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ORIGINAL ARTICLE

Metabolism of amino acids during hyposmotic adaptation in the whiteleg shrimp, *Litopenaeus vannamei*

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Abstract The penaeid prawn, *Litopenaeus vannamei*, was employed to investigate intracellular isosmotic regulation in situations where invertebrates encounter hyposmosis. Hemolymph osmolality was first analyzed to confirm osmoregulatory conditions in the experimental animals, followed by analysis of amino acids in muscle and hemolymph using high-performance liquid chromatography. Total muscle amino acid levels decreased when hemolymph osmolality was extremely low, whereas glycine and L-serine levels increased in the hemolymph. These results suggest that tissue amino acids were released into the hemolymph to lower the osmolality of the tissues for purposes of low-salinity adaptation. Next, oxygen consumption and ammonia excretion rates were examined, and the O/N ratio was determined. Oxygen consumption levels and ammonia excretion rates increased, and the O/N ratio decreased when the animals were exposed to low salinity. These results suggest that amino acids were abundantly consumed as an energy source when animals were exposed to low salinity. To confirm the consumption of particular amino acids, the specific activity of L-serine ammonia lyase was also examined. Specific activity was highest when L-serine levels in the hemolymph were highest. Thus, it appears that L-serine levels increased under hyposmotic conditions due to the consumption of L-serine as an energy source. It was concluded that particular amino acids as osmolytes are likely metabolized as energy sources and consumed for purposes of hyposmotic adaptation.

Keywords Crustacea · D-Alanine · Intracellular isosmotic osmoregulation · Invertebrates · Respiratory metabolism

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Introduction

Osmoregulation is indispensable in controlling the composition of body fluids and tissues. To adapt to the osmotic effects of the environment, most organisms possess appropriate osmoregulatory mechanisms, thus allowing them to adapt to their natural habitats (Richard et al. 2008; Péqueux 2008). Crustaceans are the most diverse and successful aquatic invertebrates that have invaded freshwater bodies from the sea (Greenaway 2001; Freire et al. 2008a). Furthermore, crustaceans form a major group of aquatic invertebrates that includes many commercially important species (Li et al. 2007). Hence, the unique osmoregulatory mechanisms of crustaceans have been well studied. The main two means of carrying out osmotic control are anisosmotic extracellular regulation (AER) to maintain the osmolality of the body fluid, and intracellular isosmotic regulation (IIR) which regulates particular amino acids as osmolytes in the tissues (Péqueux 1995; Augusto et al. 2007a, b).

In crustaceans, the former mechanism, AER, is performed using the gills and antennal glands. Both organs possess ion channels and secondary active transport

proteins, which are mainly driven by negative electrical potential produced by primary active transport proteins in the plasma membrane, such as Na^+/K^+-ATPase (Ahearn et al. 1999; Towle and Weihrauch 2001; Freire et al. 2008b; Charmantier et al. 2009). The gills function in osmoregulation via active ion transportation of sodium and chloride, and adaptive water permeability (Augusto et al. 2007a; Freire et al. 2008b). The antennal glands are organs that excrete urine to regulate inorganic ion levels in the hemolymph. They also function in calcium regulation, reabsorption of sodium and chloride in freshwater species, and secretion of magnesium and sulfate in marine and estuarine species (Wheatly et al. 2002; Ahearn et al. 2004; Freire et al. 2008b). These mechanisms work towards maintaining stable hemolymph osmolality, but it is rare that hemolymph osmolality is maintained to be completely stable under excessive change in environmental osmolality.

The latter mechanism, IIR, regulates particular amino acids, and can be considered to support AER. When the osmolality of the body fluid changes due to changing environmental osmolality, cells at first rapidly regulate their volume by controlling levels of inorganic ions. Subsequently, potassium in particular is regulated as an intracellular osmotic effector (Gilles and Péqueux 1981; Augusto et al. 2007a). Thereafter, cells accumulate or degrade quantities of particular nonessential free amino acids as osmolytes to control their osmolality and ion composition for purposes of volume readjustment (Gilles and Péqueux 1981). This type of osmoregulation has been well studied by utilizing decapod crustaceans as experimental animals (Péqueux 1995; Abe et al. 2005a). Free amino acids utilized in this manner differ slightly according to species, but glycine, glutamine, proline, and alanine are the most commonly utilized osmolyte species in crustacean muscle (Abe et al. 1999; Fujimori and Abe 2002; McNamara et al. 2004; Augusto et al. 2007a, b, 2009; Faria et al. 2011). Not only L-amino acids, but also D-amino acids, are utilized in the above mechanism (Okuma and Abe 1994a; Abe et al. 1999; Fujimori and Abe 2002; Abe et al. 2005a). In crustaceans, D-alanine is commonly used in IIR to accumulate alanine in tissues without inhibiting fundamental enzymatic activities due to the presence of excessive L-alanine (Abe et al. 2005b). Many studies have therefore been recently performed to elucidate these systems of IIR for adaptation to high levels of salinity. However, there have been very few recent studies of adaptation to low-salinity levels.

We, therefore, conducted this study to better understand this amino acid-based osmoregulatory system of low-salinity adaptation. We chose a penaeid shrimp, *Litopenaeus vannamei*, as the experimental animal. *L. vannamei* is native to the eastern Pacific Ocean, from the Mexican state of Sonora as far south as northern Peru, where temperature and salinity ranges of 15–28 °C and 5–45 ‰ have been reported (Bett and Vinatea 2009). It has been reported that this species quickly alters its amino acid levels in tissues in response to environmental salinity change (McCoid et al. 1984), and is thus suitable for use in studying IIR. Our results suggest that not only do amino acids function as osmolytes, but also that some amino acids may serve as energy sources during the process of low-salinity adaptation.

Materials and methods

Experimental animals

Male whiteleg shrimp *L. vannamei* were purchased from International Mariculture Technology, Co. Ltd. (Myoko, Nigata, Japan). Carapace length was 25.2 ± 1.4 mm (mean ± SD) and wet weight was 11.9 ± 2.2 g (mean ± SD). The shrimp were stocked in a 3-t tank at approximately 20 °C in artificial seawater (Sea Life, Marinetech, Tokyo, Japan) initially at the salinity level of seawater.

Experimental procedures

Experimental animals were individually acclimatized for 7 days at 28 °C in 40-L tanks at a salinity of 28 parts per thousand (ppt) in accordance with Huong et al. (2010). During the acclimation, animals were feed dry food pellets (Gold Prawn, Higashimaru Co. Ltd., Kagoshima, Japan) until saturation each day, except in the 24 h before the experiments. Remaining feed was removed from each tank 5 h after feeding, and 50 % of the rearing water was exchanged daily.

After the acclimatization, premolt or intermolt individuals were exposed to low salinity. The molt stage was C_2 to D_2 in accordance with Chan et al. (1988). The total exposure time was set to 24 h, in accordance with the results of studies showing that internal changes are completed in about 24 h after the animals encounter acute salinity reduction (McCoid et al. 1984; Dalla Via 1986; Huong et al. 2010). Animals were exposed to experimental salinities of 3, 7, 14, and 28 ppt (control) at 28 °C for 8 h. Next, individuals were moved into an apparatus described by Shinji et al. (2009). The apparatus was a 1.1-L dual chamber consisting of analytical and blank chambers; all water was circulated at a flow rate of 4.8 L h^{-1}. Animals were kept in the analytical chamber of the apparatus at 28 °C for 16 h. After A 24-h total exposure time, inflow and outflow water were collected from the apparatus for the analysis of oxygen consumption and ammonia excretion rates. Experimental animals were anesthetized by being frozen on ice soon after the water sampling, and were then

dissected thereafter. Hemolymph and muscle were used in subsequent analyses. Collected tissues were stored at −80 °C until analysis.

Analysis of hemolymph osmolality

Hemolymph samples were centrifuged at 4 °C and 9,000*g* for 5 min to precipitate coagulated protein, and the supernatants collected. Fifteen microliters of each supernatant was analyzed using a 110 Osmometer (Fiske, Norwood, MA, USA).

Analysis of inorganic ions and free amino acids

Inorganic ions and free amino acids in the muscles were extracted in accordance with the methods of Okuma and Abe (1994b), with several modifications. Approximately 1 g of muscle (wet weight) was homogenized with 15 % perchloric acid in a fourfold volume. The samples were then centrifuged at 4 °C at 18,000*g* for 10 min, and the supernatants were collected. Part of the supernatant was neutralized with an equal amount of 1.6 M potassium hydrogen carbonate before analyzing inorganic ions (except potassium). Six hundred microliters of the remaining supernatant was neutralized in a solid-phase column (IC Maxi-Clean Cartridge IC-OH, Grace Davison Discovery Sciences Co. Ltd., MD, USA) and used for potassium analysis. Hemolymph samples were diluted 50 and 200 times with Milli-Q water and used directly to analyze inorganic ions. Free amino acids in the hemolymph were extracted using acetonitrile. Stored hemolymph samples were centrifuged at 4 °C at 9,000*g* for 5 min. After collection of the supernatant, an equal amount of acetonitrile was added to each sample. The samples were centrifuged at 4 °C at 18,000*g* for 10 min, and the supernatants were collected for analysis.

Inorganic ions in each extract were quantified using an IA-100 ion analyzer (Toa DKK, Tokyo, Japan). Sodium, potassium, magnesium, and calcium were separated on an analytical column for cation analysis equipped with a guard column both provided by the manufacturer (Toa DKK, Tokyo, Japan), and chloride and sulfate were handled similarly for anion analysis. Both analyses were performed with eluent specific for cation or anion analysis also purchased from the manufacturer (Toa DKK, Tokyo, Japan). Total free L-amino acid levels were analyzed with a commercial kit (BioVision, Mountain View, CA, USA). Individual free amino acids were analyzed by high-performance liquid chromatography (HPLC). The Shimadzu LC-10 HPLC system was used for precolumn derivatization according to the methods of Nimura and Kinoshita (1986). A Develosil ODS-UG-5 (6.0 × 200 mm, Nomura Chemical, Aichi, Japan) was used as an analytical column, and a Develosil ODS-UG-5 (6.0 × 35 mm, Nomura Chemical, Aichi, Japan) was used as a guard column. A Shim-pack GRD-ODS (4 × 35 mm, Shimadzu, Kyoto, Japan) was used as a precolumn to guard the analytical column, in accordance with the manufacturer's manual. A binary system consisting of 50 mM of sodium acetate and methanol were used as the mobile phases. Eighty mg of *o*-phthalaldehyde (OPTA) and 100 mg of *N*-acetyl-L-cysteine (AcCys) were dissolved into 10 mL of methanol and used as a derivatization reagent. The samples, together with 0.1 M sodium tetraborate and OPTA-AcCys reagent, were mixed in a ratio of 1:3:2 and injected into the HPLC system after 4 min of reaction at room temperature. Each derivatized amino acid was separated at 28 °C according to the time program in Table S1, were made to emit fluorescence using a wavelength of 350 nm, and were detected at a wavelength of 450 nm. Each amino acid was quantified by utilizing an external standard where approximately 700 mg of each amino acid was dissolved in 0.1 M hydrochloric acid to 1 L total. In the process of quantification, asparagine and lysine were standardized against L-asparagine and L-lysine, because these amino acids could not be separated into each chiral isomer. All of the asparagine and lysine were regarded as L-amino acids, because D-asparagine and D-lysine have not been found in crustacean tissues (Okuma et al. 1995). The quantities of other L-amino acids that could not be analyzed by HPLC were determined by subtracting all L-amino acids analyzed by HPLC from the total L-amino acids.

Analysis of oxygen consumption rate, ammonia excretion rate, and O/N ratio

Dissolved oxygen and ammonia–N concentrations in the water samples collected as described above were analyzed according to a method described by Shinji et al. (2009). Dissolved oxygen was analyzed by the Azide–Winkler titration method. Manganous sulfate reagent was prepared with 120 g of manganese (II) sulfate tetrahydrate, 1 mL of sulfuric acid, and 250 mL of distilled water. Alkaline iodate solution was prepared with 90 g of sodium hydroxide, 25 g of potassium iodide, 5 g of sodium azide, and 250 mL of distilled water. After water had been sampled from the apparatus described above, 1 mL of manganous sulfate reagent and 1 mL of alkaline iodate solution were added to each water sample (100 mL). The resulting precipitant was reacted with 20 % hydrochloric acid, and the quantity of dissolved oxygen was estimated by titration with 0.01 N sodium thiosulfate. The ammonia–N concentration was determined using commercial salicylate and cyanurate reagents for ammonia analysis in accordance with the protocol provided by the manufacturer (Hach, Loveland, CO, USA). Twenty-five milliliters of

water sample and salicylate reagent provided as a package were mixed and incubated for 3 min at room temperature. Thereafter, pre-packed cyanurate reagent was added and incubated for 15 min at room temperature. The absorbance at 655 nm was measured by a spectrophotometer (DU 530 Life Science UV/Vis Spectrophotometer, Beckman Coulter Inc., CA, USA), and ammonia–N concentration was calculated from the standard curve of an NH_4Cl standard for ammonia–N analysis (Hach, Loveland, CO, USA). Oxygen consumption rate and ammonia excretion rates were calculated on the basis of the differences between the analytical and blank chambers, as follows (Cech 1990):

Oxygen consumption rate (mg g^{-1} h^{-1}):

$$\frac{\{O_2^{in}\,(mgL^{-1}) - O_2^{out}\,(mgL^{-1})\} \times \text{flowrate}(Lh^{-1})}{\text{weight (g)}}$$

Ammonia excretion rate (μg g^{-1} h^{-1}):

$$\frac{\{N^{out}\,(\mu gL^{-1}) - N^{in}\,(\mu gL^{-1})\} \times \text{flowrate}(Lh^{-1})}{\text{weight (g)}}$$

The O/N ratio was calculated from the molar rates of oxygen consumption per ammonia–N excretion.

L-Serine ammonia lyase assay

Approximately 100 mg of muscle was homogenized with 50 mM Tris–HCl (pH 8.3) at ninefold volume. Homogenates were centrifuged at 20,000*g* for 20 min, and the supernatants were collected. The quantity of protein in the supernatants was analyzed using Micro BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). The activity of L-serine ammonia lyase was analyzed by the following method. The supernatants were diluted to 10 times their volume with 50 mM Tris–HCl (pH 8.3), and 90 μL of diluted supernatant and 10 μL of 100 μg μL^{-1} L-serine were added together. The mixtures were incubated at 30 °C for 30 min for enzymatic reaction, and then heated at 105 °C for 10 min to deactivate enzymes. Enzymatic activities were estimated by analyzing pyruvate, which is the main product of L-serine ammonia lyase. Pyruvate was analyzed with a commercial pyruvate assay kit (BioVision, Mountain View, CA, USA). The L-serine ammonia lyase activity that produced 1 μmol min^{-1} of pyruvate was defined as 1 U.

Statistical analysis

Statistical methods were selected according to the homogeneity of variance examined by the Bartlett test. The significance of variation in all analytical values among the different salinities was examined using one-way analysis of variance (ANOVA) or the Kruskal–Wallis test. Differences between the means were compared using a Bonferroni-type multiple comparison.

Results

Relationship of hemolymph osmolality to environmental salinity

The lowest experimental salinity was set to 3 ppt, because our previous study reported that *L. vannamei* could not remain alive for more than 6 h with a salinity reduction from 28 ppt to salinities lower than 3 ppt (Huong et al. 2010). No mortality was observed during the experiments. Animals showed significant decreases in hemolymph osmolality in all experimental salinities; the largest decrease was observed between the salinities of 3 and 7 ppt (Fig. 1; $P < 0.05$), and showed a decrease concomitant with that of the environmental osmolality between the salinities of 3 and 7 ppt.

Composition of inorganic ions and free amino acids in muscle

During low-salinity exposure, there were no significant differences in sodium, potassium, magnesium, calcium, chloride and sulfate (data not shown). Consequently, the total quantity of these ions among different salinities did not significantly change (Fig. 2a; $P > 0.05$). In contrast, concentrations of total free amino acids were significantly lower at 3 ppt than at all the other salinities (Fig. 2a; $P < 0.05$); this was the same salinity at which the greatest, parallel reduction in hemolymph osmolality against environmental osmolality was observed. The quantity of the decrease compared with the control was 80 mmol kg^{-1}, corresponding to the approximately 40 % decrease in

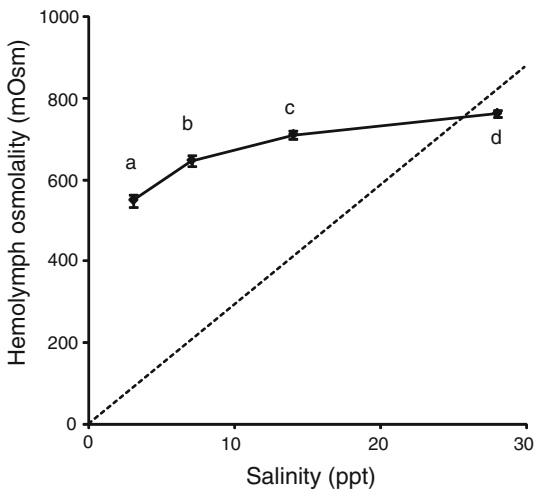

Fig. 1 Changes in hemolymph osmolality with salinity in subadult male *Litopenaeus vannamei*. *Bars* correspond to the SE of the average value. The *broken line* represents the osmolality of each salinity treatment. *Different letters* indicate significant difference ($n = 7$ each; $P < 0.05$)

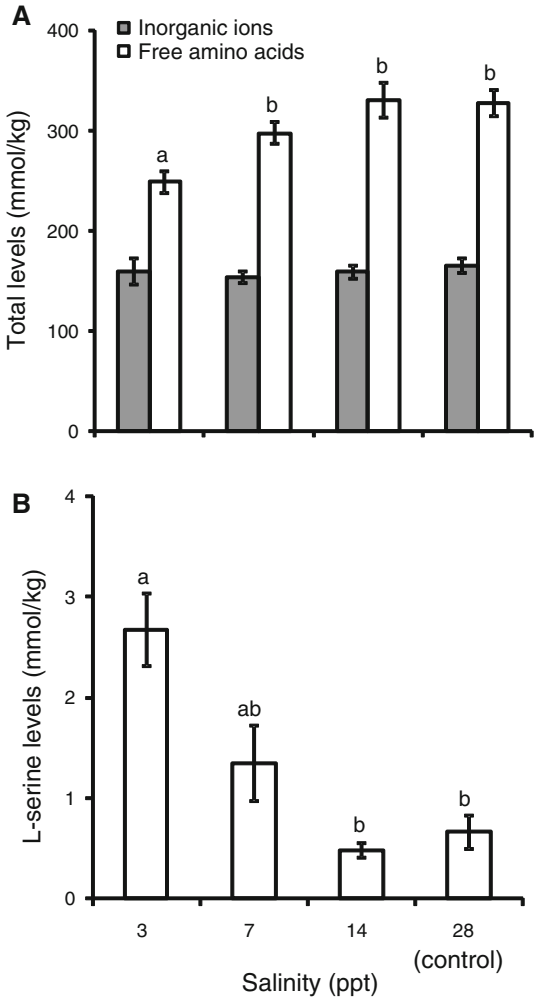

Fig. 2 Levels of total inorganic ions and free amino acids (**a**) and L-serine (**b**) in the muscle at differing salinities. Total inorganic ions are the total levels of Na^+, K^+, Mg^{2+}, Ca^{2+}, Cl^-, and SO_4^{2-} in whole muscle. *Vertical lines* indicate SE ($n = 7$ each). *Different letters* indicate significant difference ($P < 0.05$). Details of the composition of amino acids in the muscle are given in Table S2

hemolymph osmolality. Among the free amino acids, concentrations of 10 amino acids and other L-amino acids that could not be analyzed by HPLC decreased significantly (Table S2; $P < 0.05$), whereas the concentrations of the remaining free amino acids were maintained against the environmental salinity reduction ($P > 0.05$). L-Glutamine, D-alanine, L-alanine, and other L-amino acids accounted for 19.8, 9.1, 9.4 and 56.0 % of total decrease of amino acids, and comprising most of the free amino acids that decreased in concentration in the muscle. Total quantities of D- and L-alanine reached approximately a fifth of the decrease of total amino acids, and the ratio of D-alanine to the sum of D- and L-alanine decreased significantly only at 3 ppt (Table 1; $P < 0.05$). Most of the decreases in concentrations of free amino acids were the lowest at 3 ppt, at which salinity the hemolymph osmolality decreased at the same rate as the environmental osmolality. Although the concentrations of many free amino acids decreased at low salinity, L-serine showed completely different dynamics from the other amino acids. L-serine concentrations increased significantly at low salinity and became remarkably high at 3 ppt (Fig. 2b; $P < 0.05$).

Composition of inorganic ions and free amino acids in hemolymph

Total inorganic ion levels in the hemolymph declined significantly as environmental salinity declined (Fig. 3a; $P < 0.05$). Although the levels of various inorganic ions in the hemolymph decreased as salinity decreased (Table S3; $P < 0.05$), potassium levels did not decline ($P > 0.05$). Moreover, the concentration of calcium was maintained at levels greater than those of magnesium, although it decreased significantly at 3 ppt ($P < 0.05$). Whereas the concentrations of various inorganic ions decreased, those of glycine and L-serine increased significantly (Fig. 3b, c;

Table 1 Changes in free amino acid concentrations in the muscle of *L. vannamei* exposed to low salinities (mean ± SE)

	Salinity (ppt)			
	3	7	14	28 (control)
Free amino acids (mmol L^{-1})				
L-Gln	10.47 ± 2.63[a]	20.23 ± 1.83[b]	20.08 ± 2.11[b]	26.15 ± 2.60[b]
Gly	106.38 ± 14.31	87.54 ± 7.18	96.24 ± 13.43	100.34 ± 17.75
L-Arg	44.93 ± 2.86	46.21 ± 2.02	49.12 ± 4.05	49.36 ± 1.31
L-Ala	6.18 ± 1.51[a]	13.38 ± 1.62[b]	15.12 ± 1.60[b]	13.64 ± 1.88[b]
D-Ala	4.44 ± 1.36[a]	11.40 ± 1.51[b]	13.19 ± 1.46[b]	11.60 ± 1.82[b]
Minor L-amino acids	19.52	21.43	24.24	25.69
Other L-amino acids	56.47 ± 7.36[a]	97.22 ± 9.60[b]	111.48 ± 8.46[b]	100.71 ± 11.91[b]
D/D + L (%)	39.59 ± 1.70[a]	45.73 ± 0.51[ab]	46.51 ± 0.31[b]	45.45 ± 0.68[ab]

Minor L-amino acids are the sum of L-amino acids present in small quantities. Other L-amino acids indicate the sum of L-amino acids that could not be analyzed by HPLC. D/D + L indicates the ratio of D-alanine to the sum of D- and L-alanine. Different letters indicate significant difference ($n = 7$ each; $P < 0.05$). Details of the composition of free amino acids in the muscle are given in Table S2

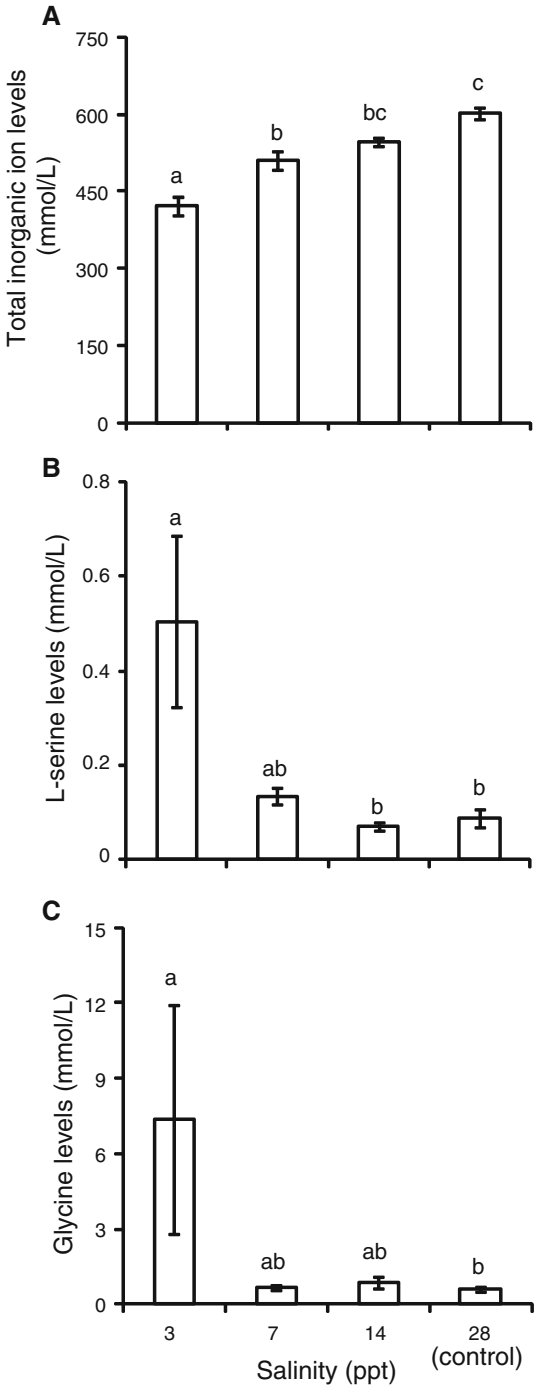

Fig. 3 Levels of total inorganic ions (**a**), L-serine (**b**) and glycine (**c**) in hemolymph at differing salinities. Total inorganic ions are the total levels of Na^+, K^+, Mg^{2+}, Ca^{2+}, Cl^-, and SO_4^{2-} in the hemolymph. *Vertical lines* indicate SE ($n = 7$ each). *Different letters* indicate significant difference ($P < 0.05$). Details of the compositions of inorganic ions in the hemolymph are given in Table S3

$P < 0.05$). The dynamics of both of these free amino acids showed trends contrary to those of most of the amino acids in the tissues, except the case of L-serine in the muscle. However, the other 17 amino acids and taurine did not significantly change in level ($P > 0.05$). The increase in total amino acid levels at low-salinity conditions was not significant, and the total quantity remained at only 2.5 % of the total quantity of osmolytes.

Oxygen consumption rate, ammonia excretion rate, and O/N ratio

Oxygen consumption rate increased as environmental salinity levels decreased and was significantly higher at 3 ppt than at 28 ppt (Fig. 4a; $P < 0.05$). Ammonia excretion rate did not change at 14 ppt, but showed a significant increase when animals were exposed to a salinity of 3 ppt (Fig. 4b; $P < 0.05$). This extreme increase was different from the gradual increase in oxygen consumption rate. In light of the increase in ammonia excretion rate, the O/N ratio (the molar ratio of consumed oxygen and excreted nitrogen), showed a significant decrease only at 3 ppt (Fig. 4c; $P < 0.05$).

L-Serine ammonia lyase activity

The specific activity of L-serine ammonia lyase in muscle increased at salinities lower than 7 ppt and became significantly higher at 3 ppt than in the controls (Fig. 5; $P < 0.05$). This increase corresponded to that of L-serine in the muscle and hemolymph.

Discussion

IIR by particular free amino acids is a system of adaptation to fluctuations in body fluids (Gilles and Péqueux 1981), which implies that IIR occurs in relation to the osmolality of the extracellular fluid (Faria et al. 2011). In this study, hemolymph osmolality decreased as environmental osmolality decreased. The osmotic decrease in the hemolymph between 3 and 7 ppt paralleled that of the surrounding environment, and at 3 ppt, reached approximately 200 mOsm (Fig. 1). These results correspond to those of our previous study in that *L. vannamei* cannot tolerate such a large decrease in hemolymph osmolality, and survive at salinities of <3 ppt (Huong et al. 2010). Hence, it was suggested that at 3 ppt, IIR is required for adaption to critical decreases in hemolymph osmolality.

Crustacean muscle contains high levels of free amino acids (Che Mat and Potts 1985; Dooley et al. 2000). In this investigation, free amino acid levels decreased at 3 ppt, whereas the levels of all inorganic ions were maintained (Fig. 2a). This suggests that the decrease in total free amino acids helped to regulate the osmotic difference between hemolymph and muscle, and probably maintained the concentrations of inorganic ions. This idea is supported by the results of our analysis of D-alanine ratios (Table 1).

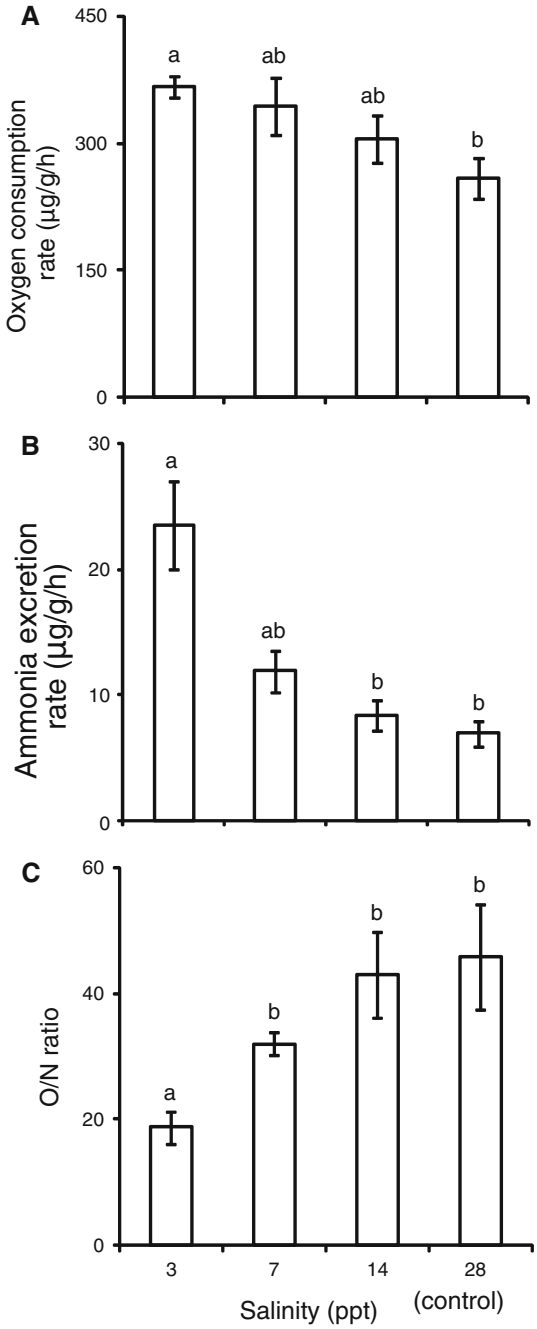

Fig. 4 Mean oxygen consumption rate (**a**), mean ammonia excretion rate (**b**), and changes in O/N ratio at differing salinities (**c**). *Vertical lines* indicate SE ($n = 7$ each). *Differing letters* indicate significant difference ($P < 0.05$)

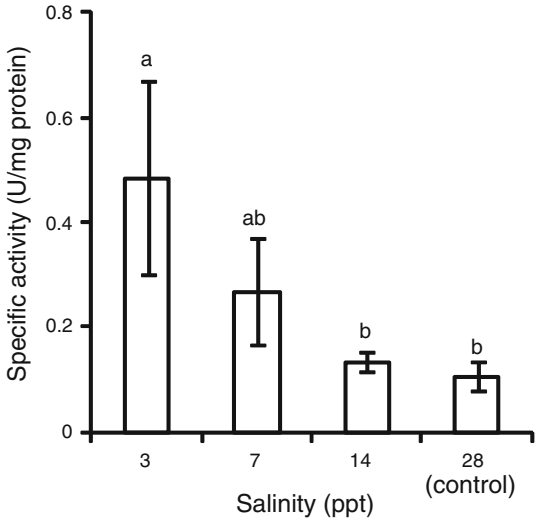

Fig. 5 Changes in the specific activity of L-serine ammonia lyase at differing salinities. *Bars* correspond to the SE of each average value ($n = 4$–7). *Differing letters* indicate significant difference ($P < 0.05$)

Alanine is considered to be an important osmolyte for purposes of IIR in crustaceans; however, excessive amounts of L-alanine inhibit important enzymes involved in cellular metabolism. These enzymes include alanine aminotransferase, alanine dehydrogenase and pyruvate kinase. Hence, crustaceans are considered to possess large quantities of D-alanine in particular tissues, and are known to alter the ratio of D-alanine to the sum of D- and L-alanine to control overall alanine levels in the process of IIR (Abe et al. 2005b). Therefore, the decrease in the ratio of D-alanine in the muscle at 3 ppt can be regarded as a typical response during the process of IIR. L-glutamine, D-alanine, L-alanine, and other L-amino acids that could not be analyzed by HPLC accounted for most of the decrease in free amino acids in the muscle (Table 1). The identities of the other L-amino acids were not clear. However, it may be assumed that L-proline accounted for most of these, because *L. vannamei* contains abundant proline (McCoid et al. 1984), and proline in the muscles of crustaceans is present in the L-form (Okuma et al. 1995; Abe et al. 1999; Fujimori and Abe 2002). Except in the case of glycine, our data match those of previous reports showing that crustaceans commonly regulate glycine, L-glutamine, L-proline, and D- and L-alanine levels in the process of IIR (Abe et al. 1999; Fujimori and Abe 2002; McNamara et al. 2004). Although the concentrations of many free amino acids decreased at low salinity, L-serine showed completely different dynamics from the other amino acids (Fig. 2b). The role of L-serine is therefore likely not that of an osmolyte, because if it had been an osmolyte its concentration would have decreased. Therefore, our data suggest that the increasing L-serine levels at 3 ppt play other osmoregulatory roles in the muscle—for example, as an energy source, as discussed later in this section.

There are three basic mechanisms by which the quantities of free amino acids in the tissues are varied for purposes of IIR: (1) changes in efflux and uptake through the plasma membrane; (2) displacement of the equilibrium between synthesis and degradation of proteins; and (3) changes in the rates of synthesis and degradation of amino acids (Gilles 1977; Boone and Schoffeniels 1979; Gilles

and Péqueux 1981; Neufeld and Wright 1996; Foster et al. 2009). In our study, whereas the levels of inorganic ions in the hemolymph decreased, those of particular free amino acids (glycine and L-serine) increased significantly (Fig. 3; Table S3). Our results mainly support the first mechanism, namely the efflux of amino acids from tissues to the body fluids. However, glycine and L-serine, the levels of which increased significantly in the hemolymph at low salinity, showed no decrease in the muscle. To shed light on this contradiction, it was necessary to focus on the dynamics of L-serine that increased in both muscle and hemolymph at low salinity. Animals were starved before the experiments, so that they could not take in nitrogenous substances. This suggests that the source of L-serine in the hemolymph was from nutritional stocks in tissues. Furthermore, the levels of L-serine in the muscle were maintained higher than those in the hemolymph. It is known that facilitated diffusion along a concentration gradient is a major system in which the transport of amino acids occurs in many organisms. Therefore, it is likely that L-serine in the muscle could move to the hemolymph along this gradient during the process of IIR. Hence, the increases in levels of L-serine in the muscle and hemolymph suggest that L-serine was synthesized in muscle at low salinity and then excreted into the hemolymph. Glycine levels increased only in the hemolymph and did not fluctuate in the muscle. Hence, we can assume that the glycine in the hemolymph came from other tissues. We therefore hypothesize that when animals are required to perform IIR in hyposmotic situations, they synthesize the appropriate forms of amino acids in the tissues for excretion into the body fluid.

Next, we focused on respiratory catabolism to further examine the functioning of amino acids in the hemolymph. Respiration is a mechanism for producing energy by consuming oxygen, and the oxygen consumption rate is often used as an indicator of energy consumption (Cech 1990; Chen and Nan 1993; Li et al. 2007). Ammonia is the main breakdown product of nitrogenous substances in many aquatic organisms (Spaargaren 1982; Regnault 1987). Hence, the ammonia excretion rate is an indicator of the consumptions of nitrogenous substances (Chen et al. 1994; Chen and Chia 1996; Lemos et al. 2001; Setiarto et al. 2004). Furthermore, a low O/N ratio, as calculated from the rates of oxygen consumption and ammonia excretion, indicates a high level of dependency on nitrogenous substances for purposes of aerobic metabolism (Corner and Cowey 1968; Chen and Lai 1993). The changes in ammonia excretion and O/N ratio at 3 ppt suggest that increased amounts of nitrogenous substances were consumed as energy sources by respiration at 3 ppt. This activated energy consumption likely occurs to satisfy the needs of active ion transport, as described in previous studies (Piller et al. 1995; Setiarto et al. 2004; Curtis and McGaw 2010). In crustaceans, most of the excreted ammonia is the product of the catabolism of amino acids (Haberfield et al. 1975; Regnault 1987; Chen and Chia 1996; Lemos et al. 2001). In this study, the dynamics of the ammonia levels corresponded to those of L-serine and glycine in the hemolymph. This is indicative of the mechanism previously reported in aquatic invertebrates, including crustaceans, by which glycine is metabolized to L-serine, and the L-serine is used as an energy source through the conversion of pyruvate and ammonia for use in the TCA cycle (Abe et al. 2005a). Hence, we can assume that the increased concentrations of L-serine and glycine in the hemolymph were metabolized as energy sources, decomposed to ammonia, and then excreted.

This assumption is supported by our results for the specific activity of L-serine ammonia lyase, which is an important enzyme in energy production and converts L-serine to pyruvate and ammonia (Ogawa et al. 1999). Specific activity of L-serine ammonia lyase increased in line with increases in the levels of L-serine in the muscle and hemolymph (Fig. 5). Considering the dynamics of L-serine in the muscle and hemolymph, our results suggest that part of the L-serine synthesized in the muscle was utilized as an energy source, and the remainder was moved to the body fluid to be consumed in other organs. Although we did not determine the specific activities of the enzymes involved in the metabolism of glycine, it is possible that glycine is also used as an energy source, because it is one of the major glucogenic amino acids. Therefore, those amino acids that increased in concentration in the hemolymph at low salinity are likely to decrease tissue osmolality via their efflux and to be utilized as energy sources in osmoregulation.

We can summarize our hypotheses concerning IIR in low-salinity adaptation as follows. When the shrimp encounter a sudden decrease in salinity, they attempt to regulate their body fluids as stably as possible. If they cannot maintain the osmolality of the body fluid within the proper range, they regulate intracellular osmolytes to adapt to the decrease in osmolality of the body fluid. Consequently, particular free amino acids are synthesized as energy sources in the tissues and are excreted to the body fluid. The amino acids that are released into the body fluid are likely metabolized by respiration, decomposed to ammonia, and then excreted. This system is likely to be part of the overall mechanism of IIR. It may be common in adaptation to hyposmotic conditions in many aquatic invertebrates, because both IIR and increasing nitrogenous excretion are widely observed under these conditions (Gilles and Péqueux 1981; Regnault 1987; Neufeld and Wright 1996; Navarro and Gonzalez 1998; Abe et al. 2005a; Watanabe et al. 2005; Richard et al. 2008). To support these hypotheses, it is important to trace in more

detail the metabolism of amino acids in the body. Further studies will shed light upon this aspect of osmoregulation mechanism.

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