (1914) as a universal effect.

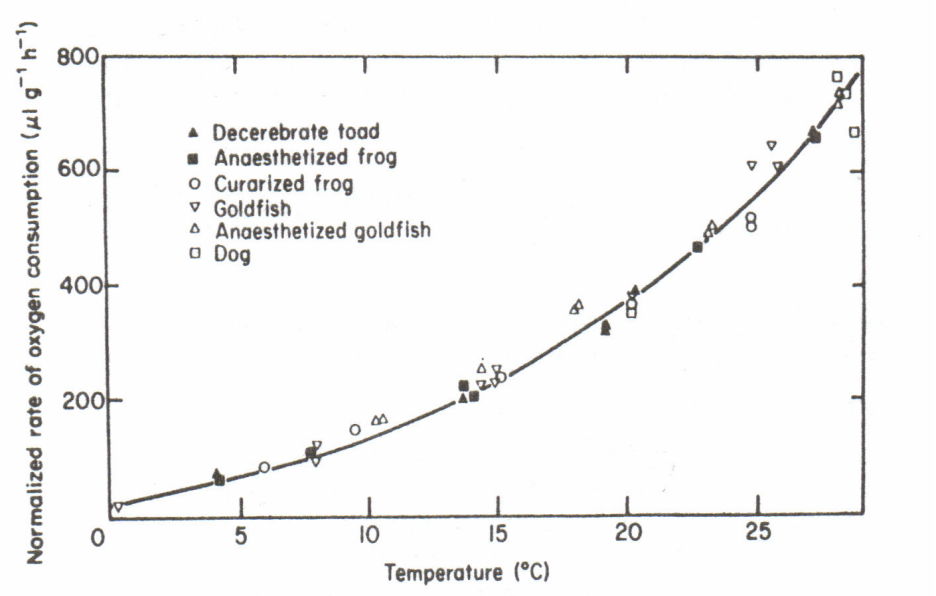


Figure 3: Original Q_{10} curve measured by Krogh for oxygen consumption. Oxygen consumption rates are normalized between the different species, and all follow the same relationship (Cossins and Bowler 1987).

The Q_{10} can be calculated for:

A 10° interval: $Q_{10} = R_2T+10℃ / R_1T$ (R is rate, T is temperature at that rate)

or

for any temperature interval: $Q_{10} = (R_2 / R_1)^{(10/ T2 -T1)}$ (Cossins and Bowler 1987).

Under normal conditions animals living in the subtidal zone are not subjected to large temperature changes within small periods of time. Therefore, the changes that occur in the processes of these animals are relatively slow and give the crustaceans enough time to adequately adapt to the changes in temperature when they do occur. These adaptations include, for example, behavioral changes, the expression of different protein isoforms, adjustment of

the mitochondrial density, and others (for review see Cossins and Bowler 1987). Other crustaceans that live, for example, in the intertidal zone, experience larger daily temperature changes. Therefore they need more adaptive capabilities to endure the internal changes that occur with temperature, as well as the external changes in the environment that occur in response to temperature, such as oxygen concentration or the salinity in the water (Taylor 1988).

Critical Temperatures

When the temperature increases above the physiological range, crustaceans among other species, switch from aerobic metabolism to anaerobic metabolism even though there is sufficient oxygen available in the environment. The switch to anaerobiosis occurs due to a mismatch between the oxygen demand of the issues and the supply being delivered. With an adequate supply of oxygen in the environment, it means that the inadequate supply of oxygen to the tissues must be due to an inadequacy in the processes that are responsible for delivering the oxygen to the tissues. The temperature at which the onset of anaerobic metabolism occurs, and end products of anaerobic metabolism accumulate, is defined as the critical temperature (Tc) (Zielinski and Portner 1996) (Figure 4).

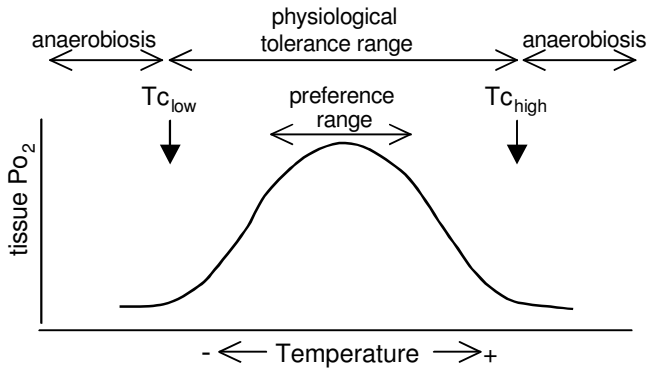


Figure 4: Temperature tolerance model based on Shelford's law of tolerance (1931). Animals live mainly in a temperature preference range with maximum oxygen availability to their tissues. The animals can endure higher and lower temperature with in their physiological tolerance range. At the critical temperatures (Tc_{high} and Tc_{low}) metabolism switches to anaerobiosis and survival beyond these thresholds temperatures is limited to very short periods of time. (adapted from Frederich and Portner 2000).

Critical temperatures were first described in the worm, *Sipunculus nudus,* which lives in the sediments of the intertidal zone (Zielinski and Portner 1996). The aim of that study was to look at how the worm's energy and acid-base status changed with temperature. The animals in the study were exposed to low temperatures of 4°C and 0ºC for a period of 8 days. Oxygen consumption, intracellular pH and anaerobic end products (acetate, succinate and propionate) were measured. The critical temperature was characterized between 4°C and 0ºC by the increase in anaerobic end products. Additionally, right before the Tc was reached the ATP concentration in the tissues and oxygen consumption decreased rapidly.

A subsequent study to describe Tc used the lugworm, *Arenicola marina,* and similarly looked at temperature dependent changes in energy metabolism and acid-base status (Sommer *et al.* 1997). Two different populations from two different environments (North Sea, Germany and White Sea, Russia) were

compared and the results showed that the different populations had two different critical temperatures, characterized by the accumulation of the anaerobic end product acetate. In addition to a low critical temperature, a high critical temperature was also characterized in both populations. This study confirmed the general concept of critical temperatures and showed that the actual Tc can shift with temperature adaptation.

More recent studies confirmed the concept of critical temperatures in gastropods (*Limopsis marionensis, Laterula elliptica*, and *Littorina saxatilis*), cephalopods (*Pareledone charcoti* and *Lolliguncula brevis*), crustaceans (*Maja squinado*), and even in vertebrates (eelpouts, *Zoarces viviparous* and *Pachycara brachycephalum*) (for references see table below). All of these studies found different critical temperatures in different species, but confirmed the general concept of Tc.

Table 2: Critical temperatures characterized by the onset of anaerobiosis as described in invertebrates and vertebrates.

Species **Common Name**	Critical Temperature (Tc) Low High ˚C	Accumulation of Anaerobic End Product	Reference
Sipunculus nudus **Sipunculid**	0-4	Acetate, succinate, propionate	(Zielinski and Portner 1996)
Arenicola marina **Lug worm, polychaeta**	5 20	Acetate, propionate	(Sommer *et al.* 1997)

Lolliguncula brevis **Squid**	26-31	Octopine	(Portner *et al.* 1996)
Maja squinado **Spider crab**	1 30	Lactate, succinate	(Frederich and Portner 2000)
Limopsis marionensis **Bivalve**	2-4	Succinate, acetate	(Portner *et al.* 1999)
Littorina saxatilis **Gastropod**	28	Succinate	(Sokolova and Portner 2003)
Laterula elliptica **Bivalve**	6	Succinate	(Peck *et al.* 2002)
Zoarces viviparous **Eelpout**	21-24	Succinate	(Van Dijk *et al.* 1999)
Pachycara brachycephalum **Eelpout**	9	Succinate	(Van Dijk *et al.* 1999)

Depending on the species studied, different anaerobic end products were measured because the pathways of anaerobic metabolism vary with species. All species break down glucose through the process of glycolysis and end with pyruvate. The pathway of anaerobic metabolism from pyruvate to the respective end products differs between species. Whether the anaerobic end product is lactate, succinate, propionate or octopine depends on the specific enzymes in the biochemical pathways. However, the accumulation of the respective anaerobic end product always indicates the onset of anaerobiosis and, during temperature stress, the critical temperature.

AMP-activated Protein Kinase

In response to temperature increases, animals switch to anaerobic metabolism, which means that production of ATP, the basic unit of cellular energy, is drastically decreased. The major ATP producing pathways, Krebs cycle and subsequent oxidative phosphorylation, require oxygen which is no

longer accessible to the cells at temperatures beyond Tc. A recent study (Melzner *et al*.. 2006) investigating critical temperatures in the cephalopod, *Sepia officinalis*, actually measured ATP concentrations at various temperatures ranging from 8℃-26℃. Their results indicate that ATP is conserved throughout the temperature increase until about 5 degrees before the critical temperature, at which time there is a decrease in the ATP concentrations.

While the regulation of the individual pathways of energy metabolism is standard textbook knowledge, the integrative regulation of all the pathways was poorly understood until recently. Work on mammalian systems showed that AMP-activated protein kinase (AMPK) plays an important role. AMPK is a protein/enzyme, found in the cytosol and the nucleus of the cell, that plays a crucial role in monitoring and adjusting the energy levels within a cell (Winder 2001).

AMPK is activated, both during times of short-term metabolic changes, and also during chronic adaptations. AMPK is activated by an increase in AMP which directly correlates to a decrease in ATP through the adenylate kinase reaction: the hydrolysis of ATP leads to ADP and P_i. Two ADP in turn are converted by the adenylate kinase enzyme into another ATP and an AMP.

ATP -> ADP + Pi (ATP hydrolysis)

2ADP -> ATP + AMP (adenylate kinase reaction)

A breakdown of ATP therefore leads directly to an increase in AMP, which makes AMP a good indicator of ATP turnover.

AMPK contains three subunits (α, β, γ). The α-subunit contains the catalytic activity, the beta and gamma subunits have regulatory functions (Ruderman and Prentki 2004). AMPK gets activated allosterically by binding AMP to the gamma subunit. Additionally, AMPK can be activated via phosphorylation by AMPK-kinases (AMPKK). The AMPKK are also activated by AMP. One AMPKK was recently identified as LKB1, a tumor suppressor (Woods *et al.* 2003). Details of the LKB1 activation and its relevance for AMPK are currently under investigation. AMPK is more susceptible to phosphorylation by AMPKK if AMP is already bound to the γ-subunit. Maximum AMPK activity is achieved through AMP binding to AMPK and phosphorylation of AMPK through AMPKK (Ruderman and Prentki 2004, for review see Kemp *et al.* 2003).

The purpose of activated AMPK is to decrease the amount of ATP lost to processes unnecessary during stressful situations (i.e. digestion), and therefore, to save ATP for the critical organs and tissues. This is done by inhibiting ATP-consuming pathways (i.e. glycogen, protein and fatty acid synthesis) and activating ATP-producing pathways (i.e. glucose uptake, glycolysis, fatty acid oxidation). AMPK affects each pathway in a different way, mostly by inhibition or activation of rate-limiting enzymes. AMPK can also induce chronic changes to the pathways by effecting gene expression (for review see Hardie and Sakamoto 2006). AMPK is often referred to as a “metabolic master switch” or “low fuel gauge” (Hardie & Carling 1997, Winder & Hardie 1999)

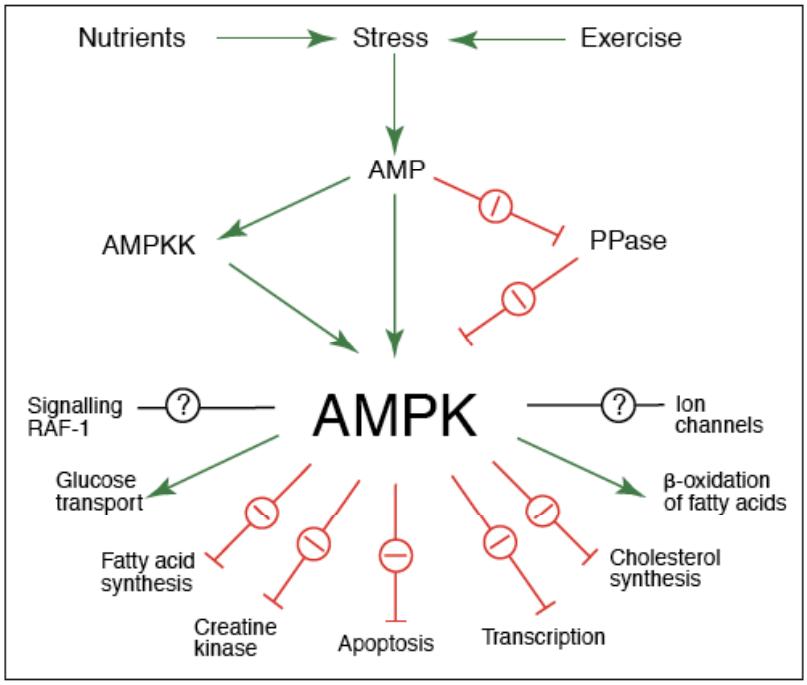


Figure 5: Activation and regulation of AMPK. Cellular stress leads to an increase in the AMP concentration, AMP in turn activates AMPK and AMPKK. Activated AMPK activates ATP producing pathways and inhibits ATP consuming pathways. Question marks represent unknown downstream targets of AMPK for the specific pathway (Kemp *et al.* 1999).

At this point, AMPK regulation of energy metabolism is only described in mammals such as rats, mice and humans, but little is known about its role in invertebrates. AMPK has been highly conserved during evolution so we hypothesize that it plays a crucial role, not only in vertebrates, but also in invertebrates. A recent study demonstrated AMPK activation in frogs (Bartrons *et al.* 2004). This study shows a significant increase in the activation of AMPK during hypoxia and low temperature. To my knowledge this is the only study showing AMPK activation through temperature stress. There are no published studies that address the AMPK levels in crabs, and none that investigate what changes occur in response to a drastic increase in environmental temperature.

Heat Shock Proteins

Heat shock proteins (HSPs) are molecular chaperones that play an important role throughout normal cell life and as responders during stress. HSPs are a diverse group that vary, both in their genetic makeup, and molecular mass. The different families of HSPs are named according to their molecular weight in kilodaltons (i.e. heat shock proteins with a molecular mass around 70 kDa all belong to the HSP70 family) (Hochachka and Somero 2002). There are 7 families that range in molecular mass from 10 - 110 kDa (Snyder and Rossi 2004). Under normal conditions HSPs help to maintain proper folding and interactions of new proteins, as well as help with the correct placement of that specific protein within the cell. When the cell is stressed (i.e. in response to increasing temperatures, chemicals, hypoxia, etc…) proteins can be altered or denatured and heat shock proteins play a vital role in preventing aggregation, as well as, aiding in the refolding of the denatured proteins back to their functional shapes. During times of high environmental stress the synthesis of heat shock proteins increases, this is known as the heat shock response (Hochachka and Somero 2002).

During a heat shock response, there is an increase in the synthesis of HSPs, both those that are stress-induced, and those that are constitutively synthesized during normal cell life. Heat shock responses have been observed in almost all animals, except for two cold water fish that live in environments below 0°C (Hoffman *et al.* 2000). However, these two Antarctic fish species lacking

HSPs live in an absolutely stable environment with temperature ranges of less than +/-2℃. Therefore, they do not need a response to changing temperatures. The entire heat shock response is still not completely understood. A cellular thermometer most likely triggers one of several heat shock transcription factors which induces the increase in HSP synthesis. Following this synthesis there must also be a regulatory cascade that causes the increase in synthesis to return to a normal range (Katschinski 2004). The heat shock response is not a fix for all stressful situations, because not all proteins that are damaged in response to stress are refoldable, which explains why long term environmental stress on an animal can still lead to permanent injury or death.

Induction of HSP synthesis can still occur during normal body temperature ranges in ectotherms (Hochachka and Somero 2002). When aquatic animals are being studied it is important to establish, whether or not, physical handling and experiments cause enough stress within themselves to initiate a heat shock response. Every species responds differently to various stressors, with responses that depend on their specific genetic makeup, development, environment, and even what family of HSP is being synthesized in response to the stress (Iwama *et al.* 2004).

HSP70 is one of the most abundant and highly conserved heat shock proteins that responds to stress in animals. They are important during increasing temperatures because they can bind to the hydrophobic surfaces of denatured, or partially denatured proteins. In this way they can help in the refolding of these proteins, and also help by keeping the open sites of the proteins from interacting

with other proteins or any other surfaces in their vicinity (Katschinski 2004).

A recent study investigated the HSP70 expression in intertidal benthic organisms, specifically the anemone, *Anthopleura elegantissim* (Snyder and Rossi 2004). The aim of the study was to compare the animals internal temperature to the expression levels of HSP70. This study was unique in that all the experiments were done in the animals normal habitat, as oppose to in a laboratory. These organisms are found in the intertidal zone, where there are large daily temperature changes. The conclusion of the study was that on sunny days, with warmer temperature, changes the HSP70 levels were significantly higher than the HSP70 expression on foggy days with lower temperatures. They also found higher HSP70 levels in animals that were emerged and then immersed in water with the tides, compared to those that were immersed for the whole time. Another study, that explored HSP70 expression with respect to temperature, actually named the onset temperature for heat shock proteins as T_{on} (Tomanek 2005). The researcher mentioned that T_{on} varies among species, and can even change within a species as there are seasonal acclimations to changing environments. Since T_{on} indicates the time of stress, I am interested whether or not there is any correlation between the onset of heat shock response and the onset of anaerobiosis or the critical temperature.

In summary, exposure of marine invertebrates to high temperature leads to failure of the heart, and an onset of anaerobic metabolism, despite the presence of sufficient oxygen in the environment. At critical temperatures cellular

energy is depleted and the circulatory system shows signs of failure. It is likely that the stress from high temperatures and hypoxia will activate the metabolic master switch, AMPK, to help prevent further ATP depletion. Additionally, thermal thresholds are described which are characterized by increased HSP70 protein levels. This leads to the question, whether at high temperatures, the heart fails to pump oxygenized hemolymph to the peripheral tissues, which then in turn leads to anaerobiosis in these peripheral tissues, or whether the heart receives deoxygenated hemolymph and then fails, due to anaerobiosis in the heart.

The hypothesis for the current project is that failure of the heart is responsible for lack of sufficient oxygen in the peripheral tissues, causing the switch from aerobic to anaerobic metabolism at critical temperatures. In addition I hypothesize that the protective cellular mechanisms of AMPK activity and HSP70 protein levels increase at the critical temperature.

Materials and Methods:

Whole animals (n=20) and semi-isolated hearts (n = 30) were incubated and exposed to temperature increases and decreases. Throughout the temperature changes the heart rate was monitored continuously. At specific temperatures the animals were sacrificed, and tissue samples obtained, to measure lactate, AMPK activity and HSP levels.

Animals

Large male, *Cancer irroratus,* were caught with crab traps off the shores of the Mount Desert Island Laboratories in Salisbury Cove, Maine in June-July 2004 and June-July 2005. They were kept in a flow through seawater tank in the Marine Science Center at the University of New England. The crabs were fed frozen fish twice a week. Complete tank cleanings were done when necessary.

Temperature Incubations

In order to determine the Tc of the animals they were exposed to a progressive temperature increase from 18˚C to approximately 32˚C over a 2-3 hour time period (n= 40). Throughout the temperature increase the heart rate was monitored (see below). The temperature at which the maximum heart rate was reached was likely close to Tc, which gave me a range to work with during the next experiment. In the next set of experiments lactate measurements (see below) were made at 18˚C, 25˚C, 30˚C, and if the crab was still alive, at 32℃.

Animals (n=10) were also exposed to a progressive decrease in temperature until about 5℃ was reached, which was likely prior to the lower critical temperature, to get a better scale of the various heart rates. No lactate measurements were made at these low temperatures.

I chose to identify a higher critical temperature, because it is easier and cheaper to heat something up than it is to cool it down, especially for animals who live in relatively cold environments to begin with.

At the indicated temperatures the described tissues were quick-frozen with pliers, pre-cooled in liquid nitrogen, and stored at -80˚C. Doing this preserved the metabolic state of the tissue, i.e. the lactate concentration and the AMPK activity.

Magnesium Concentration in Crab Hemolymph

The magnesium concentration in crab hemolymph was measured in order to accurately mimic the crab ringer in which semi-isolated hearts were placed. It is important to have a very accurate magnesium concentration because the amount of magnesium in the hemolymph correlates with activity levels of crustaceans, specifically it correlates to the cardiac activity. An incorrect magnesium concentration in the ringer solution would affect the heart rate (Walters and Uglow 1981, Frederich *et al.* 2000).

Hemolymph samples were obtained with a needle inserted into the articular membrane of the legs of five male *Cancer irroratus*. Magnesium concentration was measured with a photometric assay (Pointe Scientific Inc.). In this assay a dye specifically binds to magnesium, therefore the measured absorbency at 530 nm correlates with the magnesium concentration.

Semi-isolated Hearts

In order to see if the limitations were the same for the heart and the whole animal, we compared the Tc for both. To make these comparisons directly with the circulatory system, I compared results from both whole animals and isolated

hearts of animals; the semi-isolated heart preparation described by Wilkens and McMahon (1994) was used. The cerebral ganglion of the animals were destroyed by cutting with scissors into the head of the animals. After that, the legs, the tail, the ventral side of the animal, and the interior organs (stomach, liver, testes/ovaries) were carefully removed until only the heart remained attached to the dorsal carapace (n = 30). The carapace, with the heart, was placed into a temperature controlled chamber filled with a solution that mimiced the ion composition of the animal's hemolymph (in mM: Na^+ 460, K^+ 10, Ca^{2+} 10, Mg^{2+} 25, Cl^- 490, HEPES 5, glucose 10, pH 7.4). For measuring the heart rate a photoplethysmograph (see below) was positioned below the semi-isolated heart. The semi-isolated hearts were exposed to the same temperature incubations as described for the whole animal.

Pacing of Isolated Hearts

The isolated hearts were paced to mimic the increasing heart rate seen in whole animals in response to increasing temperature. This was done using the same isolation process and buffer solution as described above. Once the heart was in the buffer, electrodes were placed in contact with the heart. The electrodes were attached to an electrical stimulator (BIOPAC Systems Inc., Goleta CA). The stimulation voltage was adjusted between 5 and 10 V, to ensure a continuous pacing of the heart. With each degree of temperature change the stimulator was set to the corresponding rate needed to mimic the average speed

of the whole animals' hearts at that temperature. To determine the respective rate for the different temperatures a regression line was fitted through the whole animal heart rate data. The equation from that regression line (Y = 8.1437X – 9.3418, Y= BPM X= Temperature ℃) was used to calculate the rate in Hz needed to mimic the BPM at corresponding temperature (n= 15).

Heart Rate

To test if the circulatory system is what fails at critical temperatures, the animal's heart rate was monitored, during all incubations, with a photoplethysmograph that was connected to a digital recording device (BIOPAC Systems Inc., Goleta, CA). The photoplethysmograph emits an ultraviolet (UV) light signal that penetrates the carapace of the animal and is then partially reflected by the heart. The amount of reflected light is then measured by UV sensors. As the heart beats, it changes shape, and therefore the amount of reflected UV light changes, depending on the shape of the heart. This change in reflection leads to a change in the signals detected by the digital recording device. The signal changes detected, equal the number of heart beats. This non-invasive method was first described by Depledge (1984) and is superior to the other commonly used invasive method which requires implanting electrodes into the animal (e.g. Cumberlidge and Uglow 1977). When anaerobiosis sets in, the heart rate decreases which helped in determining the animal's critical temperature.

Lactate Assay

To determine when the switch from aerobic to anaerobic metabolism takes place, the lactate concentration was measured in tissue samples from the heart, claw muscle, and hepatopancreas. Lactate was assayed by a photometric enzyme test (Bergmeyer 1985). These tissues were easily accessible and represent tissues with different levels of metabolic activity. For this photometric test the tissue samples were ground under liquid nitrogen and the tissue powder then treated with perchloric acid to precipitate protein. The lactate concentration in the remaining sample was measured photometrically. The assay uses the reaction: lactate + NAD^+ -> pyruvate + NADH. In this reaction one NADH molecule is generated for one lactate molecule. The reaction was catalyzed by the enzyme lactate dehydrogenase (LDH). The increase in NADH is measured by the photometer with a pH-stabilizing buffer, NAD^+ and LDH, at a wavelength of 340nm (n = 2-5).

The lactate concentration was normalized to the protein concentration in the extract measure by the Bradford method (Bradford 1976).

AMPK levels

The activation of AMPK was measured using a western blot. The same tissues were ground under liquid nitrogen and homogenized in a buffer containing phosphatase inhibitors to prevent dephosphorylation of AMPK. Fifty

µg protein of the homogenate (determined by the Bradford method, Bradford 1976) was separated on an 8 % polyacrylamide/SDS gel at 200 V for 30 min. The proteins were transferred to a nitrocellulose membrane at 100 V for 1 hour. Primary rabbit anti pT172 antibodies (Upstate, NY) and secondary anti-rabbit antibodies (BIORAD) probed for a colorimetric signal, after blocking the membranes with 3 % non-fat dry milk. The membranes were scanned and the bands quantified using the NIH-image software (n=1-2).

Heat Shock Protein 70 Levels

The HSP70 protein levels were measured using the same tissue extracts and western blot methodology that is described above. The primary antibodies were mouse anti HSP70 (Sigma). The HSP70 sequence is highly conserved and therefore it is possible to use the mouse antibody in the crab (Frederich, O'Rourke, unpublished data) (n=1-2).

Statistics

The changes in lactate, HSP70 and AMPK activity between the three groups (whole animal, unpaced isolated hearts, paced isolated hearts) were compared by a one-way nonparametric Kruskal-Wallis ANOVA (GraphPad Prism Software). An unpaired t-test was used to determine the significance of changes that occurred within one group. A p-value of < 0.05 was considered significant.

Results:

Magnesium

The magnesium concentration in the hemolymph of male *Cancer irroratus* was measured as 25.4 $\pm$ 2.6 mM, n=10 (mean $\pm$ stdev).

Heart Rate

The heart rate of the whole animal steadily increased through out the temperature increase. The lowest heart rate, 49 $\pm$ 21 BPM, occurred at 6.6°C, the resting heart rate at 18.6°C was 146 $\pm$ 39 BPM, and the maximum heart rate reached was, 189 $\pm$ 31 BPM, which occurred at 25°C. The heart rate increased from 6.6°C – 25ºC, with a Q_{10} of 2.1. Above 25°C the heart rate decreased until death, which occurred at 30°C (Figure 6).

The heart rate of the isolated heart started at 32 BPM at 3.5°C, and increased to 73 $\pm$ 8 BPM at 10°C. The Q_{10} between 3.5°C and 10°C was 5.5. Above 10°C the heart rate remained relatively constant until 31°C, resulting in a Q_{10} of 1.2. At the control temperature of 18°C the heart rate was 67 $\pm$ 19 BPM, and the maximum heart rate reached was 90 $\pm$ 10 BPM at 26°C. At 30.5°C the heart rate was 75 $\pm$ 37 BPM and by 31°C the heart rate decreased to 33 $\pm$ 17 BPM and than continued to drop, until death at 32°C (Figure 7).

The heart rate for the paced, isolated hearts was calculated using the regression equation Y= 8.1437 X – 9.3418 (where X is temperature and Y is Hz), based on a line fitted as a linear regression through the whole animal data.

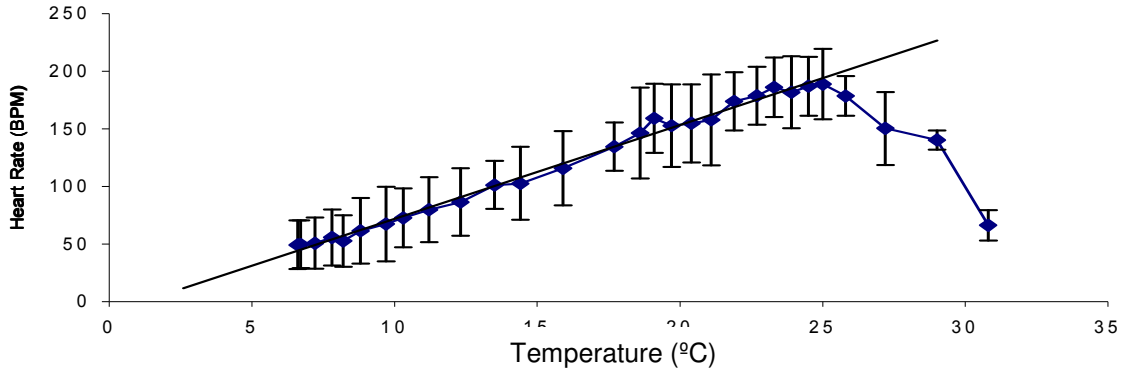
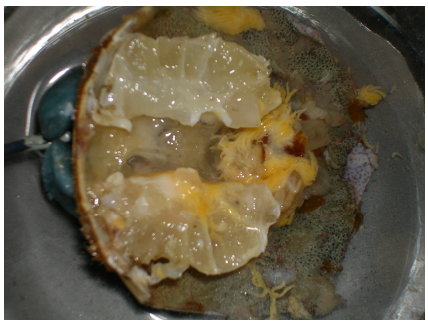


Figure 6: Heart rate (BPM) of whole male animals as a function of temperature (°C). A linear regression line was fitted to the whole animal data between 6.6°C and 25.4°C to find a Q_{10} of 2.1. (mean ± stdev) (n= 5).



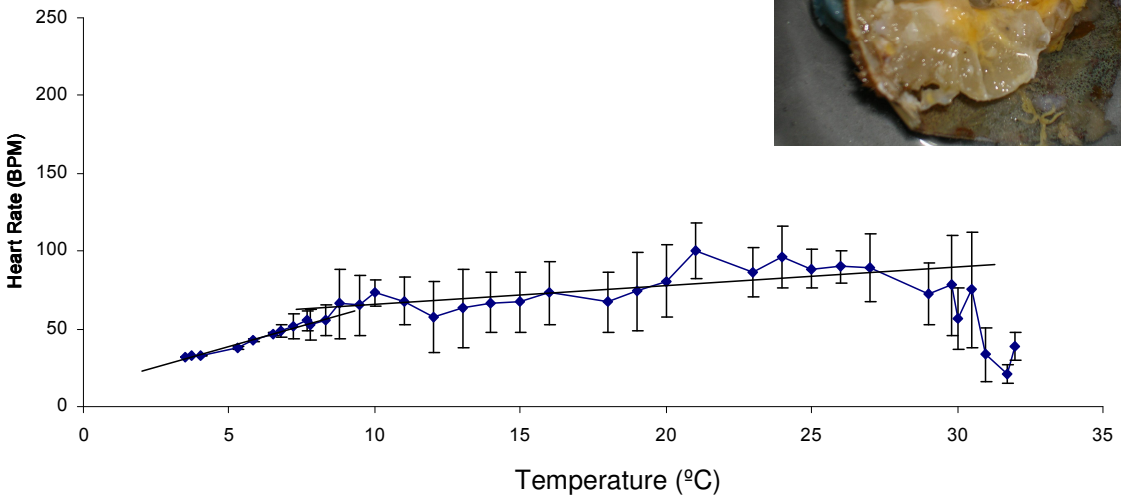


Figure 7:Heart rate (BPM) of male semi-isolated hearts as a function of temperature (°C). A linear regression line (Y = 5.365X + 12.036) was fitted through the data from 3.5°C-7.8°C to find a Q_{10} = 5.5; a second linear regression line (Y = 1.2071X + 53.747) was fitted through the data from 8.8°C-30°C to find a Q_{10} of 1.2. (mean ± stdev) (n= 3-5).

Lactate

In the heart of the whole animals, the lactate concentration, at the control temperature, 18℃, was 0.11 $\pm$ .04 mmol/g protein. At 25℃, the lactate concentration increased to 0.22 $\pm$ .08 mmol/g protein. At 30℃, the temperature of death, lactate concentration measured 1.15 $\pm$ .32 mmol/g protein. There was no significant difference between the 18℃ and 25℃ but there was a significant difference between 25℃ and 30℃ (ANOVA, $p < 0.05$) (Figure 8, Table 3).

At a control temperature ,of 18℃, the unpaced isolated hearts had a lactate concentration of 0.11 $\pm$ .07 mmol/g protein. At 30℃ the lactate remained the same, measuring at 0.10 $\pm$ .04 mmol/g protein. The lactate concentration at death (32℃) was 0.52 + .31 mmol/g protein. There was no significant difference between any of the lactate concentrations (ANOVA, $p < 0.05$) (Figure 8, Table 3).

At a control temperature, of 18℃, the lactate concentration of the paced isolated hearts was 0.08 $\pm$ .03 mmol/g protein. At 25℃, lactate concentration dropped to 0.06 $\pm$ .05 mmol/g protein. The lactate concentration then increased to 0.34 $\pm$ .14 mmol/g protein, with an increase of temperature to 30℃. At 32℃ the lactate concentration was still 0.34. ANOVA statistics show no significant difference between any of the concentrations (Figure 8, Table 3).

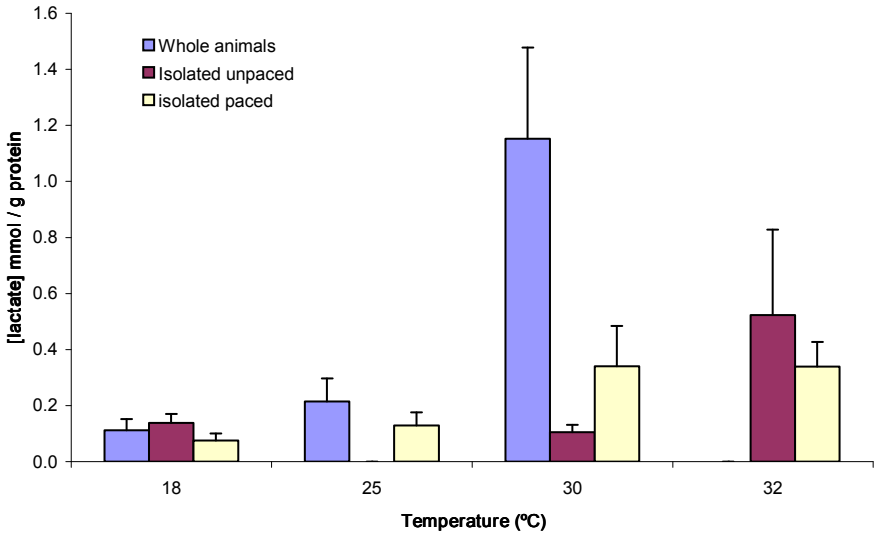


Figure 8: Lactate concentration as a function of temperature (°C) in the whole animals, isolated unpaced hearts, and isolated paced hearts. (mean ± stdev) (n= 2-5)

Table 3: Summary of unpaired t-test results comparing different groups ($p<0.05$). (WA= whole animal, IUP= isolated unpaced, IP= isolated paced, 18, 25, 30 or 32 = temperature (°C)).

groups	**p<0.05**
W18 vs. IUP18	No
W18 vs. IP18	No
IUP18 vs. IP18	No
WA25 vs. IP25	No
WA30 vs. IUP30	Yes
WA30 vs. IP30	Yes
IUP30 vs. IP30	No
IUP32 vs. IP32	No

AMPK

Western blot data (Figure 9) was put into relative units for comparisons to be made. In the whole animal, the AMPK activity increased, from a low activity of 66 (relative unit) at 18℃, to a higher activity 255 (relative unit) at 25℃, and remained high at 30℃ with 258 (relative unit) activity. In the paced isolated heart, the AMPK activity was very high at, 357 (relative unit) at 18℃, and than decreased at 25℃ to 186 (relative unit), and than again at 30℃ to 121 (relative unit). It increased to 265 (relative unit) between 30℃ and 32℃. Due to the low n (1-2 per group) no statistical significance could be found (Figure 10).

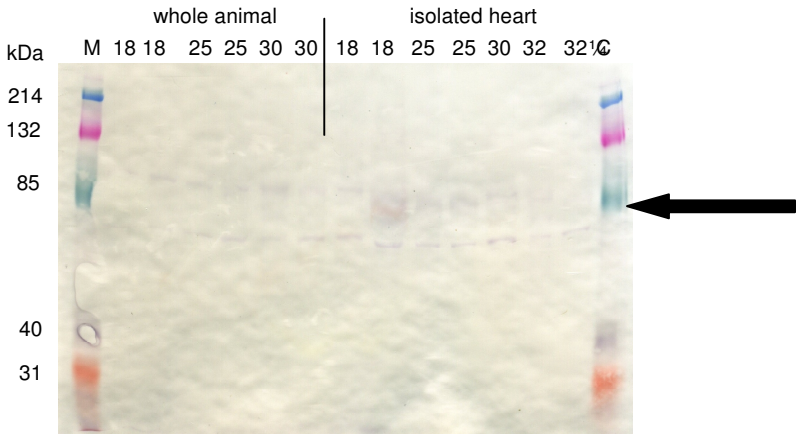


Figure 9: Western blot for AMPK showing a protein marker (M) with the respective molecular weight (kDa) and paced isolated heart samples as well as hearts from whole animal incubations. Arrow indicates AMPK bands at 65 kDa.

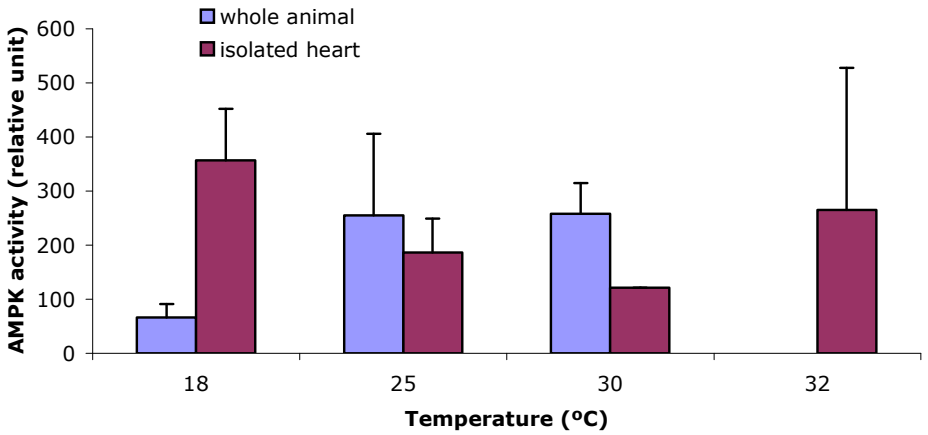


Figure 10: AMPK activity as a function of temperature (°C) in both the whole animal and isolated paced hearts. No significant differences were identified with the respective groups ($p>0.05$) (mean $\pm$ stdev) (n=1-2).

Heat Shock Proteins

In the whole animal, the HSP70 protein levels increased by a 2.2 fold, from 2787 $\pm$ 211 (relative unit) at 16°C, to 5994 $\pm$ 1250 (relative unit) at 25°C. From 25°C to 30°C, the protein levels decreased 1.8 fold, to 3275 $\pm$ 400 (relative unit) (Figure 12). In the isolated hearts, the HSP70 protein levels increased 1.8 fold, from 503 (relative unit) to 892 $\pm$ 27 (relative unit) between 18°C and 25°C, and continued to increase, 1.3 fold to 1197 (relative unit) at 30°C. The protein levels decreased between 30°C and 32°C, to 928 + 221 (relative unit) (Figure 13). It is important to note, the whole animal relative unit and the isolated heart relative unit can not be directly compared. They depend on the individual western blot (Figure 11). Due to the low n in both groups (1-2 per

group) no statistical significance could be found within or between the two groups.

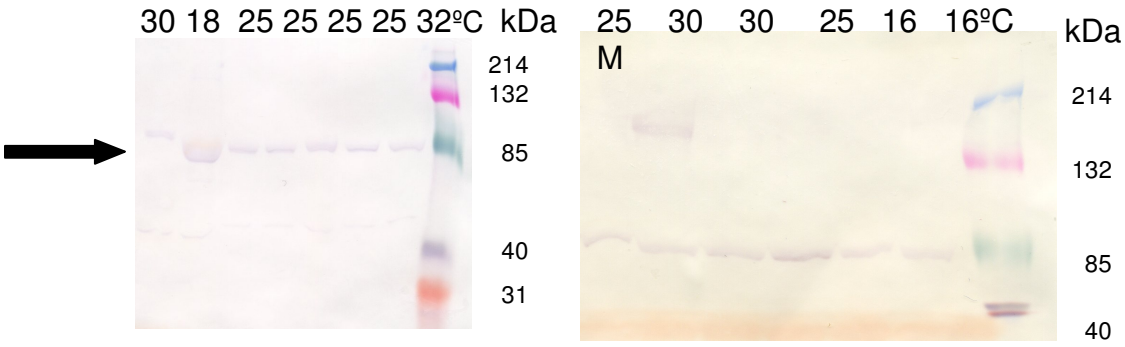


Figure 11 Western blots for HSP70 showing a protein marker (M) with the respective molecular weight (kDa) and isolated paced heart samples (left), as well as hearts from whole animal incubations (right) . The arrows indicated the HSP70 bands at 85 kDa .

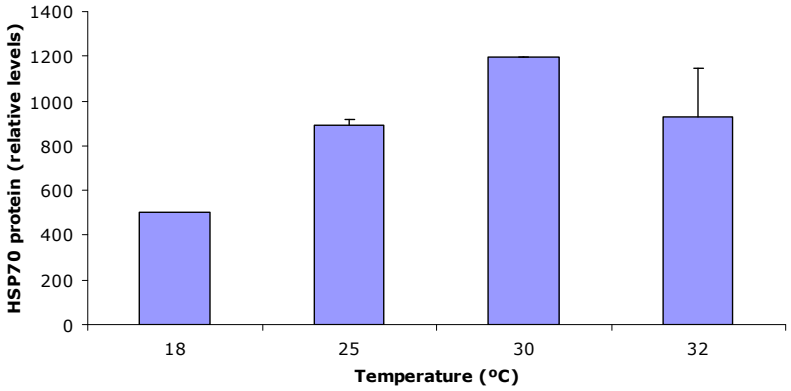


Figure 12: HSP70 protein of the isolated heart as a function of temperature (°C). No significant differences shown between protein levels ($p>0.05$) (mean $\pm$ stdev) (n=1-2).

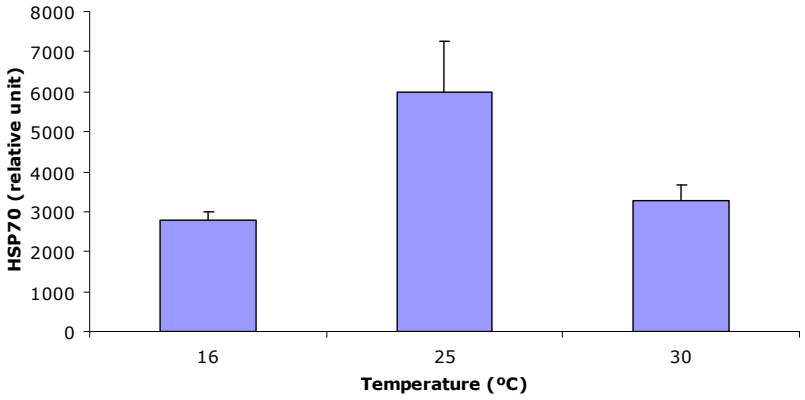


Figure 13: HSP70 protein of the whole animal hearts as a function of temperature (℃). No significant differences shown between protein levels ($p>0.05$) (mean $\pm$ stdev) (n=2).

Discussion:

Critical temperatures have been observed in several invertebrate species, using different parameters as indicators, such as heart rate, ventilation rate, or the accumulation of various anaerobic end products (Zielinski and Portner 1996, Sommer *et al.* 1997, Portner *et al.* 1996, Frederich and Portner 2000, Portner *et al.* 1999, Sokolova and Portner 2003, Peck *et al.* 2002, Van Dijk *et al.* 1999, Shillito *et al.* 2006). This study shows that the high critical temperature of the rock crab, *Cancer irroratus,* can be defined using heart rate and lactate measurements, not only in the whole animals, but also in semi-isolated hearts. The results showed that the whole animal has an upper critical temperature in the range between 25 and 30℃. In contrast, the upper critical temperature of the semi-isolated hearts was between 30 and 32℃.

The biological processes, e.g. heart rate, of invertebrates increase within the physiological temperature range at a consistent Q_{10} between 2 and 3. In this

study the whole animals' heart rates increased at a Q_{10} of 2.1. Ahsanullah and Newell (1971) calculated Q_{10} of 1.48-2.85 in a related species, the green crab, *Carcinus maenas.* Their study also demonstrated that the Q_{10} of one process can vary within species, depending on variation in gender, size, and weight which accounts for the range given. In this experiment all the subjects used were male and were about the same size and shape so changes were not seen.

In this study the measured heart rates were also consistent with other decapod studies that investigated heart rate. The *Carcinus maenas* study by Ahsanullah and Newell (1971) measured heart rate at various temperatures with crabs of various sizes. Their results indicated that at 20℃ the heart rate ranges from 60-120 BPM, at 25℃ the heart rate range increased to 70-150 BPM, the majority of animals heart rates at this temperature were in the 100-150 BPM range, and at 30℃ there was a decrease in the heart rate range, back down to 50-100 BPM. Another study on *Carcinus maenas* (Cumberlidge and Uglow 1977) backs up these heart rate findings at 11℃. Their study also went as far as to characterize the various ranges of heart rate as resting, active, or elevated. They concluded that the resting heart rate range of *Carcinus maenas* is 25-45 BPM, the active level heart rate range was 50-75 BPM, and elevated levels are anything greater than 80 BPM. Similar heart rates were also reported by Frederich and Portner (2000) in the sea spider, *Maja squinado,* with a heart rate of approximately 50 BPM at 15℃ and a peak in heart rate at approximately 105 BPM at 30C. McGaw *et al.* (1995) reported a resting heart rate of 77 $\pm$ 3 BPM at 12° $\pm$ 1° C in the Dungeness crab, *Cancer magister*.

In my study the heart rate of *Cancer irroratus* ranges from 49 ± 21BPM at 6.6℃, 146 ± 39 BPM at 18.6℃, to a maximum heart rate of 189 ± 31 BPM, reached at 25℃. These heart rates are higher than the heart rates reported in the above studies. A possible explanation for the differences in heart rate between the various species is their different marine habitats. The physiological temperature range for each species is different, therefore temperature will affect the heart rate of each species differently. One species may show only minor heart rate changes with temperature increase, because they are acclimated to a broader physiological temperature range. In contrast, others, like *Cancer irroratus*, will show large changes in heart rate with increasing temperatures, because they are acclimated to a narrower physiological temperature range.

It is also important to note that the control temperature of 18℃ is about 5 degrees higher than the average daily temperatures they live in naturally. I used 18℃ as the control temperature because initially it was easier to maintain for long periods of time because I lacked sufficient cooling equipment to maintain a constant 12℃. In response, the reported heart rate at this temperature is slightly higher than their actual normal resting heart rate. However, because 18℃ still falls within their physiological temperature range, and both the whole animal and isolated hearts were run within the same temperature range, it is still possible to compare the two in order to investigation the mechanisms of critical temperature.

McMahon (1999) concluded that in the majority of cases, crustacean heart rate measurements are not a good measure of cardiac performance. His results, among others, have shown that in crustacean's, cardiac output remains constant

even as the heart rate increases because the stroke volume adapts to maintain it. Cardiac output can be measured in crustaceans using a Doppler flow meter (Frederich *et al.* 2000, McMahon 1999, McGaw 2005, McGaw *et al.* 1995). However, these studies were done within the physiological temperature range and they were assessing the actual cardiac performance. My study focuses not on cardiac performance, or the physiological temperature range, but on the transition into the non-physiological temperature range. Various other studies (Zielinski and Portner 1996, Sommer *et al.* 1997, Portner *et al.* 1996, Frederich and Portner 2000, Portner *et al.* 1999, Sokolova and Portner 2003, Peck *et al.* 2002, Van Dijk *et al.* 1999) have shown that heart rate is a good indicator for this transition into anaerobic metabolism which was clearly identified in my study at 25℃.

Current work, on semi-isolated hearts, has found two significant differences between hearts *in vivo* and those *in vitro*, a lower actual heart rate in the isolated hearts and a lower Q_{10}. A decreased heart rate in semi-isolated hearts was discussed in several studies (McGaw *et al.* 1995, DeWachter and Wilkens 1996, Wilkens and McMahon 1994). Wilkens and McMahon (1994) assessed the cardiac performance of semi-isolated hearts of the crab, *Carcinus maenas.* Their results showed that immediately after isolation the resting heart rates are 15 – 25 BPM lower than that of the heart *in vivo*. They also found that 15-20 min after isolation, heart rate decreased by another 10-20 BPM to a stable average of 49.7 $\pm$ 2.8 BPM. Their semi-isolated hearts maintained this average heart rate for approximately four hours. Other mechanical properties, such as

systolic pressure, persist with slight decreases, or without any change during the four hour duration. McGaw *et al.* (1995) reported a decrease from an average of 77 $\pm$ 3 BPM to 57 $\pm$ 3 BPM after isolation, in the Dungeness crab, *Cancer magister,* at 12° + 1°C. DeWachter and Wilkens (1996) reported similar decreases in the Dungeness crab heart rate following isolation. Worden *et al.* (2006) reported a decrease in the heart rate of semi-isolated hearts in comparison to whole animal heart rates in the American lobster, *Homarus americanus,* but the degree of the decrease was significantly less than those observed in crabs. These respective differences between the crab and lobster are discussed later in context with $Q_{10.}$ The results of this study correlate with the patterns identified in the above studies. *In vivo,* the control heart rate at 18°C was 146 $\pm$ 39 BPM, whereas *in vitro* heart rates at 18°C were only 67 $\pm$ 19 BPM. I performed all of the experiments *in vivo* and *in vitro* within a three hour time period to avoid having compromised results because of the decline in the stability of the semi-isolated hearts, which was reported to occur after four hours. One possible reason for the isolated hearts to beat slower, is because the ion composition in the buffer contains magnesium. Magnesium concentrations in the blood have been linked directly to activity, and therefore also heart rate (Walters and Uglow 1981). However, this is not the explanation in our study, because magnesium concentration in the crabs' blood was carefully measured; results gave a concentration of 25.4 $\pm$ 2.6 mM. This amount was used in the buffer solution and it is in the same range as magnesium concentrations found in related species, e.g. *Carcinus maenus* - 16.2 mM, *Cancer pagurus* - 26 mM

(Tentori and Lockwood 1990), and *Cancer magister* - 32 mM (Terwilliger and Brown 1993). One more likely reason for the decrease in heart rate in semi-isolated hearts is the lack of circulating neurohormones, as explained in the introduction. Neurohormones play an important role in stimulating the heart. Another likely factor, is the decrease in stimulation of the heart directly from the central nervous system, since much of that has been destroyed in the isolation procedure. The only pacing mechanism that the isolated hearts still have completely intact is the cardiac ganglion (McGaw *et al.* 1995, Wilkens and Kuramoto 1998).

The second major difference in semi-isolated hearts, unpaced compared to *in vivo* hearts, is a change in the Q_{10}. Unlike heart rate this is not universally seen in all species. Initially the Q_{10} of the isolated hearts is above normal, at 5.5, but between 10°C and 30°C Q_{10} is below normal, at 1.2. Q_{10}s higher than normal have been observed in various species, when the temperature approaches extreme levels (reviewed by Cossins and Bowler 1987). However, the temperature range of 3.5°C – 7.8°C does not represent an extreme temperature for *Cancer irroratus.* Therefore this difference is likely caused by the isolation procedure. My study (above 10°C), and others, on various species have shown that there is little, if any, response in the heart rate to increasing temperatures, yielding Q_{10}s around 1 (DeWachter and Wilkens 1996, Jury and Watson 2000). DeWachter and Wilkens (1996) Dungeness crab study reported that the Q_{10} of semi-isolated hearts remained under 2, and it continued to decrease with increased temperature, to a Q_{10} of 1 at higher temperatures. Jury and Watson

(2000) reported results, similar to those seen in the isolated hearts of crabs, in the American lobster, *Homarus americanus.* DeWachter and Wilkens (1996) explained that in whole animals, stroke volume and cardiac output decrease, with increasing temperatures, and the initial increase in heart rate is the systems' way of compensating for the decrease in stroke volume and cardiac output. As the temperature continues to increase, some unknown extrinsic factors, e.g. neurohormones, continue to compensate by further increasing the heart rate. In semi-isolated hearts, the stroke volume and cardiac output still decrease in response to a temperature increase, and the heart rate still initially increases to compensate. However, in contrast, the heart rate of the semi-isolated hearts does not continue to increase, as in the whole animals, because the extrinsic factors discussed above are not available to the semi-isolated hearts, which is why they only show a small initial increase, and then remain constant throughout the temperature increase. The cardiac ganglion, the main pacemaker of the crustacean heart, seems to not be affected by temperature. The neurophsiological details are beyond the scope of the present study.

Recently Worden *et al.* (2006) reported that the Q_{10} of semi-isolated hearts in lobsters remains close to the same as the Q_{10} measured in whole animals, which ranged from 1.0 to 3.5. Crabs and lobsters are both decapod crustaceans, however they evolved from different lineages, and vary in some aspects of their physiology, e.g. Frederich *et al.* (2000) discussed differences in magnesium levels in the blood and the varying effects it has on the cardiac function of various systematic groups. There are a number of differences

between the two species circulatory systems that could be responsible for their different heart rates *in vivo*, but never the less, all the crab studies support my findings of a low Q_{10} in most isolated hearts.

These, largely different, heart rates of whole animals and semi isolated hearts made it difficult to conclude anything correlated to critical temperatures. Any difference in Tc could be attributed to different cardiac workloads. In order to determine if the whole animal and semi-isolated hearts really did have two different critical temperatures, I paced the isolated hearts to match the heart rate in the whole animals. I also used lactate data from the paced hearts to compare with the whole animals.

Lactate accumulation indicates the onset of anaerobic metabolism. Therefore, measuring lactate at various temperatures allows us to identify the temperature range at which the switch from aerobic to anaerobic metabolism occurs, in turn, identifying the critical temperature. In this study, lactate concentration was measured in the heart muscle, as oppose to the more common measurements taken from the blood. With failure of the heart at Tc, blood is not circulating anymore, and therefore, lactate buildup occurs in the tissues. This makes correlating our data with others difficult, because commonly lactate concentrations are taken from the blood, which contains much less lactate than the muscles themselves. Lactate still proved to be a reliable indicator, because, at the control temperature of 18℃, both the hearts of the whole animals and the isolated hearts had the same low concentration, implying that both were using aerobic metabolism, and therefore there wasn't any stress from

the isolation procedure that affected lactate concentrations. In the whole animals the lactate significantly increased between 25℃ and 30℃. Also, at 30℃, or death of the whole animal, there was a significant difference in lactate concentrations between the heart of the whole animal and both the paced and unpaced isolated hearts. The lactate in the isolated hearts had still not increased significantly from the concentration at 25℃ by 32℃, when the animals died.

At the end of this study the AMPK and HSP data is preliminary because time and methodological constraints limited the number of replicates that could be run. Due to low *n* no statistical tests can be done accurately. Therefore, no statements about the significance of the differences seen are made. However, my data, in conjunction with current publications, still gives us clues into the use of these parameters when measuring critical temperatures. The preliminary AMPK activity and HSP levels both showed an increase between 18℃ and 25℃ in the whole animals. These results indicate that at 25℃ the animal is stressed and attempting to compensate, by increasing ATP production, and decreasing ATP consumption, by increasing AMPK activity. The HSPs attempt to compensate by preventing denaturing and aggregation of proteins. An increase in AMPK activity due to temperature has been shown only in one vertebrate, the frog (Bartrons *et al.* 2004), but never before in an invertebrate. Further details, about AMPK activity, in invertebrates are necessary to determine if temperature induced hypoxia is the likely cause of an increase in AMPK activity in invertebrates. An increase in HSP levels has been shown in marine invertebrates, e.g. snails (Tomanek 2005). This study had similar results, an

increase, followed by a decrease of HSP70. The decrease in HSP levels in my study occurred between 25℃ and 30℃.

The preliminary data suggests that AMPK activity and HSP levels can be useful measures in determining the critical temperature of the whole animals. The HSP level data showed an increase at the same temperature as lactate, indicating that the T_{on}, described by Tomanek (2005), does correspond directly with Tc. The preliminary data for the isolated hearts seems to indicate that these parameters would not be useful in determining the Tc for isolated systems. It is possible that the stress of the isolation process causes unusual patterns of AMPK activity and HSP levels. Nevertheless, further experiments need to be done to utilize this data as part of my final results.

No other study, to my knowledge, has investigated critical temperatures of *Cancer irroratus,* therefore no direct comparison can be made to the critical temperature range I found, of 25-30℃. However, other studies have investigated critical temperatures in other marine invertebrates, and found critical temperatures ranging from 20-30℃; Van Dijk *et al.* 1999 (*Zoarces viviparous* and *Pachycara brachycephalum)*, Sommer *et al.* 1997 (*Arenicola marina*), Frederich and Portner 2000 (*Maja squinado*). The species with low Tcs were marine animals that live in the Antarctic and in the North Sea, therefore, it makes sense that their critical temperatures are lower than the rock crab, because they live in colder daily environments. The species at the top of that range are from the Mediterranean, and therefore have higher critical temperatures than the rock crab, because their daily environmental temperature range is higher. Other

studies have investigated changes of heart rate in response to temperature and observed drastic drops, but didn't go as far as to state a critical temperature. One such study by DeWachter and Wilkens (1996) investigated heart rate in *Cancer magister* and found a significant decrease after 20℃, supporting my data for *Cancer irroratus*.

Several studies have also investigated lower critical temperatures, (see table 1 in the introduction). It is important to note that *Cancer irroratus* also has a low critical temperature, which most likely can also be identified through lactate accumulation. However it is difficult to measure the determining parameters, because, with a decrease in temperature, there is also a decrease in metabolism, and all other parameters. Therefore, the actual changes in heart rate and lactate concentration are small, making identification of low the critical temperature very difficult. This study was more interested in the mechanism of the setting of critical temperatures, which is most likely the same at both the low and high Tc. Therefore, the responsible system for setting critical temperatures can be identified, by investigating just the high critical temperature of a species.

In that context, it is important to note that the temperature increase these animals were exposed, to within a two hour time period, is much faster than any temperature increase that occurs in their natural environment. In the normal ocean environment temperature increases occur slowly, allowing the animals time to acclimate to the temperatures and adjust their physiological functioning range accordingly. These animals are exposed to seasonal temperature changes and some larger daily temperature fluctuations if they move through different

temperature ranges on the ocean floor. The fast artificial temperature increase for this study was chosen because we were not, primarily interested, in physiological range of functioning, but instead, in pushing them past their functional range, to identify what physiological process is responsible for setting this range. This fast temperature increase might shift the actual Tc, but still will allows us to identify the involved mechanisms and systems. I did not allow the crabs time to make any adaptations.

In conclusion, my results indicate that the whole animals, and the isolated hearts, have different upper critical temperatures. The whole animals have an upper critical temperature between 25℃ and 30℃, this was indicated by the decrease in heart rate at 25℃, and the increase of lactate concentration in the heart between 25℃ and 30℃. The isolated hearts proved to have a higher upper critical temperature than the whole animals. At 30℃, the whole animals lactate concentration was significantly higher than both the paced isolated, and unpaced isolated hearts. The isolated hearts did not only survive past 30℃, but they beat steady until 32℃, where the heart rate dropped and was soon followed by death. The unpaced isolated hearts had no significant changes in the lactate concentrations at any of the temperatures including 32℃, indicating that a switch to anaerobiosis had not occurred. The paced isolated hearts showed significant increases in lactate concentration between 18℃ and both 30℃ and 32℃, and between 25℃ and 30℃. However, at 30℃ and 32℃, the lactate concentration was the same, and the increase in lactate between 25℃ and 32℃ was not significant. With the compiled data, of both the paced and unpaced isolated

hearts, it appears the critical temperature might be slightly above 32℃, which is as high as seven degrees above that of the whole animal.

Finally, because the circulatory system of these animals is complex, and because heart rate can not be directly correlated with all parameters, this study lacks enough evidence to state that the circulatory system is, or is not, the limiting physiological process of critical temperatures. However, this study can strongly conclude, that the heart muscle itself is not the responsible mechanism for setting the critical temperatures, in the rock crab. The critical temperature of the heart muscle is higher than that of the whole animal. This means, that the lack of sufficient oxygen, and the subsequent onset of anaerobic metabolism, is not caused by the heart muscle's lack of ability to pump the blood.

Other factors within the circulatory system, e.g. oxygen carrying capacity of the blood, arterial flow, etc… might be responsible for setting Tc. It is also possible that failure of a completely different system involved in oxygen intake and delivery, e.g. the ventilatory system, is responsible for setting critical temperatures. If the ventilatory system is responsible, it would prevent uptake of oxygen from the environment into the hemolymph. Therefore, the heart switches to anaerobiosis, in turn not delivering oxygenated hemolymph to all other tissues.

There is also evidence that AMPK activity does increase with temperature in *Cancer irroratus.* This could be an indicating parameter for Tc, but only for whole animals, not isolated hearts. The isolation procedure itself appears to cause an increase in AMPK activity. HSP levels seems to also be an indicative parameter of Tc in the whole animal, but not in the isolated heart. More

experimentation needs to be done to statistically demonstrated the preliminary AMPK activity and HSP level data.

As ectothermic animals, marine invertebrates are greatly affected by temperature changes in general. This is not only important with seasonal or daily changes, but also in the context of climate change and global warming. As this study has shown, marine invertebrates are able to tolerate a broad temperature range. However, the switch to anaerobiosis at Tc occurs within a very narrow temperature increment. This means, while one degree more or less within the physiological tolerance range has no major consequences, the same small temperature change, close to Tc, can lead directly to death of the animal. Those animals living close to the outer most limits of their physiological tolerance range will be most affected by temperature changes. Through this physiological mechanism, climate change and global warming can have a direct effect on the biogeography of marine invertebrates. *Cancer irroratus* can serve as a model species to investigate this physiological mechanism, which then can be applied to other, more commercially important species.

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