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Evolutionary transition to freshwater by ancestral marine palaemonids: evidence from osmoregulation in a tide pool shrimp

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ABSTRACT: The transition from marine/brackish waters to freshwater habitats constitutes a severe osmotic and ionic challenge, and successful invasion has demanded the selection of morphological, physiological, biochemical and behavioral adaptations. We evaluated short-term (1 to 12 h exposure) and long-term (5 d acclimation), anisosmotic extracellular (osmolality, [Na^+, Cl^-]) and long-term isosmotic intracellular osmoregulatory capability in *Palaemon northropi*, a neotropical intertidal shrimp. *P. northropi* survives well and osmo- and ionoregulates strongly during short- and long-term exposure to 5–45‰ salinity, consistent with its rocky tide pool habitat subject to cyclic salinity fluctuations. Muscle total free amino acid (FAA) concentrations decreased by 63% in shrimp acclimated to 5‰ salinity, revealing a role in hypoosmotic cell volume regulation; this decrease is mainly a consequence of diminished glycine, arginine and proline. Total FAA contributed 31% to muscle intracellular osmolality at 20‰, an isosmotic salinity, and decreased to 13% after acclimation to 5‰. Gill and nerve tissue FAA concentrations remained unaltered. These tissue-specific responses reflect efficient anisosmotic and anisoionic extracellular regulatory mechanisms, and reveal the dependence of muscle tissue on intracellular osmotic effectors. FAA concentration is higher in *P. northropi* than in diadromous and hololimnetic palaemonids, confirming muscle FAA concentration as a good parameter to evaluate the degree of adaptation to dilute media. The osmoregulatory capability of *P. northropi* may reflect the potential physiological capacity of ancestral marine palaemonids to penetrate into dilute media, and reveals the importance of evaluating osmoregulatory processes in endeavors to comprehend the invasion of dilute media by ancestral marine crustaceans.

KEY WORDS: Freshwater invasion · Marine–freshwater transition · Physiological adaptation · Osmotic and ionic regulation · Free amino acids · Palaemonid shrimp · *Palaemon northropi*

INTRODUCTION

The invasion of freshwater by the decapod Crustacea began some 600 million years ago (Ruppert & Barnes 1994), a process often inextricably associated with increasing terrestriality. To illustrate, some sesarminid (Schubart & Diesel 1998, 1999) and ocypodidid (Thurman 2003) brachyurans occupy freshwater secondarily, having penetrated from the marine supralittoral into semiterrestrial riverbank habitats, using this medium for gill wetting, molting, larval release and refuge. Other taxa such as palaemonid shrimp, however, have taken a more direct route via brackish waters like coastal lagoons and estuaries, followed by penetration into freshwater habitats (Freire et al. 2003).

The interface between the marine and freshwater habitats presents an osmotic and ionic barrier that few crustacean taxa have been able to penetrate. While

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the extracellular body fluids of most marine species approximate seawater in composition and are essentially isosmotic and mainly isoionic with respect to Na^+ and Cl^-, such ion concentrations are minimal in freshwater. Consequently, freshwater species must expend more energy on the anisosmotic and anisoionic regulatory mechanisms that maintain their steep osmotic and ionic gradients against the surrounding medium, sustaining their essential life processes. The successful invasion of dilute media includes the selection of favorable morphological (Taylor & Taylor 1992, Freire & McNamara 1995, Onken & McNamara 2002), physiological (Péqueux 1995, Kirschner 2004, Weihrauch et al. 2004, Tsai & Lin 2007, Freire et al. 2008) and biochemical (Leone et al. 2005, Mendonça et al. 2007) adaptations, and those ancestral crustaceans that surmounted this barrier evidently evolved efficient mechanisms of hyperosmotic and ionic regulation, allowing survival in such habitats (Schubart & Diesel 1998, 1999).

The principal mechanisms of ionic and osmotic regulation in freshwater crustaceans include active salt absorption across the specialized gill epithelia (Lima et al. 1977, McNamara & Lima 1997, McNamara & Torres 1999, Weihrauch et al. 2004, Belli et al. 2009) and low water permeability that, together with the excretion of isosmotic or dilute urine by the antennal glands (Denne 1968, Augusto et al. 2007a), allows the anisoionic and anisosmotic regulation of hemolymph ions and water essential to optimal cell and organ metabolism (Freire et al. 2003, 2008). In estuarine or diadromous crustaceans that inhabit brackish or dilute media, a second mechanism, isosmotic intracellular regulation, adjusts cell volume by means of osmotic effectors like K^+, small peptides and, particularly, free amino acids (FAA). Adjustment in the intra- and extracellular FAA pools depends on alterations in FAA synthesis and oxidation rates, shifts in flux equilibria across the cell membranes and/or changes in protein metabolism (Tan & Choong 1981, Péqueux 1995). Further, tissue FAA concentrations can be used as good indicators of the recency of freshwater invasion, given their reduced concentration in freshwater crustaceans, compared to marine crustaceans (Potts & Parry 1964, Mantel & Farmer 1983, Augusto et al. 2007a,b).

Studies on the invasion of freshwater are sparse and are not well resolved with regard to subjacent physiological mechanisms. The transition to freshwater has occurred not only on a macroevolutionary scale, but also recently, continuing to the present day in many diadromous palaemonids, particularly within the genus *Macrobrachium* (Moreira et al. 1983, Jalihal et al. 1993, Pereira & Garcia 1995, Freire et al. 2003, Murphy & Austin 2005). Lee & Bell (1999) suggest that freshwater invaders might originate more readily from ancestors that occupied habitats exhibiting ample temporal and/or spatial salinity ranges since broad salinity tolerance can develop under these circumstances. Additionally, diurnal or seasonal salinity fluctuations are important as these lesser time-scales determine acclimation response rates. Of particular interest in the study of the conquest of freshwater are intertidal species, confronted daily by notable, cyclic salinity fluctuations, and whose osmoregulatory abilities may still reflect those of ancestral species that invaded dilute media, offering an excellent opportunity to examine basal traits and physiological adaptation in progress. We hypothesize that the colonization of freshwater by the palaemonid shrimps may have come about by means of a brackish water ancestor frequently exposed to a wide salinity range. This ancestral species must have acquired osmotic and ionic regulatory capability in dilute media, which would furnish the sustaining adaptations requisite for the subsequent invasion of freshwater.

As an experimental model, we employ *Palaemon northropi*, an intertidal, palaemonid shrimp distributed from the Bermuda archipelago in Central America to Uruguay in southern South America (Holthuis 1952). This species inhabits rocky tide pools where it frequently confronts widely variable salinities as a function of tides, precipitation, evaporation and seasonal temperature variation. Here we evaluate the osmoregulatory physiology of *P. northropi*, the chief process underpinning the conquest of dilute media. We examine responses like survival, osmotic and ionic regulatory capability and muscle tissue hydration and a role for FAA in intracellular volume regulation in shrimp exposed to a wide salinity range, during both short-term exposure (hours) such as might occur during a daily tidal cycle, and after long-term exposure (days) to examine adjustments in intracellular FAA titers.

MATERIALS AND METHODS

Specimens of the neotropical intertidal shrimp *Palaemon northropi*, measuring from 1.5 to 4.0 cm total length (modal length 3.0 cm, N ≈ 600), were collected at low tide from tidal pools on the rocky shores of Barequeçaba and Guaecá beaches near São Sebastião on the northern coast of São Paulo State, Brazil (23° 47′ 32″ S, 45° 35′ 54″ W). Salinity and temperature at the collection sites varied significantly ($p < 0.001$), ranging from 3 to 33‰ and 24 to 31°C, respectively, both parameters dependent on pool location on the shore.

In the laboratory, shrimp were held in 60 l plastic tanks containing aerated seawater from the collection sites for 3 d prior to beginning experiments. Holding

salinity was 31 to 33‰, stocking density was 2 l ind.$^{-1}$ and water temperature was maintained at ~23°C. The shrimp were fed on alternate days in the evening with fragments of fish muscle and checked daily in the morning for excess. Seawater was changed every 2 to 3 d when necessary, evaluated by accompanying water transparency.

To estimate the approximate lethal salinity limit, 3 groups of 10 shrimp each were directly exposed for a 10 d period to experimental saline media of <0.5 (freshwater), 1, 1.5, 2, 5, 20 or 45‰ salinity. Adult *Palaemon northropi* survived <2 h after direct transfer from seawater to fresh (<0.5‰) or brackish (1‰) water. However, at 1.5‰ and above (5, 20 and 45‰), all shrimp survived for at least 10 d. Subsequent experiments were based on these findings.

To examine the effect of acute exposure to saline media on anisosmotic extracellular osmotic and ionic regulatory capability and on tissue hydration, groups of 12 to 13 intermolt shrimp were exposed to salinities of 5, 20 or 50‰ for 1, 6 or 12 h to simulate a tidal cycle. To evaluate the effect of long-term exposure to saline media on osmotic and ionic regulatory ability, isosmotic intracellular regulatory capacity and tissue hydration, groups of 30 intermolt shrimp were directly exposed to 5, 20 or 45‰ salinity for 5 d. Shrimp for these analyses measured 3 to 4 cm total length. Saline media (<0.5 to 20‰) were prepared by diluting seawater from the collection sites with freshwater or by diluting the first thaw of frozen seawater (for 45 and 50‰), and were verified using a hand-held optical refractometer (Model 10419, American Optical).

After each exposure period, hemolymph samples were taken from the pericardial region located at the dorsal/posterior margin of the cephalothorax using an insulin syringe coupled to a #25-8 needle. Each pooled hemolymph sample (~50 µl) consisted of the hemolymph from 4 to 5 individual shrimp. Hemolymph osmolality was measured in 10-µl samples using a vapor pressure osmometer (Model 5500, Wescor). Hemolymph Na^+ concentration was measured by atomic absorption spectrophotometry (Model 932AA, GBC Scientific Equipment) in 10-µl hemolymph samples diluted 1:15 000 in distilled water for shrimp exposed to 5‰; 1:15 000 or 1:25 000 for those exposed to 20 and 35‰; and 1:20 000 or 1:50 000 for those exposed to 45 and 50‰. Hemolymph Cl^- concentration was measured in 10-µl hemolymph samples using a microtitrator (Model E485, Metrohm AG), employing mercuric nitrate as the titrant and s-diphenylcarbazone as the indicator (Schales & Schales 1941).

The isosmotic and isoionic points were calculated by solving quadratic equations produced from the equations for the hemolymph/external medium isosmotic or isoionic lines ($y = a + bx$) and the respective second order polynomial equations ($y = ax^2 + bx + c$) that describe the function between hemolymph osmolality or ionic concentration and that of the external medium. Resolution of these quadratic equations for $y = 0$, i.e. the intercepts (real roots) of the 2 equations, provides the respective isosmotic or ionic points. Osmotic and ionic regulatory capabilities are expressed as the ratio of variation in hemolymph osmotic or ionic concentration (Δ hemolymph mOsm kg^{-1} H_2O or mmol l^{-1} Na^+ and Cl^-) as a function of variation in the osmotic or ionic concentration of the external medium from the respective values in 5 and 45‰ salinity. A ratio of 1 indicates no regulation; values close to 0 indicate excellent regulatory capability (Freire et al. 2003).

To identify and quantify tissue FAA, muscle tissue samples (~50 mg) were taken from the abdominal muscle, free of nerve cord and intestine. Nerve tissue samples consisted of the ventral nerve cord and optic, thoracic and supraesophageal ganglia (~10 mg). The gill tissue sample consisted of gill pairs 6 to 8 (~2 mg). The fresh tissue samples were carefully weighed after standard blotting (±10 µg precision, Ohaus APD 250 electronic balance), oven-dried at 60 °C for 24 h and quickly reweighed. Tissue hydration (%) was calculated as: [(fresh weight – dry weight)/fresh weight] × 100.

For FAA analyses, the dried tissue samples were homogenized in distilled water, protein being precipitated with 80% ethanol (v/v). An internal standard of 6.24 nmol α-aminobutyric acid was added and the samples were derivatized with triethylamine and phenylisothiocyanate, forming FAA/phenylthiocarbamil derivatives (Bidlingmeyer et al. 1987). The individual FAA were identified and quantified by HPLC (Milton Roy) using a Picotag C18 Column (Waters Corporation) according to Freire et al. (1995) and Augusto et al. (2007a). FAA contribution to intracellular osmolality was estimated by converting the total FAA concentration obtained in nmol mg^{-1} dry weight (DW) to mmol kg^{-1} fresh weight, and assuming osmotic equilibrium between the intra- and extracellular media and negligible hemolymph FAA titers in a 30% hemolymph space for muscle and nerve tissue (50% for gill tissue).

The effects of exposure time to the different salinities on hemolymph osmolality, Na^+ and Cl^- concentrations, tissue hydration and FAA titers were evaluated using 1- or 2-way ANOVA (salinity, salinity/time and salinity/tissue) followed by the Student-Newman-Keuls (SNK) multiple means test to locate statistically significant groups. All statistical analyses were performed after ascertaining normality of distribution and equality of variance using the Sigma Stat 2.03 software package (SPSS), employing a minimum significance level of $p = 0.05$. Data are expressed in the text as mean ± SE.

RESULTS

Osmotic and ionic regulation

Hemolymph osmolality and Na^+ and Cl^- concentrations during long-term (5 d) acclimation and short-term (1 to 12 h) exposure of *Palaemon northropi* to a wide salinity range are provided in Figs. 1 to 3.

Palaemon northropi is a strong long-term hyper-/hypo-osmoregulator with an osmoregulatory capability (Δ hemolymph osmolality/Δ medium osmolality) of 0.32 and an isosmotic point of 566 mOsm kg^{-1} H_2O (18.3‰) (Fig. 1A). Control shrimp held at 35‰ maintained a hemolymph osmolality of 714 ± 7 mOsm kg^{-1} H_2O, decreasing to 599 ± 12 in 20‰ and to 548 ± 12 in 5‰; osmolality increased to 948 ± 31 mOsm kg^{-1} H_2O on acclimation to 45‰ (Fig. 1A).

During short-term exposure of shrimp initially held at 35‰ (Fig. 1B), hemolymph osmolality remained unchanged in those exposed to 20‰, but decreased moderately after 6 h (555 ± 7 mOsm kg^{-1} H_2O) and 12 h (605 ± 15 mOsm kg^{-1} H_2O) in shrimp exposed to 5‰. On exposure to 50‰, osmolality increased sharply to 981 ± 2 mOsm kg^{-1} H_2O after 6 h and to 1044 ± 17 mOsm kg^{-1} H_2O by 12 h (Fig. 1A). Hemolymph osmolality was hyper-regulated in 5 and 20‰ and hyporegulated in 50‰ during all short-term exposures; after 1, 6 and 12 h exposure to these media, isosmotic points gradually increased (655 mOsm kg^{-1} H_2O [22.3‰], 659 mOsm kg^{-1} H_2O [22.5‰] and 662 mOsm kg^{-1} H_2O [22.6‰], respectively), while osmoregulatory capabilities decreased (0.14, 0.32 and 0.33, respectively).

Hemolymph $[Na^+]$ in shrimp held for 5 d at 35‰ was 201 ± 12 mM and remained unchanged after transfer to 5 (249 ± 32), 20 (221 ± 20) or 45‰ (268 ± 4 mM Na^+). $[Na^+]$ was hyper-regulated up to the isoionic point at 201 mM (14.3‰) and was strongly hyporegulated at higher salinities (Fig. 2A). Na^+ regulatory capability was 0.03.

During short-term exposure of shrimp kept at 35‰, hemolymph $[Na^+]$ increased markedly after 1 h at all salinities (5, 20 or 50‰) (Fig. 2B). In 20‰, $[Na^+]$ rapidly

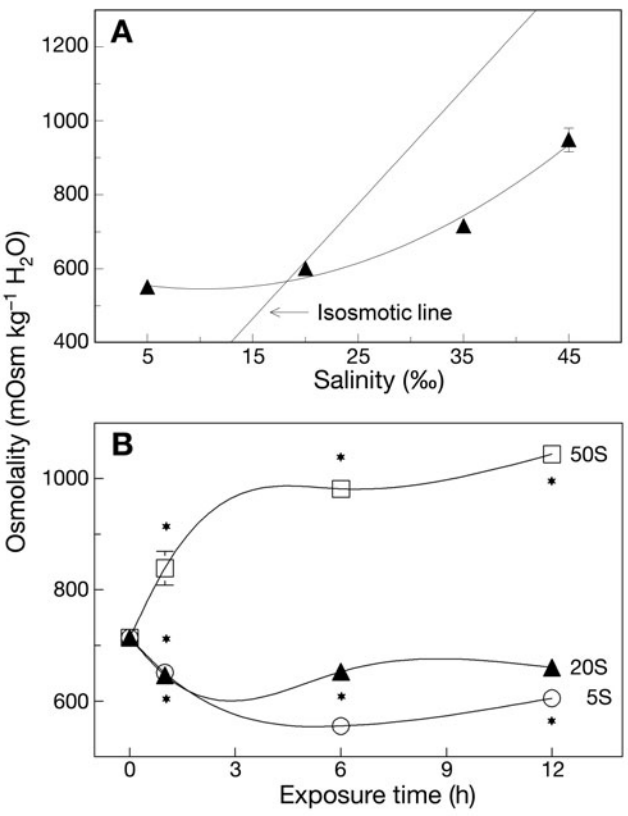

Fig. 1. *Palaemon northropi*. (A) Hemolymph osmoregulatory capability after 5 d acclimation to 5 to 45‰ salinity range. All means are significantly different from each other ($0.001 < p \leq 0.04$, $y = 0.32x^2 - 6.72x + 580.13$, $R^2 = 0.98$). (B) Changes in hemolymph osmolality during exposure from 35‰ (time = 0 h) to hypo- (5 and 20‰) and hyperosmotic (50‰) media. Values are mean ± SE (N = 6). * Significantly different from preceding value ($p \leq 0.05$)

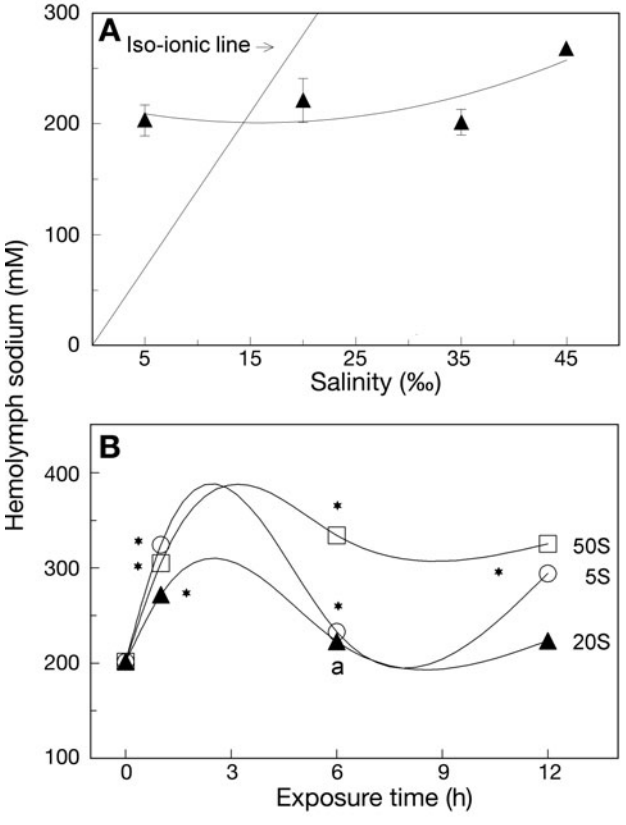

Fig. 2. *Palaemon northropi*. (A) Hemolymph Na^+ concentration after 5 d acclimation to 5 to 45‰ salinity. $[Na^+]$ is unaffected by salinity ($p = 0.228$, $y = 0.07x^2 - 2.13x + 217.94$, $R^2 = 0.63$). (B) Changes in hemolymph $[Na^+]$ during exposure to hypo- (5 and 20‰) and hyperosmotic (50‰) media. Values are mean ± SE (N = 6). * Significantly different from preceding value ($p \leq 0.05$); [a]not significantly different from time = 0 h

decreased to initial values within 6 h, but remained significantly elevated after 12 h at 50‰; however, in 5‰, after a decrease at 6 h, [Na^+] increased after 12 h exposure. Hemolymph [Na^+] was hyper-regulated in 5‰ for all short-term exposures, and hyporegulated in all other salinities. Isoionic points after 1, 6 and 12 h were 273 (19.9‰), 231 (16.9‰) and 236 mM Na^+ (17.2‰), respectively. Na^+ regulatory capability at 1, 6 and 12 h was 0, 0.16 and 0.05, respectively.

Hemolymph [Cl^-] in shrimp held at 35‰ was 283 ± 13 mM, decreasing slightly after 5 d exposure to 20‰ (247 ± 9); in 5 and 45‰, concentrations decreased (209 ± 1 mM Cl^-) or increased (367 ± 9 mM Cl^-), respectively (Fig. 3A). [Cl^-] was hyper-regulated up to the isoionic point at 217 mM (13.6‰) and strongly hyporegulated at higher salinities. [Cl^-] regulatory capability was 0.25.

During short-term salinity exposure (1, 6 and 12 h) (Fig. 3B), hemolymph [Cl^-] was unchanged in 20‰, but increased steadily with time in 50‰. In 5‰, [Cl^-] decreased sharply after 1 and 6 h, recovering initial values by 12 h. [Cl^-] was hyper-regulated at 5‰ and strongly hyporegulated at all other salinities. The isoionic points after 1, 6 and 12 h were 270 (17‰), 278 (17.4‰) and 266 mM [Cl^-] (16.6‰), respectively, while [Cl^-] regulatory capabilities progressively decreased (0.15, 0.28 and 0.3, respectively).

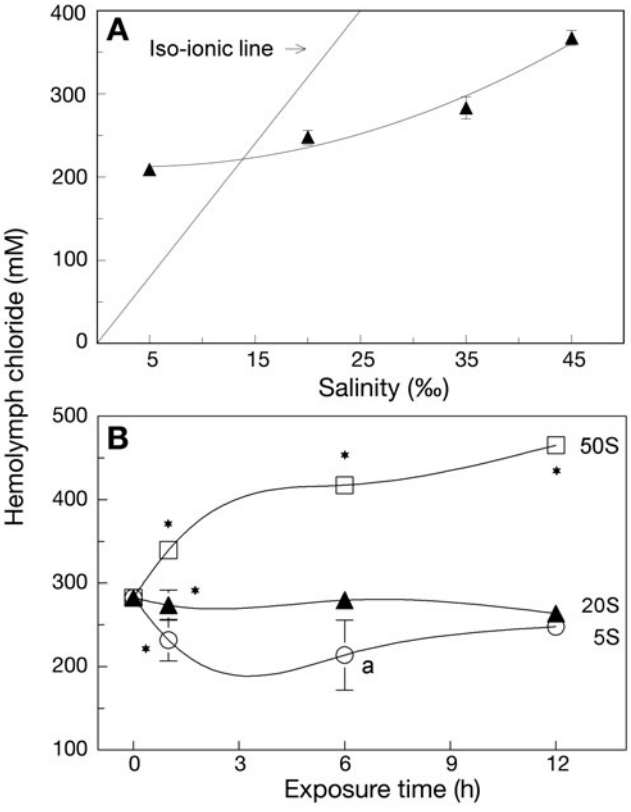

Fig. 3. *Palaemon northropi*. (A) Hemolymph Cl^- concentration after 5 d acclimation to 5 to 45‰ salinity. All means are significantly different from each other ($0.001 < p \leq 0.002$, $y = 0.09x^2 - 0.65x + 213.69$, $R^2 = 0.97$). (B) Changes in hemolymph [Cl^-] during exposure to hypo- (5 and 20‰) and hyperosmotic (50‰) media. Values are mean ± SE (N = 6). * Significantly different from preceding value; [a]significantly different from control (time = 0 h) ($p \leq 0.05$)

Tissue hydration and FAA concentrations

There was no change in muscle tissue hydration (84.6% at 35‰, time = 0) in shrimp exposed to 5‰ between 1 and 24 h (Fig. 4). In 20‰, hydration increased to a maximum within 1 h, declining to seawater values after 6 h. In 50‰, hydration rapidly decreased within 1 h, reaching a minimum by 6 h which was sustained up to 24 h. However, by 120 h, muscle hydration was similar at all salinities (82%), although less than initial values in seawater. Gill and nerve tissue hydration in *Palaemon northropi* held at 20‰ was also ~84%, unchanged after 5 d acclimation to 5 or 45‰ salinity.

Total FAA concentrations in the muscle tissue alone decreased by 65%, from 143 ± 12 to 53 ± 6 mmol kg^{-1} wet weight (WW) (Fig. 5A), after acclimation from 20 to 5‰ for 5 d. In the hemolymph, total FAA increased by 150%, from 4.6 ± 1.2 mmol l^{-1} at 20‰ to 11.5 ± 2.0 mmol l^{-1} in 5‰ after 5 d (Fig. 5B). There were no changes in total FAA in the gill and nerve tissues after acclimation to any salinity (Fig. 5A).

The decrease in muscle total FAA on acclimation to 5‰ is mainly a consequence of diminished glycine (–71%), arginine (–95%) and proline (–66%) (Table 1). In the hemolymph, the increase in total FAA concen-

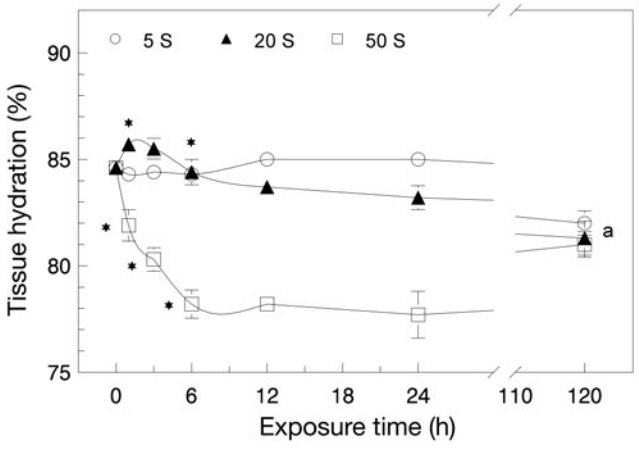

Fig. 4. *Palaemon northropi*. Effect of direct acclimation to salinities of 5, 20 or 50‰ on muscle tissue hydration. Values are mean ± SE (N = 6). [a]Significantly different from 35‰ (control, time = 0 h); * significantly different from preceding value ($p \leq 0.05$)

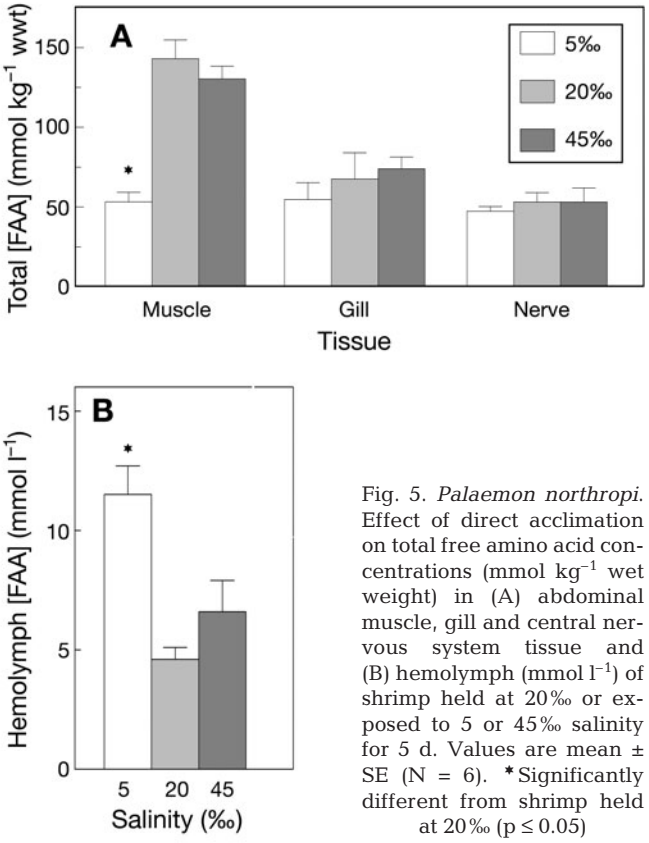

Fig. 5. *Palaemon northropi*. Effect of direct acclimation on total free amino acid concentrations (mmol kg^{-1} wet weight) in (A) abdominal muscle, gill and central nervous system tissue and (B) hemolymph (mmol l^{-1}) of shrimp held at 20‰ or exposed to 5 or 45‰ salinity for 5 d. Values are mean ± SE (N = 6). * Significantly different from shrimp held at 20‰ ($p \leq 0.05$)

tration after exposure to 5‰ is mainly due to alanine (+384%) and proline (+216%) (Table 1).

Total FAA contribution to intracellular osmolality decreased in the muscle tissue alone, from 31% in shrimp at 20‰, to 13 and 18% in 5 and 45‰, respectively. The contribution of FAA to intracellular osmolality in gill (14%) and nerve tissue (10%) was similar at all 3 salinities.

DISCUSSION

Many intertidal marine and estuarine crustaceans confront cyclic fluctuations in salinity, temperature and oxygen concentration, and exhibit notable acclimatory capability (Mantel & Farmer 1983). A case in point, *Palaemon northropi* survives remarkably well in media ranging from 1.5 to 45‰ salinity, and for up to 24 h in 50‰, much like *P. affinis* (Kirkpatrick & Jones 1985), *P. longirostris* (Campbell & Jones 1989) and *P. pandaliformis* (Freire et al. 2003). Such survival ability, underpinned by effective hyper-/hypo-osmoregulatory mechanisms, may have allowed the penetration of ancestral euryhaline marine or estuarine palaemonids into brackish and fresh water, a considerable advantage compared to stenohaline marine decapods, confined to a restricted salinity range. Marine and brackish water palaemonids also show fairly similar osmoregulatory capabilities with isosmotic points close to their modal salinities a little above the middle of their osmoregulatory ranges (Table 2), a characteristic typical of species inhabiting variable salinity environments (Parry 1954, Denne 1968, Spaargaren 1972, Kirkpatrick & Jones 1985, Morris et al. 1988, González-Ortegón et al. 2006).

Although *Palaemon northropi* cannot absorb salt against fresh (<0.5‰) or very dilute brackish (1.0‰) water, succumbing within a few hours (see estimating lethal limits in 'Materials and methods'), it strongly hyper-/hyporegulates hemolymph osmolality during both direct, short-term exposure and after 5 d acclimation at 5 to 45‰ salinity. Such regulatory capacity is consistent with the species' occurrence in rocky tide pools where the measured summer salinity ranges from 3 to 33‰ (see 'Materials and methods') as a function of tides, evaporation and precipitation. Hemolymph Na^+ and Cl^- concentrations are also efficiently hyper-/hyporegulated during short-term exposure and

Table 1. *Palaemon northropi*. Principal individual and total free amino acid concentrations in abdominal muscle, gill and nerve tissue (mmol kg^{-1} wet weight) and hemolymph (µmol l^{-1}) of the intertidal shrimp *P. northropi* held in 20‰ or exposed to 5 or 45‰ salinity for 5 d. Data are mean ± SE (N = 6). *Significantly different from same tissue in 20‰ ($p < 0.05$, 1-way ANOVA, Student-Newman-Keuls multiple means test). nd: not determined

Tissue	Salinity (‰)	Glycine	Taurine	Arginine	Alanine	Proline	Other FAA	Total
Muscle	5	19.5 ± 1.2*	7.8 ± 0.5	0.6 ± 0.2*	3.5 ± 0.4	5.0 ± 0.7*	55	53.3 ± 6.0*
	20	68.0 ± 16.8	18.4 ± 5.2	12.5 ± 1.3	6.9 ± 2.1	14.6 ± 8.1	22	143 ± 12.0
	45	65.3 ± 4.1	14.7 ± 0.8	13.6 ± 0.2	11.2 ± 1.7	9.7 ± 5.9	15	130.3 ± 7.5
Gill	5	4.7 ± 1.1	nd	13.5 ± 2.7	9.0 ± 1.8	8.4 ± 1.5	26.7	54.7 ± 10.5
	20	16.4 ± 1.6	nd	17.9 ± 4.3	6.4 ± 1.5	5.0 ± 0.9	21.9	67.6 ± 16.4
	45	25.8 ± 1.7	nd	12.3 ± 1.7	8.2 ± 0.8	7.3 ± 1.3	20.4	74 ± 7.3
Nerve	5	4.1 ± 0.3	16.6 ± 0.04	nd	5.3 ± 0.3	5.9 ± 0.1	15.5	47.4 ± 2.8
	20	4.9 ± 0.6	12.8 ± 1.5	0.4 ± 0.1	5.8 ± 0.6	5.2 ± 0.5	24	53.1 ± 5.8
	45	7.0 ± 1.2	11.5 ± 2.0	nd	9.9 ± 1.7*	11.1 ± 1.9*	13.6	53.1 ± 8.8
Hemolymph	5	2045.1 ± 243.8	1225.0 ± 202.0	1.6 ± 1.1*	1333.7 ± 330.8	1616.8 ± 237.5*	5247.8	11470 ± 1971*
	20	1435.5 ± 54.5	838.5 ± 11.7	48.5 ± 4.8	347.1 ± 120.2	510.7 ± 191.3	1426.7	4607 ± 1169
	45	1879.2 ± 373.1	664.8 ± 99.4	242.1 ± 50.5	956.1 ± 203.9	890.3 ± 163.6	1964.5	6597 ± 1321

Table 2. Species, predominant biotope and hemolymph osmolality (at modal salinity indicated) and isosmotic point (both in Osm kg^{-1} H_2O) for palaemonid shrimp from marine, brackish or freshwater environments. Hemolymph osmolality and isosmotic points clearly decline with occupation of dilute media among brackish water *Palaemon* and *Palaemonetes*, as also seen within brackish water, diadromous and hololimnetic *Macrobrachium* species

Species	Predominant biotope	Hemolymph osmolality	Hemolymph isosmotic point	Source
Palaemon serratus	Marine/brackish	770 (25‰)	685	Parry (1954)
Palaemon affinis	Marine/brackish	750 (20‰)	629	Kirkpatrick & Jones (1985)
Palaemon macrodactylus	Brackish	725 (23‰)	657	Born (1968)
Palaemonetes varians	Marine/brackish	620 (22‰)	602	Potts & Parry (1964)
Palaemon northropi	Marine/intertidal	599 (20‰)	566	Present study
Palaemon longirostris	Brackish	573 (17‰)	579	González-Ortegón et al. (2006)
Palaemonetes pugio	Brackish	550 (17‰)	465	Roesljadi et al. (1976)
Palaemon pandaliformis	Brackish	450 (25‰)	364	Freire et al. (2003)
Palaemonetes argentinus	Brackish/freshwater	410 (1‰)	600	Charmantier & Anger (1999)
Macrobrachium equidens	Brackish	555 (29‰)	492	Denne (1968)
Macrobrachium rosenbergii	Brackish/freshwater	480 (<0.5‰)	520	Sandifer et al. (1975)
Macrobrachium acanthurus	Brackish/freshwater	440 (<0.5‰)	640	Moreira et al. (1983)
Macrobrachium amazonicum	Freshwater (diadromous)	403 (<0.5‰)	602	Augusto et al. (2007a)
Macrobrachium potiuna	Freshwater (hololimnetic)	418 (<0.5‰)	562	Freire et al. (2003)
Macrobrachium brasiliense	Freshwater (hololimnetic)	412 (<0.5‰)	521	Freire et al. (2003)
Macrobrachium olfersi	Freshwater (diadromous)	336 (<0.5‰)	428	Freire et al. (2003)
Macrobrachium nipponense	Freshwater (hololimnetic)	330 (<0.5‰)	450	Wang et al. (2004)

acclimation: Na^+ and Cl^- increase after 12 h exposure to 50‰ from 35‰, such as might occur during evaporation at low tide. However, hemolymph [Na^+] is unchanged after 5 d acclimation at 5, 20 or 45‰, while [Cl^-] increased modestly with increasing salinity, inferring uncoupled regulatory mechanisms for these ions. Such osmoregulatory characteristics suggest that *P. northropi* is well adapted to fluctuating salinities, and its hyper-/hyporegulatory capability reflects a pattern typical of brackish water palaemonids (cf. González-Ortegón et al. 2006). Conspicuously, however, very few *Palaemon* species actually inhabit freshwater: *P. paucidens*, a salt-tolerant (30‰), semi-hololimnetic, Indo-West Pacific species, is the only documented example (Fidhiany et al. 1991). Thus osmoregulatory capacity within the genus *Palaemon* may reflect the potential physiological capability of ancestral marine palaemonids to penetrate dilute media, characteristics that appear to have been lost during the subsequent radiation of diadromous and hololimnetic species into the freshwater biotope.

Crustacean cells, when exposed *in vivo* to hypo- or hyperosmotic media, do not simply swell or shrink like good osmometers, but rather recover their volume during a slow readjustment period. The overall volume regulation mechanism consists of a rapid phase limiting cell swelling or shrinkage, followed by a slower phase of volume readjustment (Gilles & Péqueux 1981). In *Palaemon northropi*, muscle water contents are identical after 5 d acclimation to 5, 20 or 45‰; however, during short-term exposure to 20‰, tissue water increased very briefly and transiently (3 h), but decreased notably and persistently (up to 24 h) in 50‰, revealing much better regulation in hyposmotic than in hyperosmotic media, attesting to a notable capability to withstand dilute media at the tissue level. The efficient regulation of tissue hydration after acclimation is consistent with life in an unstable environment and constitutes an effective mechanism of osmotic regulation.

FAA are important intracellular osmotic effectors in crustaceans and many other organisms (Edwards 1982, Biagini et al. 2000, Frick & Wright 2002, McNamara et al. 2004, Augusto et al. 2007a,b). Such regulation is a slow, temporally asymmetrical process occurring within 24 h of exposure to hypoosmotic media but requiring several days in hyperosmotic media (Gilles & Péqueux 1983). Tissue total FAA concentrations also constitute a useful parameter to evaluate the degree of adaptation to dilute media (Potts & Parry 1964), since they are 2- to 4-fold greater in marine compared to freshwater species. FAA decrease by 63% in muscle tissue of *Palaemon northropi* acclimated to 5‰, revealing a role in hypoosmotic cell volume regulation; however, gill and nerve tissues did not respond. The effective contribution of total FAA to intracellular osmolality subsequent to alteration in hemolymph osmolality also decreased to 13% in muscle tissue after exposure to 5‰ compared to shrimp held at 20‰ (31% contribution). Such tissue-specific responses evidently reflect the efficient hemolymph osmotic and ionoregulatory mechanisms and reveal the dependence of muscle tissue on intracellular osmotic effectors like FAA. This response is consistent with the regulatory physiology

of a species that inhabits rocky tide pools and is submitted daily to widely varying salinities. In species invading dilute media and freshwater, evolutionary pressures may have selected for rapid response mechanisms like anisosmotic extracellular regulation to the detriment of much slower responses like isosmotic intracellular regulation.

Glycine, proline, alanine and arginine are the main effectors of intracellular isosmotic regulation in most crustaceans and constitute 40 to 60% of total FAA concentration, independently of salinity, ontogenetic stage or habitat (Dalla Via 1986, Freire et al. 1995, Haond et al. 1999, Huong et al. 2001, Wang et al. 2004, Augusto et al. 2007a,b). In *Palaemon northropi*, the decrease in muscle total FAA on acclimation to 5‰ is mainly a consequence of diminished glycine, arginine and proline, confirming that these nitrogenous components play a significant role in regulating intracellular volume. The 1.5-fold increase in hemolymph total FAA concentration after 5 d at 5‰ may derive from FAA efflux from the muscle tissue. Investigations in *Eriocheir sinensis* (Gilles 1977), *Panulirus japonicus* (Shinagawa et al. 1995) and *E. japonicus* (Abe et al. 2002) reveal that hemolymph FAA increase on exposure to dilute media and may be stored as hemolymph proteins like hemocyanin (Mantel & Farmer 1983), which may increase the amount of oxygen available to cells (Gilles & Péqueux 1981).

Our data confirm that FAA concentrations in muscle tissue constitute an effective parameter to evaluate the degree of adaptation to dilute media. FAA titers were higher in *Palaemon northropi* than in diadromous freshwater palaemonids like *Macrobrachium amazonicum* and *M. olfersi* (164 and 188 nmol mg^{-1} DW, respectively; Augusto et al. 2007a) and *M. rosenbergii* (~390 nmol mg^{-1} DW; Huong et al. 2001). Further, in *M. brasiliense*, a hololimnetic inhabitant of inland continental rivers that exhibits abbreviated larval development, FAA concentrations (142 nmol mg^{-1} DW) are lower than in other *Macrobrachium* species (Faria et al. 2008). FAA concentrations in *P. northropi* muscle may thus be similar to those of ancestral marine palaemonids. Although part of a separate clade, *P. northropi* is a more basal species than many sympatric *Macrobrachium* species (Murphy & Austin 2005).

The capability to adjust survival limits to changing environmental salinities would be of immense adaptive value during the evolutionary invasion of freshwater. The euryhaline intertidal shrimp *Palaemon northropi* exhibits many physiological adaptations such as efficient short-term hypo- and hyperosmotic and ionic regulation, rapid acclimation to salinity change and elevated yet labile muscle FAA concentrations, all of which are well suited to underpin the conquest of dilute media. This mosaic of characteristics suggests that the physiology of *P. northropi* and similar species may be crucial to understanding the invasion of dilute media by ancestral marine crustaceans many millions of years ago.

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