Is AMP activated protein kinase expression in *Cancer irroratus* a better signal for temperature stress then HSP70?

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Invertebrates of the intertidal zone and coastal waters are often exposed to rapid temperature fluctuations. Exposure to too high or too low temperatures leads to onset of anaerobic metabolism despite sufficient oxygen in the environment [7]. This mismatch of oxygen demand and oxygen supply in the tissues leads to depletion of cellular ATP and finally to death of the animals. A central regulator of cellular ATP concentrations and energy metabolism in mammals is 5'AMP-activated protein kinase (AMPK). AMPK has been described as a "metabolic master switch" that preserves ATP during stress by accelerating ATP producing pathways (glycolysis, fatty acid ß-oxidation, glucose uptake) and decelerating ATP consuming pathways (cholesterol-, protein-, fatty acid synthesis) (for review see [5, 12]). AMPK is highly conserved during evolution and was shown in invertebrate species such as *Drosophila melanogaster* [6], *Caenorhabditis elegans* [2], as well as in plants such as cauliflower and tobacco [4]. However, little is known about the effect of temperature on AMPK in general, and specifically in invertebrates. We tested the hypothesis that temperature stress increases AMPK mRNA levels in a decapod crustacean, and compared this increase to the temperature effect on heat shock protein (HSP70) expression.

To design degenerate primers AMPK gamma 2 subunit sequence data from several arthropods and vertebrates (*Apis*, accession number XP_395185, *Drosophila* EAL28803, *Anopheles* EAA01730, *Xenopus* AAH60444, *Mus* NP_663376, *Callinectes* CO897275) were aligned using the MultiAlign tool (http://prodes.toulouse.inra.fr/multalin/multalin.html) and degenerate forward and reverse primers MF-F2 5' AAY GGN GTN MGN GCN GCN CCN YTN UGG 3', and MF-R4 5' YTC NGC NGC NAR RTT DAT NAC RTC RAA YTT 3' were designed based on highly conserved regions. RNA was extracted from *Cancer irroratus* heart, claw muscle, and hepatopancreas using the RNAgents© Total RNA Isolation System (Promega) and quantified with an Agilent 2100 Bioanalyzer. Two micrograms of RNA were reverse transcribed into cDNA using the Superscript II first strand kit (Invitrogen). These methods are described in detail by Towle et al [10]. PCR with the degenerate primers and sequencing of the PCR products at the MDIBL sequencing core facility revealed a partial cDNA sequence for AMPK gamma 2 in *Cancer irroratus* (Figure 1). Specific primers (MF-F11 5' ATC ACT GAC TTT ATC CGC ATT 3', MF-R71 5' TAG GAT GGA GGG CTT AGG AA 3') for quantitative real-time PCR were designed based on this sequence using NetPrimer software. The same approach was used to identify an HSP70 sequence in *Cancer irroratus*. The specific primers MF-F41 5' CAA GAG GCT TAT TGG TAG G 3' and MF-R44 5' TTG TCA AGA CCG TAG GAG 3' were based on the sequence shown below (Figure 1) that was obtained using degenerate primers designed for copepods and published by Voznesensky et al [11] (F2: 5' GCN AAR AAY CAR GTN GCN ATG AA 3'and R2: 5' YTT YTC NGC RTC RTT NAC CAT 3').

Rock crabs, *Cancer irroratus*, were incubated for two days at 11, 24 or 27°C before sampling tissue from heart, hepatopancreas and claw muscle. RNA was extracted and reverse transcribed into cDNA as described above. Quantitative real-time PCR (Stratagene Brilliant SYBR Green QPCR Kit,

Stratagene, La Jolla, CA) measured relative mRNA expression levels for AMPK and HSP70.

Figure 1
Partial cDNA sequence of the AMPK gamma 2 subunit and HSP70 for *Cancer irroratus*:

AMPK gamma 2

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   1   G CCT TCT TTG CGC TGG TGT ACA ATG GTG TCC GCG CAG CCC CAC TCT GGG ACT CTG GGC   58
   1     P  S  L  R  W  C  T  M  V  S  A  Q  P  H  S  G  T  L  G                    19
  59 GGG CGC AGT TTC ATC GGA ATG CTT ACC ATC ACT GAC TTT ATC CGC ATT TTA CAA AAT TTC  118
  20   G  R  S  F  I  G  M  L  T  I  T  D  F  I  R  I  L  Q  N  F                    39
 119 TAC AAC TCT CCC AAC CGC AAG ATG GAA GAA TTG GAA GAT CAC AGA CTG GAG ACT TGG AGA  178
  40   Y  N  S  P  N  R  K  M  E  E  L  E  D  H  R  L  E  T  W  R                    59
 179 ACC GTG TTG AAG GAT GAG GCG CGT CCC CTG ATC AGC ATC CGG CCT GAT GAG TCT CTA TAC  238
  60   T  V  L  K  D  E  A  R  P  L  I  S  I  R  P  D  E  S  L  Y                    79
 239 GTT GCC ATC CGC TCA CTC ATC CAC CAC AAG ATT CAT CGT TTA CCC GTC ATT GAC CCG GCC  298
  80   V  A  I  R  S  L  I  H  H  K  I  H  R  L  P  V  I  D  P  A                    99
 299 ACA GGA AAT GTA CTT TAT ATT GTC ACT CAC AAA AGG ATT CTC AAG TTC CTC TAT CTC TAT  358
 100   T  G  N  V  L  Y  I  V  T  H  K  R  I  L  K  F  L  Y  L  Y                   119
 359 ATC AAT GAG CTT CCT AAG CCC TCC ATC CTA CAC AAG TCT CTC AAG GAC ATG GAC ATC GGA  418
 120   I  N  E  L  P  K  P  S  I  L  H  K  S  L  K  D  M  D  I  G                   139
 419 ACT TAC AAC AAC ATC GAG ACA GCC CGT GAA GAC ACT CTC ATT ATA GAA GCG CTT AAT AAG  478
 140   T  Y  N  N  I  E  T  A  R  E  D  T  L  I  I  E  A  L  N  K                   159
 479 TTT GTG GAA CGT AGG ATC TCA GCG CTG CCT ATT GTT GAT GCA GAT GGC AAG CTG GTG GAT  538
 160   F  V  E  R  R  I  S  A  L  P  I  V  D  A  D  G  K  L  V  D                   179
 539 ATC TAC GCC AAA TTC GAN GTT AT                                                  561
 180   I  Y  A  K  F  X  V
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HSP70

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   1  GG TTC GAT GCC AAG AGG CTT ATT GGT AGG ANG TTT AAT GAC CAC AAT GTG CAG TCT GAC   59
   1     F  D  A  K  R  L  I  G  R  X  F  N  D  H  N  V  Q  S  D                    19
  60 ATG AAG CAC TGG CCN TTC GAG GTC TTT GAT GAT AGC NCA AAG CCN AGG NTC AGG GTG GAN  119
  20  M  K  H  W  P  F  E  V  F  D  D  S  X  K  P  R  X  R  V  X                    39
 120 TAC AAG GGA GAA AAA AAG TCT TTC TAC CCT GAG GAG ATC TCT TCC ATG GTG CTC ANT AAG  179
  40   Y  K  G  E  K  K  S  F  Y  P  E  E  I  S  S  M  V  L  X  K                   59
 180 ATG AAG GAA ACT GCN GAA GCA TAC CTT GGT GCC GAA GTA AAG GAT GCT GTC ATC ACT GTC  239
  60  M  K  E  T  A  E  A  Y  L  G  A  E  V  K  D  A  V  I  T  V                    79
 240 CCA NCT TAC TTT AAC GAT TCC CAG CGT CAA GCC NCC AAA GAC GCA NGC ACN NTC TCC NGT  299
  80  P  X  Y  F  N  D  S  Q  R  Q  A  X  K  D  A  X  T  X  S  X                    99
 300 GTC AAT GTG CTG CGT ATC ATT AAT GAA CCC ACC GCT GCC GCC NTC TCC TAC NGT CTT GAC  359
 100  V  N  V  L  R  I  I  N  E  P  T  A  A  A  X  S  Y  X  L  D                   119
 360 AAG AAA GTG GGT GGT GAG CGC AAT GTG CTC ATC TTC GAT CTT GGC GGT GGG ACC TTC AAT  419
 120  K  K  V  G  G  E  R  N  V  L  I  F  D  L  G  G  G  T  F  N                   139
 420 GTA TCC ATC CTG ACC ATC GAG GAT GGC ATC TTT GAG GTG AAG TCA ACT GCA NGA GAC ACT  479
 140   V  S  I  L  T  I  E  D  G  I  F  E  V  K  S  T  A  X  D  T                   159
 480 CAT TTG GGT GGA GAA GAC TTC GAC NAC NGA ATG GTA AAC CAC TTC CAT CAT GAA TTC      536
 160  H  L  G  G  E  D  F  D  X  X  M  V  N  H  F  H  H  E  F                      178
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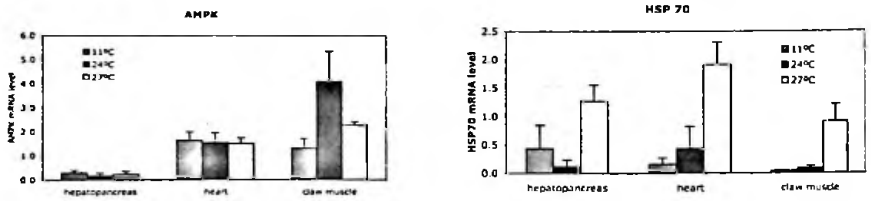
Temperature had no effect on AMPK mRNA expression in hepatopancreas and heart but expression was 7.2 fold higher in the heart at all temperatures. AMPK expression in the claw muscle at 11°C was similar to the heart, but increased significantly 3.1 fold at 24°C. An insignificant 1.7 fold increase was observed at 27°C. HSP70 expression remained low in all three tissues at 11 and 24°C but increased significantly at 27°C (4.6, 6.6 and 14.5 fold, heart, hepatopancreas, claw muscle, respectively) (Figure 2).

Figure 2:
mRNA expression of AMPK and HSP70 in hepatopancreas, heart and claw muscle of *Cancer irroratus* after incubation at 11, 24 and 27°C for 2 days.



To our knowledge these are the first data that show an influence of temperature on AMPK expression in an invertebrate. The increase in AMPK expression in the claw muscle at 24°C could be interpreted as an adaptation to a higher energy demand at higher temperatures. It is not yet clear why this increase is not observed in the heart or the hepatopancreas. Tissue specific responses of AMPK activity to other stresses have been reported [8] and highlight the complexity of the regulation of energy metabolism by AMPK. The lack of any AMPK response at 27°C was unexpected. However, exposure of *Cancer irroratus* to 27°C for longer than 2 days resulted in death of the animal. Therefore, these animals were already very close to dying and probably not representative for a stress that these animals are adapted for.

Heat shock proteins with a molecular size around 70kDa (HSP70) are often used as a general stress indicator. Various studies compare the heat shock response between closely related species and draw conclusions on respective temperature tolerance [9]. Iwama et al [3] acknowledges that HSPs are good indicators for general cellular stress, but points out that due to the variety of possible triggers of HSP expression specific conclusions might not be drawn from HSP up- or down regulation. Our data show that only very high, sub-lethal temperatures lead to HSP70 expression in *Cancer irroratus*. In contrast to the AMPK response, HSP70 is upregulated in all three investigated tissues. However, the energetic stress inflicted by high temperatures in the claw muscle leads to an increase in AMPK expression at a lower temperature than the general stress response observed for HSP70. Thus, for claw muscle, AMPK expression seems to be a better indicator of cellular stress than HSP70. Future experiments will elucidate this differential response after shorter temperature stress to investigate the time course in more detail.

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