© Indian Academy of Sciences

RESEARCH ARTICLE

CrossMark

Adenosine monophosphate-activated protein kinase from the mud crab, *Scylla paramamosain*: cDNA cloning and profiles under cold stress

CHENCUI HUANG[1], KUN YU[1], HUIYANG HUANG[1,2] and HAIHUI YE[1,2]*

[1] *College of Ocean and Earth Sciences, Xiamen University, Xiamen 361102, People's Republic of China*
[2] *Collaborative Innovation Center for Development and Utilization of Marine Biological Resources, Xiamen 361102, People's Republic of China*

Abstract

Adenosine monophosphate-activated protein kinase (AMPK), an important energy sensor, is crucial for organism survival under adverse conditions. In this study, the roles of this gene under cold stress in a warm-water mud crab, *Scylla paramamosain* was investigated. The full-length cDNA (*SpAMPK*) was 1884 bp and its open reading frame of 1566 bp was isolated and characterized. The expressions of *SpAMPK* detected by quantitative real-time PCR (qRT-PCR) in various tissues revealed that the highest expression was in the hepatopancreas. The profiles of *SpAMPK* gene in the hepatopancreas, chela muscle and gill were detected when the subadult crabs were exposed to the four temperature conditions of 10, 15, 20 and 25°C. The results showed that the expression patterns of *SpAMPK* mRNA in the three tissues were significantly higher when crabs were exposed to 15°C than the other three temperature treatments, while at 10°C treatment, the *SpAMPK* mRNA was lowest among the four temperature treatments. These findings suggested that the high expression of *SpAMPK* mRNA might initiate ATP-producing pathways to generate energy to cope with cold stress at 15°C treatment, which was slightly below the range of optimum temperatures; while treatment at 10°C, far lower than optima, the low expression of *SpAMPK* mRNA could reduce the energy expenditure and thus induce the crabs into cold anesthesia. The results of *SpAMPK* in this study might contribute to the understanding of the molecular mechanism of acclimation to cold hardiness in *S. paramamosain*.

[Huang C., Yu K., Huang H. and Ye H. 2016 Adenosine monophosphate-activated protein kinase from the mud crab, *Scylla paramamosain*: cDNA cloning and profiles under cold stress. *J. Genet.* **95**, 923–932]

Introduction

For poikilotherms, such as crustaceans, one of environmentally relevant stressors is temperature change, which has been discovered to impact on multiple physiological processes, such as growth, development and reproduction (Aguilar-Alberola and Mesquita-Joanes 2014; Thongda *et al.* 2015), and even survival (Hochachka and Somero 2002). Especially, cold temperature can lead to the destruction of tissues. For example, cold stress could cause damnification to the balance of oxidation–antioxidant in tissues, and bring about the oxidation damage of DNA (Jia *et al.* 2009). Moreover, studies have shown that the stress associated with cold temperature affects the inflammatory response, neuroendocrine regulation and energy metabolism (Hangalapura *et al.* 2004; van den Brand *et al.* 2010).

When exposed to cold stress, poikilotherms initiate adaptive tactics, such as mechanisms to compensation for energy (Hochachka and Somero 2002). Other protective mechanisms, such as antioxidant defense, heat shock response and an increase in mitochondrial density are also important for organisms to maintain cellular homeostasis at low temperatures (Wang *et al.* 2007; Kong *et al.* 2008; De Gobba *et al.* 2014; Zhang *et al.* 2014). It is well known that cold stress would lead to disturbance of aerobic metabolism and reduction of ATP (Hochachka and Somero 2002). Therefore, some animals have to initiate anaerobic metabolism to meet part of energy needs (Costanzo *et al.* 2004; Colson-Proch *et al.* 2009).

Adenosine monophosphate-activated protein kinase (AMPK), a highly conserved serine/threonine kinase, plays a pivotal role in regulating cellular energy metabolism (Hardie 2004). ATP-generating catabolic pathways, such as oxidations of amino acid, fatty acid and glycolysis are

*For correspondence. E-mail: haihuiye@xmu.edu.cn.

Keywords. *SpAMPK* gene; cold stress; mRNA transcripts; *Scylla paramamosain*.

switched on by AMPK. Synchronously, AMPK shuts off ATP-consuming anabolic processes, such as the synthesis of lipids, proteins and glycogen (Hardie and Sakamoto 2006). Due to its function in energy metabolism regulations, AMPK has become an increasingly important research object in thermal stress, including high and cold temperature stresses. In vertebrates, such as fish (Olsvik *et al.* 2013), common frog (Bartrons *et al.* 2004), chicken (Zhang *et al.* 2014), rat (Hardie and Sakamoto 2006), mouse (Mulligan *et al.* 2007) and pig (Faure *et al.* 2013), the expressions of AMPK were upregulated during heat or cold exposure. In invertebrates, the effects of heat temperature stress on the expression of AMPK have been carried out, in the brine shrimp, *Artemia franciscana* (Zhu *et al.* 2007); rock crab, *Cancer irroratus* (Frederich *et al.* 2009); intertidal limpet, *Cellana toreuma* (Han *et al.* 2013). However, the effects of cold temperature stress on the expression of AMPK in invertebrates are still indefinite.

The mud crab, *Scylla paramamosain* is widely distributed in intertidal and subtidal unstructured soft sediments in the coastal area of southeast China, and has been a commercially important species in aquaculture (Le Vay *et al.* 2008; Ye *et al.* 2011). Although it can survive between 7 and 37°C, the optimum temperature for its growth and development are approximately 18–30°C (Shelley and Lovatelli 2012). It was reported that the biological zero of *S. paramamosain* is 12.19°C, and the embryos cannot complete its development below this temperature (Hamasaki 2002). Cold temperatures in winter generally brings about large-scale mortality in this species, in aquaculture. In consideration of its important economic value, the effects of cold temperatures on diversely physiological adaptations for sustaining metabolism in mud crab have been valued increasingly (Kong *et al.* 2012; Yu *et al.* 2014b).

In the present study, the full-length cDNA of *S. paramamosain AMPK* (*SpAMPK*) was identified and characterized. The effects of cold temperature on *SpAMPK* in the three tissues of subadult crabs were detected by quantitative real-time PCR (qRT-PCR) and revealed the potential of *SpAMPK* to be applied as a biomarker for stress responses in *S. paramamosain*.

Material and methods

Experimental animals

The experimental, *S. paramamosain* were obtained from a commercial source located in Xiang'an, Xiamen, Fujian, China. Three female adult crabs with carapace length of 8.5 ± 0.6 cm and body weight of 370 ± 35 g were selected for the acquisition of full-length *SpAMPK* and analysis of tissue distribution. Besides, 105 subadult crabs, averaging 5.1 ± 0.7 cm in carapace length and 90 ± 8 g in body weight were chosen for cold stress experiments. All crabs in this study were healthy with both claws and appendages intact. Soon after the crabs were transported to the laboratory, they were acclimated to common aquaculture conditions (salinity 25, 28 ± 0.5°C) for two days, during which the crabs were fed with live clam, *Ruditapes philippinarum*.

Low temperature stress and sample preparation

We assigned five temperature regimes 5, 10, 15, 20 and 25°C, by using five temperature-controlled incubators. Subadult crabs were randomly placed into these incubators. Each crab was placed in an individual round plastic culture vessel (diameter 10 cm; height 12 cm). Filled with about one third of volume of filtering seawater (salinity 25), each vessel was kept in the controlled temperature for 24 h to achieve the desire levels, respectively. After that, the subadult crabs were transferred into the vessels at random. At 0, 1, 3, 6, 12, 24 and 48 h of cold exposure, for each cold exposure, three crabs were selected, respectively. The hepatopancreas, chela muscle and gill were dissected from each crab and frozen immediately in liquid nitrogen and stored in −80°C.

Nucleic acid extraction and cDNA synthesis

Total RNA was isolated from the samples of *S. paramamosain* by using RNAizol reagent (Invitrogen, Carlsbad, USA) based on the manufacturer's protocol. The RNA integrity and concentration were detected by 1.5% agarose gel electrophoresis and by using a NanoDrop 2000 spectrophotometer (NanoDrop Technologies, Wilmington, USA), respectively.

For first-strand cDNA synthesis, 2 μg total RNA was treated with RNase-free DNase I (TaKaRa, Kyoto, Japan) to remove genomic DNA, then reversely transcribed by using the Revert Aid^{TM}. First-strand cDNA synthesis kit (Fermentas) and random primers were used for cDNA synthesis.

Cloning of SpAMPK from S. paramamosain

Based on expressed sequence tags (ESTs) from cDNA libraries of *S. paramamosain*, which was homologous to *AMPK* of the rock crab *Cancer irroratus*, the brine shrimp *Artemia franciscana* and some other species, specific primers *SpAMPK*-F1 and *SpAMPK*-R1 (table 1) were designed to verify it. By using rapid amplification of cDNA ends (RACE), the 3′- and 5′-end cDNA sequences of *SpAMPK* were acquired. The 5′ *SpAMPK*-R1 and 5′ RACE primer F, 3′ *SpAMPK*-F1 (table 1) and 5′ RACE primer R were used for the first round 5′-end and 3′-RACE-PCR. The RACE-PCR performed as follows: initial denaturation at 94°C for 3 min, 11 cycles of 94°C for 30 s, 65°C for 30 s (decreasing by 1°C per cycle) and 72°C for 1.5 min, followed by 20 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 1.5 min and final elongation at 72°C for 10 min. The conditions for the second round 5′-end and 3′-RACE-PCR using 5′ *SpAMPK*-R2 and 5′ RACE primer F, 3′ *SpAMPK*-F2 and 5′ RACE primer R (table 1) were the same as the first round. Finally, the full-length cDNA of *SpAMPK* was verified by specific primers *SpAMPK*-F2 and *SpAMPK*-R2.

Table 1. Primers used in cloning and characterizing the gene of *SpAMPK*.

Name	Sequence (5′–3′)	PCR objective
SpAMPK-F1	CTTTTCCGTCATCCCCACATC	Sequence verifying
SpAMPK-R1	GCTTCCCAGGAGTTCCTTTCG	Sequence verifying
3′ *SpAMPK*-F1	GAAGCGAGCCACCATTGAAGA	Genomic cloning
3′ *SpAMPK*-F2	GTGGAATGCCGAAAGGAACTCC	Genomic cloning
5′ *SpAMPK*-R1	AATGCGTTCTGGATGTGGTTT	Genomic cloning
5′ *SpAMPK*-R2	CGCTTCATTGGGTCCACCAT	Genomic cloning
SpAMPK-F2	GCACATTCTCACAGGCACCA	Validation of full-length cDNA
SpAMPK-R2	ACAGGGTAGTGCTGAGGTTAGGC	Validation of full-length cDNA
SpAMPK-F3	AAAGTGGCACCTCGGTATTCG	Real-time PCR
SpAMPK-R3	CTCCTGTTATCGGGTTCTTGC	Real-time PCR
18S rRNA-F	CAGACAAATCGCTCCACCAAC	Internal control
18S rRNA-R	GACTCAACACGGGGAACCTCA	Internal control
GAPDH-F	AATGCCATCACAATAGAAAAATC	Internal control
GAPDH-R	GGAACAATCAACACTACCACACC	Internal control
β-actin-F	GAGCGAGAAATCGTTCGTGAC	Internal control
β-actin-R	GGAAGGAAGGCTGGAAGAGAG	Internal control

Bioinformatics analysis

After the open reading frame (ORF) was gained by the ORF finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html), the coding region sequences were translated into amino acid sequences. The AMPK protein sequences from other species were determined using a GenBank database search with basic local alignment search tool (BLAST) program (http://blast.ncbi.nlm.nih.gov/Blast.cgi). ClustalW program was used for performing the multiple sequence alignment. According to the deduced amino acid sequences of *Sp*AMPK and other known AMPK proteins, a phylogenic tree was constructed by the neighbour-joining (NJ) method using the software of MEGA 5.0. To determine the confidence of tree branch positions, bootstrap analysis of 1000 replicates was carried out.

qRT-PCR analysis

At present studies have shown that the housekeeping genes could be influenced by several factors such as different tissues and development stages, leading a potential discrepancy to the results. In view of these factors, it is necessary to select two or three appropriate housekeeping genes for the analysis of gene expression (Radonić *et al.* 2004; Czechowski *et al.* 2005; Yang *et al.* 2013). Thus, three housekeeping genes: *18S* rRNA (GenBank ID: FJ774906), *β-actin* (GenBank ID: GU992421) and *GAPDH* (GenBank ID: JX268543) were used as control for normalizing *SpAMPK* mRNA transcripts. The qRT-PCR was employed to detect the *SpAMPK* mRNA levels in various tissues (cerebral ganglion, eyestalk ganglion, heart, stomach, gill, hepatopancreas, thoracic ganglion, chela muscle, epidermis and hemocytes). While crabs were exposed to the four low temperature treatments, *SpAMPK* mRNA levels in the three tissues (hepatopancreas, chela muscle and gill) were detected by qRT-PCR. Gene-specific primers (table 1) were designed to amplify a 101 bp fragment of coding sequence. The reaction mixture of 20 μL in total, contained 10 μL of 2 × SYBR Premix Ex Taq$^{\text{TM}}$ (TaKaRa), 1.2 μL of diluted cDNA template, 0.8 μL of each 10 μM primers, and 6.4 μL of ddH$_2$O. Real-time PCR conditions were 95°C for 10 min, 40 cycles of 95°C for 20 s, 54°C for 30 s, 72°C for 30 s. The sample was carried out for three replicates and normalized by the average of *18S* rRNA, *β-actin* and *GAPDH* (Yang *et al.* 2013). The gene expression levels were calculated by the $2^{-\Delta\Delta \text{Ct}}$ comparative threshold cycle (Ct) method.

Statistical analysis

The data were determined using one-way ANOVA followed by Duncan's multiple range tests, and statistical analysis was performed on SPSS 16.0 software (SPSS, Chicago, USA). Differences were considered as significant, when P value <0.05.

Results

Cloning and identification of SpAMPK cDNA

The full-length cDNA of *SpAMPK* was successfully cloned by 5′-RACE and 3′-RACE. *SpAMPK* cDNA (GenBank accession no. KP161206) was 1884 bp, containing an ORF which encoded a putative protein with 521 amino acids, a 29 bp 5′-untranslated region (UTR) and a 289 bp 3′-UTR with a polyadenylation signal sequence 'aataa' and a poly(A) tail (figure 1). Molecular weight and theoretical isoelectric point (pI) of *SpAMPK* were 58.89 and 7.31 kDa, respectively.

Homology analysis of cDNA

We investigated identities of AMPKα subunit amino acid sequences of *S. paramamosain* and several other species derived from the NCBI GenBank database through multiple sequence alignment on ClustalW. As shown in figure 2,

```
1    ACAGCTGACGGACGGACGCAGCAGCAGCCATGGATACACCAGTCCAAGGACAAGCGCCTGGCTCCCAGACCATTACCTTAATGAAAATAG
                                  M  D  T  P  V  Q  G  Q  A  P  G  S  Q  T  I  T  L  M  K  I
91   GCCATTATCAGATTGGTAATACATTTGGTGCTGGAACCTTCGGAAAAGTGAAATATGGGGAGCACATTCTCACAGGCACCAAAGTTGCCA
     G  H  Y  Q  I  G  N  T  F  G  A  G  T  F  G  K  V  K  Y  G  E  H  I  L  T  G  T  K  V  A
181  TAAAAATCCTCAACCGCAAGACCATTAAAAACTTGGACATGGTCAGTAAAATAAAGCGAGAGATTACCAACCTCGAGCTTTTCCGTCATC
     I  K  I  L  N  R  K  T  I  K  N  L  D  M  V  S  K  I  K  R  E  I  T  N  L  E  L  F  R  H
271  CCCACATCATCAAGTTATATCAAGTGATCAGTACACCAACAGACATTTTCATGGTGATGGAATATGCTTCTGGAGGAGAACTGTTTGATT
     P  H  I  I  K  L  Y  Q  V  I  S  T  P  T  D  I  F  M  V  M  E  Y  A  S  G  G  E  L  F  D
361  ACATCAAGCAGAAAAGCAAGCTCAAAGAATCAGAAGCACGGAGGTTCTTCCAGCAGATTATCTCTGGTGTGGATTATTGCCATAGACATA
     Y  I  K  Q  K  S  K  L  K  E  S  E  A  R  R  F  F  Q  Q  I  I  S  G  V  D  Y  C  H  R  H
451  TGGTGGTTCACCGGGACCTTAAGCCAGAAAACCTCCTCCTGGATCACAACTTGCATGTCAAGATTGCAGACTTTGGTTTGTCAAACATAA
     M  V  V  H  R  D  L  K  P  E  N  L  L  L  D  H  N  L  H  V  K  I  A  D  F  G  L  S  N  I
541  TGGTTGATGGAGAATTCCTTCGTACTAGTTGTGGATCTCCTAATTATGCTGCTCCTGAAGTCATATCTGGAAAGCTGTATGCTGGTCCAG
     M  V  D  G  E  F  L  R  T  S  C  G  S  P  N  Y  A  A  P  E  V  I  S  G  K  L  Y  A  G  P
631  AGGTGGATGTGTGGTCTTGTGGTATTATCCTGTATGCCCTACTATGTGGCACACTTCCTTTTGATGATGAGCACGTCCCATCACTTTTCC
     E  V  D  V  W  S  C  G  I  I  L  Y  A  L  L  C  G  T  L  P  F  D  D  E  H  V  P  S  L  F
721  GCAAGATAAAATCGGGTGTGTTTCAGATCCCAGACTACCTTAATCAGAGTGTAGTACGCCTGCTATTGCACATGTTGATGGTGGACCCAA
     R  K  I  K  S  G  V  F  Q  I  P  D  Y  L  N  Q  S  V  V  R  L  L  L  H  M  L  M  V  D  P
811  TGAAGCGAGCCACCATTGAAGATATCAAGAAGCATGAATGGTTCCAGAAAGACCTACCAGCATATCTCTTCCCTCCACCATATGATCATG
     M  K  R  A  T  I  E  D  I  K  K  H  E  W  F  Q  K  D  L  P  A  Y  L  F  P  P  P  Y  D  H
901  ACAATTCTGTGATAGATCAAGAGGCAATAACTGAAGTTTGTGAGAAATTCCAAGTTGAAACAGCAGAAGTTCAGAGTGCCATTCTGTCAG
     D  N  S  V  I  D  Q  E  A  I  T  E  V  C  E  K  F  Q  V  E  T  A  E  V  Q  S  A  I  L  S
991  AAGATCAGCATAACCAACTAAAAATTGCCTACAATCTGATTGTTGACAACAAACGTTTTGCTGATGCCAGTGCCATGTACAGTATATCTG
     E  D  Q  H  N  Q  L  K  I  A  Y  N  L  I  V  D  N  K  R  F  A  D  A  S  A  M  Y  S  I  S
1081 CTTTCTATACTGGTGTGTCCCCTCCCCCTGCTGTGCCCACTCCTGCCTTCAGCCCCTCAGACTCAAGCCCAAGCCCTTTCAAACCACATC
     A  F  Y  T  G  V  S  P  P  P  A  V  P  T  P  A  F  S  P  S  D  S  S  P  S  P  F  K  P  H
1171 CAGAACGCATTGCACCATTGAGGGAACGAGCACTTAGTGGAGACCGTGGAATGCCGAAAGGAACTCCTGGGAAGCGTGCAAAGTGGCACC
     P  E  R  I  A  P  L  R  E  R  A  L  S  G  D  R  G  M  P  K  G  T  P  G  K  R  A  K  W  H
1261 TCGGTATTCGGTCCCAAAGTAAACCCTTGGATATCATGAGTGAAGTCTATAAGGCTATGAAGGTTTTAGGTTTTGAGTGGAAGGTTGTAA
     L  G  I  R  S  Q  S  K  P  L  D  I  M  S  E  V  Y  K  A  M  K  V  L  G  F  E  W  K  V  V
1351 ATCCATTCCATGTGCGTGTGCGCCGCAAGAACCCGATAACAGGAGGTTGTGTACAGATGGCCCTGCAGCTCTATCAAGTTGATTACAGAT
     N  P  F  H  V  R  V  R  R  K  N  P  I  T  G  G  C  V  Q  M  A  L  Q  L  Y  Q  V  D  Y  R
1441 CACATCTCCTAGATTTCAAGATCATCTGCAATGAAGGTGATATAGCACAGGTTGAAAAGAAAGGTTTGGAGGAGGAAGAGACTGGGCCTA
     S  H  L  L  D  F  K  I  I  C  N  E  G  D  I  A  Q  V  E  K  K  G  L  E  E  E  E  T  G  P
1531 CATCTCATCATGTCATGGAATTTTTTGAGATGTGTGCTGCCTTGATCACTGAACTGGCACGCTGAgctcttcaagcagaactgcctaacc
     T  S  H  H  V  M  E  F  F  E  M  C  A  A  L  I  T  E  L  A  R  *
1621 tcagcactaccctgttattcactggctaagaactgactgactgctccatgtttcccttaatccccttcaccatgcttctgcactgcctgg
1711 tgatgtatactgtgcattctccaccacaaaccatttacaagcaagaaatggaaatccttggcttgatatgagcattgctattccagaatt
1801 tagtgcctatgggtcagtgacaacatttttgctgcctcattgcaaataatgaaaactttgtaaaaaaaaaaaaaaaaaaaaaaa
```

Figure 1. The full-length cDNA sequence of *SpAMPK* and deduced amino acid sequence. The nucleotide sequence is numbered from the 5′ end and the single-letter amino acid code is shown above each corresponding codon. Uppercase letters indicate the translated region and lowercase letters indicate the untranslated region. The start code ATG and the termination code TGA are shown in bold. The polyadenylation signal 'aataa' is underlined.

*Sp*AMPK had the typical conserved domains of AMPKs: a catatytic domain, a USA-like autoinhibitory domain and ATP-binding sites. The major site on AMPK phosphorylated by upstream kinases was found to be Thr-172, and it was conserved. Compared with other arthropods and vertebrates, *Sp*AMPK exhibited relatively a high degree of identity: 71% with *A. franciscana*, 63% with the insect *D. melanogaster*, and 60% with *D. rerio* and *M. musculus*.

The NJ phylogenic tree (figure 3) was constructed according to the reported AMPKα amino acid sequences from some vertebrate and invertebrate species, by the software of MEGA 5.0. On the whole, the phylogenic tree of AMPK included two large clades: vertebrates and invertebrates groups. In the invertebrate clade, *Sp*AMPK clustered first with *C. irroratus* to form an independent clade, and was close to the clade of insects.

Tissue distributions of SpAMPK

The qRT-PCR was carried out to determine the expression levels of *SpAMPK* in various tissues. Compared with the control tissue (chela muscle), *SpAMPK* mRNA was abundantly expressed in the hepatopancreas, and moderately distributed in the eyestalk ganglion, thoracic ganglion and heart (figure 4).

Expression of SpAMPK mRNA in the hepatopancreas, chela muscle and gill under low temperatures

In this study, we observed that the mud crabs could move freely at 25 and 20°C, slightly at 15°C and could only move when they were disturbed at 10°C. At 5°C, they were already in the state of cold anesthesia and became motionless, and died after the 12 h cold stress.

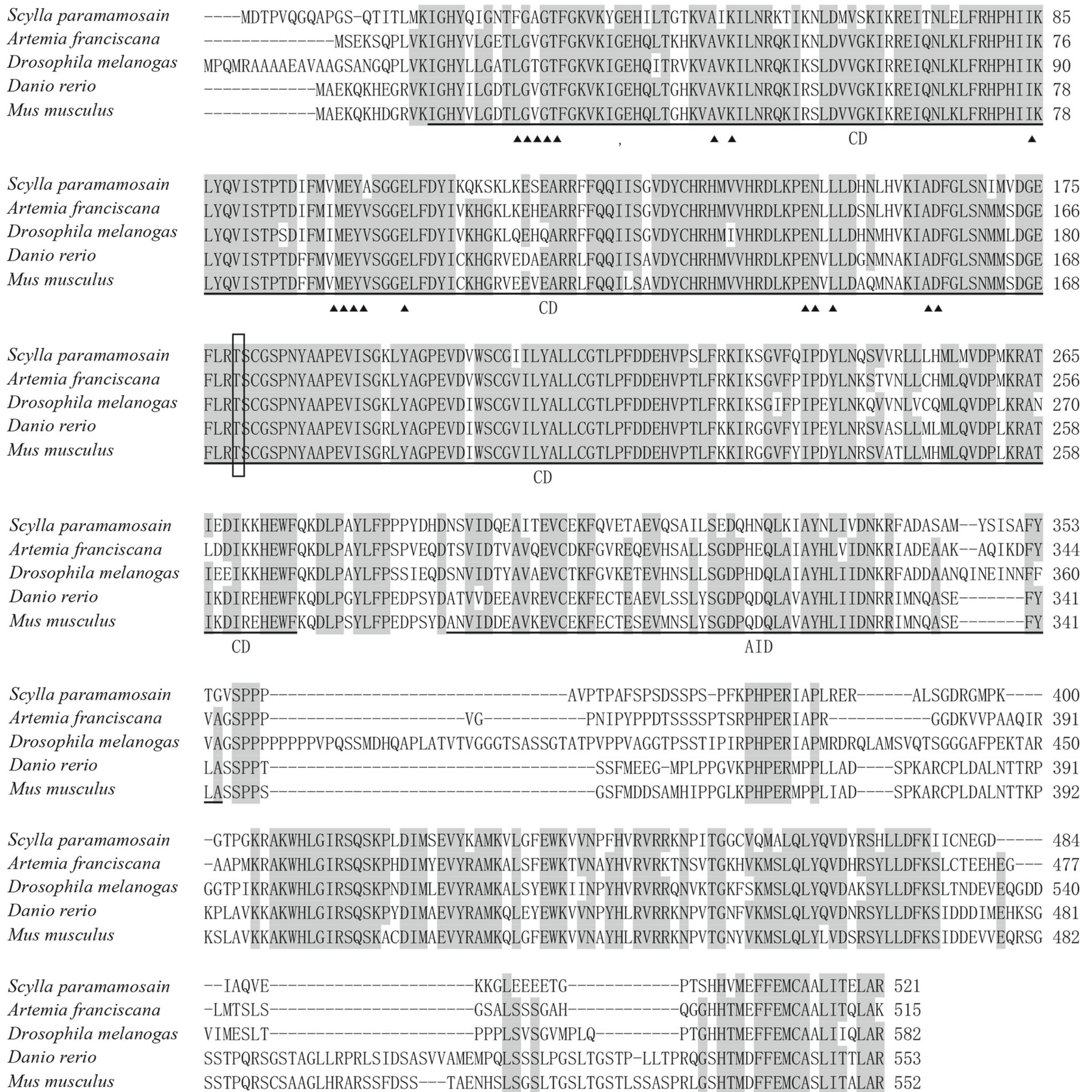

Figure 2. ClustalW alignment of AMPKα amino acid sequences for some vertebrate and invertebrate species. The sequence for *S. paramamosain* is from this study, and others were obtained from GenBank (*Artemia franciscana* ABI13783.1, *Drosophila melanogaster* NP_726730.1, *Danio rerio* XP_700831.4, *Mus musculus* NP_835279.2). The shaded regions indicate conservative residues. The flanking areas indicate the large conservative region T172 that activates the AMPK protein. ▲, ATP-binding site; CD, catalytic domain; AID, autoinhibitory domain.

In the hepatopancreas (figure 5a), the expression of *SpAMPK* mRNA at each point did not markedly change at both 25 and 20°C ($P > 0.05$), similar to that of control temperature (28°C) at 0 h. While at 15°C treatment, the abundance of *SpAMPK* mRNA increased dramatically ($P < 0.05$) from 0 to 6 h, reaching a peak of approximately 2.2-fold at 0 h, then decreased from 6 to 12 h, followed by stable expressions lasting till the end of the experiment. The *SpAMPK* mRNA at 15°C treatment was obviously higher than other three treatments ($P < 0.05$) at each time of sampling. At 10°C treatment, the expression of *SpAMPK* mRNA declined rapidly and was significantly lower than other temperature regimes at 6–48 h ($P < 0.05$).

In the chela muscle (figure 5b), compared with 25°C treatment, the expression levels of *SpAMPK* mRNA at 6 and 24 h were higher at 20°C, and no obvious variation was detected

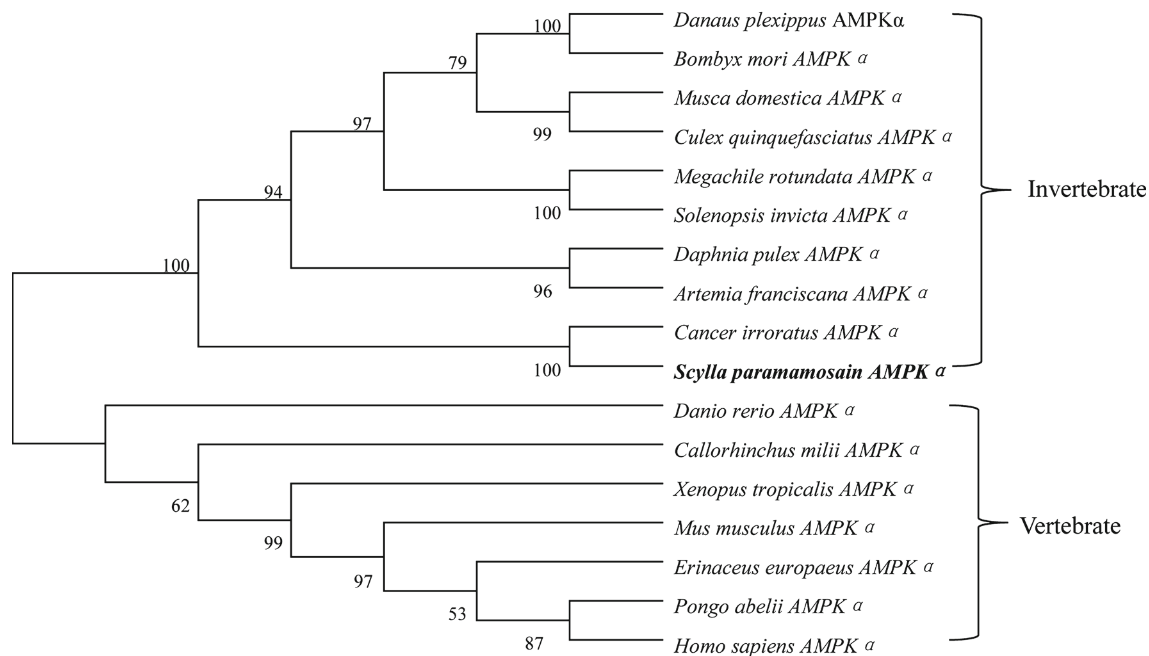

Figure 3. NJ phylogenetic tree of representative vertebrate and invertebrate *Sp*AMPK amino acid sequences. Bootstrap values supporting branch points are expressed as the percentage of 1000 replicates. The following organisms with AMPK GenBank-reported sequences were included in the analysis: *Danaus plexippus* (EHJ71225.1), *Bombyx mori* (NP_001093315.1), *Musca domestica* (XP_005180024.1), *Culex quinquefasciatus* (XP_001844429.1), *Megachile rotundata* (XP_003707119.1), *Solenopsis invicta* (EFZ13258.1), *Daphnia pulex* (EFX87591.1), *Artemia franciscana* (ABI13783.1), *Cancer irroratus* (ACL13568.1), *Scylla paramamosain* (KP161206), *Danio rerio* (XP_700831.4), *Callorhinchus milii* (XP_007901898.1), *Xenopus tropicalis* (NP_001135554.1), *Mus musculus* (NP_835279.2), *Erinaceus europaeus* (XP_ 007518085.1), *Pongo abelii* (NP_001125173.1) and *Homo sapiens* (NP_006243.2).

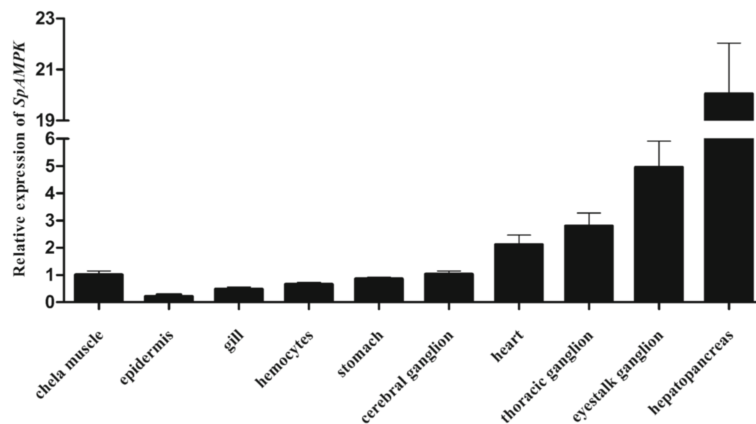

Figure 4. Expression levels of *SpAMPK* gene normalized to *18S* rRNA, *β-actin* and *GAPDH* in the chela muscle, epidermis, gill, hemocytes, stomach, cerebral ganglion, heart, thoracic ganglion, eyestalk ganglion and hepatopancreas of *S. paramamosain*. Gene expression levels are relative to *SpAMPK* expression in chela muscle, values are the mean ± SE ($n = 3$).

at any other time at 20°C treatment. At 15°C treatment, however, it showed significantly higher *SpAMPK* mRNA levels than other treatments ($P < 0.05$): *SpAMPK* mRNA level was rapidly and significantly upregulated and reached a peak at 1 h after cold challenge, with expression level about 2.3-fold than at 0 h. At 10°C treatment, the expression of *SpAMPK* mRNA markedly decreased at 1 h ($P < 0.05$), and increased slightly from 1 to 6 h, then no distinctive changes took place till the end. On the whole, the expression of *SpAMPK* mRNA at each time at 10°C treatment was lower than the other treatments ($P < 0.05$).

In the gill (figure 5c), except a transient increase at 3 h at 20°C ($P < 0.05$), *SpAMPK* expressions at 20°C treatment were not significantly different from those at 25°C treatment.

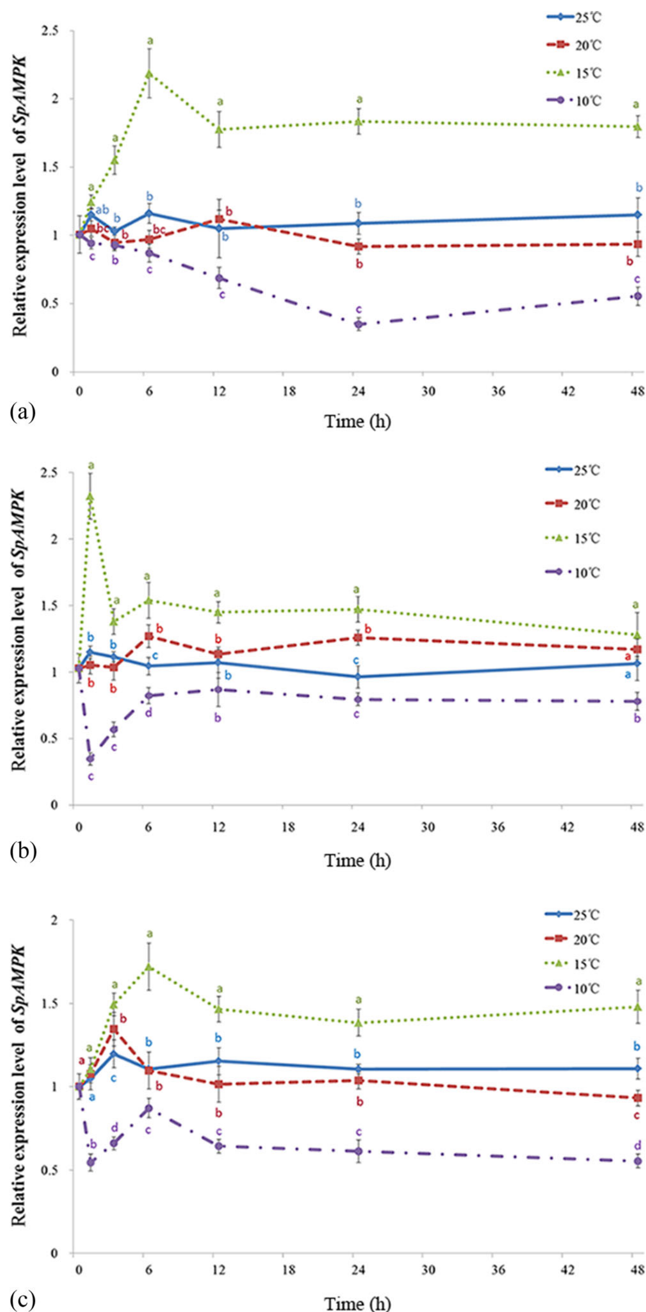

Figure 5. The relative expression of *SpAMPK* in three tissues of *S. paramamosain* at four temperatures. Each point represents the mean value from three determinations with standard error. Significant differences in different low temperature groups are indicated by different small letters on the top of break-points ($P < 0.05$): one-way ANOVA, $N = 3$. (a) Relative expression of *SpAMPK* mRNA in the hepatopancreas. (b) Relative expression of *SpAMPK* mRNA in the chela muscle. (c) Relative expression of *SpAMPK* mRNA in the gill.

At 15°C treatment, obviously higher expression levels were observed at 3 h ($P < 0.05$), and the mRNA expression reached maximum at 6 h, and then dropped slightly at 12 h, which showed much higher than other temperatures ($P < 0.05$). In contrast to 25, 20 and 15°C treatments, the 10°C group crabs showed significantly lower *SpAMPK* mRNA levels ($P < 0.05$).

In short, the expression of *SpAMPK* mRNA in three tissues (hepatopancreas, chela muscle and gill) during four temperature treatments had a similar pattern. The *SpAMPK* mRNA level was not obviously affected by 25°C treatment, and it happened to be transiently upregulated at 20°C treatment. While at slightly colder temperature of 15°C, *SpAMPK* mRNA showed a markedly higher level than the other temperatures. However, at 10°C treatment, the *SpAMPK* mRNA expression was significantly lower than the other three (15, 20 and 25°C) treatments.

Discussion

In this study, the *AMPK* genes were isolated and characterized from the mud crab, *S. paramamosain*. The full-length *SpAMPK* cDNA encoded a deduced protein with 521 amino acids, which was similar to that of the brine shrimp, *A. franciscana* (Zhu *et al.* 2007) and the mouse, *M. musculus* (Stapleton *et al.* 1996). The putative protein of *Sp*AMPK displayed a high similarity to some vertebrate and invertebrate sequences (60–71%). Like its mammalian counterparts ($\alpha 2$), *Sp*AMPK contained an N-terminal catalytic domain and a C-terminal region combining with β-subunits and γ-subunits to form an active and stable complex (Hardie 2004). The protein sequence of *Sp*AMPK showed that it had all the conserved domains necessary for kinase activity, such as the conserved threonine residue, Thr172 in the activation loop of the catalytic domain. In this study, the phylogenetic analysis supported the result that vertebrate AMPKs were paralogous to invertebrate AMPKs.

Tissue distribution detected by qRT-PCR revealed that *SpAMPK* mRNA was present in all the examined tissues in the present study. The highest level of *SpAMPK* transcript in the hepatopancreas signified the importance of this gene in energy regulation. This finding was consistent with the reports of high abundance in the liver of mouse, where AMPK was believed to play a significant role in controlling fat and glucose metabolisms (Davies *et al.* 1989; Mulligan *et al.* 2007). In mammals, AMPK$\alpha 2$ is expressed abundantly in tissues mainly involving high energy demand, such as liver, pancreas, neurons and skeletal muscles (Towler and Hardie 2007). In mud crab, metabolic enzymes are rich in the hepatopancreas, and the process of synthesis and secretion of enzymes are mainly in hepatopancreas. It is considered as a main organ in modelling physiological metabolism and responsible for the nutrient supplies of the ovary and food digestion, as well as the metabolism centre of lipid and carbohydrate (Kong *et al.* 2008; Zeng *et al.* 2010). Therefore, the hepatopancreas plays a strong role in metabolism process in *S. paramamosain*.

In this study, we observed that the subadult crabs died after 12 h at 5°C stress. Previous studies have showed that the Ca^{2+}-ATPase and Ca^{2+}/Mg^{2+}-ATPase activities in the mud crabs decreased sharply at this temperature (Kong

et al. 2012). Treatments with 5°C were considered to be beyond the critical range of adaptive low temperature, so it caused physiological disturbance in *S. paramamosain*, and the subadult crabs died at last.

At 25°C treatment, the patterns of *SpAMPK* mRNA level in all the three tissues (hepatopancreas, chela muscle and gill) did not change obviously, and they were all similar to that of control temperature (28°C). The optimum temperatures for feeding and growing of the mud crabs are approximately 18–30°C (Shelley and Lovatelli 2012), within which small-scale temperature fluctuations show little influence on the physiological progress of the mud crabs (Yu *et al.* 2014a). The stable expressions of *SpAMPK* mRNA in all the three tissues at 25°C revealed that the physiological metabolism in *S. paramamosain* was unaffected at this temperature. When the temperature dropped to 20°C, the expression of *SpAMPK* significantly upregulated in the gill (3 h) and chela muscle (6, 24 h) and only slightly changed in the hepatopancreas. This might suggest that the gill and chela muscle are more sensitive to temperature changes than the hepatopancreas. As both a respiratory and osmoregulation organ, gill directly in contact with aquatic environment, leading to prompt heat-change between gill and water. Under low temperatures, gill raises metabolism to sustain its physiological functions (Xu and Qin 2012). Chela muscle is just beneath the carapace, acutely sensing the fluctuation of temperature, with burning energy to generate heat under low temperatures (Atwood and Cooper 1995). It was reported that cold temperatures would improve aerobic metabolism in muscle by inducing AMPK (Jäger *et al.* 2007). Thus, when the subadult crabs were exposed to 20°C environment, a transient upregulation of *SpAMPK* mRNA was observed in both gill and chela muscle, which indicated that more energy was required to maintain the respiratory metabolism of gill and heat production of chela muscle.

Preliminary data showed that AMPK was elicited by cold stress in mouse and chicken hepatocytes to restore cellular energy homeostasis, which is a compensatory response for ATP-consuming processes (Corton *et al.* 1994; Zhang *et al.* 2014). It was found that AMPK could induce lot of metabolic changes, such as an increase in intake and oxidation of plasma fatty acids and glucose, besides enhancing the expressions of glucose transporter 4 (GLUT4) and hexokinase II (HKII) (Hardie and Sakamoto 2006). Further, low temperature could increase generation of reactive oxygen species (ROS) in the swimming crab *P. trituberculatus* and lead to oxidative stress (Meng *et al.* 2014). Intriguingly, recent studies showed that ROS produced by cold stress could arouse the AMPK, and then resulted in the increase of glycolysis and mitochondrial biogenesis, which in turn reduced deleterious effects caused by oxidative stress (Wu *et al.* 2014). Briefly, low temperatures induce AMPK for two purposes: one to meet energy demand, and the other to reduce deleterious effects caused by ROS.

Temperature 15°C is just below the range of optimum temperatures (18–30°C) (Shelley and Lovatelli 2012). In all the three tissues examined in *S. paramamosain*, the expression of *SpAMPK* mRNA at 15°C treatments was significantly higher than the other three temperature treatments. Besides AMPK, there were some other regulatory elements involved in cell energy supplying such as adenosine triphosphatase (ATPase) (Kong *et al.* 2008) and adenine nucleotide translocase (ANT) (Santamaria *et al.* 2004). ATPase could supply energy for facilitating ion movement across the membrane, and it was reported that activities of four kinds of ATPase at 15°C were higher than those at 27°C in *S. paramamosain* (Kong *et al.* 2012). In addition, *SpANT2* in subadult crab *S. paramamosain* was involved in transporting ATP from mitochondria to the cytoplasm and simultaneously transferring ADP to mitochondria, and its profile was significantly increased under 15°C exposure, much higher than those at the 25 and 20°C treatments (Yu *et al.* 2014b). In the estuarine crab, *Neohelice granulata*, a slight drop in temperature led to an increase in blood sugar levels followed by the descending of glycogen since glycogen was broken down into glucose for the energy supply of metabolism (Valle *et al.* 2009). At 15°C, in mud crab, the soluble sugars were higher than those at 27°C, which could supply energy and protect cells (Kong *et al.* 2008). These data unveiled that the mud crab would strengthen metabolism and generate energy to cope with the slight cold stress of 15°C. Previous researches showed that AMPK protein expression and activity increased based on the upregulation of mRNA levels when exposed to temperature stress (Frederich *et al.* 2009; Zhang *et al.* 2014). Therefore, the sharp increase of *SpAMPK* mRNA at 15°C would contribute to regulate the whole-body energy metabolism by stimulating the ATP-generating pathways which occurred as a defense against cold stress.

At 10°C treatment, however, the *SpAMPK* mRNA in the three tissues did not continue to increase, and instead, turned into significantly lower levels than those in other temperature treatments. 10°C is already lower than the biological zero (12.19°C) of *S. paramamosain* (Hamasaki 2002), under which cells divided abnormally during the growth of embryo (Zeng 2007) as well as no moult occurred (Wu 1982). An inhibition of physical activities and growth, including slow physiological processes, could happen when temperature was too low and outside the normal range for selfregulation of crustaceans (Wang and Wang 2014). It was reported that with low metabolic rate at 10°C, the *SpANT2* mRNA decreased sharply in *S. paramamosain* (Yu *et al.* 2014b). In this study, a significant reduction of *SpAMPK* mRNA levels was also observed. As both AMPK and ANT are participants in regulating energy metabolism, the downregulation of *SpAMPK* mRNA levels coincides with declined levels of metabolism at 10°C treatment. It was also suggested that cold stress at this temperature has beyond the ability of cellular protective mechanisms.

In conclusion, *SpAMPK* played a key role in cold stress for the mud crabs. Under a moderately low temperature (i.e. 15°C), the mud crabs could initiate the ATP-produced pathways through the high expression of *SpAMPK* mRNA.

However, when the cold stress was far below the adaptive limits (i.e. 10 and 5°C), the mud crabs reduced the metabolism through the low expression of *SpAMPK* mRNA. Therefore, *SpAMPK* might serve as a bioindicator for monitoring the cold stress.

Acknowledgements

This study was supported by the National Natural Science Foundation of China (nos. 31472294 and J1210050), and Spark Plan Project in Fujian Province (no. 2016S0055). The authors sincerely thank the anonymous reviewers for valuable comments on the manuscript.

References

Aguilar-Alberola J. A. and Mesquita-Joanes F. 2014 Breaking the temperature-size rule: thermal effects on growth, development and fecundity of a crustacean from temporary waters. *J. Therm. Boil.* **42**, 15–24.

Atwood H. L. and Cooper R. L. 1995 Functional and structural parallels in crustacean and *Drosophila* neuromuscular systems. *Am. Zool.* **35**, 556–565.

Bartrons M., Ortega E., Obach M., Calvo M. N., Navarro-Sabaté À. 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.

Colson-Proch C., Renault D., Gravot A., Douady C. J. and Hervant F. 2009 Do current environmental conditions explain physiological and metabolic responses of subterranean crustaceans to cold? *J. Exp. Biol.* **212**, 1859–1868.

Corton J. M., Gillespie J. G. and Hardie D. G. 1994 Role of the AMP activated protein kinase in the cellular stress response. *Curr. Biol.* **4**, 315–324.

Costanzo J. P., Dinkelacker S. A., Iverson J. B. and Lee J. R. E. 2004 Physiological ecology of overwintering in the hatchling painted turtle: multiple-scale variation in response to environmental stress. *Physiol. Biochem. Zool.* **77**, 74–99.

Czechowski T., Stitt M., Altmann T., Udvardi M. K. and Scheible W. R. 2005 Genome-wide identification and testing of superior reference genes for transcript normalization in *Arabidopsis*. *Plant Physiol.* **139**, 5–17.

Davies S. P., Carling D. and Hardie D. G. 1989 Tissue distribution of the AMP-activated protein kinase, and lack of activation by cyclic-AMP-dependent protein kinase, studied using a specific and sensitive peptide assay. *Eur. J. Biochem.* **186**, 123–128.

De Gobba C., Tompa G. and Otte J. 2014 Bioactive peptides from caseins released by cold active proteolytic enzymes from *Arsukibacterium ikkense*. *Food Chem.* **165**, 205–215.

Faure J., Lebret B., Bonhomme N., Ecolan P., Kouba M. and Lefaucheur L. 2013 Metabolic adaptation of two pig muscles to cold rearing conditions. *J. Anim. Sci.* **91**, 1893–1906.

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

Hamasaki K. 2002 Effects of temperature on the survival, spawning and egg incubation period of overwintering mud crab broodstock, *Scylla paramamosain* (Brachyura: Portunidae). *Suisanzoshoku (Japan)* **50**, 301–308.

Hangalapura B. N., Nieuwland M. G. B., Buyse J., Kemp B. and Parmentier H. K. 2004 Effect of duration of cold stress on plasma adrenal and thyroid hormone levels and immune responses in chicken lines divergently selected for antibody responses. *Poult. Sci.* **83**, 1644–1649.

Han G. D., Zhang S., Marshall D. J., Ke C. H. and Dong Y. W. 2013 Metabolic energy sensors (AMPK and SIRT1), protein carbonylation and cardiac failure as biomarkers of thermal stress in an intertidal limpet: linking energetic allocation with environmental temperature during aerial emersion. *J. Exp. Biol.* **216**, 3273–3282.

Hardie D. G. 2004 The AMP-activated protein kinase pathway—new players upstream and downstream. *J. Cell Sci.* **117**, 5479–5487.

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

Hochachka P. W. and Somero G. N. 2002 *Biochemical adaptation: mechanism and process in physiological evolution.* Oxford University Press. Oxford, UK.

Jäger S., Handschin C., Pierre J. S. and Spiegelman B. M. 2007 AMP-activated protein kinase (AMPK) action in skeletal muscle via direct phosphorylation of PGC-1α. *Proc. Natl. Acad. Sci. USA* **104**, 12017–12022.

Jia H. Y., Li J. M., Yu Q. J., Wang J. and Li S. 2009 The effect of cold stress on DNA oxidative damage of lung in chicken. *Chin. J. Appl. Physiol.* **25**, 373–376 (in Chinese).

Kong X., Wang G. Z. and Li S. J. 2008 Seasonal variations of ATPase activity and antioxidant defenses in gills of mud crab *Scylla serrata* (Crustacea, Decapoda). *Mar. Biol.* **154**, 269–276.

Kong X., Wang G. Z. and Li S. J. 2012 Effects of low temperature acclimation on antioxidant defenses and ATPase activities in the muscle of mud crab (*Scylla paramamosain*). *Aquaculture* **370**, 144–149.

Le Vay L., Lebata M. J. H., Walton M., Primavera J., Quinitio E., Lavilla-Pitogo C. *et al.* 2008 Approaches to stock enhancement in mangrove-associated crab fisheries. *Rev. Fish. Sci.* **16**, 72–80.

Meng X. L., Liu P., Li J., Gao B. Q. and Chen P. 2014 Physiological responses of swimming crab *Portunus trituberculatus* under cold acclimation: antioxidant defense and heat shock proteins. *Aquaculture* **434**, 11–17.

Mulligan J. D., Gonzalez A. A., Stewart 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.* **580**, 677–684.

Olsvik P. A., Vikeså V., Li K. K. and Hevrøy E. M. 2013 Transcriptional responses to temperature and low oxygen stress in Atlantic salmon studied with next-generation sequencing technology. *BMC Genomics* **14**, 1.

Radonić A., Thulke S., Mackay I. M., Landt O., Siegert W. and Nitsche A. 2004 Guideline to reference gene selection for quantitative real-time PCR. *Biochem. Biophys. Res. Commun.* **313**, 856–862.

Santamaria M., Lanave C. and Saccone C. 2004 The evolution of the adenine nucleotide translocase family. *Gene* **333**, 51–59.

Shelley C. and Lovatelli A. 2012. *Mud crab aquaculture: a practical manual*. Food and Agriculture Organization of the United Nations (FAO).

Stapleton D., Mitchelhill K. I., Gao G., Widmer J., Michell B. J., Teh T. *et al.* 1996 Mammalian AMP-activated protein kinase subfamily. *J. Biol. Chem.* **271**, 611–614.

Thongda W., Chung J. S., Tsutsui N., Zmora N. and Katenta A. 2015 Seasonal variations in reproductive activity of the blue crab, *Callinectes sapidus*: vitellogenin expression and levels of vitellogenin in the hemolymph during ovarian development. *Comp. Biochem. Phys. A* **179**, 35–43.

Towler M. C. and Hardie D. G. 2007 AMP-activated protein kinase in metabolic control and insulin signaling. *Circ. Res.* **100**, 328–341.

Valle S. C., Eichler P., Maciel J. E., Machado G., Kucharski L. C. and Da Silva R. S. M. 2009 Seasonal variation in glucose and neutral amino acid uptake in the estuarine crab *Neohelice granulate*. *Comp. Biochem. Phys. A* **153**, 252–257.

van den Brand H., Molenaar R., van der Star I. and Meijerhof R. 2010 Early feeding affects resistance against cold exposure in young broiler chickens. *Poult. Sci.* **89**, 716–720.

Wang G. Z., Kong X. H., Wang K. J. and Li S. J. 2007 Variation of specific proteins, mitochondria and fatty acid composition in gill of *Scylla serrata* (Crustacea, Decapoda) under low temperature adaptation. *J. Exp. Mar. Biol. Ecol.* **352**, 129–138.

Wang W. N. and Wang A. L. 2014 Changes of protein-bound and free amino acids in the muscle of the freshwater prawn *Macrobrachium nipponensein* different salinities. *Aquaculture* **233**, 561–571.

Wu Q. S. 1982 *High-yield technology for shrimps and crabs breeding*. Agriculture Press, Beijing, China (in Chinese).

Wu S. B., Wu Y. T., Wu T. P. and Wei Y. H. 2014 Role of AMPK-mediated adaptive responses in human cells with mitochondrial dysfunction to oxidative stress. *Biochim. Biophys. Acta* **1840**, 1331–1344.

Xu Q. and Qin Y. 2012 Molecular cloning of heat shock protein 60 (PtHSP60) from *Portunus trituberculatus* and its expression response to salinity stress. *Cell Stress Chaperones* **17**, 589–601.

Yang Y. N., Ye H. H., Huang H. Y., Li S. J., Zeng X. L., Gong J. *et al.* 2013 Characterization and expression of SpHsp60 in hemocytes after challenge to bacterial, osmotic and thermal stress from the mud crab *Scylla paramamosain*. *Fish Shellfish Immun.* **35**, 1185–1191.

Ye H. H., Tao Y., Wang G. Z., Lin Q. W., Chen X. L. and Li S. J. 2011 Experimental nursery culture of the mud crab *Scylla paramamosain* (Estampador) in China. *Aquacult. Int.* **19**, 313–321.

Yu K., Ye H. H., Gong J., Huang H. Y. and Lin S. H. 2014a Cloning of adenine nucleotide translocase 2 (*ANT*2) and its expression under low temperature stress in the mud crab, *Scylla paramamosain*. *J. Xiamen Univ.-Nat. Sci.* **53**, 436–442 (in Chinese).

Yu K., Ye H. H., Huang C. C., Gong J. and Huang H. Y. 2014b Effects of different temperature and salinity on the expression of adenine nucleotide translocase 2 (*ANT2*) mRNA in the mud crab, *Scylla paramamosain*. *J. Fish. Sci-China* **6**, 11 (in Chinese).

Zeng C. 2007 Induced out-of-season spawning of the mud crab, *Scylla paramamosain* (Estampador) and effects of temperature on embryo development. *Aquacult. Res.* **38**, 1478–1485.

Zeng H., Ye H. H., Li S. J., Wang G. Z. and Huang J. R. 2010 Hepatopancreas cell cultures from mud crab, *Scylla paramamosain*. *In Vitro Cell. Dev.-An.* **46**, 431–437.

Zhang Z. W., Bi M. Y., Yao H. D., Fu J., Li S. and Xu S. W. 2014 Effect of cold stress on expression of AMPKα–PPARα pathway and inflammation genes. *Avian Dis.* **58**, 415–426.

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.

Received 20 November 2015, in final revised form 28 March 2016; accepted 11 April 2016
Unedited version published online: 15 April 2016
Final version published online: 1 December 2016

Corresponding editor: Indrajit Nanda