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ORIGINAL ARTICLE

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Mud crab *Scylla paramamosain* glutamate dehydrogenase: molecular cloning, tissue expression and response to hyposmotic stress

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Abstract Glutamate dehydrogenase (GDH) is an important enzyme for the metabolism of glycine, proline and alanine, which are general osmolytes in aquatic animals. To explore the possible relationship between *GDH* expression and osmoregulation under hyposmotic stress, cDNA cloning of *GDH* and its expression, as well as free amino acid analysis in *Scylla paramamosain,* were investigated. *GDH* cDNA was first isolated from *S. paramamosain* and the 2269 bp sequence consisted of a 1622 bp open reading frame encoding 533 amino acids. *GDH* was predominantly expressed in muscle, which is a pool of amino acids. Under acute transfer from a salinity of 28 to 5 ppt, the haemolymph osmolality was significantly decreased at 6 h, and the expression of *GDH* in the muscle dropped sharply to 29.6% as compared to the control at 6 h ($P < 0.05$). Accordingly, the total free amino acid concentration decreased significantly in the muscle; this included glutamic acid, glycine, proline, glutamine and arginine, accounting for a large proportion of the total free amino acids ($P < 0.05$). In contrast, concentrations of total free amino acids in the haemolymph increased, partially compensating for a sharp reduction in extracellular fluid osmolality under hyposmotic stress, effectively maintaining the balance between extra- and intracellular osmolality. It was concluded that *GDH* plays an important role in controlling osmoregulation in *S. paramamosain*.

Keywords Glutamate dehydrogenase · *Scylla paramamosain* · Free amino acids · Osmoregulation · Hyposmotic stress

Introduction

Salinity is an important ecological factor that influences the distribution, abundance and general physiology of aquatic animals [1]. As a result, changes in environmental salinity are directly related to the osmoregulation capacity of these animals. Osmoregulation is an essential physiological process for aquatic animals to cope with the discrepancies between the ion concentrations within their bodies and environments and maintain normal life activities [1]. Most aquatic animals with appropriate osmoregulatory mechanisms can freely adapt to their habitats [2]. As the predominant aquatic animal, crustaceans can dwell in seawater and fresh water due to their various osmoregulatory mechanisms. The main two forms of osmoregulatory mechanisms are anisosmotic extracellular regulation (AER) and intracellular isosmotic regulation (IIR) [3]. AER maintains the osmolality of the body fluid within limits mainly by the regulation of gills and antennal glands, while IIR can control intracellular osmolality and maintain the balance between tissues and the haemolymph primarily by regulating non-essential amino acids (function as osmolytes)

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[4]. Under excessive changes in environmental salinity, it is difficult for body fluid osmolality to be maintained at a completely stable level by AER. At that point, it is believed IIR compensates for or supports AER by accumulating or degrading quantities of particular non-essential free amino acids as osmolytes, effectivley moderating fluctuations in extracellular osmolality and facilitating volume readjustment [4].

Free amino acids play a vital role in determining cell volume and osmotic adjustments of many organisms, including bacteria [5], algae and plants [6], and differ slightly among different species. In crustaceans, free amino acids accumulate in response to hyperosmotic stress. A number of non-essential amino acids, including taurine, proline, glutamic acid, alanine and glycine, are generally utilized as osmoeffectors in crustaceans [4, 7, 8]. Glutamate, a precursor to proline, is considered a major regulatory checkpoint in the synthesis of proline for osmoregulation [9].

It has been reported that activities of vital enzymes in the synthesis of free amino acids can be up-regulated by intracellular ions and can lead to accumulation of free amino acids in high salinity [10]. Glutamate dehydrogenase (GDH, EC 1. 4. 1. 2) is believed to be a potential control factor for the synthesis of free amino acids. Glutamate is synthesized from α-ketoglutarate via GDH. Proline and glycine are synthesized from glutamate while alanine is synthesized from pyruvate during transamination of glutamate [9]. An increase in glutamate production via GDH could enhance the synthesis of glycine, proline and alanine. The role of *GDH* in osmoregulation has been investigated via amino acid metabolism in various crustaceans [11, 12]. Two *GDH* cDNAs (EU496492, AM076955) have been characterized in whiteleg shrimp *Litopenaeus vannamei* and they are mainly expressed in muscle [13]. Under hyperosmotic stress, *GHD* shows an increased expression in the muscle of the freshwater Chinese mitten crab *Eriocheir sinensis* [14]. However, our understanding of transcriptional regulation of *GDH* in crustaceans is limited and the specific molecular mechanisms of *GDH* for osmoregulation in crustaceans are not clear.

The mud crab *Scylla paramamosain* is abundant in estuaries and coastal waters throughout the Indo-Pacific and Indian Ocean regions and can survive within a wide range of salinities [15]. It is a commercially important species farmed in in the Asian region. Throughout their prolonged culture period, the salinity of aquaculture farms often decreases rapidly with abiotic factors such as heavy rainfalls and inflow of freshwater. The drastic salinity changes may have significant impacts upon farm productivity and, in severe conditions, result in high crustacean mortality [1, 16]. Thus, information on the osmoregulation to hyposmotic environmental salinity stress is required as a fundamental component of aquaculture. We conducted this study to verify the hypothesis that *GDH* is involved in osmoregulation under hyposmotic stress via an analysis of *GDH* expression and the free amino acid concentration in *S. paramamosain*.

Materials and methods

Animals

Healthy female *S. paramamosain* weighing 160 ± 20 g (mean ± SD) were obtained from local vendors in Hangzhou Bay of Zhejiang province, China. Prior to the experiment, crabs were acclimated for 1 week in large, rectangular holding tanks with running seawater with a salinity of approximately 28 ppt at 25 ± 0.4 °C. During the acclimatization, crabs were fed fresh manila clam *Ruditapes philippinarum* every day until 24 h before the experiment.

Methods

After one week, individuals in stage C of the intermolt cycle were selected as experimental crabs according to the method described by Imayavaramban, with modifications [17]. The basal endite of the second maxilla was dissected, placed on a glass slide in a drop of clean water, and then observed under 40× magnification using a microscope (Model E100; Nikon, Tokyo, Japan). Experimental crabs were divided into four groups using a completely randomized design. For the salinity transfers, one group of high salinity (28 ppt)-acclimated crabs (90 crabs) were directly transferred to dilute 5 ppt sea water in 120 l, water-jacketed, recirculating tanks. Muscles and haemolymph were sampled at 0, 6, 12, 24, 48 and 72 h, respectively. The other three groups of high salinity (28 ppt)-acclimated crabs (15 crabs per group) were transferred to recirculating tanks with a salinity of 28 (as control), 21 and 14 ppt. Muscles and haemolymph were collected at 24 h, in accordance with previous studies [18, 19].

The haemolymph samples were withdrawn by puncturing the arthrodial membrane at the base of the swimming pereiopods with a 27-syringe. Haemolymph osmolality was then measured in 10 μl samples using a vapor pressure osmometer (Wescor 5600, Logan, UT, USA) [20]. Crabs were anesthetized by being frozen on ice before sacrifice. Gills were extracted for the *GDH* cDNA clone. Muscle, hepatopancreas, gills, haemocytes, hearts, intestines and antennal glands were sampled for tissue-specific expression of *GDH*. Claw muscle was sampled for *GDH* expression. Muscle and haemolypmh were sampled for free amino acid analysis. All target tissues were extracted separately, frozen in liquid nitrogen and stored at −80 °C until analysis.

Total RNA extraction and cDNA synthesis

Total RNA extraction was carried out according to the protocol provided with a Unizol Reagent Kit (Invitrogen, Carlsbad, CA, USA), quantified by spectrophotometry (Nanodrop 2000) and agarose-gel electrophoresis. Total RNA was reverse transcribed using a PrimeScript® RT reagent Kit (TaKaRa, Kyoto, Japan). The volume of each reaction component was as follows: 0.5 μl of oligo dT primer (50 μM); 0.5 μl of PrimeScript® enzyme mix; 0.5 μl of random 6mers (100 μM); 2 μl of 5 × PrimeScript® buffer; less than 500 ng of total RNA; and up to 10 μl of RNase free dH_2O. The reaction was conducted at 37 °C for 15 min and 85 °C for 5 s. RACE-Ready cDNA was synthesized from total RNA using a SMARTer™ RACE cDNA Amplification Kit (TaKaRa). The reaction conditions and components were performed according to the manufacturer's instructions.

Full-length *GDH* cDNA clone and sequence analysis

Based on the homologous gene cloning strategy, the primers *GDH*-F and *GDH*-R (Table 1) were designed to amplify a cDNA fragment based on the alignment of *GDH* sequences from other crustacean species. Polymerase chain reactions (PCRs) were performed as follows: 30 cycles of denaturation at 94 °C for 30 s, annealing at 52 °C for 30 s, elongation at 72 °C for 1 min, followed by a 10 min extension at 72 °C and cooling to 4 °C. The full-length cDNA sequence of the *GDH* was obtained by using the SMART™ RACE cDNA amplification kit (TaKaRa). Four gene-specific primers, *GDH*-5′ inner, *GDH*-5′ outer, *GDH*-3′ inner and *GDH*-3′ outer (Table 1), were designed based on the cDNA fragment to obtain the complete cDNA sequence. Reaction conditions were according to the manufacturer's recommendations. PCR fragments were subjected to electrophoresis on a 1% agarose gel to determine length difference and then cloned into the pMD-19T vector (TaKaRa). After transformation into the competent cells of *Escherichia coli* DH5α (TaKaRa), recombinant bacteria were identified by blue/white screening and confirmed by PCR. Three of the positive clones were sequenced in both directions, and these resulting sequences were verified and subjected to cluster analysis in the National Center for Biotechnology Information (NCBI) database.

Table 1 Primers designed for cloning and expression analysis of *GDH*

Primers	Sequences (5′ → 3′)
GDH-F	CCCMGSRACTACTCCATCAA
GDH-R	CCGCTCAAGAGACTCCTGAA
GDH-5′*GDH* outer	TGATGCCAATCATAGCCATGTAGGAGGCC
GDH-5′*GDH* inner	CACGACATCTCTCGCTCCCCGGTTCCCAA
GDH-3′*GDH* outer	CCTACATGGCTATGATTGGCATCACTCC
GDH-3′*GDH* inner	CACCTGCATTGGCGTCAAGGAAGTGGAC
GDH-RTF	GTACATTCCAGACGCCGAAG
GDH-RTR	CGCTCCTTCATCTCCTCCAC
β-actin-RTF	GCCCTTCCTCACGCTATCC
β-actin-RTR	GCGGCAGTGGTCATCTCC

Sequences were edited by SeqMan software and then transferred to the corresponding amino acid sequence by EdiSeq. Homology nucleotide and protein sequences were searched using the BLAST algorithm at the National Center for Biotechnology Information (http://www.ncbi.nlm.gov/blast). The signal peptide was determined using the Signal P 4.1 programme (http://www.cbs.dtu.dk/services.SignalP/). The potential active site was predicted using ScanProsite (http://prosite.expasy.org/scanprosite/). The potential phosphorylation site of the deduced amino acids was predicted using NetPhos2.0 (http://www.cbs.dtu.dk/services/NetPhos/). MEGA version 4.0 was used to align the *GDH* amino acid sequences of *S. paramamosain* with other species from the NCBI Gen-Bank and construct the phylogenetic tree via neighbor-joining.

Quantitative real-time PCR (qPCR) analysis

The expression of *GDH* in target tissues was detected via quantitative real-time PCR (qRT-PCR). A pair of gene-specific primers (*GDH*-RTF, *GDH*-RTR) were designed according to the target gene sequence; *β*-actin was used as the internal standard gene control (Table 1). SYBR Green RT-PCR was carried out using the StepOnePlus™ Real-Time PCR System. Each individual was run in triplicate along with the control gene, *β*-actin. *GDH* expression levels were calculated using the equation $2^{-\Delta\Delta Ct}$. The reaction was performed in a 20 μl mixture containing 2 μl of diluted cDNA template, 0.4 μl of each forward and reverse primer (10 μM), 0.4 μl of ROX reference dye (50×), 10 μl of SYBR Green Premix Ex Taq™ (2×) (TaKaRa) and 6.8 μl of dH_2O. The PCR conditions were as follows: 95 °C for 30 s; 40 cycles of 95 °C for 5 s; 60 °C for 30 s; 95 °C for 15 s; 60 °C for 60 s; 95 °C for 15 s. The specificity of PCR amplification for each primer pair was represented by a melting curve constructed after the PCR amplification.

Muscle and haemolymph free amino acids analysis

Muscle samples weighing approximately 0.2 g and haemolymph samples weighing about 100 μl were taken from crabs in each treatment group and fully homogenized in an ultrasonic cell disruption system by adding 8% sulfosalicylic acid with a quality volume (w/v) ratio of 1:2. The homogenate was centrifuged at 12,000 rad/min for 20 min

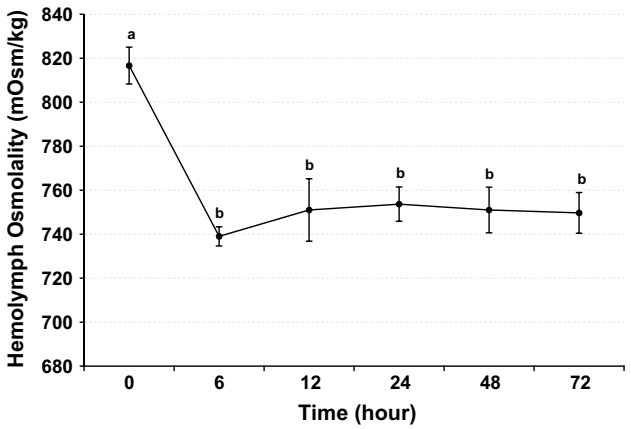

Fig. 1 Haemolymph osmolality in *Scylla paramamosain* following acute transferred from 28 to 5 ppt. *Bars* correspond to the SEM of the average value, *different letters* indicate significant difference ($n = 7$, $P < 0.05$)

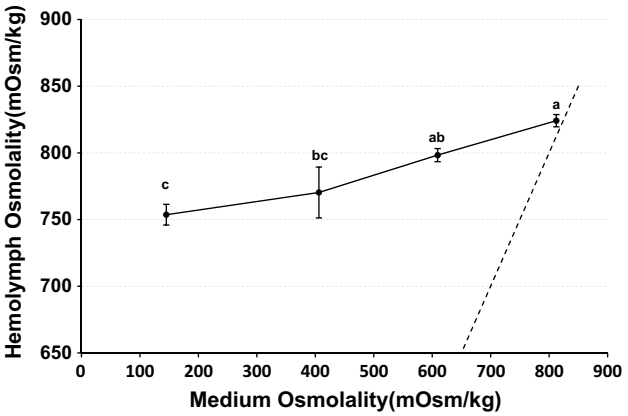

Fig. 2 Haemolymph osmolality in *Scylla paramamosain* acclimated to 28 ppt, transferred to 5, 14, 21 and 28 ppt for 24 h, respectively. The *broken line* represent the medium osmolality of each treatment. *Bars* correspond to the SEM of the average value, *different letters* indicate a significant difference ($n = 7$, $P < 0.05$)

to precipitate protein and cellular debris. The supernatants were quickly transferred to fresh Eppendorf tubes and filtered using a 0.22-μm filter membrane. The diluted samples (volume ratio of 1:10) were then analyzed using an L-8900 Amino Acid Analyzer (Hitachi, Japan).

Data analysis

All data were analyzed using SPSS software (Ver16.0) and presented as mean ±SEM. The amount of *GDH* mRNA was normalized to that of *β*-actin mRNA. Differences in *GDH* expression among tissues were analyzed by one-way ANOVA. Statistical significance was set at $P < 0.05$.

Results

Haemolymph osmolality

To confirm the osmoregulatory conditions in *S. paramamosain*, haemolymph osmolality was analyzed. The results show that when crabs are rapid transferred from a salinity of 28 ppt to 5 ppt, the haemolymph osmolality drops sharply at 6 h and then stabilizes at 751.33 mOsm/kg after 12 h (Fig. 1). In salinity gradient treatments, the mean haemolymph osmolality of crabs in the control (28 ppt) was 824.2 mOsm/kg. When the salinity was changed from 28 ppt to 21, 14 and 5 ppt, the mean haemolymph osmolality was 798.3, 770.3 and 754 mOsm/kg, respectively (Fig. 2).

Characterization of *GDH* cDNA from *S. paramamosain*

The full-length *GDH* cDNA sequence of *S. paramamosain* was 2,269 bp, containing a 1,662 bp open reading frame (ORF) encoding a 553 amino acid protein, with a 37 bp 5′UTR and a 570 bp 3′UTR (Online Resource 1); it was deposited in GenBank as KM189809. The calculated molecular weight of the deduced protein was 61.538 kDa with a theoretical isoelectric point of 6.859. The deduced protein included a 17 amino acid signal peptide, 37 potential phosphorylation sites, one dehydrogenase family signatures, one Glu/Leu/Phe/Val dehydrogenases active site and three putative N-glycosylation sites.

Sequence alignments of the *GDH* amino acids showed the identities of 93, 92, 91 and 89% to that of the *E. sinensis*, *L. vannamei GDH* b, *Fenneropenaeus chinensis* and *L. vannamei GDH* a, respectively (Fig. 3). A phylogenetic tree was constructed by the neighbor-joining method (Fig. 4) by using *GDH* homologs of *S. paramamosain* and other species. *S. paramamosain* showed a close evolutional relationship with *E. sinensis*, *L. vannamei* and *F. chinensis*.

Tissue expression of *GDH* in *S. paramamosain*

GDH cDNA transcripts relative to *β*-actin in different tissues from *S. paramamosain* are shown in Fig. 5. The expression of *GDH* was significantly higher in muscle than in other tissues ($P < 0.05$). The second highest *GDH* expression value was detected in the hepatopancreas. No significant differences were detected among the intestine, heart, antennal gland and gill. The lowest *GDH* expression value was found in the haemocytes.

GDH expression in response to hyposmotic challenges

The above results showed *GDH* was expressed predominantly in the muscle, which is a pool of amino acids that

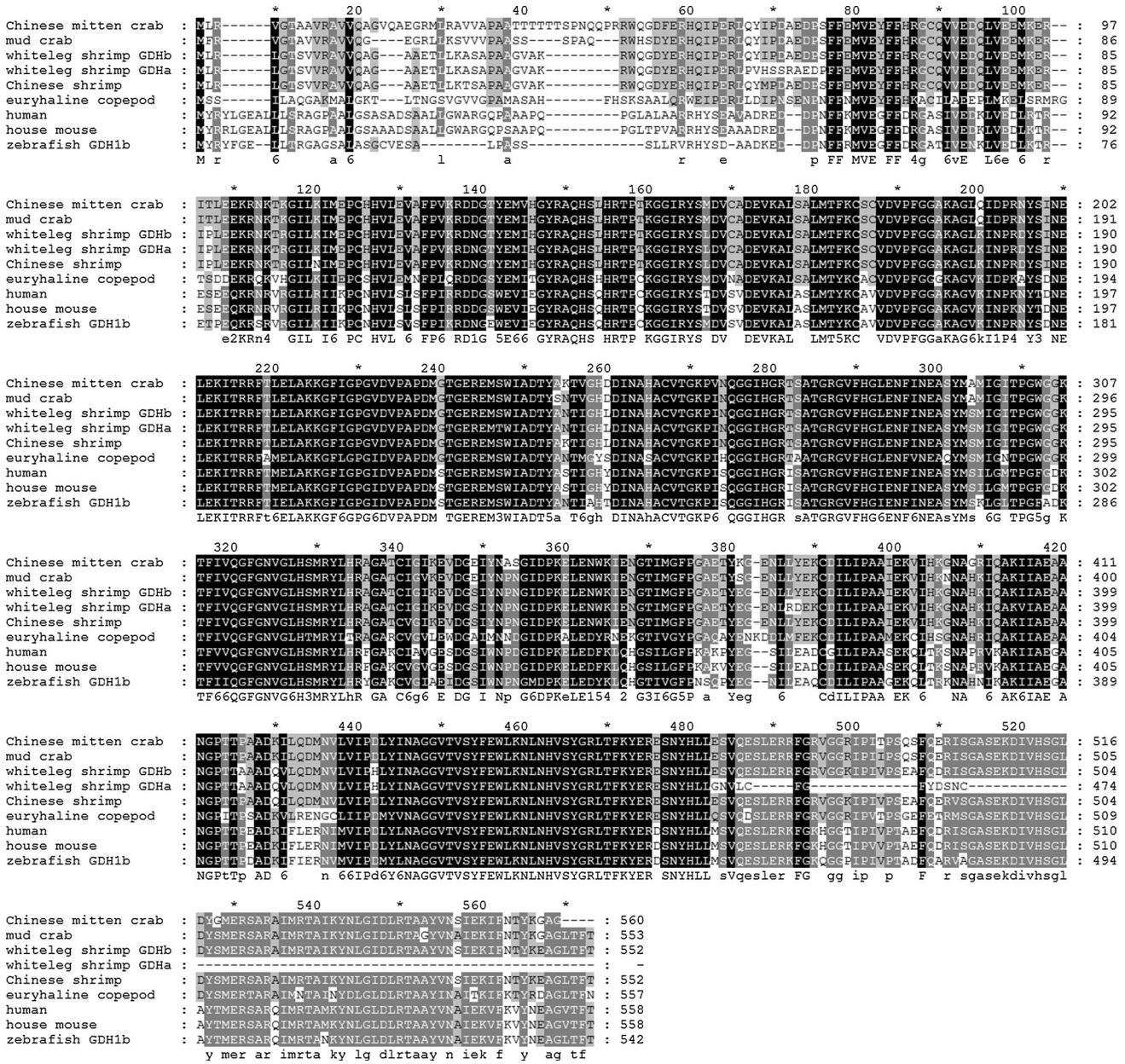

Fig. 3 Amino acid alignment of *GDH* from *Scylla paramamosain* with other species. Accession numbers of the *GDH* cDNA for each organism are as follows: Chinese mitten crab *Eriocheir sinensis* (JN628041); Chinese shrimp *Fenneropenaeus chinensis* (KF781120.1); euryhaline copepod *Tigriopus californicus* (AAP49384.1); human *Homo sapiens* (NP955839.2); mud crab *S. paramamosain* (KM189809); whiteleg shrimp *Litopenaeus vannamei GDH* a (AM076955); whiteleg shrimp *L. vannamei GDH* b (EU496492); zebrafish *Danio rerio GDH* 1b (NM_199545.4)

play a vital role in IIR. It suggest various levels of *GDH* in different salinities. To address this question, we investigated *GDH* expression in the muscle of *S. paramamosain* after being transferred from a salinity of 28 ppt to a salinity of 5 ppt. The results showed that *GDH* transcript expression decreased substantially, to the lowest level at 6 h (0.296-fold to the original value), and then gradually returned to its initial level at 24 h (Fig. 6a). However, there was no significantly difference in *GDH* expression among crabs exposed to different salinities for 24 h (Fig. 6b).

Free amino acid concentration in muscle and haemolymph under hyposmotic stress

GDH is believed to be a potential control factor for the synthesis of free amino acids that are important in osmotic adjustment, suggesting that the expression of *GDH* would

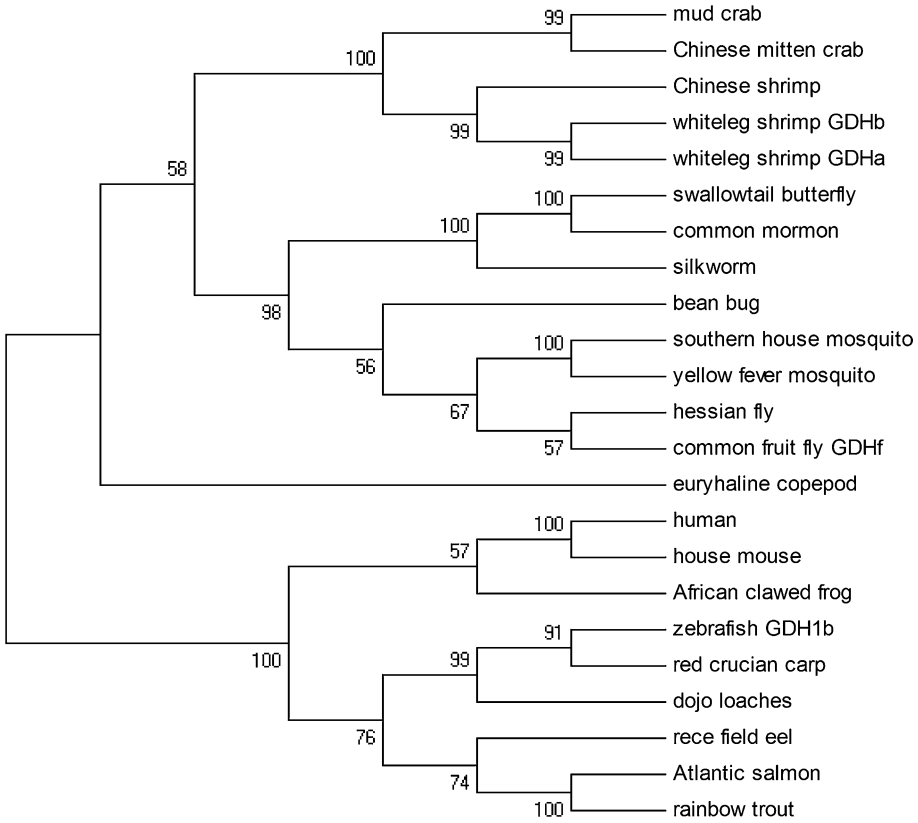

Fig. 4 Neighbor-joining phylogenetic tree of *GDH* amino acid sequences of *Scylla paramamosain* and other species. Node values represent an analysis of 1,000 bootstrap trials. Accession numbers for each *GDH* homolog are as follows: African clawed frog *Xenopus laevis* (NM_001093554.1); Atlantic salmon *Salmo salar* (AJ556995.1); bean bug *Riptortus pedestris* (AK418057.1); Chinese mitten crab *Eriocheir sinensis* (JN628041); Chinese shrimp *Fenneropenaeus chinensis* (KF781120.1); common mormon *Papilio xuthus* (AK401310.1); common fruit fly *Drosophila melanogaster GDH* f (NP996274.1); dojo loaches *Misgurnus anguillicaudatus* (JF694443.1); euryhaline copepod *Tigriopus californicus* (AY292656.1); hessian fly *Mayetiola destructo* (KF647651.1); house mouse *Mus musculus* (NM_008133.4); human *Homo sapiens* (NP955839.2); rainbow trout *Oncorhynchus mykiss* (AJ419570.1); rece field eel *Monopterus albus* (JF694445.1); red crucian carp *Carassius auratus red var* (JN634757.1); silkworm *Bombyx mori* (NM_001046780.1); southern house mosquito *Culex quinquefasciatus* (XM_001864815.1); mud crab *S. paramamosain* (KM189809); swallowtail butterfly *Papilio xuthus* (AK401310.1); whiteleg shrimp *Litopenaeus vannamei GDH* a (AM076955); whiteleg shrimp *L. vannamei GDH* b (EU496492); yellow fever mosquito *Aedes aegypti* (XM_001660811.1); zebrafish *Danio rerio GDH* 1b (NM_199545.4)

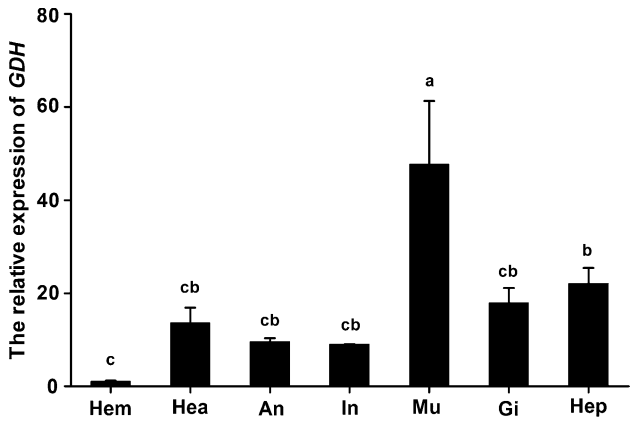

Fig. 5 Relative *GDH* expression in different tissues of *Scylla paramamosain*. *Hem* haemocytes, *Hea* heart, *An* antennal gland, *In* intestine, *Mu* muscle, *Gi* gill, *Hep* hepatopancreas. β-actin was used as the internal control. *Bars* correspond to the SEM of the average value, and *different letters* indicate significant differences ($n = 7$, $P < 0.05$)

affect the concentration of free amino acids. To explore the relationship between *GDH* expression and the regulation of free amino acids, the concentration of free amino acids in the muscle and haemolymph were analyzed.

Total free amino acid concentration in the muscle decreased significantly ($P < 0.05$) during acclimation to hyposmotic pressure (Table 2). Compared with the initial value, the concentration of total free amino acids in muscle decreased to 92.7%; it was 72.6% at 48 h ($P < 0.05$). The salinity stress response of non-essential free amino acids were similar to total free amino acids, which contributed to the reduction of the total free amino acid concentration. The concentration of alanine was the highest and, accounting for about 30% of the total free amino acids, showed a slightly decreasing trend, except at 24 h. Proline, accounting for about 8% of the total free amino acids, decreased slightly from 6 to 48 h. Compared to the initial value,

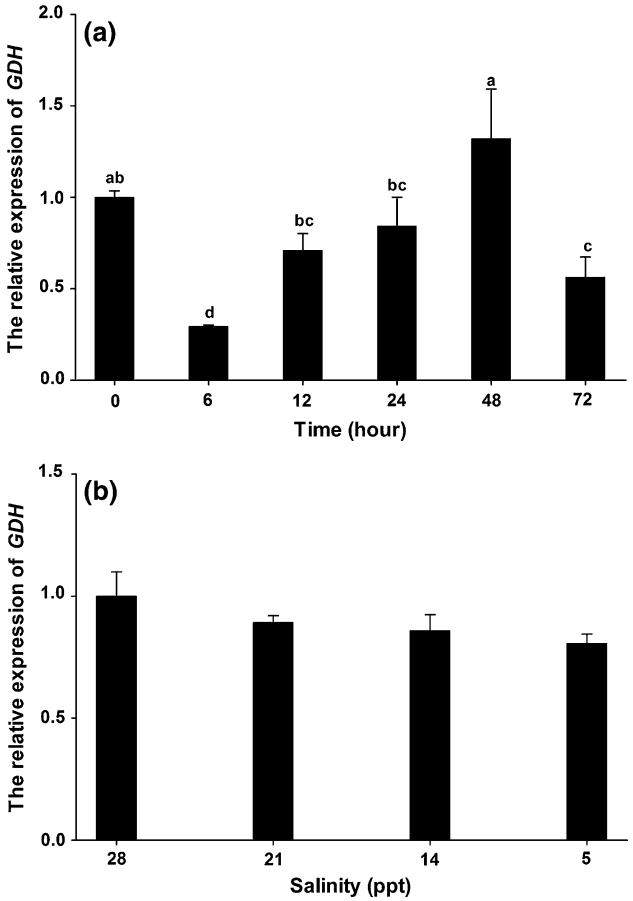

Fig. 6 **a** Relative *GDH* expression in the muscle of *Scylla paramamosain* following rapid transfer from a salinity of 28 ppt to 5 ppt; **b** Relative GDH expression in the muscle of *S. paramamosain* at different salinities. *β*-actin was used as the internal control. *Bars* correspond to the SEM of the average value, *different letters* indicate significant differences ($n = 7$, $P < 0.05$)

glutamic acid concentration decreased significantly from 6 to 72 h, reaching a minimum value at 12 h ($P < 0.05$). Glycine concentration slightly increased at 6 h and then decreased significantly from 12 to 72 h ($P < 0.05$). The concentration of glutamine decreased significantly from 24 to 72 h ($P < 0.05$). In contrast, the concentration of serine increased significantly at 6 h, subsequently dropped to the initial level, and then remained constant from 24 to 72 h ($P < 0.05$).

Whereas the concentration of most free amino acids decreased in the muscle, the concentration of free amino acids in the haemolymph increased, especially that of proline, glutamine, serine and taurine (Table 2). The concentration of serine increased significantly from 6 to 12 h, and then declined constantly to the initial level ($P < 0.05$). A similar trend was was observed for proline. The concentration of taurine increased significantly and peaked at 6 h ($P < 0.05$).

The average concentration of free amino acids in muscle under different hyposmotic treatments are shown in Table 3. The concentrations of proline and glutamine in the muscle decreased significantly after the crabs were transferred from a salinity of 28 ppt to 21 ppt and 14 ppt, separately ($P < 0.05$). In contrast, serine concentration increased significantly ($P < 0.05$). However, at 24 h, there was no significant difference in proline, glutamic acid and glutamine concentrations in the crab muscles exposed to salinities of 21, 14 and 5 ppt ($P < 0.05$).

The dynamics of most free amino acids in the haemolymph show a trend contrasting to that in the muscle (Table 4). Compared with the control, the concentration of serine increased 2.79-fold in 21 ppt, 2.52-fold in 14 ppt and 1.14-fold in 5 ppt.

Discussion

Haemolymph osmolality analysis

Haemolymph osmolality was first analyzed to confirm the osmoregulatory conditions in *S. paramamosain* [21]. For the crabs transferred from 28 to 5 ppt, haemolymph osmolality dropped rapidly at 6 h and then remained almost stable after 12 h. The results demonstrated that the experimental crabs achieved a new stable osmoregulatory condition after 12 h of hyposmotic stimulation, which is agreement with previous findings for *S. paramamosain* [20]. Similar patterns have been reported in that that 24 h is sufficient for salinity acclimation by *Macrobrachium amazonicum* [22] and *L. vannamei* [18].

In different hyposmotic treatments, haemolymph osmolality showed a decrease concomitant with a reduction in salinity, and remained hyper-regulated to the environmental salinity that had not yet reached the isosmotic point. These results support the notion that the isosmotic point of *Scylla serrata* was 962 mOsm/kg, which is equivalent to 31 ppt presented by Nicholas Romano [23].

Isolation, characterization of *GDH* cDNA

The *GDH* reaction plays a key role in the metabolism of amino acids in metazoans [9]. In this study, the *GDH* gene was cloned from *S. paramamosain*, which encoded a 553-amino acid protein. The deduced protein was conserved as compared with other species in amino acid sequences. As anticipated from the mitochondrial localization of this enzyme, its coding sequence included an N-terminal mitochondrial signal sequence peptide, which was in agreement with the discovery in *Tigriopus californicus* [9], *L. vannamei* [13] and *E. sinensis* [14]. To the best of our knowledge, this is the first characterization of the *GDH* gene from a marine crab.

Table 2 Free amino acids in *Scylla paramamosain* muscle following acute transfer from a salinity of 28 ppt to a salinity of 5 ppt (mean ±SEM)

Free amino acids			
	Time (h)	Muscle (mg/g)	Haemolymph (mg/l)
Thr	0	0.292 ± 0.019^{a}	12.804 ± 2.512
	6	0.196 ± 0.05^{b}	21.727 ± 3.71
	12	0.094 ± 0.025^{c}	13.625 ± 4.084
	24	0.101 ± 0.006^{c}	8.401 ± 1.296
	48	0.076 ± 0.022^{c}	8.755 ± 1.368
	72	0.102 ± 0.028^{c}	20.425 ± 15.812
Val	0	0.24 ± 0.019^{a}	11.04 ± 1.596
	6	0.248 ± 0.014^{a}	26.545 ± 4.967
	12	0.135 ± 0.018^{b}	18.894 ± 3.198
	24	0.169 ± 0.025^{b}	15.563 ± 2.366
	48	0.128 ± 0.027^{b}	13.372 ± 2.922
	72	0.14 ± 0.018^{b}	14.235 ± 2.323
Met	0	0.123 ± 0.01^{ab}	6.59 ± 1.962^{b}
	6	0.162 ± 0.005^{a}	22.273 ± 3.693^{a}
	12	0.103 ± 0.022^{b}	16.489 ± 0.829^{ab}
	24	0.112 ± 0.002^{b}	15.756 ± 4.57^{ab}
	48	0.098 ± 0.019^{b}	13.149 ± 6.401^{ab}
	72	0.109 ± 0.006^{b}	10.741 ± 2.197^{ab}
Ile	0	0.128 ± 0.013^{a}	3.267 ± 1.752
	6	0.063 ± 0.033^{b}	13.439 ± 3.648
	12	0.068 ± 0.009^{ab}	10.196 ± 2.021
	24	0.09 ± 0.003^{ab}	7.033 ± 1.481
	48	0.073 ± 0.025^{ab}	7.931 ± 3.141
	72	0.08 ± 0.012^{ab}	3.423 ± 1.662
Leu	0	0.156 ± 0.017^{a}	8.724 ± 2.077
	6	0.148 ± 0.014^{a}	15.85 ± 4.627
	12	0.059 ± 0.009^{b}	12.23 ± 2.85
	24	0.101 ± 0.014^{b}	9.537 ± 0.307
	48	0.067 ± 0.014^{b}	7.99 ± 2.417
	72	0.079 ± 0.01^{b}	6.297 ± 1.8
Tau	0	0.528 ± 0.09	73.155 ± 25.473^{b}
	6	0.512 ± 0.089	140.47 ± 3.051^{a}
	12	0.426 ± 0.083	110.98 ± 20.844^{ab}
	24	0.613 ± 0.102	103.36 ± 11.137^{ab}
	48	0.481 ± 0.12	127.33 ± 16.353^{a}
	72	0.692 ± 0.103	117.86 ± 3.915^{ab}
Asp	0	0.045 ± 0.003	11.79 ± 5.957^{b}
	6	0.073 ± 0.054	20.349 ± 3.653^{ab}
	12	0.154 ± 0.141	26.294 ± 4.066^{ab}
	24	0.035 ± 0.011	19.806 ± 0.961^{ab}
	48	0.033 ± 0.009	19.182 ± 8.286^{ab}
	72	0.016 ± 0.001	31.628 ± 4.844^{a}
Ser	0	0.048 ± 0.007^{c}	5.048 ± 1.354^{c}
	6	0.13 ± 0.006^{a}	14.006 ± 1.788^{ab}
	12	0.083 ± 0.006^{b}	19.73 ± 3.198^{a}
	24	0.058 ± 0.013^{c}	5.759 ± 1.827^{c}
	48	0.048 ± 0.005^{c}	10.867 ± 2.182^{bc}
	72	0.056 ± 0.003^{c}	7.926 ± 3.173^{bc}
Glu	0	0.32 ± 0.032^{a}	23.831 ± 5.381
	6	0.278 ± 0.05^{ab}	21.184 ± 2.968
	12	0.157 ± 0.047^{b}	32.911 ± 5.942
	24	0.258 ± 0.028^{ab}	27.114 ± 7.345
	48	0.171 ± 0.044^{b}	31.739 ± 4.969
	72	0.171 ± 0.028^{b}	33.372 ± 7.299
Gly	0	0.848 ± 0.141^{a}	25.168 ± 5.585
	6	0.906 ± 0.073^{a}	27.474 ± 4.311
	12	0.452 ± 0.072^{b}	18.359 ± 3.379
	24	0.761 ± 0.054^{ab}	22.243 ± 1.669
	48	0.446 ± 0.126^{b}	26.11 ± 4.72
	72	0.467 ± 0.073^{b}	22.198 ± 2.146
Pro	0	1.876 ± 0.288	56.821 ± 19.165^{b}
	6	1.319 ± 0.079	75.291 ± 11.167^{ab}
	12	1.841 ± 0.262	113.98 ± 6.936^{a}
	24	1.39 ± 0.245	80.688 ± 18.219^{ab}
	48	1.213 ± 0.376	76.256 ± 18.263^{ab}
	72	1.607 ± 0.222	79.65 ± 3.564^{ab}
His	0	0.11 ± 0.024	1.714 ± 0.991^{ab}
	6	0.111 ± 0.005	3.87 ± 2.718^{ab}
	12	0.103 ± 0.025	5.544 ± 1.327^{a}
	24	0.058 ± 0.003	0.839 ± 0.449^{ab}
	48	0.085 ± 0.008	1.982 ± 1.246^{ab}
	72	0.094 ± 0.018	0.446 ± 0.446^{b}
Lys	0	0.334 ± 0.03^{a}	8.562 ± 0.662
	6	0.187 ± 0.034^{b}	14.208 ± 4.478
	12	0.142 ± 0.026^{b}	10.267 ± 2.011
	24	0.162 ± 0.019^{b}	8.151 ± 1.369
	48	0.177 ± 0.026^{b}	10.104 ± 1.725
	72	0.175 ± 0.042^{b}	8.534 ± 2.252
Arg	0	4.932 ± 0.19	64.32 ± 9.047
	6	4.827 ± 0.233	34.246 ± 3.162
	12	4.798 ± 0.111	58.855 ± 12.719
	24	4.743 ± 0.187	34.175 ± 8.109
	48	4.141 ± 0.697	57.251 ± 21.053
	72	4.738 ± 0.188	53.21 ± 8.429
Gln	0	2.651 ± 0.273^{a}	109.839 ± 28.282^{b}
	6	2.47 ± 0.108^{a}	154.477 ± 40.41^{b}
	12	2.595 ± 0.098^{a}	304.624 ± 43.192^{a}
	24	1.769 ± 0.198^{b}	182.24 ± 31.583^{b}
	48	1.45 ± 0.377^{b}	160.045 ± 13.867^{b}
	72	1.395 ± 0.163^{b}	147.631 ± 18.86^{b}
NEFAA	0	18.332 ± 1.023^{a}	447.078 ± 93.339^{b}
	6	17.316 ± 0.646^{a}	645.323 ± 77.445^{ab}
	12	16.938 ± 0.125^{ab}	832.426 ± 70.471^{a}
	24	16.64 ± 0.491^{ab}	610.890 ± 58.736^{ab}
	48	13.564 ± 2.178^{b}	656.386 ± 104.094^{ab}
	72	15.871 ± 0.577^{ab}	621.561 ± 51.305^{ab}

Table 2 continued

Free amino acids			
	Time (h)	Muscle (mg/g)	Haemolymph (mg/l)
Ala	0	6.816 ± 0.271	87.08 ± 18.978
	6	6.58 ± 0.449	95.954 ± 16.003
	12	6.25 ± 0.196	126.33 ± 29.551
	24	6.894 ± 0.298	124.62 ± 4.776
	48	5.42 ± 0.454	136.85 ± 28.914
	72	6.526 ± 0.897	118.34 ± 3.876
Tyr	0	0.154 ± 0.027^{a}	12.059 ± 3.537
	6	0.108 ± 0.008^{ab}	18.313 ± 3.854
	12	0.076 ± 0.016^{b}	14.038 ± 1.883
	24	0.058 ± 0.001^{b}	6.045 ± 0.784
	48	0.078 ± 0.015^{b}	8.773 ± 2.864
	72	0.099 ± 0.013^{b}	8.277 ± 0.706
Phe	0	0.079 ± 0.013^{a}	5.688 ± 0.674^{b}
	6	0.032 ± 0.021^{ab}	13.021 ± 2.881^{a}
	12	0.042 ± 0.02^{ab}	4.963 ± 0.652^{b}
	24	0.036 ± 0.009^{ab}	1.665 ± 0.833^{b}
	48	0.029 ± 0.009^{b}	4.426 ± 0.759^{b}
	72	0.032 ± 0.008^{ab}	3.935 ± 0.775^{b}
Cys	0	0.005 ± 0.002	6.327 ± 4.941
	6	0.004 ± 0.001	8.105 ± 3.523
	12	0.005 ± 0.001	2.285 ± 2.285
	24	0.004 ± 0.001	4.008 ± 2.367
	48	0.005 ± 0.001	0 ± 0
	72	0.004 ± 0.001	1.023 ± 1.023
EFAA	0	1.351 ± 0.075^{a}	56.675 ± 2.557^{b}
	6	1.036 ± 0.059^{b}	132.304 ± 17.779^{a}
	12	0.642 ± 0.105^{c}	81.425 ± 3.659^{b}
	24	0.772 ± 0.026^{bc}	66.106 ± 10.812^{b}
	48	0.648 ± 0.128^{c}	65.728 ± 16.656^{b}
	72	0.717 ± 0.083^{c}	67.59 ± 24.395^{b}
TFAA	0	19.684 ± 1.077^{a}	503.753 ± 95.894^{b}
	6	18.352 ± 0.591^{a}	777.626 ± 65.04^{ab}
	12	17.58 ± 0.023^{ab}	913.851 ± 66.818^{a}
	24	17.412 ± 0.512^{ab}	677.000 ± 69.080^{ab}
	48	14.212 ± 2.305^{b}	722.113 ± 120.432^{ab}
	72	16.589 ± 0.526^{ab}	689.151 ± 74.678^{ab}

Different letters indicate significant differences ($n = 7$, $P < 0.05$)

GHD tissue expression and response to hyposmotic challenge

We found different *GDH* expressions in all tissues examined via qPCR. The highest expression of *GDH* was detected in the muscle of *S. paramamosain,* which is consistent with previous studies of *L. vannamei* [13] and *E. sinensis* [14]. This finding could be attributed to the vital role of GDH in the catabolism of glycine, proline and alanine, which account for a high proportion of the total free amino acids in the muscle [24]. Crustacean muscle is the major tissue for protein deposition and possibly the main pool of free amino acids [25]. Most amino acid metabolism occurs in the muscle. Hence, muscle can be utilized when free amino acids are needed for physiological functions [13]. Moreover, free amino acids serve as osmolytes in crustaceans. Combining the findings in *S. paramamosain*, *L. vannamei* [13] and *E. sinensis* [14], the results support the notion that muscle is the optimal site for the expression of some central enzymes in the IIR in crustaceans.

In this investigation, the expression of *GDH* in the muscle decreased significantly after crabs were transferred from high salinity (28 ppt) to low salinity (5 ppt) at 6 h ($P < 0.05$); it returned to normal after 24 h. Li et al. [12] showed that *GDH* expression in *L. vannamei* is similar to that of Na^{+}–K^{+} ATPase, a proven marker of osmoregulation capacity. The level of *GDH* expression in *E. sinensis* positively correlated with the environmental salinities [14]. However, in *T. californicus*, the expression of *GDH* and enzyme activity do not show any relationship with the regulation of proline and alanine under hyperosmotic stress [9, 26]. The differences in species and experimental design can explain the difference between *T. californicus* and *S. paramamosain*. *T. californicus* is a small species, and the whole organism was used as detected tissue to quantify *GDH* expression. *T. californicus* were transferred from 50 to 100 ppt seawater for 90 min [9]. Alternatively, in *S. paramamosain*, only the dominant tissue (muscle) was used for *GDH* expression analysis, and the exposure time was sufficient for the crabs to respond to the ambient salinity challenge. Hence, our results, together with the findings in *E. sinensis,* indicate that *GDH* plays an important role in osmoregulation in macro-crustaceans. However, no significant difference was found in *GDH* expression in the muscle of crabs that maintained stable levels in different salinity treatments for 24 h. It is known that internal changes are completed within about 24 h after the animals encounter an acute reduction in salinity [18]. This idea is supported by our results demonstrating *GDH* expression in the muscle of *S. paramamosain* was back to normal 24 h after adaptation to a salinity of 5 ppt. Hence, the results suggest *GDH* expression in S. *paramamosain* muscle may return to normal following transfer from a salinity of 28 ppt to 21 ppt and 14 ppt for 24 h, while showing no significant difference in *GDH* expression under different salinity treatments.

Free amino acid concentration in muscle

Free amino acids play an important role in intracellular osmotic regulation in crustaceans [4, 7]. In the freshwater prawn *Macrobrachium olfersii*, total free amino acid concentration reaches a maximum value by 48 h and then declines to freshwater values after exposure to a salinity of 21 ppt [27]. In this study, the total concentration of

Table 3 Free amino acids in *Scylla paramamosain* muscle under different salinity treatments (mean ± SEM)

Free amino acids (mg/g)	Salinity (ppt)			
	28 (control)	21	14	5
Ala	6.849 ± 0.24	6.852 ± 0.701	6.055 ± 0.107	6.894 ± 0.298
Arg	5.055 ± 0.079^{a}	4.135 ± 0.106^{b}	4.093 ± 0.264^{b}	4.743 ± 0.187^{a}
Gly	0.881 ± 0.109	0.718 ± 0.165	0.729 ± 0.038	0.761 ± 0.054
Glu	0.344 ± 0.015^{a}	0.224 ± 0.028^{b}	0.31 ± 0.042^{ab}	0.258 ± 0.028^{ab}
Gln	2.651 ± 0.273^{a}	1.519 ± 0.183^{b}	1.513 ± 0.098^{b}	1.769 ± 0.198^{b}
Pro	1.953 ± 0.211^{a}	1.109 ± 0.024^{b}	1.247 ± 0.202^{b}	1.39 ± 0.245^{ab}
Ser	0.052 ± 0.004^{b}	0.12 ± 0.026^{a}	0.168 ± 0.014^{a}	0.058 ± 0.013^{b}
Tau	0.545 ± 0.078	0.618 ± 0.018	0.568 ± 0.007	0.613 ± 0.102
EFFA	1.361 ± 0.07	0.649 ± 0.023	1.039 ± 0.563	0.772 ± 0.026
NEFFA	18.643 ± 0.797	15.629 ± 1.141	17.155 ± 1.232	16.64 ± 0.491
TFFA	20.005 ± 0.842^{a}	16.278 ± 1.123^{b}	18.195 ± 1.795^{ab}	17.412 ± 0.512^{b}

Different letters indicate significant differences ($n = 7$, $P < 0.05$)

Table 4 Free amino acids in *Scylla paramamosain* haemolymph under different salinity treatments (mean ± SEM)

Free amino acids (mg/l)	Salinity (ppt)			
	28 (control)	21	14	5
Ala	87.08 ± 7.559	122.39 ± 28.792	123.212 ± 3.799	124.62 ± 4.776
Arg	33.9122 ± 3.03926^{b}	56.157 ± 4.605^{a}	42.367 ± 2.860^{ab}	34.175 ± 8.109^{b}
Gly	25.171 ± 1.289^{ab}	32.711 ± 3.453^{a}	31.074 ± 1.99^{a}	22.778 ± 1.955^{b}
Glu	22.499 ± 2.324	28.236 ± 2.959	28.149 ± 3.827	27.114 ± 7.345
Gln	109.84 ± 28.282	154.04 ± 23.376	170.98 ± 23.457	182.24 ± 31.583
Pro	56.831 ± 7.643	66.868 ± 7.709	72.204 ± 11.091	80.688 ± 18.219
Ser	5.055 ± 1.382^{b}	14.102 ± 2.838^{a}	12.748 ± 0.360^{a}	5.7586 ± 1.827^{b}
Tau	79.82 ± 9.788	86.722 ± 27.791	86.414 ± 18.274	103.359 ± 11.137
EFAA	55.008 ± 1.803	54.715 ± 16.078	49.361 ± 6.936	66.106 ± 10.812
NEFAA	452.10 ± 56.772	594.41 ± 78.476	612.854 ± 59.993	611.43 ± 59.070
TFFA	508.77 ± 59.325	649.13 ± 94.531	662.214 ± 66.378	677.53 ± 69.382

Different letters indicate significant differences ($n = 7$, $P < 0.05$)

free amino acids in the muscle decreased significantly after transfer to 5 ppt from 6 to 48 h ($P < 0.05$). The results suggest that *S. paramamosain* could down-regulate the free amino acid levels in the body to reduce the intracellular osmolality, helping *S. paramamosain* to adapt to the sharp reduction in haemolymph osmolality under environmental hyposmotic stress.

Non-essential free amino acids work as osmolytes and differ slightly among different species [27, 28]. Arginine, glycine and alanine are the main organic osmolytes in decapods like *M. olfersii* [27] and *M. amazonicum* [8]. L-glutamine, D-alanine and L-alanine in muscle of *L. vannamei* decrease significantly in a low salinity of 3 ppt [19]. In the present study, the levels of arginine, glutamine, glycine, glutamic acid and proline decreased significantly and they work as osmolytes during acclimation to hyposmotic stress ($P < 0.05$). The reduction of glutamic acid, glycine and proline correlated with the decrease of *GDH* expression in *S. paramamosain*, suggesting the central role of *GDH* in the metabolism of glutamic acid, glycine and proline, which work as osmolytes. However, no significant change is found in the alanine concentration, which is consist with previous findings of the intertidal shrimp *Palaemon northropi* [29]. It has been shown that alanine synthesis is observed at a constant salinity in an intertidal copepod [26]. The preferential accumulation of alanine over proline was found in the intertidal copepod *T. californicus* when anoxia or nutritional stress was combined with hyperosmotic stress [30]. Therefore, regulation of alanine appears to be independent of that of proline and was not mediated by changes in the amount of *GDH*. In this study, there was no significant difference found in proline, glutamic acid and glutamine concentrations in the muscle of crabs exposed to salinities of 21, 14 and 5 ppt. This may be due to the reaction rate and degree of regulation of special free amino acids correlated with the level of hyposmotic stress [14]. Obviously, further research is warranted to better understand the diversity of responses and their underlying mechanisms of omolyte regulation in IIR.

Free amino acid contents in haemolymph

Free amino acids in haemolymph are directly involved in mediating the response to salinity exposure [14]. In this study, the concentrations of total free amino acids in the haemolymph increased significantly after transfer to a salinity of 5 ppt ($P < 0.05$) and showed a trend opposite to that of haemolymph osmolality. Thus, an increase in the total free amino acid concentration partially compensates for a sharp reduction in extracellular fluid osmolality under hyposmotic stress, reducing the osmotic difference between extra- and intracellular osmolality. Without supplemental dietary protein, most of the free amino acid concentrations increased in the haemolymph, especially taurine, glutamine and proline, which decreased in the muscle. The results reveal efflux of amino acids from muscle to the haemolymph.

Free amino acids can be utilized as energy substrates in physiological compensation to stressors [31, 32]. L-serine levels in *L. vannamei* were significantly increased in the haemolymph and muscle in accordance with the decreasing saline environment due to the consumption of L-serine as an energy source ($P < 0.05$) [19]. In this study, we found a significant increase in serine concentration in the muscle and haemolymph ($P < 0.05$). It suggested that serine works as an energy source, synthesized in muscle during low salinity conditions and then released into the haemolymph.

Overall, the full-length cDNA sequence of *GDH* from *S. paramamosain* was obtained and characterized in this investigation. *GDH* was primarily expressed in the muscle. Acute hyposmotic challenges down-regulated the expression of *GDH* in the muscle of *S. paramamosain* and led to a decrease in synthesis of free amino acids, especially glutamic acid, proline and glycine, to meet the requirement of osmoregulation in *S. paramamosain* under hyposmotic conditions. In summation, *GDH* plays an important role in osmoregulation in *S. paramamosain*.

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