The Journal of Experimental Biology 212, 722-730
Published by The Company of Biologists 2009
doi:10.1242/jeb.021998

AMP-activated protein kinase (AMPK) in the rock crab, *Cancer irroratus*: an early indicator of temperature stress

Markus Frederich*, Michaela R. O'Rourke, Nathan B. Furey and Jennifer A. Jost
Department of Biological Sciences, University of New England, Biddeford, MA 04005, USA
*Author for correspondence (e-mail: mfrederich@une.edu)

Accepted 27 November 2008

SUMMARY

Exposure of marine invertebrates to high temperatures leads to a switch from aerobic to anaerobic metabolism, a drop in the cellular ATP concentration ([ATP]), and subsequent death. In mammals, AMP-activated protein kinase (AMPK) is a major regulator of cellular [ATP] and activates ATP-producing pathways, while inhibiting ATP-consuming pathways. We hypothesized that temperature stress in marine invertebrates activates AMPK to provide adequate concentrations of ATP at increased but sublethal temperatures and that AMPK consequently can serve as a stress indicator (similar to heat shock proteins, HSPs). We tested these hypotheses through two experiments with the rock crab, *Cancer irroratus*. First, crabs were exposed to a progressive temperature increase (6°C h^{-1}) from 12 to 30°C. AMPK activity, total AMPK protein and HSP70 levels, reaction time, heart rate and lactate accumulation were measured in hearts at 2°C increments. AMPK activity remained constant between 12 and 18°C, but increased up to 9.1(±1.5)-fold between 18 and 30°C. The crabs' reaction time also decreased above 18°C. By contrast, HSP70 (total and inducible) and total AMPK protein expression levels did not vary significantly over this temperature range. Second, crabs were exposed for up to 6 h to the sublethal temperature of 26°C. This prolonged exposure led to a constant elevation of AMPK activity and levels of HSP70 mRNA. AMPK mRNA continuously increased, indicating an additional response in gene expression. We conclude that AMPK is an earlier indicator of temperature stress in rock crabs than HSP70, especially during the initial response to high temperatures. We discuss the temperature-dependent increase in AMPK activity in the context of Shelford's law of tolerance. Specifically, we describe AMPK activity as a cellular marker that indicates a thermal threshold, called the pejus temperature, T_p. At T_p the animals leave their optimum range and enter a temperature range with a limited aerobic scope for exercise. This T_p is reached periodically during annual temperature fluctuations and has higher biological significance than earlier described critical temperatures, at which the animals switch to anaerobic metabolism and HSP expression is induced.

Key words: AMPK, HSP70, temperature stress, critical temperatures.

INTRODUCTION

Marine crustaceans are exposed to frequent seasonal and diel temperature changes. These temperature changes have a profound impact on ectothermal animals' energy metabolism and scope for exercise. Additionally, the potential impacts of global climate change on marine ecosystems emphasize the need to understand the effects of rising sea temperatures on marine invertebrates (for a review, see Osovitz and Hofmann, 2007). To predict how species will be affected by these temperature changes, we need both an indicator of heat stress and an understanding of the underlying mechanisms. Many physiological studies investigating temperature stress identify heat stress through the expression of heat shock proteins (HSPs) (e.g. Feder and Hofmann, 1999; Hoffmann et al., 2003; Tomanek, 2005). HSPs are ubiquitously expressed proteins that act as chaperones and aid in the conformational stabilization of other proteins (Feige et al., 1996; Frydman, 2001). Though HSPs were identified and named for their activation during temperature stress, many other stressors, including osmotic shock, hypoxia and exercise, induce HSP expression (for a review, see Hochachka and Somero, 2002). Named after their molecular weight, the HSP70 family includes constitutive (or cognate) HSP73, and stress-inducible HSP72 (Reading et al., 1989; Brown et al., 1993). Tomanek and Somero have shown that a heat shock response in snails is already detectable 1–2 h after heat stress and that the temperature at which HSP70 expression occurs can change after acclimation (Tomanek and Somero, 1999). Iwama acknowledges that HSPs are good indicators of general cellular stress, but points out that due to the variety of possible triggers of HSP expression, specific conclusions about HSP up- or down-regulation may not be valid (Iwama et al., 2004). The time lag between the actual heat stress and the detectable accumulation of HSPs raises the question of whether an additional, faster mechanism exists that would aid in withstanding thermal challenges.

In mammals, AMP-activated protein kinase (AMPK) has been described as a 'low fuel sensor' (Hardie and Carling, 1997) or a 'metabolic master switch' (Winder and Hardie, 1999), which reflects AMPK's central role in cellular energy metabolism. AMPK is thought to constantly monitor the energy status of the cell and, if needed, regulate the anabolic and catabolic processes to ensure a constant ATP concentration (Hardie et al., 2006). This regulation is achieved by AMPK through phosphorylation and subsequent activation or inhibition of rate-limiting enzymes of all the major energy metabolism pathways. By decelerating ATP-consuming pathways such as glycogen synthesis, fatty acid synthesis, protein synthesis and cholesterol synthesis, and in parallel accelerating ATP-producing pathways such as glucose uptake, glycolysis and fatty acid β-oxidation, the activation of AMPK prevents ATP depletion and promotes replenishment of the ATP pool (Fig. 1) (for a review, see Hardie et al., 2006; Hardie and Sakamoto, 2006).

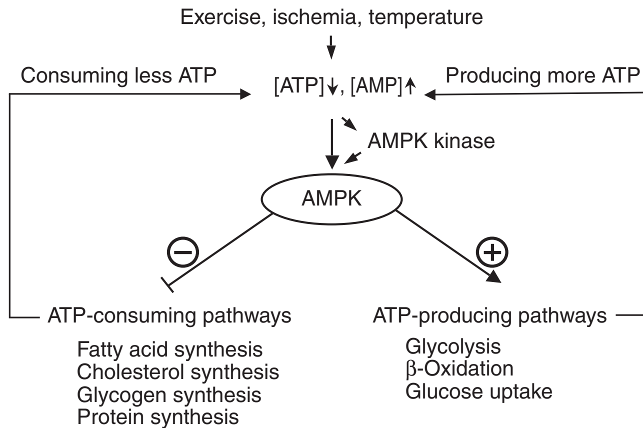

Fig. 1. Model of the AMP-activated protein kinase (AMPK) cascade. Stressors such as hypoxia, exercise or temperature lead to a decrease in cellular ATP and an increase in cellular AMP. This activates AMPK either directly, or indirectly through an upstream AMPK kinase. Once AMPK is activated, it phosphorylates multiple downstream targets, mainly rate-limiting enzymes of all energy metabolism pathways. The effect of this phosphorylation, in summary, leads to an acceleration of all ATP-producing pathways and a deceleration of all ATP-consuming pathways. Therefore, AMPK activation preserves the cellular ATP concentration.

The AMPK protein is a heterotrimer with a catalytic α-subunit and regulatory β- and γ-subunits. AMPK derives its main activation by phosphorylation of the α-subunit at threonine 172 (T172). AMP plays a major role in activating AMPK by four effects in parallel: (1) allosteric activation of AMPK kinase (AMPKK); (2) binding of AMP to AMPK, rendering it a poorer substrate for protein phosphatases; (3) binding of AMP to AMPK, making it a better substrate for the upstream kinase, AMPKK; and (4) allosteric activation of AMPK (Hardie et al., 1999). AMP is a good indicator of cellular stress because an increased ATP hydrolysis rate leads to a rapid accumulation of AMP in the cell. ATP hydrolysis first increases the cellular ADP concentration. The ADP is then converted by the adenylate kinase reaction (2 ADP→ATP+AMP) to ATP and AMP (Hardie et al., 2003). Therefore, during increased ATP use, AMP accumulates well before any changes in cellular ATP or ADP concentration occur. This is especially true for muscle tissues with the creatine phosphate or arginine phosphate system. These high energy phosphagens rapidly provide more ATP, so that the cellular ATP concentration remains constant despite a high ATP hydrolysis rate (Bessman, 1985). In a previous study on mice, we described AMPK activation under conditions of a raised AMP but constant ATP concentration (Frederich and Balschi, 2002).

Hypoxia, exercise and osmotic shock are known to activate AMPK in mammals through AMPKK activation and AMP accumulation. Cold stress has been shown to affect AMPK activity in frogs (Bartrons et al., 2004) and in the brown adipose tissue of mice (Mulligan et al., 2007); for a recent review on AMPK-activating factors see Hardie et al. (Hardie et al., 2006). However, AMPK activity and its regulation during heat stress, especially in invertebrates, have not yet been investigated thoroughly. Furthermore, most AMPK studies focus on vertebrates, especially mammals. Only a few studies have investigated AMPK in invertebrates in species such as the brine shrimp, *Artemia franciscana* (Zhu et al., 2007), and the fruit fly, *Drosophila melanogaster* (Lee et al., 2007).

AMPK is remarkably highly conserved during evolution with high sequence similarity between humans and other mammals [rat, mouse, rabbit, pig (Hardie et al., 1998)]. AMPK has also been described in the fruit fly, *D. melanogaster* (Pan and Hardie, 2002), and the nematode worm, *C. elegans* (Gao et al., 1995), as well as in plants such as cauliflower and tobacco (Kelner et al., 2004). We recently identified AMPK in the rock crab, *Cancer irroratus*, and demonstrated tissue-specific AMPK activation during hypoxia (Pinz et al., 2005). It is therefore likely that the AMPK cascade is a central mechanism for regulating energy metabolism found in most, if not all eukaryotes.

Marine invertebrates switch to anaerobiosis during heat stress (see Discussion). This anaerobic metabolism is characterized by a limited ATP yield, and an accumulation of AMP is expected due to the concomitant increase in metabolic rate. Because of the ubiquity of the AMPK cascade, we predicted that AMPK is activated during temperature stress. We tested this hypothesis in a marine decapod crustacean and compared AMPK activation with a more established marker for heat stress, the heat shock protein 70 (HSP70).

MATERIALS AND METHODS

Animals

Male rock crabs, *Cancer irroratus* (Say 1817), with an average carapace width of 101.9±9.7 mm (mean±s.d.) were obtained from a local lobster fisherman in Saco, ME, USA, kept in a flow-through seawater system in the Marine Science Center of the University of New England at 12–15°C, and fed squid and fish *ad libitum*.

Temperature incubations

Animals (five per experiment) were placed in a darkened 100 l tank at 12°C overnight. The next day animals were exposed to a fast progressive temperature increase (6°C h^{-1}) and killed at 12, 16, 18, 20, 22, 24, 26 or 28°C (30°C was also used in some trials). Animals were killed at the respective temperature with a cut through the cerebral ganglion and the heart was removed and either stored in RNAlater® solution (Ambion, Austin, TX, USA) or flash-frozen with Wollenberger tongs pre-cooled in liquid nitrogen. The flash frozen samples were stored at –80°C until analysis of AMPK activity and HSP70 protein levels.

In a second set of experiments, animals were exposed to the same progressive temperature increase up to the sublethal temperature of 26°C and temperature was then kept constant at 26°C. Animals (five per time point) were killed and tissue harvested as described above at 0, 1, 2, 4 and 6 h after reaching 26°C.

Reaction to experimental stimulation

To investigate the ability of the animals to respond to experimental stimulation at different temperatures, animals were subjected to the fast progressive temperature increase described above. At 12, 16, 18, 20, 22, 24, 26, 28 and 30°C the animals were turned upside down and placed on a flat surface underwater. The time (reaction time) to return to the upright position was monitored. Animals were counted as 'not responding' if they did not turn within 15 min.

Heart rate

To monitor the animals' heart rate during the temperature incubations, photoplethysmographs (iSiTEC, Bremerhaven, Germany) connected to a digital recording device (PowerLab, Mountain View, CA, USA) were glued to the carapace above the heart as described in detail by Depledge (Depledge, 1984) or Frederich and Pörtner (Frederich and Pörtner, 2000).

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

Target	Primer	Nucleotide sequence	References
AMPKα, degenerate	MO F1	5′-TTY ggN AAR gTN AAR gTN gg-3′	This study
	MO R6	5′-DAT NAC YTC Ngg NgC NgC RTA RTT-3′	
AMPKα, specific	AMPK F1	5′-TAT CCT CAA Tgg Tgg CTC gCT TCA-3′	This study
	AMPK R1/3	5′-TCC gCA AgA TTA AgT Cgg gTg TgT-3′	
AMPKγ, degenerate	MF F2	5′-AAY ggN gTN MgN gCN gCN CCN YTN Ugg-3′	This study
	MF R4	5′-YTC NgC NgC NAR RTT DAT NAC RTC RAA YTT-3′	
18S, specific	18S F2	5′-gCC gCA CgA gAT TgA gCA ATA ACA-3′	This study
	18S R1	5′-Agg ACA CgT TgA TCC CTT CAg TgT-3′	
HSP70, specific	MF-F41	5′-CAA gAg gCT TAT Tgg TAg g-3′	This study
	MF-R42	5′-gCT TgA CgC Tgg gAA T-3′	
HSP70, degenerate	F2	5′-gCN AA RAA YCA RgT NgC NAT gAA-3′	Voznesensky et al., 2004
	R2	5′-YTT YTC NgC RTC RTT NAC CAT-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.

Lactate

The lactate concentration in the heart tissue was measured using a photometric test according to Bergmeyer (Bergmeyer, 1985) to characterize the onset of anaerobiosis. Briefly, tissue was ground under liquid nitrogen and the frozen tissue powder transferred to 1.2 mol l^{-1} perchloric acid to precipitate the protein. After neutralization with 1 mol l^{-1} K_2HPO_4, the sample was centrifuged, and the lactate concentration was measured in the supernatant at 340 nm as NADH accumulation by the lactate dehydrogenase reaction. The lactate concentrations were normalized to the protein concentration in the extract and are presented as nanomoles per gram of protein (Bradford, 1976).

Western blots

Total HSP70 protein, inducible HSP70 protein, total AMPK protein and AMPK phosphorylation (i.e. AMPK activity) were quantified by western blots. Tissue samples were ground under liquid nitrogen and homogenized in a buffer containing phosphatase inhibitors to prevent dephosphorylation of AMPK (in mmol l^{-1}: Tris-HCl 20, NaCl 50, NaF 50, NaPPi 30, sucrose 250, $ZnCl_2$ 10, sodium vanadate 100, DTT 2, PMSF 50, pepstatin A 5 and leupeptin 5, with 0.4 mg ml^{-1} digitonin; pH 7.4 at 4°C). Proteins from the homogenate (50 µg) were separated on a 10% polyacrylamide/SDS gel at 180 V for 30 min. The proteins were then transferred to a nitrocellulose membrane at 70 V for 2 h and the membrane blocked with 3% non-fat dry milk and 0.1% gelatin. Primary rabbit anti-AMPK (total AMPK), anti-pT172 antibodies (Upstate, Lake Placid, NY, USA), mouse anti-HSP70 (Sigma, St Louis, MO, USA), anti-inducible HSP70 (Biovision, Mountain View, CA, USA) and secondary goat anti-rabbit or goat anti-mouse antibodies (Biorad, Hercules, CA, USA), respectively, were used to visualize the proteins. Mouse anti-actin antibodies (Sigma) were used as a loading control. The membranes were developed with a colorimetric kit (Biorad, Opti-4CN system). Scanned bands were quantified using Image-J software (NIH, Bethesda, MD, USA).

The AMPK activity, quantified as T172 phosphorylation, could also be interpreted as AMPKK activity rather than AMPK activity itself. Because AMPK has a multitude of downstream targets, the activity of each target (e.g. acetyl-CoA carboxylase, HMG CoA reductase, phosphofructokinase 2, protein synthase, and many others) could be used to measure AMPK activity. Each of these downstream targets, however, is regulated in its respective activity by other regulators as well and, therefore, does not reflect AMPK activity alone. Many studies, including one of ours (Frederich and Balschi, 2002), have shown that an increase in phosphorylated T172 is representative of the respective cellular effects of activated AMPK. These days, it is common to quantify AMPK activity as T172 phosphorylation.

We measured the heat shock response as both the total HSP70 and the inducible HSP70 to ensure that we did not miss any small upregulation of inducible HSP that might otherwise be masked by a constitutively expressed HSP in the cell.

Sequencing

To design degenerate primers, we searched GenBank for AMPKα and AMPKγ protein sequences from various invertebrate and vertebrate species. Obtained sequences were aligned using the MultAlin tool (http://bioinfo.genotoul.fr/multalin/multalin.html). Degenerate forward and reverse primers for PCR (see Table 1) were designed based on highly conserved areas in the aligned sequences. For HSP70, we used a primer pair that Voznesensky and colleagues (Voznesensky et al., 2004) prepared for the copepod, *Calanus finmarchicus*.

Total RNA from *C. irroratus* hepatopancreas was purified using the Total RNA Isolation System (Promega, Madison, WI, USA) and reverse transcribed (Super-Script First Strand Synthesis System, Invitrogen, Carlsbad, CA, USA). cDNA was amplified with the respective primer pairs *via* PCR with an annealing temperature of 45°C. DNA was sequenced at the Mount Desert Island Biological Laboratory (MDIBL, Salisbury Cove, ME, USA) sequencing core facility on an ABI 3100 sequencer. The DNA sequences obtained were converted to a predicted amino acid sequence using the NCBI open reading frame finder (http://www.ncbi.nlm.nih.gov), and a BLAST search confirmed the cDNA as AMPK or HSP70, respectively. These methods are described in detail by Towle and colleagues (Towle et al., 2001).

Quantitative real-time PCR

Specific primers for AMPK and HSP70 (see Table 1) were designed with the idtDNA primer design tool (www.idtDNA.com) based on the sequences obtained with the respective degenerate primers. Specific primers for 18S were designed based on Spears et al. (Spears et al., 1992) (GenBank accession no. M91050). Expression of AMPK and HSP70 mRNA was quantified in duplicate by quantitative real-time PCR using the Stratagene Brilliant SYBR Green qPCR Kit (Stratagene, La Jolla, CA, USA) on a Stratagene MX3005s instrument. After 40 cycles with an annealing temperature of 55°C, a melting curve analysis confirmed that only one DNA

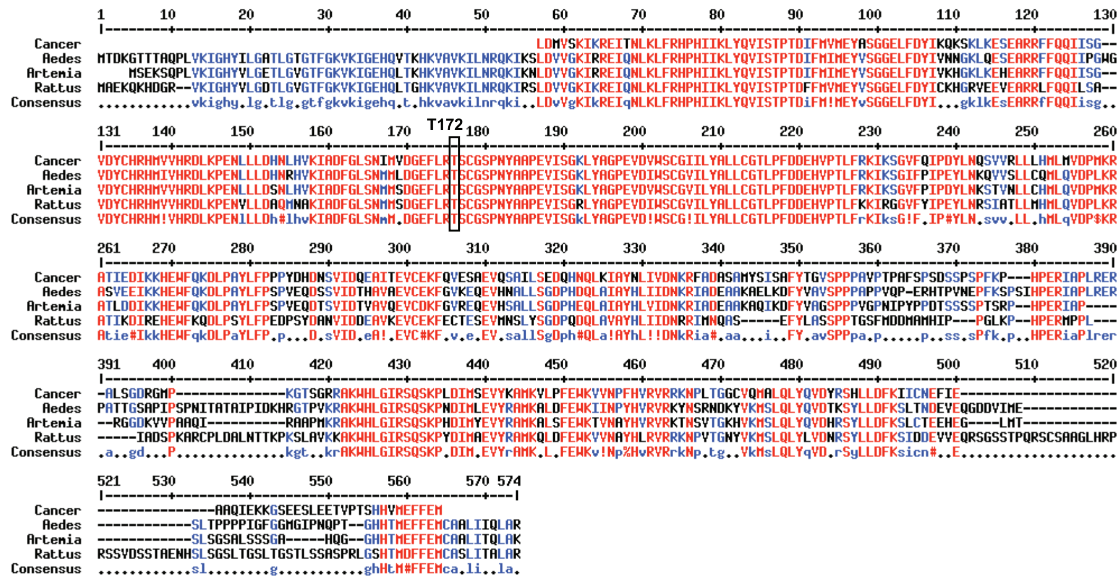

Fig. 2. Alignment of AMPKα amino acid sequences for vertebrate and invertebrate species. The sequence for *Cancer irroratus* is from this study. The remaining sequences were obtained from GenBank (*Aedes aegypti* AAX20150, *Artemia franciscana* ABI13783, *Rattus norvegicus* NM_023991). Sequence conservation is indicated as: black, no conservation; blue, some conservation; and red, complete conservation among the compared species. More than 60% of the obtained *Cancer irroratus* sequence (453 amino acids) is conserved in this comparison. A large region of conservation is found in the area flanking the T172 region that activates the AMPK protein. T172 lines up in this sequence comparison at position 176 because it was identified and named in the rat sequence, but shifts slightly when compared with other species.

product was amplified. The 18S gene was used as a reference gene and one sample (undiluted and diluted 1:10, 1:100, 1:1000) with high HSP70 mRNA expression served as an internal standard. AMPK and HSP70 mRNA levels are shown as relative increase above the AMPK or HSP70 mRNA level, respectively, for heart tissues at the control temperature of 12°C.

Statistics

Data were tested for significant difference by ANOVA and a Tukey *post-hoc* analysis or repeated measures ANOVA, dependent on the data set (GraphPad InStat; www.graphpad.com). $P<0.05$ was considered significant. Data are shown as means ± s.e.m.

RESULTS

AMPK sequences are highly conserved

The partial amino acid sequence for the AMPKα subunit for *C. irroratus* (GenBank submission no. FJ496868) confirmed the high conservation that has been reported for several other species (Hardie et al., 1998). More than 60% of the obtained *C. irroratus* sequence (453 amino acids) is conserved in comparison to other arthropods and vertebrates (Fig. 2). We could not determine whether the *C. irroratus* sequence more closely resembles the mammalian AMPKα1 or α2 subunit based on our partial sequence. However, more importantly, the region flanking the regulatory threonin 172 (T172) site where the AMPKα subunit is phosphorylated by an upstream AMPKK is highly conserved. The region flanking the T172 position for *C. irroratus* contains the amino acid sequence VDGEFL-R**pT**SCGSPNY, compared with rat SDGEFLR**pT**SCGSPNY. The antibodies used to quantify AMPK phosphorylation in *C. irroratus* (see below) were raised against the rat specific antigen KDGEFLR**pT**SCGSPNY. Except for the very first amino acid, rat and crab sequences are identical. The antigen used also varies in this first position. Exactly the same *Cancer irroratus* sequence of 15 amino acids flanking the T172 position was identified for *Carcinus maenas*, *Homarus americanus* and *Calanus finmarchicus* (data not shown). With this high sequence conservation of the peptide, the use of heterologous antibodies is not problematic. Similarly high conservation was observed in the 180 amino acid sequence of the AMPKγ subunit (GenBank submission no. FJ496867, 54% similar to mouse, NM_153745). As expected, the obtained HSP70 sequence of 178 amino acids (GenBank submission no. FJ496866) is highly conserved as well and shows more than 80% similarity with the mouse HSP70 sequence (AAC84170).

Effect of progressive temperature increase

Reaction to experimental stimulation

Between 12 and 18°C, the crabs needed 4.1±0.9 s to right themselves after being turned upside down (Fig. 3A). The crabs became slower at 20, 22 and 24°C (28.5, 58.9 and 46.2 s, respectively). With a high variability (between 1 and 280 s) this increase was not statistically significant. At 26°C, the average reaction time decreased to 20.3±9.8 s. However, 20% of the crabs did not return to the upright position (Fig. 3B) and are not included in this time average. At 28°C, 80% were not responding at all, and the remaining animals needed 157.7±20.6 s to react ($P<0.05$, repeated measures ANOVA). None of the animals were able to right themselves at 30°C.

Heart rate

During the progressive temperature increase, the heart rate increased, following a Q_{10} of 2.2 between 12 and 26°C, reaching a maximum of 153±27 beats min^{-1} at 26°C. Heart rate remained constant between 26 and 30°C, and then dropped at temperatures above 30°C (Fig. 3C).

Lactate

Lactate concentration in the heart remained constant at 7.3±2.6 nmol g^{-1} protein between 12 and 26°C, but increased

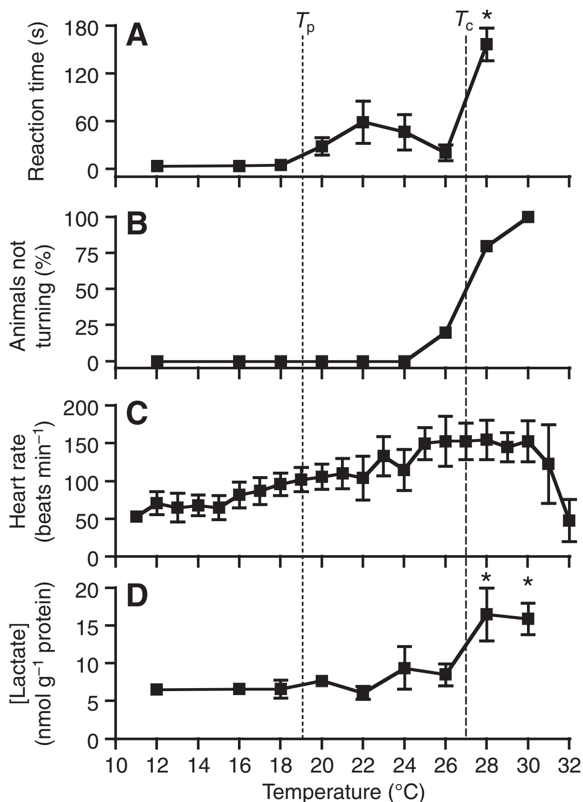

Fig. 3. (A) Reaction time after experimental stimulation in *Cancer irroratus* at increasing temperatures. Animals slowed down slightly in their response above 18°C, and became significantly slower at 28°C (N=15, *P<0.05, repeated measures ANOVA). The percentage of animals not responding at all is shown in B: all animals righted themselves between 12 and 24°C, no animal was able to turn at 30°C. (C) Heart rate of not experimentally stimulated *Cancer irroratus* increased with a Q_{10} of 2.2 between 12 and 26°C and leveled off at 153±27 beats min^{-1} at 26°C before decreasing again above 30°C (N=6). (D) Lactate concentration in the heart tissue increased significantly above 26°C (N=6, *P<0.05, ANOVA). The vertical dashed line indicates the critical temperature (T_c), the vertical dotted line indicates the pejus temperature (T_p).

significantly (P<0.05, ANOVA) 2.2-fold to 16.1±5.6 nmol g^{-1} protein above 26°C (Fig. 3D). The concordance between the lack of scope for exercise, the maximum heart rate (see above) and an increase in lactate accumulation indicates that the animals reached their critical temperature (T_c, see Discussion) between 26 and 28°C, as indicated by the dashed line in Fig. 3. However, a reduction in the scope for exercise is already evident between 18 and 20°C, indicated by the dotted line in Fig. 3 (T_p).

AMPK and HSP70

AMPK activity (western blot for T172 phosphorylation) did not differ between 12 and 18°C. AMPK activity started to increase above 18°C and reached a maximum at 28°C [9.9(±2.3)-fold, P<0.05, ANOVA; Fig. 4A]. The high variability of the data is consistent with the high variability of the scope for exercise of the individual animals (as shown by the reaction time data in Fig. 3A,B). To test for a discontinuity in the data, a Q-BASIC program to identify critical points (Yeager and Ultsch, 1989) was used. We identified two significantly different linear regressions (y=1.21–5.26E–03x, R^2=0.8659, P<0.05; y=–12.76+0.75x, R^2=0.8067, P<0.05). The two regressions intersect at 18.5°C. The increase in AMPK activity above 18°C coincides with the decrease in reaction time (Fig. 3A). AMPK protein (Fig. 4B) and AMPKα mRNA levels (Fig. 4C) showed the same small decreases and increases, which remained statistically insignificant (P>0.05, ANOVA) during the progressive temperature increase.

The increase of AMPK activity above 18°C occurred before any significant changes in HSP70 protein or mRNA levels were detected (Fig. 4D–F). A non-significant upward trend of inducible HSP70 protein and HSP70 mRNA can be seen at 28°C. This might indicate the onset of the heat shock response, which would coincide with the inability of most animals to respond after being turned on their backs (Fig. 3B) and the onset of anaerobic metabolism (Fig. 3D).

Effect of constant temperature stress

The fast, progressive temperature increase described above elicits a quick and immediate cellular response to thermal stress, as shown by the rapid phosphorylation of AMPK. To test whether AMPK and HSP70 are affected differentially during prolonged thermal stress, we exposed the animals for various periods of time to the sublethal temperature of 26°C. Exposure to 26°C for up to 6 h led to a constantly high heart rate above 150 beats min^{-1} (Fig. 5A). The lactate concentration in the heart peaked after 4 h at 23.4±8.5 nmol g^{-1} protein and remained above control levels (15.3±0.8 nmol g^{-1} protein) after 6 h (Fig. 5B). AMPK activity remained constant at the high level that was reached at 26°C (Fig. 5C). AMPKα mRNA levels increased continuously throughout exposure to 26°C, reaching 5.6±2.2 times more after 6 h at 26°C than in the 12°C controls, but reached statistical significance after 4 and 6 h, only at the P<0.1 level, ANOVA (Fig. 5D). HSP70 protein levels (total and inducible) remained constantly low with no significant changes (Fig. 5E,F). HSP70 mRNA levels rose constantly throughout the 6 h, up to 6.8(±1.7)-fold above control (Fig. 5G). Thus, after this prolonged exposure to high temperatures, both AMPKα mRNA and HSP70 mRNA increased. The HSP70 protein did not follow the same trend as HSP70 mRNA. Our experimental protocol most likely did not account for the time lag of protein synthesis from mRNA. However, this was not the intention of the experiment and, with the AMPK activity rising before either HSP70 protein or mRNA increased, was not followed further.

DISCUSSION

AMPK is the subject of active research in the medical community, which focuses on AMPK functions in homoeothermic mammals in response to cellular ischemia and metabolic stress. AMPK plays a role in obesity (Kola et al., 2008), heart failure (Hardie, 2008) and diabetes (Koh et al., 2008). In all of these diseases, the cellular ATP homeostasis is disturbed. Because AMPK is an important regulator of ATP homeostasis, it plays a major role in developing or actually treating the respective disease. However, little is known about AMPK in invertebrates and the effects of temperature on AMPK activity. As expected, we found a high sequence conservation for the AMPK protein in crustaceans. The sequence conservation across diverse phyla indicates an important function in cellular metabolism, and maintenance of cellular ATP concentration is probably the most important form of homeostasis. We have shown that AMPK is expressed in *C. irroratus*, that temperature stress leads to AMPK activation, and that this activation occurs well before the well-characterized heat shock response with HSP70.

It is likely that a deeper understanding of the physiological processes involved in withstanding temperature stress will allow for predictions of the potential impacts of temperature change on animals. Once stress markers have been identified, they can be used

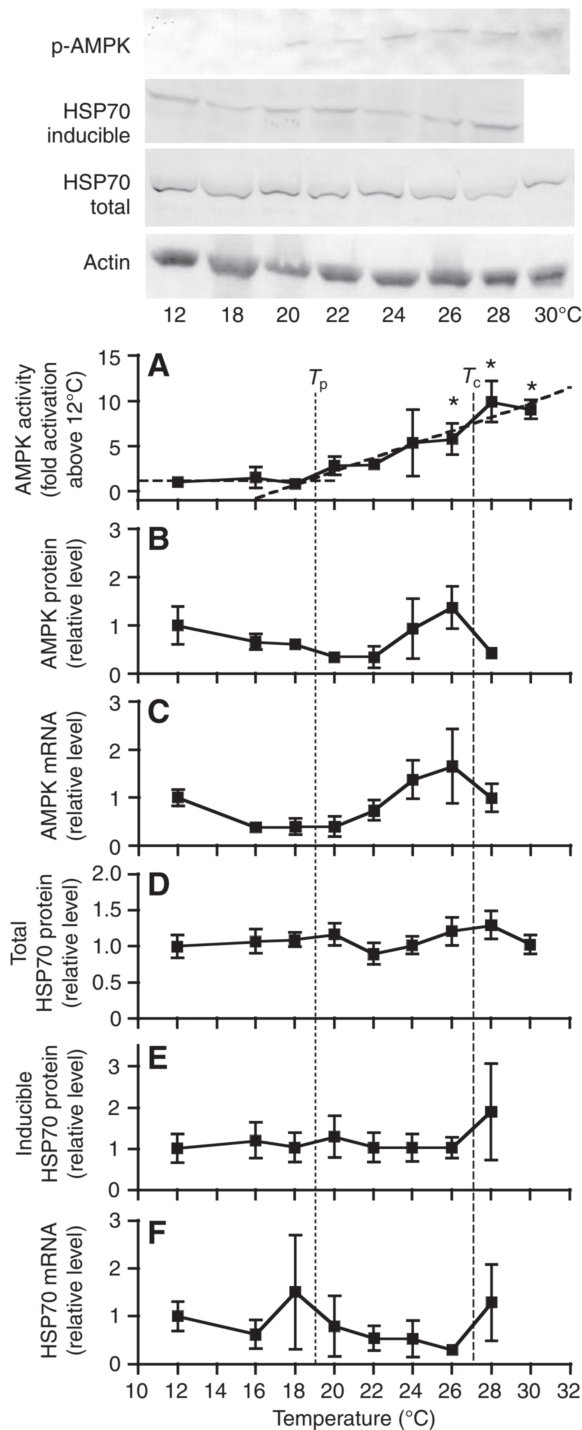

Fig. 4. Representative western blots and the respective quantification for phosphorylated and therefore activated AMPK (p-AMPK), heat shock protein 70 (HSP70 inducible and total) and the loading control actin, for heart tissue of *Cancer irroratus* at temperatures between 12 and 28 or 30°C during a fast and progressive temperature increase. (A) AMPK activity remained constant between 12 and 18°C, increased above 18°C and reached significance at 26°C. Two significantly different linear regressions (dashed lines, for equations see text) were fitted using a Q-BASIC program to identify critical points (Yeager and Ultsch, 1989). The two regressions intersect at 18.5°C. (B) Total AMPKα protein remained constant during the fast progressive temperature increase. (C) Total AMPKα mRNA remained constant during the fast progressive temperature increase. (D) Total HSP70 protein did not show any significant changes during the temperature stress. (E,F) Inducible HSP70 protein and mRNA did not show any significant changes during the temperature stress. However, the slight increase at 28°C might indicate the onset of the heat shock response. For all figures: error bars show ±1 s.e.m., N=4–6 per data point, *P<0.05 *vs* 12°C, ANOVA. The vertical dashed line indicates the critical temperature (T_c), the vertical dotted line indicates the pejus temperature (T_p).

to evaluate the stress level of the respective animal. Because extreme temperatures lead to anaerobiosis in the tissues, temperature tolerance and hypoxia tolerance are related. Therefore, elucidating the cellular mechanisms involved enhances our understanding of how common changes in the environment, such as temperature and oxygen concentration, will affect marine crustaceans' survival and their geographical distribution range. This is especially important in the context of global climate change or increasing hypoxic benthic areas (e.g. Diaz and Rosenberg, 2008).

We chose the rock crab, *C. irroratus*, as a model species because it can easily be obtained and maintained. It is one of the three major decapod species of economic importance in the Gulf of Maine (Palma et al., 1999), and the crab fishery is currently expanding, especially in Canada (Gendron et al., 2001). A decline in the rock crab population could also have a negative impact on the lobster fisheries in the Gulf of Maine because rock crabs play an important role in the diet of the American lobster, *Homarus americanus* (Gendron et al., 2001). A multitude of earlier studies have characterized the physiological response of decapod crustaceans to temperature stress. The current study builds on the wealth of existing knowledge and contributes to an enhanced understanding of the cellular and molecular processes affected by temperature stress. Preliminary data from an earlier study showed that hypoxia affects AMPK activity (Pinz et al., 2005), and long-term temperature stress affects AMPKγ mRNA expression differentially in different tissues of *C. irroratus* (Frederich et al., 2006). For the current, more comprehensive study, we chose to focus on the heart only, because the temperature-induced effects on heart rate are well described and can easily be monitored by a heart rate sensor. We are aware that heart rate is a sub-optimal measure of cardiac workload. However, for the purpose of this study, the temperature-induced increase in heart rate indicates increased performance and, therefore, increased energy demand of this organ.

As outlined in the Introduction, AMPK is phosphorylated and activated through AMPKK by a change in the cellular free AMP concentration. The AMP concentration does not change only in the context of temperature stress. Potentially every stress that affects cellular energy metabolism and ATP hydrolysis rates, such as exercise, hypoxia, salinity stress and many others, will lead to changes in cellular AMP and, consequently, affect AMPK activity. Preliminary data show that salinity stress affects AMPK mRNA expression and AMPK activity in salmon, *Salmo salar*, as well as AMPK activity in the green crab, *Carcinus maenas* (M.F. and J.A.J., unpublished observations). Jibb and Richards (Jibb and Richards, 2008) show in their recent study that 0.5 h of hypoxia in goldfish leads to a 5.5-fold increase in AMPK activity in the liver. Consequently, we expect further studies to show that AMPK, as a central regulator of cellular energy metabolism, is involved in many kinds of stress response. Furthermore, we expect a dual mechanism to achieve this regulation. First, a fast and immediate response through AMPK activation by phosphorylation of AMPKα at the T172 site. This provides increased AMPK activity within seconds. Second, a slower but longer lasting response through increased

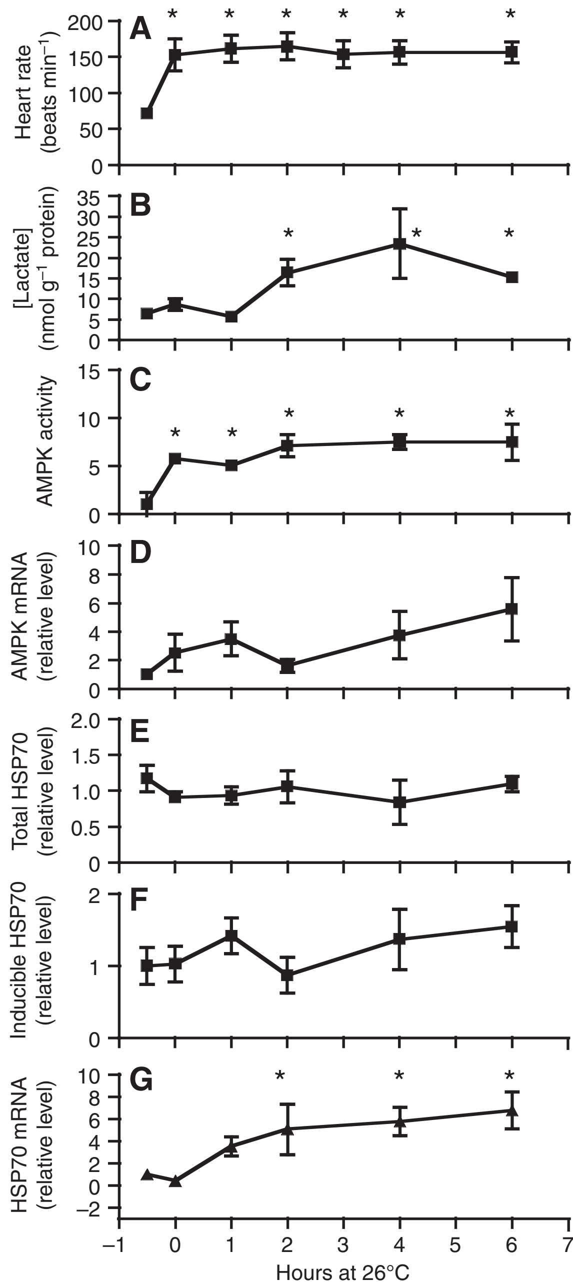

Fig. 5. (A) Keeping *Cancer irroratus* for 6 h at 26°C led to a constantly high heart rate of 160.9±11.9 beats min^{-1}. (B) Lactate in the heart peaked after 4 h and remained elevated at 6 h. (C) AMPK remained activated throughout the temperature stress. (D) AMPKα mRNA levels showed an upward trend over the 6 h but reached statistical significance only at the $P<0.1$ level (ANOVA). (E–G) HSP70 protein (total and inducible) remained constant while HSP70 mRNA levels increased slowly and reached significance at 2, 4 and 6 h. For all figures, the very first data point in each graph represents the value at 12°C for each respective parameter before the temperature increase. N=5–6 per data point, $^{*}P<0.05$ *vs* 12°C, ANOVA.

AMPK mRNA and consequently AMPK protein expression. This provides a long-term adjustment to varying energy demand. Both mechanisms are supported by our study. A third possibility is a differential expression of AMPK subunit isoforms. Whether invertebrates express the same set of isoforms as mammals (see Introduction) is currently not clear. We are aware of only one invertebrate study that claims to demonstrate two different AMPKα isoforms, in the brine shrimp, *Artemia franciscana* (Zhu et al., 2007).

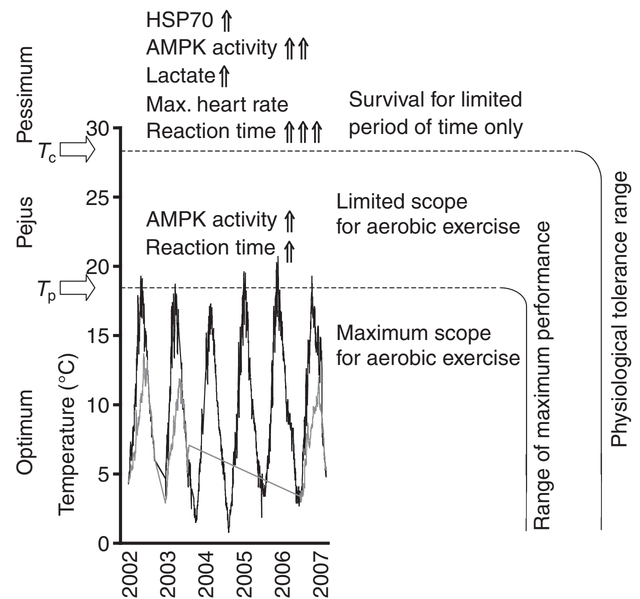

Fig. 6. Water temperatures in Casco Bay, where the crabs of this study were caught (buoy GoMOOS CO2 at a depth of 2 m, black, and 20 m, grey). Adapted from Shelford's law of tolerance and the adaptation by Frederich and Pörtner (Frederich and Pörtner, 2000); we indicate the optimum range with a maximum scope for exercise, limited by an upper pejus temperature, T_p. When the animals are exposed to temperatures above T_p they enter the pejus range with a limited scope for exercise and AMPK activity increases to ensure an adequate cellular ATP concentration. Further temperature increase leads to the critical temperature, T_c, indicated by the onset of anaerobic metabolism, lactate accumulation and HSP70 expression. Survival time in this pessimum range is limited. Therefore, the first measured marker for cellular stress through temperature is increased AMPK activity. For details see text.

AMPK and threshold temperatures

The activation of AMPK through heat stress can be viewed in the broader theoretical framework of Shelford's law of tolerance (Shelford, 1931) and critical temperatures. Shelford describes an optimum and a pessimum range for animals (and plants) for each respective environmental parameter. The ability to survive is obviously at a maximum in the optimum range and gradually decreases towards the upper and lower pessimum range. The following describes how physiological parameters can identify thresholds between those ranges and how AMPK activity can be used in a new way to characterize an additional range in this model.

Exposure of marine invertebrates to extremes of high and low temperature causes a mismatch of oxygen (O_2) demand and O_2 supply in the tissues of the animals, despite sufficient O_2 availability in the environment (Frederich and Pörtner, 2000). The thresholds for these effects have been defined in crustaceans and other invertebrates as the critical temperature (T_c) (Zielinski and Pörtner, 1996; Frederich and Pörtner, 2000; Pörtner, 2002). Critical temperatures are characterized by the onset of anaerobic metabolism and the subsequent accumulation of anaerobic end-products such as lactate, as well as the failing of ventilatory and circulatory activity (e.g. Zielinski and Pörtner, 1996; Frederich and Pörtner, 2000; Pörtner, 2002; Peck et al., 2002; Braby and Somero, 2006).

Prolonged exposure to temperatures above the upper T_c or below the lower T_c results in anaerobic metabolism that finally leads to death due to energy depletion (Frederich and Pörtner, 2000; Pörtner, 2002).

We detected an accumulation of lactate between 26 and 28°C in *C. irroratus*, concomitant with attaining the maximum heart rate. Inducible HSP70 protein, measured by western blot, as well as HSP70 mRNA, measured by quantitative real-time PCR, showed a small but statistically insignificant upward trend at this threshold. AMPK activity, however, was already well elevated at T_c, with the increase commencing between 18 and 20°C. The response time after experimental stimulation increased significantly above 26°C and coincided with lactate accumulation, maximum heart rate, AMPK activity and an upward trend in HSP. The critical temperature is therefore likely to occur between 26 and 28°C.

The threshold for the onset of increased AMPK activity coincides with the temperature at which the response time first slowed down (18°C). This initial slowing of response time was not statistically significant. However, it might be biologically significant for a crab in its environment because the ability to escape from a predator is crucial for survival. With a high variability among individuals, some animals will be affected by heat stress earlier and will be more vulnerable, as reflected in the higher standard error in response time as well as AMPK activity. The onset of increased AMPK activity coincides with the average maximum summer temperature in the area where the animals were caught as well. Records of the Gulf of Maine Ocean Observing System (www.gomoos.org) from 2002 to 2007 for Casco Bay (buoy CO2) at depths of 2 and 20 m show daily average temperatures peaking in July at approximately 19°C and hourly maximum temperatures of 20°C (Fig. 6). Animals used in the experiments were caught between late June and early September and experiments were performed in the fall. The animals therefore had a thermal history of maximum temperatures between 19 and 20°C. This is very close to the observed onset of AMPK activity between 18 and 20°C.

In an earlier study (Frederich and Pörtner, 2000), we identified critical temperatures in the spider crab, *Maja squinado,* and also an earlier threshold that we called 'pejus temperature, T_p' (pejus; latin for 'getting worse'). The upper and lower T_p encompass the range of maximum performance and coincide with the normal habitat temperature. Animals are exposed to temperatures in the pejus range, between T_p and T_c, only occasionally. A recent study by Pörtner and Knust (Pörtner and Knust, 2007) shows that eelpout in the North Sea are exposed briefly every summer to temperatures above their pejus temperature, but rarely to temperatures above their critical temperature. However, a reduction in growth rate and relative abundance was clearly correlated to temperatures in the pejus range, between T_p and T_c. Therefore, the pejus temperature represents an important threshold that describes the upper limit of regular function for an animal. Fig. 6 summarizes the optimum, pejus and pessimum range, as well as T_p, T_c and the respective cellular processes described in the present study. Habitat temperature data indicate why the cellular processes at the T_p are more often relevant for the animals' survival than the processes at T_c. Specifically, processes within the pejus range, such as AMPK activity and the subsequent increased ATP synthesis and reduced ATP use in anabolic pathways, help animals to save ATP.

The actual value for T_p in *C. irroratus*, here shown to be between 18 and 20°C, is likely to change with temperature adaptation. During the winter, when ambient water temperatures are around 3°C, *C. irroratus* cannot survive much longer than 24 h at 23°C (M.F., personal observation). While the actual temperatures for T_c and T_p shift with seasons and probably vary among populations, depending on specific thermal conditions, the mechanism is likely to remain the same. Animals are fairly tolerant to temperature variations within their range of optimum performance between the upper and the lower T_p. At the upper pejus temperature, coinciding with the average seasonal maximum temperature, AMPK activity increases to maintain an adequately high cellular ATP concentration. When animals are exposed to much higher temperatures, anaerobic metabolism sets in and survival is limited to a very short period of time. When exposed to temperatures between T_p and T_c for longer periods of time (in this study 6 h) the traditional heat shock response starts, as shown by the increase in HSP70 mRNA after several hours at 26°C (Fig. 5G).

Conclusion

In conclusion, to our knowledge this is the first study that specifically investigates the effects of heat stress on AMPK activity in any animal. We have shown that a fast, progressive temperature increase activates AMPK well before the heat shock response can be observed *via* HSP70. The temperature-related increase in AMPK activity coincides with a decrease in responsiveness and therefore might be part of a mechanism that has significant implications for the survival of the animals in their environment. We suggest that the AMPK cascade represents a cellular mechanism to conserve ATP, which allows the animals to endure short exposures to temperatures above the average maximum temperature of their habitat. AMPK therefore, may be a potential early cellular marker for heat stress in an animal.

LIST OF ABBREVIATIONS

AMPK	AMP-activated protein kinase
AMPKK	AMP-activated protein kinase kinase
HSP70	heat shock protein 70
T_c	critical temperature
T_p	pejus temperature

Supported by a New Investigator Award from the Maren Foundation at MDIBL, a Career Enhancement Award from the American Physiological Society, NSF IOB-0640478 to M.F., and a summer INBRE fellowship to M.R.O. The authors thank David Towle and Chris Smith from MDIBL for help with the quantitative real-time PCR methods and sequencing, Elizabeth Mitchell and Christina Bucicchia for technical assistance, Phil Yund for his help with the manuscript, and lobster boat captain Roger Collard for generously providing the animals. This is contribution number 18 from UNE's Marine Science Center.

REFERENCES

Bartrons, M., Ortega, E., Obach, M., Calvo, M. N., Navarro-Sabate, A. and Bartrons, R. (2004). Activation of AMP-dependent protein kinase by hypoxia and hypothermia in the liver of frog *Rana perezi. Cryobiology* **49**, 190-194.

Bergmeyer, H. U. (1985). *Methods for Enzymatic Analysis*, 3rd edn. Weinheim, Germany: Verlag Chemie.

Bessman, S. P. (1985). The creatine-creatine phosphate energy shuttle. *Annu. Rev. Biochem.* **54**, 831-862.

Braby, C. E. and Somero, G. N. (2006). Following the heart: temperature and salinity effects on heart rate in native and invasive species of blue mussels (genus *Mytilus*). *J. Exp. Biol.* **209**, 2554-2566.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248-254.

Brown, C. R., Martin, R. L., Hansen, W. J., Beckmann, R. P. and Welch, W. J. (1993). The constitutive and stress inducible forms of hsp 70 exhibit functional similarities and interact with one another in an ATP-dependent fashion. *J. Cell Biol.* **120**, 1101-1112.

Depledge, M. H. (1984). Photoplethysmography-A non-invasive technique for monitoring heart beat and ventilation rate in decapod crustaceans. *Comp. Biochem. Physiol. A* **77**, 369-371.

Diaz, R. J. and Rosenberg, R. (2008). Spreading dead zones and consequences for marine ecosystems. *Science* **321**, 926-929.

Feder, M. E. and Hofmann, G. E. (1999). Heat shock proteins, molecular chaperones, and the stress response: Evolutionary and ecological physiology. *Annu. Rev. Physiol.* **61**, 243-282.

Feige, U., Morimoto, R. I., Yahara, I. and Polla, B. S. (1996). *Stress Inducible Cellular Responses.* Berlin: Birkhäuser Verlag.

Frederich, M. and Balsch, J. A. (2002). The relationship between AMP-activated protein kinase activity and AMP concentration in the isolated perfused rat heart. *J. Biol. Chem.* **277**, 1928-1932.
Frederich, M. and Pörtner, H. O. (2000). Oxygen limitation of thermal tolerance defined by cardiac and ventilatory performance in spider crab *Maja squinado. Am. J. Physiol.* **279**, R1531-R1538.
Frederich, M., O'Rourke, M. and Towle, D. W. (2006). Is AMP activated protein kinase expression in *Cancer irroratus* a better signal for temperature stress then HSP70? *MDIBL Bull.* **45**, 37-39.
Frydman, J. (2001). Folding of newly translated proteins *in vivo*: the role of molecular chaperones. *Annu. Rev. Biochem.* **70**, 603-649.
Gao, G., Widmer, J., Stapleton, D., Teh, T., Cox, T., Kemp, B. E. and Witters, L. A. (1995). Catalytic subunits of the porcine and rat 5′-AMP-activated protein kinase are members of the SNF1 protein kinase family. *Biochim. Biophys. Acta* **1266**, 73-82.
Gendron, L., Fradette, P. and Godbout, G. (2001). The importance of rock crab, *Cancer irroratus*, for growth, condition and ovary development of adult American lobster, *Homarus americanus. J. Exp. Mar. Biol. Ecol.* **262**, 221-241.
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., Carling, D. and Carlson, M. (1998). The AMP-activated/SNF1 protein kinase subfamily: metabolic sensors of the eucaryotic cell? *Annu. Rev. Biochem.* **67**, 821-855.
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., Scott, J. W., Pan, D. A. and Hudson, E. R. (2003). Management of cellular energy by the AMP-activated protein kinase system. *FEBS Lett.* **546**, 113-120.
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.
Hochachka, P. W. and Somero, G. N. (2002). *Biochemical Adaptations, Mechanism and Process in Physiological Evolution.* New York: Oxford University Press.
Hoffmann, A. A., Sørensen, J. and Loeschke, V. (2003). Adaptation of *Drosophila* to temperature extremes: bringing together quantitative and molecular approaches. *J. Therm. Biol.* **28**, 175-216.
Iwama, G. K., Afonso, L. O. B., Todgham, A., Ackerman, P. and Nakano, K. (2004). Are hsps suitable for indicating stressed states in fish? *J. Exp. Biol.* **207**, 15-19.
Jibb, L. A. and Richards, J. G. (2008). AMP-activated protein kinase activity during metabolic rate depression in the hypoxic goldfish, *Carassius auratus*. *J. Exp. Biol.* **211**, 3111-3122.
Kelner, A., Pekala, I., Kaczanowski, S., Muszynska, G., Hardie, D. G. and Dobrowolska, G. (2004). Biochemical characterization of the tobacco 42-kD protein kinase activated by osmotic stress. *Plant Physiol.* **136**, 3255-3265.
Koh, H. J., Brandauer, J. and Goodyear, L. J. (2008). LKB1 and AMPK and the regulation of skeletal muscle metabolism. *Curr. Opin. Clin. Nutr. Metab. Care* **11**, 227-232.
Kola, B., Grossman, A. B. and Korbonits, M. (2008). The role of AMP-activated protein kinase in obesity. *Front. Horm. Res.* **36**, 198-211.
Lee, J. H., Koh, H., Kim, M., Kim, Y., Lee, S. Y., Karess, R. E., Lee, S. H., Shong, M., Kim, J. M., Kim, J. et al. (2007). Energy-dependent regulation of cell structure by AMP-activated protein kinase. *Nature* **447**, 1017-1021.
Mulligan, J. D., Gonzalez, A. A., Steward, A. M., Carey, H. V. and Saupe, K. W. (2007). Upregulation of AMPK during cold exposure occurs via distinct mechanisms in brown and white adipose tissue of the mouse. *J. Physiol.* **582**, 677-684.
Osovitz, C. J. and Hofmann, G. E. (2007). Marine macrophysiology: studying physiological variation across large spatial scales in marine systems. *Comp. Biochem. Physiol. A.* **147**, 821-827.
Palma, A. T., Steneck, R. S. and Wilson, C. J. (1999). Settlement-driven, multiscale demographic patterns of large benthic decapods in the Gulf of Maine. *J. Exp. Mar. Biol. Ecol.* **241**, 107-136.
Pan, D. A. and Hardie, D. G. (2002). A homologue of AMP-activated protein kinase in *Drosophila melanogaster* is sensitive to AMP and is activated by ATP depletion. *Biochem. J.* **367**, 179-186.
Peck, L. S., Pörtner, H. O. and Hardewig, I. (2002). Metabolic demand, oxygen supply, and critical temperatures in the antarctic bivalve *Laternula elliptica. Physiol. Biochem. Zool.* **75**, 123-133.
Pinz, I., Perry, D. and Frederich, M. (2005). Activation of 5′-AMP activated protein kinase during anaerobiosis in the rock crab, *Cancer irroratus. MDIBL Bull.* **44**, 31-32.
Pörtner, H. O. (2002). Physiological basis of temperature-dependent biogeography: trade-offs in muscle design and performance in polar ectotherms. *J. Exp. Biol.* **205**, 2217-2230.
Pörtner, H. O. and Knust, R. (2007). Climate change affects marine fishes through the oxygen limitation of thermal tolerance. *Science* **315**, 95-97.
Reading, D. S., Hallberg, R. L. and Myers, A. M. (1989). Characterization of the yeast HSP60 gene coding for a mitochondrial assembly factor. *Nature* **337**, 655-659.
Shelford, V. E. (1931). Some concepts of bioecology. *Ecology* **12**, 455-467.
Spears, T., Abele, L. G. and Kim, W. (1992). The monophyly of brachyuran crabs: a phylogenetic study based on 18S rRNA and rDNA sequences. *Syst. Biol.* **41**, 446-461.
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. and Somero, G. N. (1999). Evolutionary and acclimation induced variation in the heat-shock responses of congeneric marine snails (genus *Tegula*) from different thermal habitats: Implications for limits of thermotolerance and biogeography. *J. Exp. Biol.* **202**, 2925-2936.
Towle, D. W., Paulsen, R. S., Weihrauch, D., Kordylewski, M., Salvador, C., Lignot, J. H. and Spanings-Pierrot, C. (2001). Na^+-K^+-ATPase in gills of the blue crab *Callinectes sapidus*: cDNA sequencing and salinity-related expression of a-subunit mRNA and protein. *J. Exp. Biol.* **204**, 4005-4012.
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.
Winder, W. W. and Hardie, D. G. (1999). AMP-activated protein kinase, a metabolic master switch: possible roles in type 2 diabetes. *Am. J. Physiol.* **277**, E1-E10.
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, X. J., Feng, C. Z., Dai, Z. M., Zhang, R. C. and Yang, W. J. (2007). AMPK alpha subunit gene characterization in *Artemia* and expression during development and in response to stress. *Stress* **10**, 53-63.
Zielinski, S. and Pörtner, H. O. (1996). Energy metabolism and ATP free-energy change of the intertidal worm, *Sipunculus nudus*, below a critical temperature. *J. Comp. Physiol.* **166B**, 492-500.