Available online at www.sciencedirect.com
ScienceDirect
Comparative Biochemistry and Physiology, Part A 147 (2007) 254–259
CBP
www.elsevier.com/locate/cbpa

Hemolymph patterns of free amino acids in the brine shrimp *Artemia franciscana* after three days starvation at different salinities

Kaworu Nakamura *, Koichi Iwaizumi, Shoji Yamada

Faculty of Fisheries, Kagoshima University, 4-50-20 Shimo-Arata, Kagoshima 890-0056, Japan

Received 13 October 2006; received in revised form 26 December 2006; accepted 3 January 2007
Available online 25 January 2007

Abstract

The hemolymph pattern of free amino acids was examined in the brine shrimp, *Artemia franciscana* (Great Salt Lake origin). After one-month acclimation to 35 or 60 ppt salinity at 27 °C, the animals were transferred to 10, 35 or 60 ppt salinities to continue acclimation for 3 days without feeding at 27 °C. The osmolarity of one of the new media was raised by glycerol addition. In the hemolymph, 8 amino acids such as taurine, alanine, threonine, serine, lysine, glycine, arginine and leucine, comprised approximately 70% of the total content of free amino acids. This pattern suggested internal proteolysis due to starvation at high temperature. The total content of free amino acids significantly increased at 10 and 60 ppt salinities in comparison to 35 ppt. The hemolymph patterns from the 10 ppt and glycerol-added media showed a singularly high peak of taurine or alanine.

Keywords: Alanine; Artemia; Glycerol; Hemolymph; Salinity; Starvation

1. Introduction

Among zooplankton, the brine shrimp is a useful and important larval food in fish aquaculture around the world, due to both its convenience and its nutritional value for larvae (Bengtson et al., 1991). It belongs to the branchiopod Crustacea and there are several species and local strains (Lenz and Browne, 1991; Helland et al., 2000). Among local strains, the Great Salt Lake strain of *Artemia franciscana* is known to be very tolerant to high temperatures (Vanhaecke and Sorgeloos, 1989). Furthermore, every brine shrimp species shows an extremely wide range of environmental salinity tolerance (Vanhaecke et al., 1984). It is known to be a hypo-osmoregulator in more concentrated saline media than seawater and a hyper-osmoregulator in saline medium less than approximately 0.9% NaCl (Croghan, 1958). Furthermore, its NaCl adjustment has been studied with chloride cells of the phyllopodia, with a similar function to those of teleostean fish that live both in freshwater and seawater (Holliday et al., 1990).

For osmoregulation with other osmolytes, hemolymph free amino acids (FAAs) are known in crustacean species as being comparable to those of vertebrate species (Claybrook, 1983). They are generally present in the range of 20–80 mg/100 mL, differing from the high value of 700 mg/100 mL in insects (Jeuniaux, 1971). Some amino acids have been observed in crustacean hemolymph as the intracellular study by Duchateau and Florkin elucidated their function as major osmoeffectors (Gilles, 1997). For example, in the Japanese spiny lobster and giant freshwater prawn, hemolymph FAAs were examined in relation to changes in environmental salinity and glycine and alanine respectively were indicated as major osmolytes (Shinagawa et al., 1995; Huong et al., 2001).

However, there is limited information on hemolymph FAAs in the brine shrimp related to acclimation to various saline media. The present study aimed to reveal the composition and content of hemolymph FAAs together with hemolymph NaCl (the sum of Na^+ and Cl^- concentrations) in the brine shrimp acclimated to different salinities. Taking into consideration the various salinities of the habitats of this species, long-term acclimation at two different salinities was conducted before introducing short-term acclimation without feeding for three days at high temperature.

* Corresponding author. Tel.: +81 99 286 4160; fax: +81 99 286 4133.
E-mail address: kaworu@fish.kagoshima-u.ac.jp (K. Nakamura).

1095-6433/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.cbpa.2007.01.008

2. Materials and methods

2.1. Animals, rearing and sampling

Adults of the brine shrimp, *A. franciscana*, were used. The cysts, Great Salt Lake in origin, were previously brought sealed in an aluminium-foil bag. After hatching in saline media at 35 and 60 ppt salinities at 27 °C, nauplii were transferred to 3-L flasks containing the respective saline media. The reason for choosing such a temperature was due to the high survival and specific growth rate of this strain at 27.5 °C (Vanhaecke and Sorgeloos, 1989).

The mass-culture continued for about one month by daily feeding with the diatom, *Chaetoceros gracilis*. The strain was obtained 5 years ago from the Fishery Research Center of Kagoshima Prefecture, and since then has been inoculated and raised in our laboratory. Half of the volume of each brine shrimp medium was changed every 2 days.

For preparation of the incubation media at 35 and 60 ppt salinities, seawater from Kagoshima Bay was used. After filtration through 63 μm mesh, it was diluted with distilled water or boiled down to obtain the respective media of low or high salinity. The salinity was measured using a refraction-type salinometer (S-mil E, Atago, Tokyo).

The diatom used as a food for the brine shrimp was collected daily from 75 to 250-mL flasks of brown diatom-containing water by centrifugation at 2000 ×*g* for 15 min. The brown water contained diatoms at an approximate density of $3–6\times10^6$ cells/mL. This brown water was sampled from a 3-L source medium of Provasoli's enriched seawater. The 3-L medium was alternatively prepared in 2 flasks after inoculation with a time lag of about one week.

The diatom incubation was conducted with aeration at a 14L:10D (14 h light of 3000 lx: 10 h dark) photoperiod in an incubation room at 23 °C. The flasks containing the nauplii were put in a 27 °C incubator and provided with aeration and the above-mentioned photoperiod. Taking into consideration the convenience of sight-monitoring and postulated demerit of using strong light, the illumination intensity was reduced to 50–100 lx. The postulation was that darkness would lower the swimming activity together with the energy consumption and in this way allow faster growth (Lavens and Sorgeloos, 1991).

When sex differentiation was observed, which was about 2 weeks later, the brine shrimp rearing method was changed into separate rearing of males and females. After rearing for 2 weeks, the following experiment was introduced to examine the effect of short-term acclimation on the hemolymph parameters. The individuals acclimated at 35 ppt salinity were put into salinities of 10, 35 and 60 ppt (respective treatments symbolized as 35/10, 35/35 and 35/60). Other individuals acclimated at 60 ppt salinity were similarly put into media at 35 and 60 ppt salinities (60/35, 60/60).

Besides 35/10, to distinguish the effect of osmolarity from that of salinity on the above-mentioned hemolymph parameters, another 10 ppt salinity medium was prepared by dissolving glycerol at 7.9 g/100 mL. A number of the individuals previously acclimated for 30 days at 35 ppt salinity were transferred into this new medium (35/10 g). At 27 °C, successive acclimation of the above 6 groups continued for 3 days without feeding.

Hemolymph was individually sampled from males and females. Before bleeding, the adult was slightly washed with deionized water to ensure only hemolymph sampling without adhering medium. They were then wiped with tissue paper on the stage of a low-power binocular microscope. Then, a notch along the dorsal midline was made with dissecting scissors. The thinned tip of a capillary glass pipette (ϕ1.5 mm × L90 mm) was put on the notch. Without coagulation, the flowing hemolymph was drawn into it by sucking through a silicon tube connected to the pipette.

Male and female hemolymph of the same group was pooled separately in one or 2 silicon microtubes while cooled in iced water. Each tube contained hemolymph collected from more than 100 individuals, with the exception of 2 groups, namely 60/35 and 60/60. They were stored in a freezer at −80 °C until measurement at a later date. Concerning the 2 groups of 60/35 and 60/60, hemolymph from males and females were mixed just before Na^+Cl^- measurement due to insufficient individual quantities.

2.2. Measurement of hemolymph NaCl

Hemolymph NaCl was measured separately as Na^+ and Cl^- ions with a portable clinical analyzer (i-Stat, Abbott Lab. Inc., East Windsor). The seawater used for the incubation medium was also measured to obtain the environmental value of NaCl. Before measurement, concentrated hemolymph and seawater were diluted with distilled water to adjust their Na^+ and Cl^- concentrations to a level suitable for application to the instrument. About 80 to 100 μL of the specimen was injected into an exclusive cartridge provided with Na^+ and Cl^- electrodes. The original value was later calculated from the instruments' digital data.

2.3. Analysis of hemolymph free amino acids (FAAs)

A 50 μL volume from the pooled hemolymph was homogenized in 6.5% perchloric acid solution in a total volume of 250 μL. It was centrifuged at 8000 ×*g* for 10 min and then FAAs in the supernatant were separated and determined using a Shimadzu LC-6A amino acid analysis HPLC system (Kyoto, Japan). The sample was injected (20 μL) onto a column (7 μm, 4.0 mm × 150 mm, Shimadzu Shim-pack ISC-07/S1504Na) with a guard column (7 μm, 4.0 mm × 50 mm, Shimadzu, ISC-07Na). The column was eluted at 55 °C with the following gradient program using 2 sodium citrate buffers (buffer A: pH 3.20, buffer B: pH 10.0) at a total flow rate of 0.3 mL/min: 0–15 min, 0% buffer B; 15–35 min, a linear gradient from 0% to 16% buffer B; 35–40 min, 16% buffer B; 40–50 min, a linear gradient from 60% to 100% buffer B; 50–60 min, 100% buffer B; 60–65 min, 0.2 N NaOH for washing; 65–90 min, 0% buffer B for equilibration. Amino acids were detected with a Shimadzu RF-535 fluorescence HPLC monitor (Ex. 348 nm, Em. 450 nm) after derivatization with the

Table 1
NaCl-concentration of hemolymph from different groups (mM)

Group*	Hem. NaCl	Averaged value
35/10 f	331.3	326.3±7.1(n=2)
35/10 m	321.3	
35/10 gf	266.3	265.0±1.8 (n=2)
35/10 gm	263.8	
35/35 f	370.0	
35/35 m	351.3	368.4±16.4 (n=3)
60/35 fm	384.0	
35/60 f	394.5	
35/60 m	401.0	408.8±19.4 (n=3)
60/60 fm	430.8	

*f; female; fm, female and male; m, male.

following reagents (reagent 1: 0.216 M H_3BO_3/0.384 M Na_2CO_3/0.108 M K_2SO_4 buffer, pH 10.0, containing 0.002% NaClO; reagent 2: the same buffer, pH 10.0, containing 0.08% o-phthalaldehyde, 0.1% *N*-acetyl-L-cysteine, 0.04% Brij-35 and 1.4% ethanol) mixed at 55 °C at a flow rate of 0.2 mL/min each. Both glutamine and asparagine peaks did not separate from the threonine peak on the HPLC system used in the present study.

2.4. Statistical analysis

The values belonging to the same group and/or same salinity at the final sampling were averaged. Taking into consideration the small sample number, non-parametric analysis of variance by Kruskal–Wallis test and then Tukey–Kramer test were employed to analyze changes in the concentration of hemolymph FAAs as a consequence of salinity treatment.

3. Results

3.1. Hemolymph NaCl

The hemolymph NaCl for males and females from different groups are shown in Table 1. Their averages were 326±7.1, 265±1.8, 368±16.4 and 409±19.4 mM for the 35/10, 35/10 g, 35/35 merged with 60/35 and 60/60 merged with 35/60, respectively. Thus, the NaCl value of 35/10 g was distinctly low in comparison to the others.

The NaCl value of the medium at 35 ppt salinity was 955±7.1 mM (n=3). At 10 and 60 ppt salinities, values of 273 and 1641 mM were estimated for each respective media. Then, the average of hemolymph NaCl was calculated which corresponded with 39% of the environmental value at 35 ppt salinity. Those values corresponding to acclimation at 60 and 10 ppt salinities corresponded to 25 and 119% of each environmental value, respectively. Hemolymph was estimated to have isotonicity with approximately 330 mM NaCl.

3.2. Hemolymph FAAs

Table 2 shows the averaged concentrations of hemolymph FAAs from the groups acclimated to 3 different salinities (each n=4). Only the total value of 35 ppt salinity showed a significantly lower difference to those of 10 and 60 ppt salinities (p<0.05).

Table 2
Averaged concentrations of free amino acids in hemolymph from different groups (mM)

FAA	35/10+35/10 g	35/35+60/35	35/60+60/60
Asp	0.28±0.19	0.18±0.05	0.29±0.02
Thr*1	1.24±0.23	1.38±0.37	1.65±0.15
Ser	0.78±0.43	0.95±0.21	1.13±0.17
Glu	0.51±0.29	0.29±0.06	0.54±0.04
Pro	0.45±0.13	0.33±0.07	0.43±0.11
Gly	0.48±0.20	0.41±0.15	0.63±0.10
Ala	2.53±2.41	0.85±0.35	1.06±0.16
Val	0.45±0.18	0.34±0.11	0.49±0.01
Met	0.13±0.08	0.13±0.04	0.22±0.04
Ile	0.29±0.14	0.18±0.05	0.35±0.04
Leu	0.51±0.28	0.30±0.09	0.66±0.13
Phe	0.23±0.16	0.22±0.05	0.45±0.11
His	0.25±0.06	0.20±0.05	0.25±0.01
Lys	0.63±0.27	0.32±0.19	0.88±0.20
Arg	0.51±0.31	0.36±0.06	0.65±0.15
Tau	1.74±0.64	1.31±0.25	1.49±0.27
U.com*2	0.80±0.36	0.47±0.08	0.53±0.47
Total*3	12.29±2.00[a]	8.40±1.85[a,b]	11.87±1.13[b]

[a,b] Values followed by the same superscript are significantly different (p<0.05). For symbols, see the text. *1, including Thr, Gln and Asn; *2,unidentified compound; *3, including other minor components.

The averaged compositions of hemolymph FAAs from the groups as 35/10, 35/10 g, 35/35 merged with 60/35 and 60/60 merged with 35/60 are shown in Fig. 1. Among FAAs, taurine, alanine, threonine, serine, lysine, glycine, arginine and leucine were abundant and they comprised approximately 70% of the total FAAs. Amino acids such as taurine in 35/10 and alanine in 35/10 g showed a singularly high peak with respect to FAA patterns (Fig. 1).

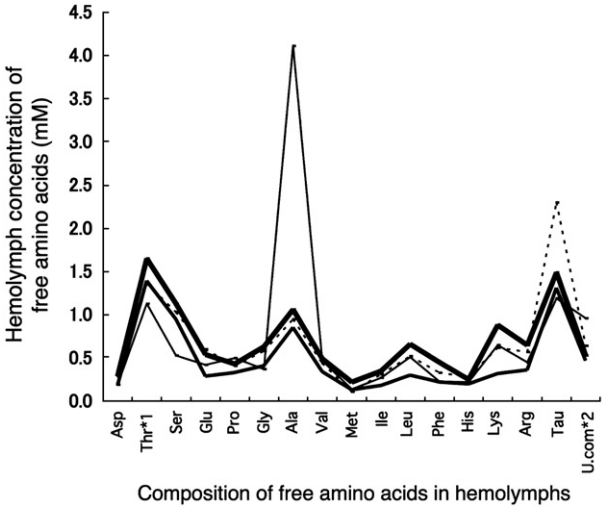

Fig. 1. Hemolymph composition of free amino acids from different groups. Thr*1, containing Thr, Asn and Gln; U.com*2, unidentified compound. Bold line, 60/60 group merged with 35/60 group; dotted line, 35/10 group; fine line, 35/10 g group; medium-fine line, 35/35 group merged with 60/35 group.

4. Discussion

Previously, Croghan (1958) examined hemolymph ions of the brine shrimp, *Artemina salina*, using the capillary-bleeding method similar to that employed in the present study. Then the hemolymph of the brine shrimp was confirmed as being hypotonic and hypertonic in saline media above and below approximately 0.9% NaCl, respectively. Furthermore, the averaged concentration of hemolymph NaCl was shown to be 1.30% in the saline medium containing 3.32% NaCl. This percentage is low compared to the values calculated from the hemolymph NaCl in the present study: 368 mM NaCl should yield 2.1% NaCl at 35 ppt salinity. This higher percentage by 0.8% than the Croghan's value may be due to concentration by hemolymph evaporation that would have inevitably occurred during bleedings in the present study.

The total concentration of hemolymph FAAs ranged from 8.4 to 12.3 mM in the present study. The values obtained in the present study exceeded the general range of 2–6 mM known for crustacean species, although high values of 10–17 and 37 mM have been observed in a few species such as the marine isopods *Sphaeroma serratum* and *Uca pugilator* (Claybrook, 1983). The above excess includes on average an approximate 8% content of the total FAAs belonging to other minor components (Table 2). Some part may be shared by the hemolymph concentration due to evaporation during sampling.

Concerning the raised values of FAAs at both low and high salinities, a report by Shinagawa et al. (1995) on the Japanese spiny lobster, *P. japonicus*, acclimated for 24 h to 75, 100 and 125% seawater showed the concentration of hemolymph FAAs to be 24.73, 2.93 and 4.18 mM at each respective media. The higher values than that of 100% seawater were recognized for both 75 and 125% seawater. The large increase in FAA concentration at 75% seawater was interpreted as being attributable to extrusion from the tissues during adaptation to low salinity.

The high concentrations of hemolymph FAAs obtained both at lower and higher salinities than seawater, even after large increases, corresponded to 4% of the hemolymph NaCl at most. In the brine shrimp, therefore, Na^+Cl^- is suggested as having a major role, greater than FAAs in hemolymph osmoregulation. The same consideration has been emphasized in the giant freshwater prawn, *M. rosenbergii*, as follows: the overall contribution of FAAs to hemolymph osmolarity levels is small compared with that of major hemolymph ions (Huong et al., 2001).

Such inferior roles of FAAs as osmolytes than NaCl in the hemolymph are evidently different from the case of the cells of crustacean tissues. Gilles (1997) reviewed that the intracellular FAAs have been major osmoeffectors in crustacean cells since the study on the Chinese crab *Eriocheir sinensis* by Duchateau and Florkin in 1955. Shaw (1958) then confirmed that the FAAs, taurine and trimethyamine oxide accounted for over 60% of the total osmotic pressure in osmoregulation of the muscular fibers from the shore crab *Carcinus maenas*.

Concerning the FAA composition of the brine shrimp, cysts and nauplii of *A. franciscana* and *Artemia* populations from different localities were examined using homogenized samples (Helland et al., 2000). The content and pool composition of FAAs were generally modulated in populations. Of total FAAs, 70% contained taurine, glutamic acid, alanine, glycine and arginine. Approximately 50% of the total content of FAAs was occupied by taurine. Furthermore, taurine, alanine, glycine and proline were considered to be the major osmolytes.

There are some differences in appearance even in the same *Artemia* species between the above-cited results and the present study. Concerning hemolymph FAAs in the present study, 70% of the total FAAs comprised 8 amino acids: taurine, alanine, threonine, serine, lysine, glycine, arginine and leucine. Except for the threonine peak, because it contained asparagine and glutamine, amino acids such as serine, lysine and leucine disagreed with the previous results. The hemolymph composition was similar to the tissue FAA composition of the copepod *Calanus finmarchius* (Helland et al., 2003). In the latter, glycine, taurine, arginine, alanine, lysine and leucine comprised 80–85% of the total FAAs. The inconsistency in the FAA composition was also observed in similarly hatched nauplii of the same strain as follows (Seidel et al., 1980): glutamic acid, aspartic acid, leucine, arginine, lysine and phenylalanine accounted for approximately half of the total FAA content.

These inconsistencies seem to have been caused by some differences between materials or methods. In the present study, the animals were deduced to have suffered from proteolysis due to starvation at high temperature, although the acclimation period was short. That is because generally the internal adjustment for acclimation does not seem to require a long period in crustacean species. For example, hemolymph osmolarity and Na^+ required only 24 h after transference to acquire stable maxima in the freshwater shrimp *M. olfersii* exposed to 21 ppt salinity (McNamara et al., 2004). In *M. rosenbergii*, hemolymph osmolarity, Na and Cl concentrations reached a constant level within 3 days after direct exposure from freshwater to 21 ppt salinity (Cheng et al., 2003). Furthermore, in Penaeidea, such as *P. monodon* (Ferraris et al., 1986) and *P. chinensis* (Chen and Lin, 1994), hemolymph osmolarity of each prawn transferred from 32 to 8–40 ppt or 30 to 10–40 ppt both reached constant levels within 2 days. The physiological adjustment during acclimation should participate in the energy metabolism and force the rapid turnover upon hemolymph FAAs.

The second reason is the support by the presence of similar patterns in blood FAAs from other animals. The blood FAAs of the lamprey *Lampetra fluviatilis*, which increased after prolonged starvation, had the following sequence: alanine, β-alanine, leucine, isoleucine, glycine, lysine, valine and threonine (Emelyanova et al., 2004). This composition, with the exception of isoleucine and valine agrees with that of the present study (Fig. 1). Similar increases and patterns of hemolymph FAAs were also seen in the snail *Helix pomatia*, subjected to starvation at low temperature (Iskierko and Gorski, 1974). The FAA sequence was leucine, valine, alanine, glutamic acid, lysine, histidine and arginine.

Concerning the hemolymph osmolytes, Shinagawa et al. (1995) examined FAAs in tissues and hemolymph of the

Japanese spiny lobster, *P. japonicus*. They found that hemolymph contained large amounts of glycine and alanine. Especially the content of glycine in both tissues evidently changed in relation to the concentration of the external saline media. For this, glycine was thought to be one of the most important osmoeffectors.

Similar results with glycine were obtained from homogenized samples of the shrimp, *Penaeus stylirostris* (Cobb et al., 1975) and *Palaemon elegans* (Dalla Via, 1989). In *P. stylirostris*, only glycine and proline showed increasing and decreasing levels corresponding to changes in salinity. In *P. elegans*, glycine, taurine, proline, arginine and alanine showed high concentrations in seawater. Furthermore, with the exception of arginine, the above amino acids lowered the total free amino acid pool by 44% after acclimation to 10 ppt saline media.

McAllen (2003) examined the quantitative change of intracellular FAAs in the supralittoral rockpool copepod, *Tigriopus brevicornis*, using homogenized specimens. Over a 3-day acclimation to 5, 35 and 70 ppt salinities, proline, alanine and lysine were the major contributors to the intracellular free amino acid pool. Furthermore, significant decreases and increases in alanine were shown for 70 and 5 ppt saline media, respectively.

Abe et al. (1999) inferred that alanine including L- and D-types was the only common amino acid osmolyte that increased with increasing salinity in the Japanese mitten crab, *E. japonicus*. In the giant freshwater prawn, *M. rosenbergii* (Huong et al., 2001), hemolymph FAAs showed a concentration range from 0.85 to 2.1 mM corresponding to the increase in salinity and its increase depended on glycine, arginine, alanine, proline and lysine. They occupied a 45 or 70% content of the total FAAs at low or high salinity, respectively. Especially, alanine showed a 5- to 6-fold increase in high salinity media.

Thus, the relatively large content and specific appearance of alanine have been detected hitherto in hemolymph or tissues of other crustacean species related to osmoregulation or acclimation process to various salinities. In the present study, hemolymph alanine showed 4.1 mM at the 10 ppt salinity medium with dissolved glycerol (this medium should have almost equal osmolarity to the 35 ppt salinity medium). Contrary to this medium, the 10 ppt salinity medium without glycerol showed an almost equal value of alanine to that of 60 ppt salinity. For this 10 ppt medium, only taurine showed the high value similar to the case of postlarvae in the seawater shrimp *Penaeus aztecus* exposed to 37% seawater (Bishop and Burton, 1993).

In earlier studies, free glycerol has been defined as the major carbohydrate component in the dormant embryo of the brine shrimp (Clegg, 1962). It could be easily absorbed into the hemolymph through the gut and may perform as one of the osmolytes. This theory seems to be supported by the evident decrease in hemolymph NaCl in 35/10 g compared to that of 35/10 (Table 1). The phenomenon would be attributable to a kind of trade-off adjustment, in terms of unquantifiable meaning, of Na^+Cl^- for the hemolymph osmolarity by alanine and possible other organic osmolyte(s) such as glycerol.

Another word similar and confusable to the above-mentioned trade-off is known as the compensatory adjustment proposed in the intracellular osmoregulation by Gilles at 1987. In this case, the organic compounds osmotically compensate for the intracellular decrease in ion concentration after a hyperosmotic shock (Gilles, 1997). It is well known that glycerol could be metabolized to pyruvate. Pyruvate could be then further catabolized into CO_2 and H_2O or, in certain cases, converted to alanine. Then, for the present 35/10 g, alanine in the hemolymph may owe partly its singular and large increase to glycerol conversion.

Acknowledgement

The authors thank Mr. Miguel Vazquez Archdale from the Fishing Technology Lab. of Faculty of Fisheries, Kagoshima University, for his kind improvements to this manuscript.

References

Abe, H., Okuma, E., Amano, H., Noda, H., Watanabe, K., 1999. Effects of seawater acclimation on the levels of free D- and L-alanine and other osmolytes in the Japanese mitten crab *Eriocheir japonicus*. Fish. Sci. 65, 949–954.

Bengtson, D.A., Leger, P., Sorgeloos, P., 1991. Use of *Artemia* as a food source for aquaculture. In: Browne, R.A., Sorgeloos, P., Trotman, C.N.A. (Eds.), Artemia Biology. CRC Press, Boston, pp. 255–285.

Bishop, J., Burton, R.S., 1993. Amino acid synthesis during hyperosmotic stress in *Penaeus aztecus* postlarvae. Comp. Biochem. Physiol. A 106, 49–56.

Chen, J.-C., Lin, J.-L., 1994. Osmolality and chloride concentration in the hemolymph of subadult *Penaeus chinensis* subjected to different salinity levels. Aquaculture 125, 167–174.

Cheng, W., Liu, C.-H., Cheng, C.-H., Cheng, J.-C., 2003. Osmolality and ion balance in giant river prawn *Macrobrachium rosenbergii* subjected to changes in salinity: role of sex. Aquac. Res. 34, 555–560.

Claybrook, D.L., 1983. Nitrogen metabolism. In: Bliss, D.E. (Ed.), The Biology of Crustacea, vol. 5. Academic Press, New York, pp. 163–213.

Clegg, J.S., 1962. Free glycerol in dormant cysts of the brine shrimp, *Artemia salina*, and its disappearance during development. Biol. Bull. 123, 295–301.

Cobb, B.F., Conte, F.S., Edwards, M.A., 1975. Free amino acids and osmoregulation in penaid shrimp. J. Agric. Food Chem. 23, 1172–1174.

Croghan, P.C., 1958. The osmotic and ionic regulation of *Artemia salina* (L.). J. Exp. Biol. 35, 219–233.

Dalla Via, G.J., 1989. The effect of salinity on free amino acids in the prawn *Palaemon elegans* (Rathke). Arch. Hydrobiol. 115, 125–135.

Emelyanova, L.V., Koroleva, E.M., Savina, M.V., 2004. Glucose and free amino acids in the blood of lampreys (*Lampetra fluviatilis* L.) and frog (*Rana temporaria* L.) under prolonged starvation. Comp. Biochem. Physiol. A 138, 527–532.

Ferraris, R.P., Parado-Estepa, F.D., Ladja, J.M., de Jesus, E.G., 1986. Effect of salinity on the osmotic, chloride, total protein and calcium concentrations in the hemolymph of the prawn *Penaeus monodon* (fabricius). Comp. Biochem. Physiol. A 83, 701–708.

Gilles, R., 1997. "Compensatory" organic osmolytes in high osmolarity and dehydration stresses: History and perspectives. Comp. Biochem. Physiol. A 117, 279–290.

Helland, S., Triantaphyllidis, G.V., Fyhn, H.J., Evjen, M.S., Lavens, P., Sorgeloos, P., 2000. Modulation of the free amino acid pool and protein content in populations of the brine shrimp *Artemia* spp. Mar. Biol. 137, 1005–1016.

Helland, S., Nejstgaard, J.C., Fyhn, H.J., Egge, J.K., Bamstedt, U., 2003. Effects of starvation, season, and diet on the free amino acid and protein content of *Calanus finmarchicus* females. Mar. Biol. 143, 297–306.

Holliday, C.W., Roye, D.B., Roer, R.D., 1990. Salinity-induced changes in branchial Na^+/K^+-ATPase activity and transepithelial potential difference in the brine shrimp *Artemia salina*. J. Exp. Biol. 151, 279–296.

Huong, D.T.T., Yang, W., Okuno, A., Wilder, M.N., 2001. Changes in free amino acids in the hemolymph of giant freshwater prawn *Macrobrachium rosenbergii* exposed to varying salinities: relationship to osmoregulatory ability. Comp. Biochem. Physiol. A 128, 317–326.

Iskierko, J., Gorski, A., 1974. Effect of starvation and low temperature on the content of free amino acids in the leg tissue and hemolympha of *Helix pomatia*. Acta Physiol. Pol. 25, 371–375.

Jeuniaux, C., 1971. Hemolymph-arthropoda. In: Florkin, M., Scheer, B.T. (Eds.), Chemical Zoology, vol. 6. Academic Press, New York, pp. 63–118.

Lavens, P., Sorgeloos, P., 1991. Production of *Artemia* in culture tanks. In: Browne, R.A., Sorgeloos, P., Trotman, C.N.A. (Eds.), Artemia Biology. CRC Press, Boston, pp. 317–350.

Lenz, P.H., Browne, R.A., 1991. Ecology of *Artemia*. In: Browne, R.A., Sorgeloos, P., Trotman, C.N.A. (Eds.), Artemia Biology. CRC Press, Boston, pp. 237–253.

McAllen, R., 2003. Variation in the free amino acid concentrations of the supralittoral rockpool copepod crustacean *Tigriopus brevicornis* during osmotic stress. J. Mar. Biol. Assoc. U.K. 83, 921–922.

McNamara, J.C., Rosa, J.C., Greene, L.J., Augusto, A., 2004. Free amino acids pools as effectors of osmostic adjustment in different tissues of the freshwater shrimp *Macrobrachium olfersii* (Crustacea, Decapoda) during long-term salinity acclimation. Mar. Freshw. Behav. Physiol. 37, 193–208.

Seidel, C.R., Kryznowek, J., Simpson, K.L., 1980. International study on *Artemia*. XI. Amino acid composition and electrophoretic protein patterns of *Artemia* from five geographical locations. In: Persoone, G., Sorgeloos, P., Roels, O., Jaspers, E. (Eds.), Ecology, Culturing, Use in Aquaculture, vol. 3. Universa Press, Wetteren, pp. 375–382.

Shaw, J., 1958. Osmoregulation in the muscle fibres of *Carcinus maenas*. J. Exp. Biol. 35, 920–929.

Shinagawa, A., Suzuki, T., Konosu, S., 1995. Preliminary studies on the effects of salinity on intracellular nitrogenous osmolytes in various tissues and hemolymph of the Japanese spiny lobster, *Panulirus japonicus* (von Siebold, 1824). Crustaceana 68, 129–137.

Vanhaecke, P., Sorgeloos, P., 1989. International study on *Artemia*. XLVII. The effect of temperature on cyst hatching, larval survival and biomass production for different geographical strains of the brine shrimp *Artemia* spp. Ann. Soc. R. Zool. Belg. 119, 7–23.

Vanhaecke, P., Siddall, S.E., Sorgeloos, P., 1984. International study on *Artemia* XXXII. Combined effects of temperature and salinity on the survival of *Artemia* of various geographical origin. J. Exp. Mar. Biol. Ecol. 80, 259–275.