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Adaptive Shifts in Osmoregulatory Strategy and the Invasion of Freshwater by Brachyuran Crabs: Evidence from *Dilocarcinus pagei* (Trichodactylidae)

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ABSTRACT To evaluate putative adaptive changes underpinning the invasion of freshwater by the Brachyura, this investigation examines anisosmotic extra and isosmotic intracellular osmoregulatory capabilities in *Dilocarcinus pagei*, a neotropical, hololimnetic crab, including its embryonic and juvenile phases. All ontogenetic stages show a remarkable ability to survive a high salinity medium (25‰, 750 mOsm/kg H_2O, 350 mm Na^+, 400 mM Cl^-). Adults hyper-regulate hemolymph osmolality up to isosmoticity at 744 mOsm kg/H_2O (24‰), $[Na^+]$ and $[Cl^-]$ becoming isoionic at 449 (22‰) and 256 mM (16‰), respectively. Hemolymph (420 ± 39 mOsm/kg H_2O) and urine (384 ± 44 mOsm/kg H_2O) are isosmotic in adults held in freshwater, and after 5-days exposure to 25‰ (787 ± 9 mOsm/kg H_2O and 777 ± 43 mOs/kg H_2O, respectively); *D. pagei* does not produce dilute urine. Total free amino acid (FAA) concentrations in embryos (14.9 ± 1.2), juveniles (32.8 ± 0.1) and adult muscle (10.9 ± 2.1 mmol/kg wet weight) in freshwater are 30-fold less than in brackish/marine Crustacea, suggesting that FAA constitute a useful parameter to evaluate adaptation to freshwater. On acclimation to 25‰, total FAA increase by $\approx 100\%$ in embryos and in adult muscle and nerve tissue and hemolymph, owing to large increases in proline, arginine and/or alanine. However, effective FAA contribution to intracellular osmolality increases only in embryos, from 3 to 4.5%. These findings suggest that gill-based, anisosmotic extracellular regulation has supplanted isosmotic intracellular regulatory mechanisms during the conquest of freshwater by the Brachyura, and indicate that *D. pagei* may be an old, well-adapted inhabitant of this biotope. *J. Exp. Zool. 307A:688–698, 2007.*

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The order Decapoda is constituted essentially by marine crustaceans of which a few taxa, including the trichodactylid, potamoid and grapsid crabs, and several caridean shrimp groups, have become fully adapted to the freshwater environment where they spend their entire life cycle (Susanto and Charmantier, 2001). Although the invasion of freshwater by the Decapoda began some 600 million years ago (Ruppert and Barnes, '94), and by the brachyuran crabs some 65–30 million years ago (Sternberg and Cumberlidge, 2001), it continues to the present day and can be seen in diadromous palaemonid shrimps of the genera *Macrobrachium*, like *Macrobrachium olfersi*,

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Macrobrachium amazonicum, *Macrobrachium acanthurus*, (Freire et al., 2003) and *Palaemon*, such as *Palaemon pandaliformis,* (Freire et al., 2003) and *Palaemonetes paludosus*, (Turner et al., '75) and in varunid and portunid crabs like *Eriocheir sinensis,* (Anger, '91) and *Callinectes danae,* (Branco and Masunari, 2000) that require salt water to complete their life cycle. Truly freshwater or hololimnetic crustaceans share important features such as a life cycle independent of salt water; production of a few lecithotrophic eggs; very abbreviated or direct larval development; reduced hemolymph osmolality in freshwater and a low isosmotic point; and a low total intracellular free amino acid (FAA) concentration compared with marine species (Claybrook, '83; Read, '84; Lee and Bell, '99; Augusto et al., 2007). Among the key adaptive factors underlying the invasion of freshwater is the capability of the early ontogenetic stages to regulate their internal fluids in this biotope, and adequate osmotic protection by the embryonic membranes in the absence of adult-like regulatory competence (Charmantier, '98).

Although marine crabs do not encounter a substantial osmotic gradient as their hemolymph is nearly isosmotic with seawater, freshwater species confront severe challenges of diffusive salt loss and osmotic water uptake across their body surfaces, for which they compensate by active salt absorbtion across the gill epithelium and adaptive low water permeability, respectively (Onken et al., '95; Onken and Putzenlechner, '96; Weihrauch et al., 2004; Freire et al., 2007). Freshwater crabs also conserve salt by producing a diminished flow of isosmotic urine (Morris and Van Aardt, '98; Rathmayer and Siebers, 2001). These mechanisms are collectively termed anisosmotic extracellular regulation (see Péqueux, '95). Another important group of osmoregulatory mechanisms present in the Crustacea is intracellular isosmotic regulation (IIR), which adjusts intracellular osmotic concentration and volume, maintaining a steady state equilibrium with the extracellular fluid (Gilles and Péqueux, '81; Péqueux, '95; Lang and Waldegger, '97; Wehner et al., 2003). This is a plesiomorphic mechanism encountered in all euryhaline invertebrates (Claybrook, '83). The principal long-term organic effector of IIR in the Decapoda is the intracellular pool of nonessential FAA like glutamic acid, glycine, alanine and proline (Haond et al., '99; Huong et al., 2001; McNamara et al., 2004; Augusto et al., 2007), the adjustment of which depends on rates of FAA synthesis and degradation, shifts in efflux/influx equilibria across the cell membrane and/or changes in protein synthesis/catabolism (Gilles, '77; Boone and Schoffeniels, '79; Tan and Choong, '81).

Physiological investigations of the invasion of freshwater by the Brachyura are few, with little discussion of adaptive osmoregulatory issues (see *Potamon niloticus*: Shaw, '59; *Potamon fluviatilis*: Harris and Micallef, '71; *Armases robertii*: Schubart and Diesel, '98). *Dilocarcinus pagei*, a hololimnetic freshwater crab, endemic to the Amazon and Paraguay/Paraná river basins of South America (Mello, 2003), maintains strong hemolymph osmotic and ionic gradients in freshwater (Onken and McNamara, 2002). Its mechanisms of gill Na^+ and Cl^- transport, including Na^+/K^+- and V-ATPase activities and mRNA expression in the uniquely asymmetrical gill epithelia have been investigated in detail (Onken and McNamara, 2002; Weihrauch et al., 2004); passive salt loss is reduced in the peculiar abdominal and thoracic hindguts, which show very low transepithelial conductances and notable ion selectivities (McNamara et al., 2005). Amado et al. (2006) have accompanied the effect of lead contamination on hemolymph and tissue osmotic and ionic responses to low salinity exposure. However, the osmoregulatory capabilities of *D. pagei* and of its main ontogenetic stages are unknown.

In the present investigation, we evaluate adaptive capability for anisosmotic extracellular adjustment in *D. pagei*, analyzing hemolymph and urine osmotic and/or ionic regulatory ability. Further, we examine survival, tissue hydration and the role of FAA in intracellular isosmotic regulatory capability in embryos and juveniles and in adult tissues during exposure to elevated salinities. The physiological significance of the osmoregulatory mechanisms disclosed is discussed in terms of the conquest of freshwater by the Brachyura.

MATERIALS AND METHODS

Ovigerous and nonovigerous, female freshwater crabs, *D. pagei* (5–7 cm carapace width, 40–50 g wet mass), were collected by hand from grassy vegetation on the banks of the Mogi River near Barrinha (21° 11′ 37″ S; 48° 09′ 50″ W), in northeastern São Paulo State, Brazil. In the laboratory, the crabs were maintained in 60-L tanks containing freshwater (<0.5‰) at room temperature, and were fed minced beef, orange,

banana and carrot on alternate days. All crabs were in the intermolt stage when used.

Groups of six adult crabs each were acclimated directly for a 10-day period to experimental media of 5, 10, 15, 20, 25, 30 or 35‰ salinity, or for 1, 2, 5 or 10 days to 25‰ salinity to estimate approximate lethal salinity limit, time course of osmotic and ionic regulatory capability and hemolymph iso-osmotic and isoionic points. Data for 35‰ refer to live crabs surviving for at least 24 hr. Control crabs were kept in freshwater. Urine osmolality was measured in crabs kept in freshwater or 25‰ for 5 days.

To evaluate a role for FAA in IIR during the ontogenetic sequence, groups of five adult crabs each were exposed to 25‰ for 1, 2, 5 or 10 days; control crabs were kept in freshwater. Eggs containing crab embryos with visible eye pigments were exposed directly to 25‰ for 2 days by transferring ovigerous females with eggs attached to their pleiopods to an aerated aquarium. Ovigerous females held in freshwater provided control embryos. Embryonic heartbeat was checked with a dissecting microscope. Female crabs carrying newly hatched juveniles ($\approx$2.5 mm length) adhering to their pleiopods were directly exposed to 25‰ or to freshwater for 2 days; samples of six eggs or juveniles each were then removed from each female. Embryos and juveniles removed from the female crabs and exposed directly to freshwater or saline medium died within 24 hr.

Hemolymph samples ($\approx$100 μL each) were taken from adult crabs through the articulation membrane at the base of the fifth pereiopod using an insulin syringe and 25-8 needle. Urine samples ($\approx$100 μL each) were obtained by inserting a polyethylene cannula coupled to an insulin syringe a short distance through the nephropores. Muscle tissue samples ($\approx$2 g) were taken from the chela. The nervous tissue sample consisted of the ventral nerve cord and thoracic ganglion ($\approx$1 g). The gill tissue sample consisted of the eighth posterior gill pair ($\approx$1 g). The embryos and juveniles were used intact.

The fresh samples of embryos, juveniles and adult tissues were weighed ($\pm$10 μg precision, Ohaus APD 250 electronic balance, Ohaus, Nanikon, Switzerland), oven dried at 60°C for 24 hr, and quickly reweighed. Tissue hydration (%) was calculated as [(fresh weight−dry weight)/fresh weight] × 100.

Individual FAA were identified and quantified by high performance liquid chromatography (HPLC). The dried tissue samples were homogenized individually in 1.5-mL Eppendorf tubes (Steinheim, Germany) with fine glass powder and 100 μL of distilled water for $\approx$5 min and centrifuged (Fanem, Excelsa 206R, São Paulo, Brazil) at 7,000 rpm for 30 min. Supernatants were transferred to fresh Eppendorf tubes and the volume completed to 100 μL for the gill and nervous tissues, or 400 μL for muscle tissue. The tubes were then sealed and the undiluted hemolymph samples and homogenates maintained at −25°C until analysis.

After protein precipitation using ethyl alcohol (80% v/v), an internal standard of 6.24 nmol α-aminobutyric acid was added, and the FAA in 15-μL hemolymph aliquots and tissue homogenates were derivatized with triethylamine and phenylisothiocyanate according to Freire et al. ('95) to form free amino acid/phenylthiocarbamyl derivatives (Bidlingmeyer et al., '87). The phenylthiocarbamyl-amino acid derivatives in each tissue homogenate or hemolymph sample, suspended in 5 mM sodium phosphate buffer containing 5% acetonitrile, were then separated and quantified by UV (254 nm) in duplicate 20-μL aliquots using an automated Milton Roy LDC MP3000 HPLC system (Milton Roy, Ivyland, PA) and Picotag C18 5-μm column (Waters Corporation, Milford, MA) according to Freire et al. ('95). Samples were eluted at 1 mL/min at 38°C using a gradient of 0.14 M sodium acetate and 0.05% triethylamine, pH 6.0, as solution A, and acetonitrile:H_20 (3:2) as solution B.

A standard solution containing 2.5 nmol of each amino acid (Pierce, Standard H) containing α-aminobutyric acid was derivatized and analyzed together with the samples. Recovery of the standard was 100$\pm$5%. The system gave a linear response ($\leq$8%) for standard amino acids of 50–1,000 pmol at injection with a precision of $\leq$8%. The elution times of the phenylthiocarbamyl-amino acids were reproducible to $\pm$0.02 min ($\pm$1 SD), allowing the unambiguous identification and quantification of all the common free amino acids except asparagine that coelutes with serine and is quantified as such; glutamine that is similarly inseparable from glycine; and tryptophan that coelutes with reaction by-products. All data have been corrected for mechanical and systematic losses based on recovery of the α-aminobutyric acid added at the ethyl alcohol precipitation step, and are expressed as millimoles FAA per kilogram wet tissue weight or μmoles FAA per liter hemolymph.

Total FAA contribution to intracellular osmolality was estimated as millimoles per kilogram fresh weight, and assumes osmotic equilibrium

between the intracellular and extracellular media and negligible hemolymph FAA titers in a 30% hemolymph space. The FAA titers in the embryos refer to the whole eggs as the embryos themselves were not dissected out.

Hemolymph and urine osmolalities were measured in 10-μL samples using a vapor pressure osmometer (Wescor, Model 5500, Wescor Inc., Logan, Utah). Hemolymph sodium concentration was measured by atomic absorption spectrophotometry (GBC, Model 932AA, GBC Scientific Equipment Pty Ltd, Victoria, Australia) in 10-μL hemolymph samples diluted 1:15,000 in distilled water for crabs held in freshwater, and 1:25,000 for those exposed to saline media. Chloride concentration was measured in 10-μL hemolymph samples using a microtitrator (Metrom AG, Model E485, Herisau, Switzerland), employing mercuric nitrate as the titrant and s-diphenylcarbazone as the indicator (Schales and Schales, '41).

The isosmotic and isoionic points were calculated by producing quadratic equations from the equation 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 two equations, provides the respective isosmotic or ionic points. Osmotic and ionic regulatory capabilities are expressed numerically as the ratio of variation in hemolymph osmotic or ionic concentration (Δ hemolymph mOsm/kg H_2O or mmol/L Na^+, Cl^-) as a function of variation in osmotic or ionic concentration of the external medium (Δ medium mOsm/kg H_2O or mmol/L Na^+, Cl^-) from the respective values in freshwater up to the isosmotic or isoionic point. A ratio of 1 indicates no regulation, whereas values close to 0 indicate excellent regulatory capability (Freire et al., 2003).

To evaluate the effect of exposure to the different saline media or acclimation time on hemolymph osmolality and sodium and chloride concentrations, and on tissue hydration, one-way analyses of variance (ANOVAs) were performed followed by the Student–Newman–Keuls multiple means test to locate statistically significant groups. The effect of exposure to 25‰ during the various time intervals on FAA concentration in the different tissue types and ontogenetic stages was evaluated using a two-way ANOVA followed by the Student–Newman–Keuls multiple means test. Student's *t*-test was used to compare the effect of salinity (freshwater or 25‰) on urine osmolality.

All statistical analyses were performed after ascertaining normality of distribution and equality of variance using the Sigma Stat 2.03 software package (SPSS Inc., Chicago, Illinois), employing a minimum significance level of $P = 0.05$. Data are expressed throughout the text as the mean ± standard error of the mean.

RESULTS

Mortality and osmotic and ionic regulation

Adult *D. pagei* survived at least 10 days without mortality in saline media ranging from 5 to 20‰ (150 to 600 mOsm/kg water). In 25‰, mortality reached 20% after 8-days exposure; in 30‰, mortality attained 50% within 9 days and at 35‰ most crabs died within 24 hr. Embryos and juveniles survived well in freshwater and at 25‰ for at least 2 days (maximum exposure period).

Hemolymph osmolality increased significantly in adult crabs exposed to 25‰ for 10 days (753 ± 6 mOsm/kg H_2O) compared with those held in freshwater (420 ± 39 mOsm/kg H_2O) (Fig. 1), and was hyper-regulated up to the isosmotic point at 744 mOsm/kg H_2O (24‰). Osmoregulatory

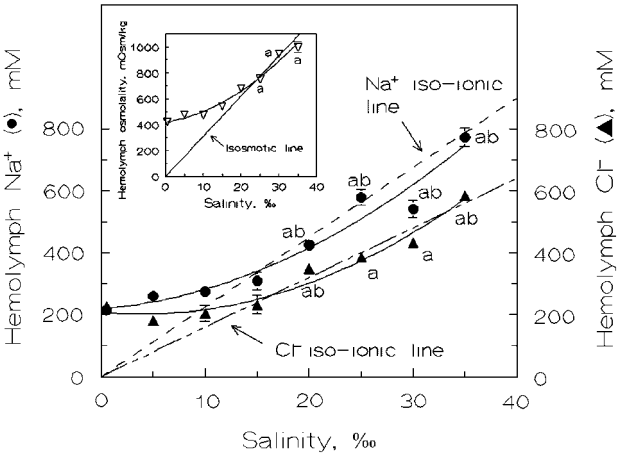

Fig. 1. Effect of acclimation to saline media of 5–35‰ (1‰ = 30 mOsm/kg H_2O, 14 mM Na^+ and 16 mM Cl^-) for 10 days on hemolymph osmolality (insert, ▽, mOsm/kg H_2O), and sodium (●, mM Na^+) and chloride (▲, mM Cl^-) concentrations in the freshwater crab *Dilocarcinus pagei*. [a]Significantly different from the value for the same parameter (same curve) at the point Salinity = 0, which is freshwater; [b]Significantly different from the immediately preceding value for the same parameter (same curve).

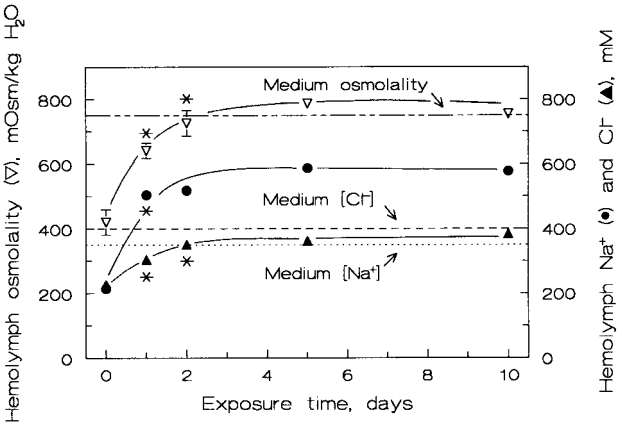

Fig. 2. Effect of acclimation to 25‰ salinity (= 750 mOsm/kg H_2O, 350 mM Na^+ and 400 mM Cl^-) for 10 days on hemolymph osmolality (▽, mOsm/kg H_2O), sodium (●, mM Na^+) and chloride (▲, mM Cl^-) concentrations in the freshwater crab *Dilocarcinus pagei*. *Significantly different from preceding value (X ± SEM, $N = 6$, $P \leq 0.05$). Curves fitted by eye.

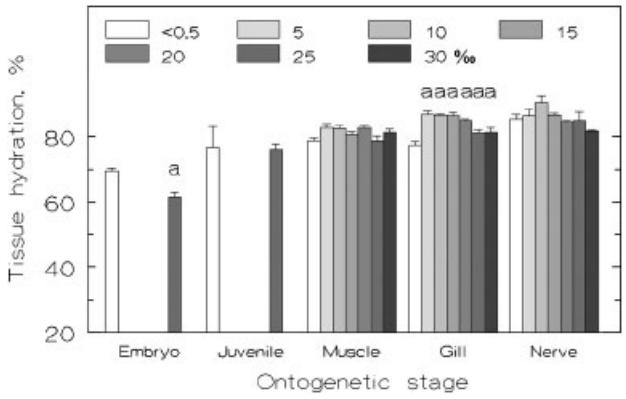

Fig. 3. Effect of exposure to saline media from 5–30‰ on water content of embryos and juveniles (2 days), and in adult tissues (10 days) of the freshwater crab *Dilocarcinus pagei*. [a]The values for gill alone are significantly higher compared to the value in freshwater (< 0.5).

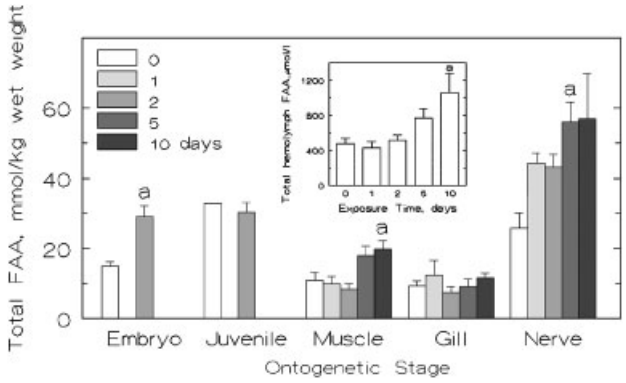

Fig. 4. Time course of acclimation to 25‰ on total free amino acid concentrations (mmol/kg wet weight) in embryos and juveniles, and in abdominal muscle, gill and central nervous tissue, and hemolymph (µmol/L, insert) of adult *Dilocarcinus pagei*. [a]Significantly different from the other values in same developmental stage (embryo, juvenile) or adult tissue (muscle, nerve).

capability (Δ hemolymph osmolality/Δ medium osmolality) was 0.47. During the 10-day time course of acclimation to 25‰ (Fig. 2), hemolymph osmolality increased to 641 ± 22 mOsm/kg H_2O within 24 hr, to 726 ± 40 mOsm/kg H_2O by 48 hr, remaining elevated thereafter at around 700 mOsm/kg H_2O.

Hemolymph sodium and chloride concentrations were 214 ± 20 mM and 226 ± 13 mM, respectively, in crabs held in freshwater, and increased significantly in 20‰ and above (Fig. 1). [Na^+] and [Cl^-] were hyper-regulated up to 15‰, conforming in higher salinities. Sodium regulatory capability was 0.46 and chloride was 0.30, with isoionic points at 449 mM Na^+ (22‰) and 256 mM Cl^- (16‰), respectively. During the time course of acclimation to 25‰, [Na^+] and [Cl^-] increased to 504 ± 16 mM Na^+ and 304 ± 9 mM Cl^-, respectively, within 24 hr, and [Cl^-] again at 48 hr (351 ± 13 mM Cl^-), remaining elevated and constant until the end of the 10-day acclimation period (Fig. 2).

Urine osmolality doubles from 384 ± 44 mOsm/kg H_2O in crabs held in freshwater to 777 ± 43 mOs/kg H_2O within 5 days of exposure to 25‰, and is isosmotic to the hemolymph in both media.

Tissue hydration and free amino acid concentrations

Water content was unchanged during exposure of juvenile crabs to 25‰ for 2 days (76.6 ± 6.6%) and in the tissues of adult crabs (≈80%) exposed to 25‰ for up to 10 days. Hydration level decreased in the embryos from 69.5 ± 0.7 to 61.5 ± 1.6% during exposure to 25‰ for 2 days (Fig. 3).

Ontogenetic stage/tissue significantly affected total FAA concentrations in *D. pagei* in freshwater (two-way ANOVA, $P < 0.001$). Total FAA concentrations were similar in embryos and adult muscle and gill tissue, but significantly greater in juveniles and adult nervous tissue (Fig. 4); hemolymph total FAA concentration in freshwater was 481 ± 56 µmol/L (Fig. 4).

Table 1 provides the principal FAA in the embryos, juveniles and in adult tissues of *D. pagei* in freshwater. Glycine and alanine constitute

TABLE 1. Principal individual and total free amino acid concentrations in whole embryos and juveniles, and in adult abdominal muscle, gill and nervous tissue (mmol/kg wet weight) and hemolymph (μmol/L) of the freshwater crab Dilocarcinus pagei held in freshwater (<0.5‰) or acclimated to 25‰ salinity for up to 10 days

Ontogenetic stage/ tissue	Salinity (‰)	Glutamic acid	Glycine	Arginine	Alanine	Proline	Others	Total FAA
Embryo	FW	0.6±0.03	1.8±0.1	0.7±0.1	2.5±0.4	0.7±0.1	8.7	14.9±1.2
	25	1.3±0.3	1.9±0.2	2.3±0.2[1]	3.9±1.0	3.2±0.5[1]	16.4	29.1±3.1[1]
Juvenile	FW	1.1±0.01	2.7±0.1	3.6±0.1	4.2±0.1	0.8±0.1	20.4	32.8±0.1
	25	1.6±0.1[1]	2.4±0.3	3.6±0.1	5.6±2.9	3.7±0.8	16.1	30.3±2.8
Adult muscle tissue	FW	0.3±0.3	3.0±0.5	1.5±0.2	1.0±0.3	0.2±0.1	3.1	10.9±2.1
	25	0.3±0.1	7.8±1.4	3.5±0.6	1.4±0.4	2.0±0.3[1]	2.8	19.7±2.4[1]
Adult gill tissue	FW	0.8±0.1	1.4±0.2	0.5±0.1	2.5±0.6	0.6±0.2	2.5	9.2±1.5
	25	1.1±0.2	2.9±0.6	1.2±0.1	2.7±0.5	1.8±0.2	2.1	11.7±1.2
Adult nervous tissue	FW	2.4±0.3	2.2±1.4	3.5±0.8	0.2±0.5	2.8±0.9	14.5	25.7±4.5
	25	4.6±0.4[1]	5.1±1.1	6.6±0.7[1]	9.2±0.5[1]	18.9±1.9[1]	9.2	56.1±5.6[1]
Adult hemolymph	FW	14.4±7.6	122.8±46.7	20.1±5.9	67.9±12.6	88.4±17.5	166.4	480.0±55.7
	25	8.2±0.4	173.4±28.8	38.2±10.4	230.0±61.8[1]	295±6.0[1]	311.2	1056.0±216.6[1]

[1]Significantly different from same stage/tissue in FW. Data for muscle and gill tissue obtained after 10-days exposure; nervous tissue after 5-days exposure ($N = 2$–6).
FAA, free amino acid; FW, freshwater.

roughly 35% of the total FAA pool in embryos and juveniles, and in adult muscle, gill and hemolymph. Arginine also prevails in juveniles and in adult muscle and nerve tissue, which also contains considerable proline and glutamic acid.

Total FAA concentrations in the ontogenetic stages of *D. pagei* after exposure to 25‰ are shown in Figure 4. Ontogenetic stage/tissue, salinity and their interactions all significantly affected total FAA concentrations (two-way ANOVA, $P < 0.001$).

On transfer to 25‰, total FAA increased by 95% in embryos (from 14.9±1.2 to 29.1±3.1 mmol/kg wet weight) mainly as a consequence of increased arginine and proline (Table 1). There were no changes in other FAA concentrations.

In the muscle tissue and hemolymph of adult crabs, despite some oscillation, total FAA concentrations increased significantly by 80 and 120%, respectively, only after 10-days exposure, mainly as a consequence of increases in nonessential FAAs like alanine and proline (Fig. 4 and Table 1). Total FAA concentration in the nervous tissue tended to increase after 24-hr exposure but increased significantly only after 5 days (≈120%, Fig. 4) and is due mainly to increased alanine, proline and arginine (Table 1). In juvenile crabs and in adult gill tissue, there were no changes in individual (except glutamic acid) or total FAA concentrations as a function of acclimation to 25‰ salinity.

Total FAA contribution to intracellular osmolality in 25‰ increased in the embryos alone, from 3.0% in freshwater to 4.5% after 2-days exposure, remaining unaltered in adult muscle (2.2 to 2.6% after 10 days), nerve (6.1 to 6.7% after 5 days) and gill (1.9% to 1.6 after 10 days) tissue. In juvenile crabs, FAA contribution decreased from 7.8 to 4.2% after 2-days exposure.

DISCUSSION

Anisosmotic and ionic extracellular regulation

Our findings show that the neotropical, hololimnetic, freshwater crab, *D. pagei*, is a strong osmotic and ionic regulator and, intriguingly, shows a remarkable ability to survive in saline media. The embryos and juveniles tolerate 25‰ well; adult crabs, although showing some mortality, survive in full strength seawater for up to 24 hr. In freshwater, the FAA concentrations in all these ontogenetic stages are much lower than in brackish and marine Crustacea, and after acclimation to 25‰, increase in the embryos and in adult tissues like muscle, nerve and hemolymph.

Various predominantly freshwater crustaceans can survive well in salinities greater than that encountered in their natural habitats. Among the Brachyura, *P. fluviatilis* survives seawater for up to 24 days (Harris and Micallef, '71), and *A. robertii*, a diadromous, semiterrestrial species, survives 48‰ for 5 days (Schubart and Diesel, '98). *E. sinensis* is anadromous, inhabiting both fresh and marine waters (Anger, '91). However, the

crayfish *Procambarus clarkii* suffers 30% mortality on exposure to 25‰, developing antennal gland necrosis (Sarver et al., '94).

Most freshwater Crustacea show similar adaptive osmoregulatory responses on exposure to elevated salinities, strongly hyper-regulating their hemolymph in freshwater and in salinities below ≈15‰, hypo-conforming at higher salinities. Adult *D. pagei* hyper-regulate hemolymph osmolality up to the isosmotic point at 24‰, much like *E. sinensis* (Schoffeniels, '70), and *Procambarus clarkii* (Sarver et al., '94) and *Cherax destructor* (Mills and Geddes, '80). The reduced hemolymph osmolality of *D. pagei*, when in freshwater (420 mOsm/kg H_2O), more resembles that of freshwater astacid crayfish and palaemonid shrimps rather than that of other freshwater Brachyura (see Table 2) and would be physiologically adaptive, decreasing hyperosmoregulatory energy expenditure by reducing passive salt loss and water gain.

Low urinary salt loss and efficient mechanisms of salt reabsorption are physiological adaptations to life in freshwater (Onken et al., '95; Freire et al., 2007). Various crayfish species produce copious, dilute urine (10–20% that of hemolymph concentration) conserving salts while excreting their osmotic water load (Mantel and Farmer, '83). Urine osmolality has been investigated in only very few freshwater crabs: in the Mediterranean freshwater crab, *Potamon edulis*, the urine (560 mOsm/kg H_2O) is virtually isosmotic to the hemolymph (Harris and Micallaef, 1971) and in the African freshwater crab, *P. niloticus*, the urine (450 mOsm/kg H_2O) is slightly hyposmotic to the hemolymph (Shaw, '59). In contrast to freshwater crayfish, *D. pagei*, like the potamoid crabs in general, has not evolved the capability to produce dilute urine in freshwater, and hemolymph and urine osmolalities are similar even at high salinity [c.f., *Potamonautes warreni* hemolymph 636 ± 33 (SEM) versus urine 488 ± 62 mOsm/L (Morris and van Aardt, '98); *P. edulis* hemolymph (Na^+ 250 mM + Cl^- 210 mM) ≈460 mOsm/kg H_2O versus urine (Na^+ 295 mM + Cl^- 275 mM) ≈570 mOsm/kg H_2O (Harris and Micallef, '71); and *P. niloticus* hemolymph (Na^+ 260 mM + Cl^- 210 mM) ≈470 mOsm/kg H_2O versus urine Na^+ 240 mM + Cl^- 210 mM) ≈450 mOsm/kg H_2O (Shaw, '59)]. As an alternative strategy, such freshwater crabs may produce a reduced flow of isosmotic urine as seen in the freshwater potamoid crabs *P. warreni* (Morris and van Aardt, '98) and *Holthuisana transversa* (Greenaway, '80), thus

TABLE 2. Hemolymph and urine osmolalities (mOsm/kg H_2O) in various freshwater crustacean taxa

Species	Hemolymph	Urine	References
Brachyura			
Dilocarcinus pagei	420 ± 39	384 ± 44	This study
	386 ± 18	—	Onken and McNamara (2002)
Potamon niloticus	500	450	Shaw ('59)
Potamon fluviatilis	538 ± 97	—	Harris and Micallef ('71)
Potamon edulis	540	560	Harris and Micallef ('71)
Eriocheir sinensis	289 ± 29	280	Rathmayer and Siebers (2001)
Paratelphusa hydrodomus	620	—	Ramamurthi ('76)
Armases robertii	678 ± 33	—	Schubart and Diesel ('98)
Astacidea			
Procambarus clarkii	387 ± 8	50	Sarver et al. ('94)
Astacus astacus	406 ± 7	—	Schubart and Diesel ('98)
Astacus leptodactylus	375	182	Khodabandeh et al. (2005)
Orconectes limosus	434 ± 26	—	Schubart and Diesel ('98)
Pacifastacus leniusculus	445	35	Kerley and Pritchard ('67)
Caridea			
Macrobrachium amazonicum	403 ± 34	—	Augusto et al. (2007)
Macrobrachium olfersi	336 ± 24	—	Freire et al. (2003)
Macrobrachium brasiliense	412 ± 15	—	Freire et al. (2003)
Macrobrachium rosenbergii	435	—	Huong et al. (2001, '68)
Macrobrachium australiense	515	25	Denne, 1968
Palaemon longirostris	510	505	Parry ('57)
Palaemonetes varians	565	545	Parry ('55)

Data are the mean ± standard error, where possible.

conserving salt and decreasing dependence on active salt absorption from freshwater by the gills, both mechanisms that counterbalance salt loss (Freire et al., 2007). Weihrauch et al. (2004) demonstrated that the posterior gills of *D. pagei* show lower F_0F_1-, V- and Na^+/K^+-ATPase activities than the diadromous crab *E. sinensis*, a difference that may reflect reduced passive salt loss in *D. pagei* and thus, lower compensatory active Na^+ and Cl^- absorption. This characteristic, perhaps typical of hololimnetic crabs, associated with a reduced flow of isosmotic urine, may constitute an efficient salt conserving strategy present in freshwater species.

Ontogeny, free amino acids and isosmotic intracellular regulation

Numerous investigations have shown that FAA play an important role in crustacean cell volume regulation (Gilles and Péqueux, '81; Haond et al., '99; Wang et al., 2004) and may be useful to evaluate the degree of adaptation to the freshwater biotope as total FAA concentrations are up to twofold to fourfold greater in marine compared with freshwater species (Mantel and Farmer, '83; McNamara et al., 2004; Augusto et al., 2007).

Our data, showing low FAA concentrations in embryos, juveniles and adult *D. pagei* (see Table 3) are of particular interest when compared with freshwater/diadromous, brackish and marine decapods as they show that the concentration of these osmotic effectors, especially in muscle tissue, constitute an effective parameter to evaluate the degree of adaptation to freshwater. This finding, together with the reduced hemolymph osmolality (420 mOsm/kg H_2O; see Table 2), as well as a life cycle entirely independent of salt water entailing abbreviated development, suggests that *D. pagei* is a well-adapted, old freshwater invader. Total FAA concentrations in the ontogenetic stages of other freshwater or diadromous species like *M. olfersi* and *M. amazonicum* (Augusto et al., 2007) are also reduced compared with marine species (Table 3).

In *D. pagei* maintained in freshwater, FAA concentrations are far higher in juveniles (32.8 mmol/kg wet weight) than in embryos (14.9 mmol/kg wet weight) or in adult muscle tissue (10.9 mmol/kg wet weight). Similarly, in the freshwater shrimps *M. olfersi* and *M. amazonicum*, total FAA concentrations are significantly greater in zoeae 1 (Augusto et al., 2007). Such an increase in the first post-embryonic phase may reflect FAA titers previously augmented in late

TABLE 3. Free amino acid titers (mmol/kg wet weight) in selected ontogenetic stages of representative decapod Crustacea from marine, estuarine and freshwater habitats

Species	Habitat	Ontogenetic stage/FAA titer		References
Homarus gammarus	Marine	Larval instar I	100 ± 8	Haond et al. ('99); Claybrook ('83)
		Larval instar II	124 ± 5	
		Juvenile	222 ± 29	
		Adult	184	
Penaeus aztecus	Marine/estuarine	Post-larvae (34‰)	125	Schoffeniels ('70); Bishop and Burton ('93)
		Adult	199	
Penaeus japonicus	Estuarine	Post-larvae (14‰)	53	Marangos et al. ('89); Dalla Via ('86)
		Juvenile (10‰)	115	
Macrobrachium amazonicum	Freshwater/diadromous	Embryo	45 ± 6	Augusto et al. (2007)
		Zoea 1 (6‰)	70 ± 9	
		Zoea 2 (6‰)	36 ± 4	
		Adult	57 ± 4	
Macrobrachium olfersi	Freshwater/diadromous	Embryo	18 ± 3	Augusto et al. (2007)
		Zoea 1	71 ± 5	
		Zoea 2 (14‰)	52 ± 7	
		Adult	38 ± 2	
Dilocarcinus pagei	Freshwater	Embryo	15 ± 1	This study
		Juvenile	33 ± 2	
		Adult	11 ± 2	

Adult FAA were quantified in muscle tissue. Where appropriate literature data have been converted from dry mass (μmol/mg dry weight) to wet weight assuming 80% water content.
FAA, free amino acid.

embryos and maintained after hatching. As the embryonic cells would be in osmotic equilibrium with their surrounding fluid, this increased FAA concentration may mirror an osmotic gradient driving water influx into the extracellular space, facilitating egg membrane rupture as seen in *H. gammarus* eggs (Rosa et al., 2005).

On exposure to high salinity, FAA concentrations in *D. pagei* increase in embryos (95%) and adult tissues like muscle, nerve and hemolymph ($\approx$100%) but are unaltered in the juveniles and adult gill tissue. Such stage- and tissue-specific responses may reflect the limited anisosmotic regulatory capability of the embryos or ineffective osmotic protection by the egg membranes, suggesting FAA participation in embryonic IIR. The development of osmoregulatory organs in juveniles, and efficient adult anisosmotic regulatory mechanisms, particularly in the gill tissue, may reduce the dependence of these stages/tissues on intracellular osmotic effectors like FAA.

Cell volume is regulated much more rapidly (hours) after exposure to hyposmotic media than to hyperosmotic media (days) (Gilles and Péqueux, '83) suggesting asymmetry of the underlying metabolic processes. Juvenile *D. pagei* show no changes in total or individual FAA concentrations on acclimation to 25‰ for 2 days, and FAA increase in adult muscle tissue only after 10-days acclimation. In the diadromous freshwater shrimps, *M. olfersi* and *M. amazonicum*, muscle tissue FAA increase after 2–5 days (Augusto et al., 2007). However, as a putatively older freshwater invader, *D. pagei* never encounters saline media in its natural habitat, and the rapid tissue FAA increase in diadromous and estuarine species may reflect their frequent exposure to saline media in nature.

Total FAA in hemolymph and muscle of adult *D. pagei* increase after 10-days exposure to 25‰, mainly consequent to rises in alanine and proline, which presumably derive from increased muscle FAA synthesis and/or protein catabolism. In *D. pagei* chela muscle tissue exposed in vitro to a hyperosmotic saline (= 17‰), ninhydrin positive substances increase by 65% after 90 min (Amado et al., 2006), which although constituting a very rapid response, and allowing for volume loss ($\approx$12%), suggests that intracellular FAAs initially increase via augmented synthesis. In contrast, there are no changes in total or individual FAA concentrations in adult *D. pagei* gill tissue during acclimation to 25‰ salinity for up to 10 days, like the estuarine crab *Panopeus herbstii* (Boone and Claybrook, '77). As the gills constitute the main site of active salt regulation in crustaceans, such stability in gill FAA during exposure to hyperosmotic medium may reflect the rapid changes occurring in intracellular osmolality as a consequence of ion transport and osmotic water movement rather than slower alterations in FAA titers. The large increase (+120%) in nerve total FAA concentration after 5-days exposure of *D. pagei* to 25‰ salinity may also reflect an overall intracellular buffering response since altered volume changes and intracellular ionic concentrations in neurons can modify membrane potential and neural signaling, affecting behavior and physiological integration.

Our findings on anisosmotic extracellular and isomotic intracellular regulation in adult *D. pagei* show that although hemolymph osmolality and $[Na^+]$ and $[Cl^-]$ increase rapidly after exposure to brackish water (see Fig. 1), increases in FAA concentrations take much longer (5–10 days), suggesting that although hemolymph ion concentrations attain a rapid equilibrium with the external medium, intracellular FAA synthesis and/or hemolymph and muscle protein catabolism follow a more protracted time course. Although total FAA concentrations in embryos and adult muscle and nerve tissue do increase after exposure to 25‰, their effective contribution to intracellular osmolality, consequent to alteration in hemolymph osmolality, increases only in the embryos from 3.0 to just 4.5%. Thus, the overall contribution of FAA to intracellular osmolality is minor compared with that of the main intracellular ions and absent in some ontogenetic stages and adult tissues. However, increases in typical intracellular inorganic ions like Na^+, K^+ and Cl^- likely compensate for the low FAA concentrations in *D. pagei*. We calculate values of 138 mmol Na^+ per kilogram wet weight and 114 mmol Cl^- per kilogram wet weight from Amado et al.'s (2006) data on extensor claw muscle of adult *D. pagei* exposed in vitro for 2 hr to a hyperosmotic saline (531 mOsm/kg H_2O, 17‰, assuming 100 mg protein/g wet weight). Thus, the intracellular concentrations of these two ions alone would account for at least $\approx$250 mOsm/kg wet weight.

The increase in total FAA in crustaceans exposed to elevated salinities results mainly from large increases in nonessential amino acids like arginine, glutamic acid, glycine, alanine and proline (Claybrook '83). In *D. pagei*, the alterations in embryo (arginine and proline), juvenile

(glutamic acid) and adult tissue (glutamic acid, arginine, alanine and/or proline) FAA profiles reflect this pattern typical of adult Crustacea and many marine invertebrates.

Clearly, the penetration and radiation of the marine and estuarine Crustacea into dilute media has implicated physiological, biochemical, morphological, reproductive and behavioral adaptations, among others. Here, we have accrued pertinent physiological findings that seem to underpin this progression. Our data suggest that, during the conquest of freshwater by the Brachyura, efficient, anisosmotic extracellular regulatory mechanisms have supplanted those adjusting cell volume by organic effectors on which hololimnetic species like *D. pagei*, including their ontogenetic stages, seem to depend very little.

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