

Characterization of CCT α and evaluating its expression in the mud crab *Scylla paramamosain* when challenged by low temperatures alone and in combination with high and low salinity

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Abstract Chaperonin containing the T-complex polypeptide-1 (CCT), which is known to be involved in intracellular assembly and folding of proteins, is a class of chaperonin omnipresent in all forms of life. Previous studies showed that CCT played a vital role in cold hardiness of various animals. In order to understand the response of the polypeptide complex to low temperature challenge and other environmental stresses, a subunit of CCT (CCT α) was cloned from the mud crab *Scylla paramamosain* by expressed sequence tag (EST) analysis and rapid amplification of cDNA ends (RACE). The full-length cDNA *SpCCT α* was of 1972 bp and contained a 1668 bp open reading frame (ORF) encoding a polypeptide of 555 amino acids with four conserved motifs. The messenger ribonucleic acid (mRNA) levels of *SpCCT α* in ten tissues of adult *S. paramamosain* was subsequently examined and the highest expression was found in muscle, followed by gill, hepatopancreas, thoracic ganglion, hemocyte, heart, cerebral ganglion, stomach, eyestalk ganglion, and epidermis. The expressions of *SpCCT α* in the muscle of sub-adult crabs (pre-acclimated to 28 °C) subjected to the challenges of both lower temperatures (25, 20, 15, and 10 °C) alone and low temperatures (15 and 10 °C) in combination with salinity of 35 and 10 were further investigated by fluorescent quantitative real-time PCR (qPCR). It was

revealed that when exposed to lower temperatures alone, the mRNA transcripts of the *SpCCT α* gene in the muscle were generally induced for significant higher expression at 10 °C treatment than the 25, 20, and 15 °C treatments; meanwhile, exposure to 15 °C also frequently led to significantly higher expression than those at 20 and 25 °C. This finding indicated that the up-regulation of *SpCCT α* was closely related to the cold hardiness of *S. paramamosain*. The results of an additional experiment challenging the sub-adult crabs with various combinations of low temperatures with different salinity conditions generally demonstrated that at both 10 and 15 °C, the expression of *SpCCT α* under the high salinity of 35 was significantly lower than that at low salinity of 10, implying that the damages caused by low temperatures with high salinity were less than that under low salinity.

Keywords *Scylla paramamosain* · CCT α · Characterization · Expression · Cold hardiness · Low temperature · Salinity

Introduction

It is well known that proteins play essential roles in various biological functions; to gain functional activity, normally proteins must fold into defined three-dimensional structures (Eisenberg 1984; Bowie et al. 1991). While proteins can fold spontaneously into their correct native state in vivo, on occasion the assistance of a special type of proteins known as molecular chaperones is essential (Kim et al. 2013). Found virtually in all cells, molecular chaperones are known to responsible for the folding and assembly of newly synthesized proteins, binding and stabilizing of non-native forms, and refolding of denatured proteins (Hartl 1996; Hartl and Hayer-Hartl 2002). Based on the sizes of subunits, molecular chaperones can largely be divided into two classes (Kim et al.

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2013). The first class of molecular chaperones are represented by GroEL from eubacteria, mitochondrial heat shock protein (Hsp60), and rubisco-subunit-binding proteins from chloroplasts, and are characterized by double-ring oligomeric protein complexes composed of 60-kDa subunits and have a central cavity that mediates adenosine triphosphate-dependent folding of polypeptides. The second class of molecular chaperones includes the thermosomes, TF55, and the chaperonin CCT (chaperonin containing the T-complex polypeptide-1) families (Somer et al. 2002; Ruano-Rubio and Fares 2007). While both thermosomes and TF55 are composed of two different monomeric subunits originating from archaeobacteria, the chaperonin CCT derives from eukaryotic cytoplasm (Trent et al. 1991; Phipps et al. 1993).

As a hetero-oligomeric chaperonin consisting of eight or nine different subunits, chaperonin CCT possess several highly conserved motifs for ATP binding despite showing only approximately 30 % amino acid homology with each subunit (Chagoyen et al. 2014). Thus, as a rule of thumb, both common ATPase function and subunit-specific function are considered to co-exist in each subunit (Yokota et al. 2000b).

Similar to other chaperonins, all CCT subunits are approximately 60 kDa and are arranged into a hexadecamer complex, forming a stacked multimeric ring with a central cavity (Muñoz et al. 2011). Depending on the cavity, CCT can facilitate folding of non-native proteins or assembling substrates with their respective partners upon binding and hydrolysis of ATP (Chagoyen et al. 2014). While the cytoskeletal proteins, actin and tubulin, are traditionally considered as the principal cellular substrates of the CCT complex (Quintá et al. 2011), in recent years an increasing number of studies have discovered that there are many other significant substrates, which are themselves encoded by essential genes and require the CCT complex to build structure themselves. These include α -transducin, the septin ring complex, firefly luciferase, histone deacetylase 3, the von Hippel-Lindau tumor suppressor, and WD-repeat β -propeller proteins, such as the Cdc20 activator of the anaphase-promoting complex (Guenther et al. 2002; Camasses et al. 2003; Dekker et al. 2008). Based on research on those already identified and many yet to be recognized substrates, the CCT complex has been confirmed to play a critical role in eukaryotic cells. A survey of literature showed that the CCT complex has been linked to cell growth (Knee et al. 2013), reproduction (Dun et al. 2012), immunity (Yokota et al. 2000a; Arockiaraj et al. 2012), and disease resistance (Posokhova et al. 2011).

Unlike eubacterial GroEL, prokaryotic Hsp60 chaperonin, and archaeobacterial Hsp60 that are induced by heat and other factors (Parsell and Lindquist 1993),

CCT has seldom been shown to vary with external environmental stress in earlier studies (Ursic and Culbertson 1992; Soares et al. 1994). However, more recently, emerging evidence has suggested that CCT is also robustly induced by heat shock in human cells (Scheda et al. 1996) or by chemical stresses in invertebrates, such as the expression of CCT γ gene induced by heavy metal (CdCl₂) exposure in the hypotrichous ciliate *Oxytricha granulifera* (Palmedo and Ammermann 1997), the expression of CCT θ induced in the ciliate *Tetrahymena pyriformis* by colchicine treatment (Domingues et al. 1999), and CCT8 expression in response to an alkylating agent in the yeast *Saccharomyces cerevisiae* (Jelinsky and Samson 1999). Moreover, underlying the increased cold hardiness, CCT α and CCT β have been reported to be up-regulated in the yeast *S. cerevisiae*, and Western blot analysis showed that cold shock induces an increase in the CCT α protein (Somer et al. 2002) and TCP-1, a subunit of chaperonin CCT, in the onion maggot *Delia antiqua* (Kayukawa et al. 2005), respectively. However, to date, there are few published studies on molecular cloning, characterization of the CCT complex, and gene expression in response to stress in crustaceans, with a few exceptions, such as a study on CCT-n in the white shrimp *Litopenaeus vannamei* (Yin et al. 2011) and chaperonin in the giant freshwater prawn *Macrobrachium rosenbergii* (Arockiaraj et al. 2012).

The mud crab *Scylla paramamosain* is a large portunid crab inhabiting intertidal and subtidal sheltered soft-sediment habitats and has a broad distribution along the coasts of southern China as well as other Indo-Pacific countries (Walton et al. 2006). With its abundance, fast growth, and high market value, the species is important for both fisheries and aquaculture in southern China (Ye et al. 2011). Unfortunately, mass mortality is common in winter with the approach of severe cold fronts to mud crab aquaculture (Xia et al. 2010). Although various mechanisms, including both behavioral and physiological ones, have been reported to be adopted by *S. paramamosain* to cope with temperature fluctuation (Kong et al. 2012; Yang et al. 2013a, b), it is necessary to further investigate on mechanisms underlying cold hardiness in the crab for both aquaculture and fisheries management of the commercially important crab species.

The present study sought to clone and sequence the full-length cDNA of CCT α (*SpCCT α*) and subsequently profile the expression levels in different tissues of *S. paramamosain*. Considering that temperature and salinity often act in concert to affect the wellbeing of marine species, both in the wild and under culture conditions, and that past studies on *S. paramamosain* have been focused on coping mechanisms on either temperature (Kong et al. 2012) or salinity alone (Gong et al.

2015), experiments were conducted to compare not only *S. paramamosain* subjected to different temperatures but also crabs to low temperatures in combination with different salinities on the *SpCCT α* expression of the species. The objective of the study is to gain better understanding on the coping mechanisms employed by *S. paramamosain* for dealing with low temperature stress, which should have implications for both natural resource management and aquaculture of the commercially important crab species.

Materials and methods

Source of the crabs

Healthy sub-adult (carapace length 5.1 ± 0.7 cm; body weight 90 ± 5 g) and adult crabs (carapace length 8.5 ± 0.6 cm; body weight 370 ± 35 g) of *S. paramamosain* were purchased from an aquaculture farm located in Xiang'an district, Xiamen city, Fujian province of China. All crabs were checked to ensure no injury or missing appendage before being transported to Xiang'an campus of Xiamen University for the experiments. Upon arriving at Xiamen University, the crabs were acclimated to the condition of temperature 28 ± 0.5 °C and salinity of 25 for 3 days in the filtered seawater, during which all crabs were fed live clam *Ruditapes philippinarum*, and gentle aeration was provided via an airstone.

Following acclimatization, adult crabs were sampled to extract total RNA to obtain the full length of *SpCCT α* and analyze tissue distribution of *SpCCT α* . Various tissues, including heart, epidermis, eyestalk ganglion, stomach, cerebral ganglion, hemocyte, thoracic ganglion, hepatopancreas, gill, and muscle, were dissected and removed from four crabs, and those tissues were processed immediately to extract RNA. Total RNAs were extracted for profiling the expression level of *SpCCT α* in these tissues.

Challenged with lower temperatures

For the challenged with lower temperatures experiment, a total of 105 sub-adult crabs were used. They were transferred from 28 °C (the temperature they acclimated to over the past 3 days) to five lower temperatures of 25, 20, 15, 10, and 5 °C (± 0.2 °C). Each temperature treatment has 21 crabs. To avoid cannibalism, crabs were kept individually in round plastic buckets (diameter 10 cm \times height 12 cm) filled with filtered seawater (salinity of 25) roughly to a third of their capacity. Every 21 buckets were placed in a temperature-controlled incubator to maintain a steady temperature. Before crabs were

transferred to each bucket, water temperature in the buckets had already been pre-adjusted to the desired levels. The crabs were put in buckets directly at 25, 20, 15, 10, and 5 °C, respectively.

At 0, 1, 3, 6, 12, 24, and 48 h of exposure, three crabs were randomly sampled from each treatment and muscle tissues from the chelipeds were dissected from each crab, which were subsequently used to extract total RNAs for profiling the expression level of *SpCCT α* .

Challenged with low temperatures in combination with high and low salinity

After the lower temperatures experiment (“Challenged with lower temperatures” section) confirmed that low temperature led to high expression of *SpCCT α* , a combined temperature with salinity experiment on a 2×2 factorial design was conducted. For this experiment, sub-adult crabs were exposed to various combinations of two low temperatures of 10 ± 0.2 and 15 ± 0.2 °C with either a low salinity (10 ± 0.1) or a high salinity (35 ± 0.1) (10 °C, 10; 10 °C, 35; 15 °C, 10; 15 °C, 35) to investigate their effects on expression of *SpCCT α* . Again, 21 sub-adult crabs were allocated randomly to each treatment with a total of 84 crabs used. To obtain the desired salinities, the natural filtered seawater (salinity of 25) was firstly adjusted to be slightly higher than salinity of 35 by adding marine salt. Then, the prepared seawater was diluted with distilled water to salinity of 35 and 10, respectively. Other experimental procedures, including sampling time, number of crabs sampled from each treatment as well as the tissue (i.e., muscle) sampled for the extraction of total RNA, were the same as the lower temperatures experiment (“Challenged with lower temperatures” section).

RNA extraction and cDNA synthesis

Tissues were dissected immediately from the crabs sampled and flash frozen in liquid nitrogen. Total RNA was extracted from the harvested tissue using Trizol RNA isolation reagent (Invitrogen) according to the manufacturer's protocol. The concentration of RNA, with genome DNA being removed by DNase I (TaKaRa) in advance, was determined spectrophotometrically and the quality was monitored by agarose gel electrophoresis. Extracted RNA was then stored in a -80 °C freezer for later use. Complementary DNA (cDNA) was synthesized from 2 μ g of total RNA using Revert AidTM First Strand cDNA Synthesis Kit (Fermentas) following manufacturer's instruction and stored at -20 °C.

Table 1 Primers used for the characterization of CCT α and profiling its expression in the mud crab *Scylla paramamosain*

Name	Sequence (5'–3')	Position	PCR objective
<i>SpCCT</i> α —F1	GTCCGTCGCTGTAAGAAGT	1000–1018	Sequence verifying
<i>SpCCT</i> α —R1	CTGGGATGAGTGGTGGTAT	1485–1503	Sequence verifying
3' <i>SpCCT</i> α —F	TAAAGAGAATAGCCAAGGCAACAG	1025–1048	Genomic cloning
5' <i>SpCCT</i> α —R	TGGCCTTCTATAAGATCCAAACC	1170–1190	Genomic cloning
<i>SpCCT</i> α —F2	AGCATCACTACCACGGAAGAAATT	32–55	Validation of full-length cDNA
<i>SpCCT</i> α —R2	CACGAACCTGGCCTTCTATAAGAT	1544–1567	Validation of full-length cDNA
<i>SpCCT</i> α —F3	AAGGCAACAGGTGCGACATT	1039–1058	Real-time PCR
<i>SpCCT</i> α —R3	ATTTGGACCGGAAGGATAA	1202–1221	Real-time PCR
18S rRNA F	CAGACAAATCGCTCCACCAAC	GenBank ID: FJ774906	Internal control
18S rRNA R	GACTCAACACGGGAACCTCA	GenBank ID: FJ774906	Internal control

Cloning full-length *SpCCT* α cDNA

Basing on the partial fragment of the *SpCCT* α derived from EST library of brain, validated primers *SpCCT* α -F1 and *SpCCT* α -R1 (Table 1) were designed to verify it. The 3' and 5' ends of the *SpCCT* α cDNA were subsequently obtained by a modified rapid amplification of ends (RACE) method using 3'-full RACE Core Set ver. 2.0 and 5'-full RACE kit (TaKaRa). Finally, validated primers of full-length cDNA of *SpCCT* α , including *SpCCT* α -F2 and *SpCCT* α -R2 (Table 1), were excogitated to rectify the mistakes of the sequence of *SpCCT* α . The PCR amplification was carried out in a reaction volume of 25 μ l containing 0.1 U of rTaq DNA polymerase (TaKaRa), 2.0 μ l cDNA, and 500 nM each of primers by the PCR procedure. The PCR procedure was performed as follows: initial denaturation at 94 $^{\circ}$ C for 3 min; followed by 32 cycles of 94 $^{\circ}$ C for 30 s, 54 $^{\circ}$ C for 45 s, and 72 $^{\circ}$ C for 30 s; and by final elongation at 72 $^{\circ}$ C for 10 min. The PCR products analyzed by 1 % agarose gel electrophoresis were subcloned using pMD19-T vector (TaKaRa) and sequenced.

Sequence analysis and phylogenetic analysis

The full-length cDNA sequence of *SpCCT* α obtained from *S. paramamosain* was analyzed for similarity by the BLAST program in NCBI database (<http://www.ncbi.nlm.nih.gov/BLAST/>). The deduced amino acid sequence was obtained using BioEdit software and motifs were predicted using ExpASY (<http://www.au.expasy.org>). Not only molecular mass but the theoretical isoelectric point was predicted using the compute pI/Mw tool (<http://web.expasy.org/protparam/>). SignalP 4.1 (<http://www.cbs.dtu.dk/services/SignalP/>)

and the TMHMM Server ver. 2.0 (<http://www.cbs.dtu.dk/services/TMHMM/>) were used to predict signal peptide and transmembrane regions, respectively. The amino acid sequences were aligned using ClustalW software, and by the software of MEGA 5.0, a dendrogram was constructed using the amino acid sequences from various organisms using the neighbor-joining method. The tree topology was evaluated by 1000 bootstrap replications.

Expression of *SpCCT* α in tissues

The total RNA extracted from each of ten tissues of adult *S. paramamosain* was reverse-transcribed with random primers into complementary DNAs and the expression of *SpCCT* α quantified by qPCR. For those sub-adult crabs subjected to stress of lower temperature or a combination of low temperature with different salinities, the time sequence of *SpCCT* α expression in the muscle dissected from chelipeds was also detected by the qPCR. In order to normalize the level of *SpCCT* α mRNA in the samples, the housekeeping gene 18S rRNA (GenBank ID FJ774906) with stable transcript abundance under diverse conditions was also amplified with the same cDNA samples using 18S rRNA F and 18S rRNA R (Table 1). Specific primers for the qPCR, *SpCCT* α -F3 and *SpCCT* α -R3 (Table 1), were designed according to the full-length sequence of *SpCCT* α to amplify the corresponding products. The qPCR was carried out in 20 μ l reaction volume containing 10 μ l of 2 \times SYBR Premix Ex Taq (TaKaRa), 0.8 μ l of each primer (10 μ M), 2 μ l of the diluted cDNA, and 6.4 μ l ddH₂O, and its shuttle PCR conditions were 30 s at 95 $^{\circ}$ C for one cycle, and 5 s at 95 $^{\circ}$ C, 30 s at 55 $^{\circ}$ C, and 30 s at 72 $^{\circ}$ C for 40 cycles.

1 GAGTACTGCGGCGAGGCGGCGGCGACAGGAAGCATCACTACCACGGAAGAAATTAGCTCGATTCCACCACA
 73 ATGACTGCCGAATCAGTGCTCAGTATCCAGGGCAAGCGTTCCCTCAGGGACGCGCTGTACGCCAGGAGAATGTTATTGCTGCCAGTTCAGTT
 M T A E S V L S I Q G K R S S G T P V R T E N V I A A S S V 30
 163 GCCAACATTGTCAAGAGCTCCCTGGGTCAGTTGGACTCGACAAAATGATTGTTGATGATGTCGGTGATGTGACAGTAACCAATGATGGA
 A N I V K S S L G P V G L D K M I V D D V G D V T V T N D G 60
 253 GCAACCATTCTAAAGCTCCTGGAGGTGGAGCACCAGCAGCTCGTGTACTGGTGGAGTTGGCACAGCTTCAGGATGAGGAGGTTGGTGAT
 A T I L K L L E V E H P A A R V L V E L A Q L Q D E E V G D 90
 343 GGCACAACCAGTGTGGTGATCATTGCTGCTGAGCTGCTCAAGTTGCTGATGAGCTTGTAAGCAGAAAAATCCATCCCACCTCCATCATT
 G T T S V V I I A A E L L K V A D E L V K Q K I H P T S I I 120
 433 GCTGGCTACAAAATTGGCTTGAAGGAAGCTTGTCCGTATATTCAGAACAACCTGACCATGAACACAGATGAGCTGGGCCGGGATTGTATT
 A G Y K L A C K E A C R Y I Q N N L T M N T D E L G R D C I 150
 523 GTTGTGCTGCCAAGACTTCCATGTCATCCAAGGTCATCTCCATTGACAGTGACTTCTTTGCCAACATGGTAGTAGATGCAGCCATGGCA
 V A A A K T S M S S K V I S I D S D F F A N M V V D A A M A 180
 613 GTGCGTACTTCTGATGGCAAGGGAGGTTATAAGTATCCCATCAAGGCTGTCAACATCCTCAAGGCACATGGCCGCTCTGCCACAGAGTCT
 V R T S D G K G G Y K Y P I K A V N I L K A H G R S A T E S 210
 703 GCAATCATCAACGGTTATGCCCTCAACTGTACCGTGGCATCACATGCAATGACCAAGAAGATCACCATGCAAAGATTGCCCTGCCTTGAC
 A I I N G Y A L N C T V A S H A M T K K I T N A K I A C L D 240
 793 TTCTCATTGAACAAGGAGAAAAATGAAGTTGGGAATTCAGGTCCTGGTGAATGATCCCGCCAAACTGGAGGCCATCCGGCAGCGTGAGGCA
 F S L N K E K M K L G I Q V L V N D P A K L E A I R Q R E A 270
 883 GATATCACAAAGGAACGTATTGAGAAAACTCTAAAGGCTGGTCAAAATGTAATCCTAACAACCTGGAGGAATTGATGACCTCTGCCTGAAG
 D I T K E R I E K I L K A G A N V I L T T G G I D D L C L K 300
 973 TACTTTGTGGAGGCTGGAGCAATGGCTGTCCGTCGCTGTAAGAAGTCAGACCTAAAGAGAATAGCCAAGGCAACAGGTGCGACATTCTTA
 Y F V E A G A M A V R R C K K S D L K R I A K A T G A T F L 330
 1063 ACAACACTGTGCAACCTTGAGGGAGAAGAATCCTTTGAACCCACTTACCTTGGCGAGGCAGCTGAGGTCACTCAGGAACGTGTGTGTGAT
 T T L C N L E G E E S F E P T Y L G E A A E V T Q E R V C D 360
 1153 GATGAGCTCATTCTCATTAAAGGGACAGTGCACGAACAGCCAGCTCTCTTATCCTTCGCGGTCCAATGATTTTTACTGCGATGAGATG
 D E L I L I K G T S A R T A S S L I L R G P N D F Y C D E M 390
 1243 GAACGTTCTCTGCATGATGCCCTGTGTGTAGTGAAGCGGGTCTGGAGAGCAGCTCCATTGTGTGGTGGAGGATCAGTGAAGCAGCA
 E R S L H D A L C V V K R V L E S S S I V C G G G S V E A A 420
 1333 CTGTCTGTCTACCTGAAAGCTTTGCCACCAGTCTTAGCTCTCGAGAACAACCTAGCAATTTGGGGCATTGTCTCAGTCTCTATTGGTCATT
 L S V Y L E S F A T S L S S R E Q L A I G A F A Q S L L V I 450
 1423 CCCAACACCCTGGCTGTCAATGCAGCTAAGGATGCAACTGACCTAGTTCCTCAAGCTTCGAGCATAACCACCTCATCCAGATCAAGACT
 P N T L A V N A A K D A T D L V P K L R A Y H H S S Q I K T 480
 1513 GAGCATTCCAGCTGAAGTATTATGGTTTGGATCTTATAGAAGGCCAGGTTCTGTACAACAAGAAAGCAGGTGCTTTGAACCAACTTTG
 E H S Q L K Y Y G L D L I E G Q V R D N K K A G V F E P T L 510
 1603 AGCAAACCTGAAGCAGCTCAAATTCGCCACAGAAGCTGCCATTACCATCCTGCGCATTGATGACATGATCAAGTTAGAACAGGAACCAAGC
 S K L K Q L K F A T E A A I T I L R I D D M I K L E Q E P S 540
 1693 AAGGAGAGTGGCTATCAGCAAGCTCTGAAATCAGGCCAGCTGGGTGAAGCTGTTAAATTACTGCTGAAAGATCTCATTTCATGGC
 K E S G Y Q Q A L K S G Q L G * 555
 1783 TATAGAACAGAAGCACAGAACAGAAAGCACTTAGCATTAAATATTGACTCTACAGGCATATTGACGTCAACATCTCCGTTAACAAGAT
 1873 ATATGGAATAATAAATTCCTTGAGTGAGCTATGTAATGTCAGCTTTGGTATGCTGTTTTTTTCTATTACTAAATAAATGCATGGCAA
 1963 AAAAAAAAAA 1972

◀ **Fig. 1** Nucleotide and deduced amino acid sequences of *SpCCTα* complementary DNA. The nucleotide sequence is numbered from the 5' end, and the single letter of amino acid code is shown below the corresponding codon. The *straight underline* represents initiation codon and termination codon, respectively. The *wavy line* under the nucleotides represents a presumable RNA instability motif (ATTTA) and the *boxed* part indicates polyadenylation signals in 3'-UTR. The shadow regions indicate the four highly conserved motifs, SLGPVG, TV/ITNDG, GDGTT, and VP/A/CGGG

Data analysis

Fold change for the gene expression relative to controls was decided by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). All quantitative values were expressed as means \pm standard deviation (SD). To meet the assumptions of ANOVA, all data were tested using Levene's homogeneity of variance test prior to the analysis. Differences in the expression levels of *SpCCTα* mRNA for the temperature treatments were analyzed using one-way analysis of variance (ANOVA) and post hoc Tukey's test used for multiple comparisons. For the expression levels of *SpCCTα* mRNA from the combined low temperature with salinity experiment, two-way ANOVA was used for analysis. All statistical analyses were performed using SPSS 16.0 (SPSS, Chicago, IL, USA). *P* values <0.05 were considered as level of significance.

Results

Cloning and sequence analysis of *SpCCTα* cDNA

The full length of *SpCCTα* cDNA that has been deposited in the GenBank database (accession no. KJ544667) was 1972 bp, containing a 5'-untranslated region (UTR) of 72 bp, a 3'-UTR of 232 bp with a canonical polyadenylation signal (AATAAA), a poly (A) tail and one destabilizing element (ATTTA), and an open reading frame (ORF) of 1668 bp encoding a polypeptide of 555 amino acids with a predicted molecular weight of 59.78 kDa and a theoretical isoelectric point (pI) of 6.16 (Fig. 1). Neither signal peptide nor transmembrane regions were found in the deduced amino acid sequence by SignalP 4.1 or the TMHMM Server ver. 2.0. Blast of the sequence with other CCTα amino acid sequence from several eukaryotes revealed that the high scoring amino acid identities were 79 % to *Locusta migratoria*, 73 % to *Sus scrofa*, 73 % to *Danio rerio*, and 72 % to *Callorhinchus milii*. In addition, several distinct function domains, including three characteristic ATP-binding motifs (SLGPVG, TV/ITNDG, and GDGTT) and an

invariable motif (VXPGGG), were identified in the deduced amino acid sequence of *SpCCTα* (Fig. 1).

Phylogenetic analysis of the *SpCCTα* protein

A phylogenetic tree was constructed (Fig. 2) to demonstrate cluster relationships based on the similarity of different CCT subunits of various organisms, including *Scylla paramamosain*, *Acromyrmex echinator*, *Camponotus floridanus*, *Apis cerana*, *Anoplophora glabripennis*, *Culex quinquefasciatus*, *Harpegnathos saltator*, *Locusta migratoria*, *Ceratitis capitata*, *Nasonia vitripennis*, *Delia antique*, and *Escherichia coli*. The dendrogram showed the order of the pairwise alignments of sequences and clusters that generated the final multiple sequence alignment together. For the construction of dendrogram, *Escherichia coli* GroEL was chosen as an outgroup, and the result indicated that *SpCCTα* belonged to the CCT family. The amino acids of CCT were highly homologous in the same subunit across species lines, but weakly isogenous among different subunits of a same species. Furthermore, the CCT subunits clearly differed from the GroEL of *E. coli*.

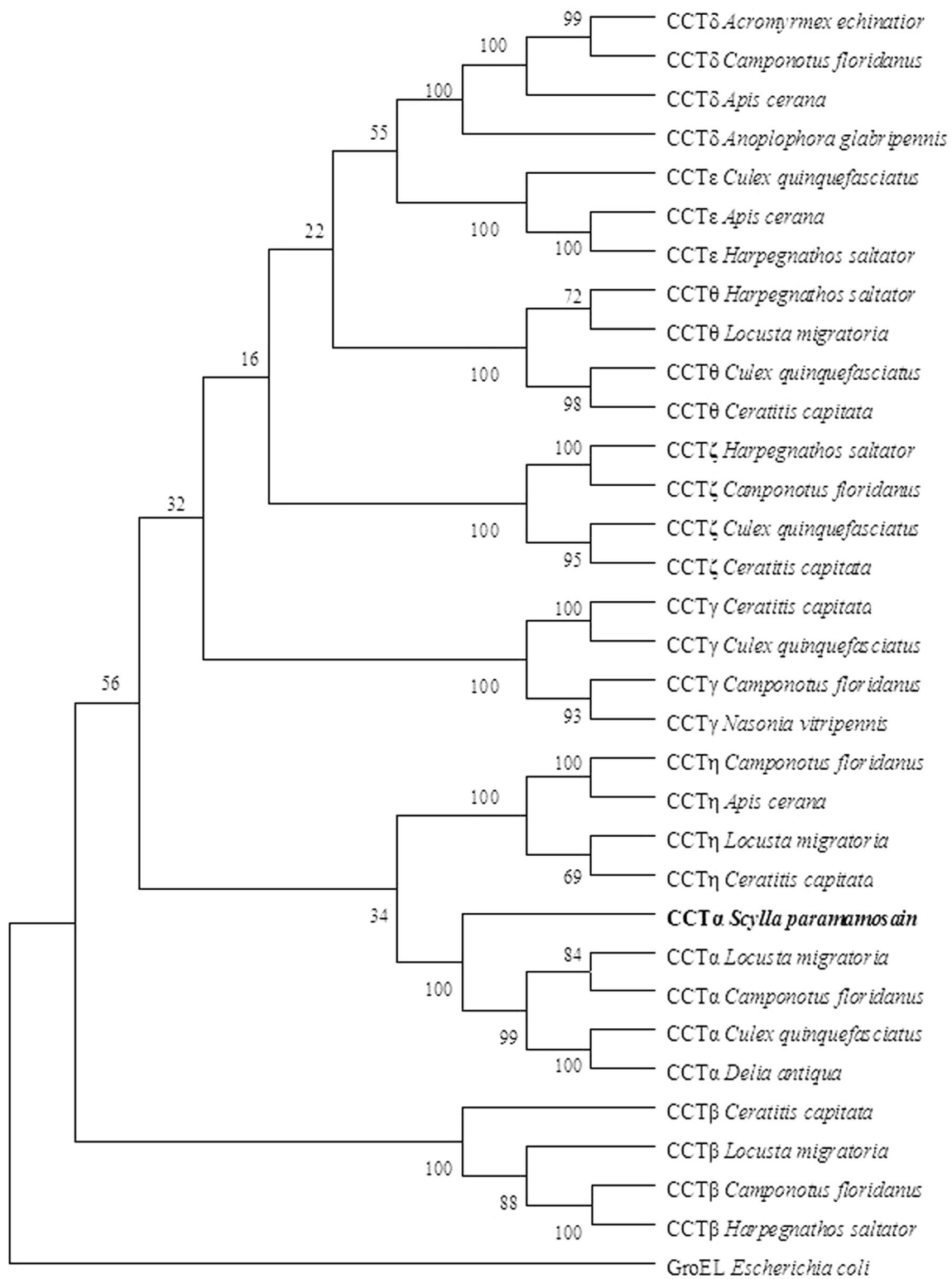
Tissue distribution of *SpCCTα*

qPCR was employed to investigate the distribution of *SpCCTα* mRNA in different tissues of *S. paramamosain* with 18S rRNA as the internal control. The result indicated that albeit with different expression levels, the *SpCCTα* transcript was ubiquitously detectable in all tissues examined, including heart, epidermis, eyestalk ganglion, stomach, cerebral ganglion, hemocyte, thoracic ganglion, hepatopancreas, gill, and muscle. Among these tissues, the highest level of *SpCCTα* transcript was detected in muscle, which was followed by the level found in gill and hepatopancreas. In contrast, substantially lower *SpCCTα* expression was detected in epidermis, eyestalk ganglion, cerebral ganglion, and stomach, respectively (Fig. 3).

SpCCTα expression in muscle following exposure to lower temperatures

All sub-adult crabs exposed to 5 °C died within 12 h, and the treatment was hence excluded from data presentation and analysis.

Among the remaining four temperature treatments, it was shown that temperature significantly influenced the expression profile of *SpCCTα* in the muscle of *S. paramamosain* (Fig. 4). When the crabs were exposed to the lowest temperature tested (10 °C), the mRNA expression of *SpCCTα* were consistently the highest



throughout the 48-h monitoring period while the second highest expression was found when the crabs were exposed to the second lowest temperature of 15 °C. The

*Sp*CCT α expression was significantly higher at 10 °C than that of the 15 °C treatment except at 48 h. Meanwhile, in most cases, the *Sp*CCT α expressions were

Fig. 2 Phylogenetic tree of *SpCCTα* protein with different CCT subunits from various organisms. Accession numbers are *Scylla paramamosain* (CCTα, KJ544667), *Acromyrmex echinator* (CCTδ, EGI63449.1), *Camponotus floridanus* (CCTα, EFN66362.1; CCTβ, EFN68281.1; CCTγ, EFN71709.1; CCTδ, EFN62007.1; CCTζ, EFN68439.1; CCTη, EFN60224.1), *Apis cerana* (CCTδ, AEY59704.1; CCTε, AEY59685.1; CCTη, AEY60160.1), *Anoplophora glabripennis* (CCTδ, JAB64915.1), *Culex quinquefasciatus* (CCTα, XP_001845710.1; CCTγ, EDS34034.1; CCTε, EDS27370.1; CCTζ, EDS39347.1; CCTθ, EDS26751.1), *Harpegnathos saltator* (CCTβ, EFN82147.1; CCTε, EFN84860.1; CCTζ, EFN82934.1; CCTθ, EFN84736.1), *Locusta migratoria* (CCTα, AHB33468.1; CCTβ, AHB33469.1; CCTη, AHB33465.1; CCTθ, AHB33466.1), *Ceratitis capitata* (CCTβ, JAB90528.1; CCTγ, JAB92150.1; CCTζ, JAC03309.1; CCTη, JAB97065.1; CCTθ, JAB90029.1), *Nasonia vitripennis* (CCTγ, NP_001177859.1), *Delia antiqua* (CCTα, BAE17115.1), and *Escherichia coli* (GroEL, AAS75782.1)

significantly higher under 10 and 15 °C as compared to those of 20 and 25 °C ($P < 0.05$). The crabs exposed to 20 and 25 °C had the lower *SpCCTα* expressions that were often not significantly different ($P > 0.05$) (Fig. 4).

SpCCTα expression in muscle following exposure to combined low temperatures with high and low salinity

A two-way ANOVA revealed a significant interaction between temperature and salinity. An overall pattern of *SpCCTα* expression in the muscle of the crabs was that under both 10 and 15 °C, significantly higher levels were detected when the crabs were simultaneously subjected to the low salinity (10) than to the high salinity (35) at 1, 3, 6, and 12 h although this appeared to fade off at 24 h and beyond ($P < 0.05$) (Fig. 5). Meanwhile, under the same salinity of either 35 or 10, the expression of *SpCCTα* was consistently significantly higher when challenged by the lower temperature of 10 °C when compared to that of

15 °C for all sampling times except at 6 h for the low salinity of 10 ($P < 0.05$) (Fig. 5).

Discussion

In the present study, a molecular chaperonin gene, the subunit of CCT complex, CCTα, was cloned from the mud crab *S. paramamosain*. Chaperonins, as a class of molecular chaperones, are a widely distributed group of protein subsets (Wang et al. 2004). While various molecular chaperones, including HSP60 and HSP70, have been elucidated for *S. paramamosain* (Yang et al. 2013a, b), the role of cytosolic HSP60 family, especially chaperonin containing the T-complex polypeptide-1 (CCT), has not yet been documented for *S. paramamosain*. Up to date, most CCTs documented are from mammals or typical model organisms (Dekker et al. 2008), and they have been manifested to play essential roles in various biological functions, including immunity, growth, reproduction, and responses to osmotic stress by assisting other significant proteins to fold and assemble properly (Jiang et al. 2000; Yamada et al. 2002; Arockiaraj et al. 2012).

The full-length cDNA of CCTα, a subunit of CCT complex, was obtained from *S. paramamosain* by this study. The 1972-bp full-length cDNA, which contains a 1668-bp ORF encoding a polypeptide of 555 amino acids, was named *SpCCTα* gene, as the amino acid sequence deduced from it shared high levels of identity to CCTα of *L. migratoria*, *S. scrofa*, *D. rerio*, and *C. millii*. Furthermore, protein signature sequence search by various tools revealed that the sequence of *SpCCTα* gene had all characteristic features of CCTα gene. In particular, the four highly conserved motifs presented ubiquitously in CCTα (SLGPVG, TV/ITNDC, GDGTT, and

Fig. 3 Mean (\pm standard error) expression levels of *SpCCTα* detected by qPCR from different tissues of *S. paramamosain* ($n=4$)

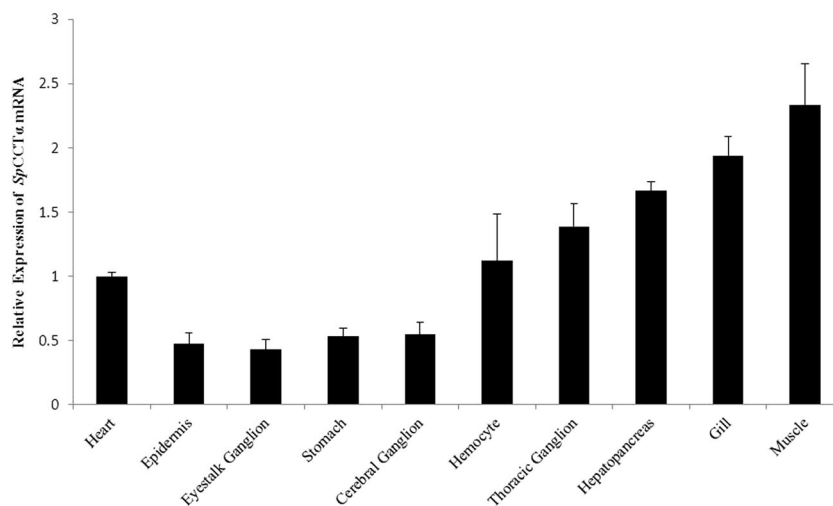
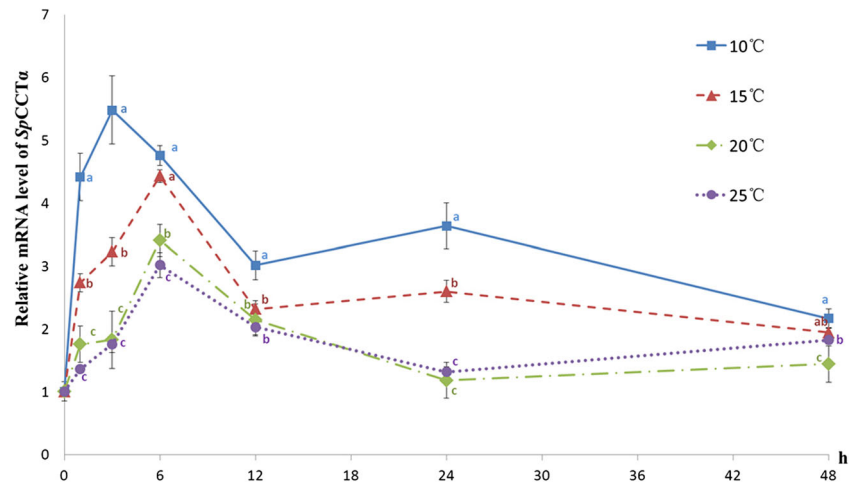


Fig. 4 Time sequence of relative mRNA transcript levels of *SpCCT α* in muscle of sub-adult *S. paramamosain* following low temperature challenges. Data are presented as mean \pm standard error ($n=3$). Different letters for data points at a same sampling time indicate significant differences ($P<0.05$)



VXPGGG) were identified with the first three being confirmed to participate mainly in ATP binding and ATP hydrolysis (Kayukawa et al. 2005) while the other motif, VXPGGG, is an invariable motif believed to be involved in protein interactions between CCT and the substrates (Palmedo and Ammermann 1997). Eukaryotic CCT α is known to consist of three domains: (1) the equatorial domain that connects N- and C-end residues of the protein and contains the ATP-binding site, (2) the apical domain that binds the peptides and formed by the central part of the protein, and (3) the intermediate domain that links the apical and equatorial domain (Muñoz et al. 2011). By analyzing the functional region of only 218 amino acids of CCT α from *E. coli*, the apical domain was demonstrated to be essential for osmotolerance and showed chaperone activity (Yamada et al. 2002). The phylogenetic analysis of *SpCCT α* with CCT subunits from various organisms revealed that amino acids are highly conserved in the same subunit across species lines, but weakly conserved in the different subunits of a same species, suggesting that it is associated with the

binding of different subunits with different polypeptides of the target proteins (Spiess et al. 2006).

The examination of the relative mRNA expression of *SpCCT α* in ten different tissues of *S. paramamosain* showed that the *SpCCT α* mRNA was expressed in all these tissues, but the highest expression level was detected in the muscle. In vertebrates, the CCT ζ -2 gene was confirmed to only show high expression in the testis of the mouse (Kubota et al. 1997); similarly, CCT β was found with overexpression in human testis (Jiang et al. 2000), both pointing to likely involvement of the CCT complex in reproduction (Dun et al. 2012). On the other hand, the gene expression of *MrChap* in the giant freshwater prawn *M. rosenbergii* was found to be the highest in the hemocytes, which was interpreted by the role that *MrChap* played in immune response (Arockiaraj et al. 2012). These results from previous research indicate tissue-specific function of the CCT complex.

Abiotic stresses, such as fluctuations of temperature and salinity, chemical reagent and environmental contaminants as well as drought stress, are serious menaces not only to the natural populations of marine organisms but also for

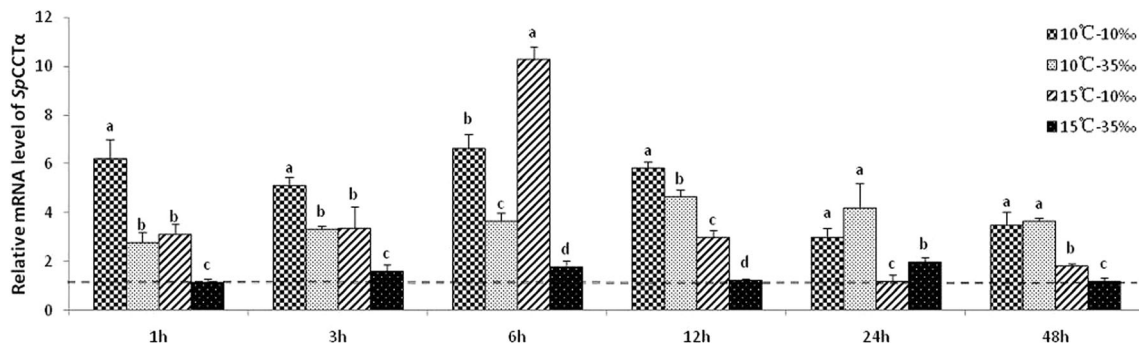


Fig. 5 Time sequence of relative mRNA transcript levels of *SpCCT α* in muscle of sub-adult *S. paramamosain* following exposure to low temperatures (10 and 15 °C) in combination with both high (35) and low salinity (10). Data are presented as mean \pm standard error ($n=3$).

Different letters for data points at a same sampling time indicate significant differences ($P<0.05$). The dotted horizontal line represents the level of *SpCCT α* expression prior to the challenge

aquaculture animals under confined conditions (Tomanek 2011). Stress response mechanisms evolved among marine organisms allow them to cope with sharp fluctuations in various abiotic factors (Paital and Chainy 2010; McGaw and Whiteley 2012). As a molecular chaperone that could assist in protein refolding under stress, the CCT complex is believed to be a key component contributing to cellular homeostasis under adverse conditions (Shimon et al. 2008). For example, CCT subunit was reportedly up-regulated in mammalian cell lines during recovery from chemical stress of sodium arsenite exposure (Yokota et al. 2000b). Similarly, CCT expression at the mRNA level was reportedly significantly improved during cell growth, especially from G1/S transition to early S phase, in the cultured cells of the mouse (Kubota et al. 1999).

Based on the result that *SpCCT α* expression was highest in the muscle among various tissues for the mud crabs *S. paramamosain*, the *SpCCT α* mRNA levels in muscle tissue were measured after sub-adult *S. paramamosain* were challenged by different lower temperatures than they have been acclimated to. The results showed significant increases in the amount of the transcript following exposure to 10 °C as compared to 25, 20, and 15 °C while exposure to 15 °C also led to mostly significantly higher levels than those of 25 and 20 °C. These results indicated that the *SpCCT α* gene was connected with the cold hardiness of *S. paramamosain*. Similarly, previous research have reported that CCT α and CCT β increased 3- to 4-fold after cold shock in the yeast *S. cerevisiae* (Somer et al. 2002) while the level of DaTCP-1 gene, a subunit of chaperonin CCT, was up-regulated in association with increased cold hardiness in *D. antiqua* (Kayukawa et al. 2005).

Recently, it was also reported that actin depolymerizing factor (ADF) was up-regulated during cold stress in the whiteleg shrimp *L. vannamei* (Fan et al. 2013). Meanwhile, it has been demonstrated that if the actin depolymerization was inhibited by the chemical reagent Latrunculin B, the subsequent cold hardiness of pupae of *D. antiqua* was compromised (Kayukawa and Ishikawa 2009). Both actin and tubulin are believed to engender depolymerization at low temperature when cytoskeleton is subjected to crumbling, which is a significant element for the occurrence of cold injuries (Upadhyya and Strasberg 1999; Kayukawa et al. 2005). Since actin and tubulin, the cytoskeletal proteins, are major substrates of the CCT complex in vivo (Quintá et al. 2011), the significant increases in CCT α expression in *S. paramamosain* exposed to low temperature of 10 °C observed in this study might be interpreted as that the *SpCCT α* gene was involved in the recovery of cytoskeleton damaged by cold stress. The mud crab *S. paramamosain* is a species with a suitable temperature range between 18 and 30 °C (Shelley and Lovatelli 2012). The biological zero, a theoretical temperature value below which metabolic processes are supposed to be negligible, for the embryonic development

of the species was estimated to be 12.19 °C (Hamasaki 2002). It was further reported that for the species at 10 °C, abnormal cell division during embryonic development occurred while at 15 °C, the initial cell division was normal but embryonic development was retarded at the gastrula stage (Zeng 2007). Therefore, 10 °C clearly represents severe thermal stress to the species, and when exposed to such a temperature, *S. paramamosain* sub-adults were likely subjected to detrimental effects of collapse of cytoskeleton and also degeneration of other proteins. The up-regulation of *SpCCT α* gene was hence likely to aid in the recovery of damaged cytoskeleton and refolding and assembling the damaged proteins. On the other hand, 20 and 25 °C are still within the suitable temperature range for the species, hence the expressions of *SpCCT α* under these temperatures were more subdued.

It has been reported that in non-hardy pupae of *D. antiqua*, cold-induced depolymerization of actin occurred while cold-hardy pupae did not, and the latter was closely related to enhanced transcript level of complex CCT in the cold-hardy larvae, demonstrating CCT could stabilize actin at low temperature (Kayukawa and Ishikawa 2009). It was also reported that plant CCT α from the mangrove tree *Bruguiera sexangula* showed a vital character related to salt- and osmotic-stress tolerance (Yamada et al. 2002). On this basis, a further experiment was conducted to investigate whether salinity might interact with low temperatures to affect the expression of *SpCCT α* in *S. paramamosain*. The results confirmed significant interactive effects between the two abiotic factors of temperature and salinity which often act in concert in marine environments. A clear pattern was shown that the expressions of *SpCCT α* under the high salinity (35) condition were generally significantly lower than those under the low salinity (10) condition when the crabs were exposed to both 10 and 15 °C. Low temperature exposure not only can lead to collapse in cellular structure and cell division but also likely to result in enhanced metabolism with increased oxygen consumption to meet higher demands for energy. The increased oxygen consumption in turn is likely to result in enhanced production of reactive oxygen species (ROS), which could cause substantial harm to the cells (Paital and Chainy 2010). Under low salinity conditions, the need to actively pump ions from the environment to the hemolymph to maintain osmotic balance requires high energy, which is likely to lead to further increased oxygen consumption, and this may help explain the significantly higher levels of *SpCCT α* expression detected under such condition as the synthesis of chaperones might have to be elevated to repair more severe cellular damages under the low salinity condition. In fact, it was reported that acute salinity drop could have severe negative impacts on both the swimming crab

Portunus trituberculatus (Lu et al. 2013) and the blue swimmer crab *Portunus pelagicus* (Romano and Zeng 2006).

On the contrary, as salinity increased, the rates of energy expenditure of crustaceans reportedly decreased, indicated by lower metabolic indicators, such as decreased ammonia-N excretion and respiration, in another mud crab species *Scylla serrata* (Chen and Chia 1996) and the whiteleg shrimp *L. vannamei* (Silvia et al. 2004). In fact, past studies have shown that acclimation to high salinity improved the ability of the copepod *Tigriopus brevicornis* to survive in frozen seawater (McAllen and Block 1997). Furthermore, an experiment assessed the combined effects of temperature and salinity on larval survival of the mud crab *S. serrata* found that while both high and low temperature led to mass mortality of newly hatched larvae at low salinities, a combination of a lower temperature of 25 °C with a high salinity of 35 actually resulted in one of the highest survival of the larvae to the postlarval megalopal stage (Nurdiani and Zeng 2007). Since it is clear that salinity could interact with the temperature to modify the impacts of temperature on aquatic organisms (Likongwe et al. 1996), better understanding of its underlying molecular mechanisms is clearly important for both natural resource management and the definition of suitable culture condition for the commercially important species, such as *S. paramamosain*.

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