RESEARCH ARTICLE

Do Osmoregulators Have Lower Capacity of Muscle Water Regulation than Osmoconformers? A Study on Decapod Crustaceans

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ABSTRACT

Decapod crustaceans occupy various aquatic habitats. In freshwater they are osmoregulators, while marine species are typically osmoconformers. Freshwater crustaceans are derived from marine ancestors. The hypothesis tested here was that osmoregulators, which can rely on salt transport by interface epithelia to prevent extracellular disturbance, would have a lower capacity of tissue water regulation when compared with osmoconformers. Four species of decapod crustaceans (the marine osmoconformer crab *Hepatus pudibundus*, and three osmoregulators of different habitats) have been exposed in vivo to a salinity challenge, for up to 24 hr. Osmoregulators were: the estuarine shrimp *Palaemon pandaliformis*, the diadromous freshwater shrimp *Macrobrachium acanthurus*, and the hololimnetic red crab *Dilocarcinus pagei*. *H. pudibundus* displayed hemolymph dilution already after 0.5 hr in 25‰, reaching ~30% reduction in osmolality, but its muscle degree of hydration did not increase. To make the different in vivo salinity challenges directly comparable, the ratio between the maximum change in muscle hydration with respect to the control value measured for the species and the maximum change in hemolymph osmolality was calculated ($\times$ 1,000): *H. pudibundus* (25‰, 8.1% kg H_2O/mOsm $\times 10^3$) > *P. pandaliformis* (2‰, 9.2) > *M. acanthurus* (30‰, 12.6) > *P. pandaliformis* (35‰, 16.7) > *D. pagei* (7‰, 60.4). Muscle slices submitted in vitro to a 30% osmotic challenge confirmed in vivo results. Thus, the estuarine/freshwater osmoregulators displayed a lower capacity to hold muscle tissue water than the marine osmoconformer, despite undergoing narrower variations in hemolymph osmolality. *J. Exp. Zool. 313A:80–94, 2010.* © 2009 Wiley-Liss, Inc.

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Decapod crustaceans are variably capable of efficient anisosmotic extracellular regulation (AER) (Florkin, '62), especially members of those groups that successfully invaded the freshwater or terrestrial habitats (reviews in Kirschner, '91, 2004; Péqueux, '95; Freire et al., 2008a). On the contrary, strictly marine species are in general osmoconformers, unable to perform AER. In these last species, ambient water variation in salinity results in extracellular fluid (ECF) osmotic disturbance (Kirschner, '91; Péqueux, '95). ECF osmotic variation necessitates cell volume regulatory mechanisms, and cells display mechanisms of osmotic adjustment referred to as intracellular isosmotic regulation (IIR) (Florkin, '62; Kirschner, '91; Péqueux, '95; Freire et al., 2008b).

Coastal species that inhabit areas under tidal influence, thus prone to salinity fluctuations, are necessarily more tolerant of salinity variation, thus in general more euryhaline than either marine osmoconformers or strictly freshwater hololimnetic

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species that may have gone stenohaline after long evolution in freshwater (Born, '68; Gilles and Péqueux, '83; Moreira et al., '83; Kirschner, '91; Péqueux, '95; Freire et al., 2003, 2008b). Estuarine crustaceans may osmoregulate more or less tightly (or may even osmoconform to some degree) in the rapidly changing salinities of their habitat (Denne, '68; Péqueux, '95; Freire et al., 2003). To live in fresh water requires the capacity for ion absorption against a steep ionic gradient, even with reduced hemolymph osmolalities when compared with marine relatives. Branchial salt absorption mechanisms of strong affinity coupled to reduced permeabilities to water and ions or the ability to produce very dilute urine result in the maintenance of the gradients (Kirschner, '91, 2004; Péqueux, '95; Rasmussen and Andersen, '96; Freire et al., 2008a).

Marine/estuarine and freshwater species may show different degrees of euryhalinity, and when tolerating either decreases or increases in salinity may show stronger or weaker capacity to osmoregulate their ECF. Time of exposure to the salinity challenge is a key factor in determining what type of responses are available and will be elicited in the animal. Although sometimes clear osmoregulatory or osmoconforming patterns can be visualized, the limits/boundaries between both types of behavior are not always clearly defined (see Péqueux, '95; Lucu et al., 2000; Freire et al., 2003, 2008a). Most osmoregulators, depending on the salinity and time of exposure, may start to display osmoconforming behavior, until eventually reaching their limit of tolerance.

Osmoconforming euryhaline species necessarily have a great capacity to maintain cellular volume, as their cells possibly face large variation in their extracellular osmolality. On the other hand, stenohaline regulators or conformers presumably display a relatively lower capacity to regulate cell volume, which then results in a narrower range of tolerance to salinity variation (Gilles, '73; Gilles and Péqueux, '83; Kirschner, '91; Péqueux, '95; Freire et al., 2008b). Uncompensated water fluxes to/from the cells then lead to irreversible cell swelling or shrinking (Gilles, '73; Burton and Feldman, '82; Herrera et al., '89; Dragolovich and Pierce, '92; Deaton, '97; Lucu et al., 2000; Souza and Scemes, 2000; Freire et al., 2008b). Cell volume regulation or IIR (Florkin, '62; Péqueux, '95; Freire et al., 2008b) is a well-conserved process, involving transmembrane transport mechanisms in species widely separated by the evolutionary process (Kinne, '93; Péqueux, '95; Wehner et al., 2003; Augusto et al., 2007b; Hoffmann et al., 2009). Reduction of ECF osmolality leads to water influx into the cells, and the cells swell. Recovery of cellular volume through regulatory volume decrease (RVD) is attained by the efflux of osmolytes, followed by water efflux (Deaton, '97; Mongin and Orlov, 2001). Conversely, increase in ECF osmolality leads to water efflux out of the cells, and the cells shrink. Recovery of cellular volume (regulatory volume increase, RVI) is attained by the uptake of osmolytes, followed by water influx. Both RVD and RVI initially (in minutes) involve the transport of inorganic ions, and later the loss or gain of organic osmolytes, mainly amino acids (Chamberlin and Strange, '89; Hoffmann and Dunham, '95; Deaton, '97; Lang and Waldegger, '97; Souza and Scemes, 2000; Wehner et al., 2003; Strange, 2004; Hoffmann et al., 2009).

This study is aimed at comparatively appraising the capacity for osmo-ionic regulation (AER) with the maintenance of muscle tissue water, when some decapod crustaceans were faced with a salinity challenge, using four species: the marine osmoconformer crab *Hepatus pudibundus*, the estuarine shrimp *Palaemon pandaliformis*, the freshwater diadromous shrimp *Macrobrachium acanthurus*, and the fully freshwater crab *Dilocarcinus pagei*. The hypothesis tested here was that the osmoregulators, species with higher capacity for AER, would display a lower capacity to regulate tissue water content (IIR capacity) when compared with the species with lowest AER capacity, the osmoconformer, when faced with a salinity challenge.

MATERIALS AND METHODS

Animals

The four species of crustaceans chosen for this study represented the occupation of different salinity habitats employing different osmoregulatory strategies. They ranged from marine to fully freshwater habitats, reflecting the evolutionary trend observed in crustaceans. Although they do not belong to a single evolutionary lineage or family, this sequence represents the invasion of fresh water by marine crustaceans (e.g., Born, '68; Morritt, '89; Harris and Bayliss, '90; Péqueux, '95; Freire et al., 2003, 2008a,b).

The marine crab *Hepatus pudibundus* Herbst, 1785 (Infra-Order Brachyura, Super-Family Calappoidea, Family Hepatidae, adults, either sex, ~6 cm carapace width) was obtained from Ipanema beach in Pontal do Paraná, State of Paraná, southern Brazil (25°37′S; 48°25′W). Crabs were purchased from local fishermen, as they are a bycatch of shrimp fisheries. The palaemonid shrimp *Palaemon pandaliformis* Stimpson, 1871 (Infra-Order Caridea, Family Palaemonidae, Sub-Family Palaemoninae, adults of either sex, 2.5–4 cm of total length) is an estuarine resident species (Teixeira and Sá, '98; Freire et al., 2003, 2008b), and was obtained using sieves, manually, from the marginal vegetation of the estuary of Perequê River, where salinity ranges from 8 to 24‰, in Pontal do Paraná, State of Paraná (25°34′S; 48°21′W). The other member of the family Palaemonidae employed, *Macrobrachium acanthurus* Wiegmann, 1836 (adults, either sex, 6–8 cm of total length), was purchased from locals who sell them as live bait for anglers, also in Pontal do Paraná. *M. acanthurus* lives in coastal fresh water streams or next to estuaries as adults, and its larvae migrate as they need more saline waters for development, being thus defined as diadromous (McNamara, '87; Teixeira and Sá, '98; Freire et al., 2008b). The freshwater hololimnetic red crab *Dilocarcinus pagei* Stimpson, 1861 (Infra-Order Brachyura, Super-Family Potamoi-

dea, Family Trichodactylidae, adults, either sex, 4–5 cm carapace width) is also sold as live bait, and was purchased in the city of Londrina, Northern State of Paraná (23°19′S; 51°08′W). The Brazilian Institute of Environmental Protection (IBAMA) formally agreed on the use of these animals in this study (Authorization number 010/2005 issued on January 27, 2005).

All crustaceans were arranged into plastic containers with water from their sampling sites, and were brought to the laboratory, either in Londrina (State University of Londrina)—*D. pagei* for the whole-animal in vivo studies—or Curitiba (Federal University of Paraná)—all other crustaceans, and *D. pagei* for the in vitro studies. Upon arrival in the laboratory, crustaceans were acclimated in aquaria (30–100 L), containing water of salinity equal to that of their habitat, under natural photoperiod, constant aeration, and water temperature of 22 ± 2°C. The marine crab *H. pudibundus* was acclimated in sea water of salinity 33‰; the estuarine shrimp *P. pandaliformis* in brackish water of salinity 12‰ (average value for the estuary, see Freire et al., 2003, 2008b); and the freshwater shrimp *M. acanthurus* and the freshwater crab *D. pagei* in fresh water of salinity <0.5‰. During acclimation, animals were fed on alternate days with ground beef or small fragments of fish filets. Aquarium water was partly replaced on alternate days, according to the need. Average time of laboratory acclimation was of at least one week.

Experiments

Control animals were directly obtained from the acclimation aquaria. After the period of acclimation to laboratory conditions in those control salinities, groups of specimens were exposed to a certain saline challenge (experimental salinity) for 0.5, 1, 2, or 6 hr, and 16–24 hr. Only one group of *M. acanthurus* was in addition sampled after 12 hr of exposure. One single experimental salinity was employed for each species, depending on its habitat and known degree of euryhalinity (Freire et al., 2003, 2008b; Amado et al., 2006), except for the estuarine shrimp *P. pandaliformis*, exposed both to salinity decrease and salinity increase. *H. pudibundus* was exposed to diluted sea water of salinity 25‰ for up to 16 hr; *P. pandaliformis* was submitted to diluted seawater of salinity 2‰ and full-strength sea water of salinity 35‰ for up to 17 hr; *M. acanthurus* was submitted to sea water of salinity 30‰ for up to 24 hr; *D. pagei* was submitted to dilute sea water of salinity 7‰ for up to 24 hr. Water salinity was measured using a refractometer salinometer (Shibuya S-28). The different salinities were obtained from natural sea water of salinity 32–33‰, using filtered tap water to dilute it accordingly, or using the first thaw of frozen sea water to slightly concentrate natural sea water up to 35‰.

Five to ten animals have been used for each control or experimental group, but mostly, *n* was of six specimens. *P. pandaliformis* was an exception; due to its small size, each hemolymph and muscle sample was generated from a pool of 3–4 animals. Samples from control animals were obtained after the period of acclimation to laboratory conditions. Experimental animals were submitted to the salinity challenge in individual beakers, rarely were two animals placed inside the same vial. All specimens of a same experimental group (time of exposure) or control were never processed in a same day. Time of experimentation during the day and season of the year were both randomized, until the desired *n* was reached.

Hemolymph and Tissue Sampling

Shrimps and crabs were anesthetized in ice. In shrimps, the ventral nerve cord was sectioned between the cephalothorax and the abdomen. Hemolymph samples were removed using a pipette, through cardiac puncture. In crabs, hemolymph was obtained using a syringe (10 mL), puncturing through the arthrodial membrane of one of the pereiopods. Hemolymph samples were vortexed to partially prevent clotting, and were immediately frozen at −20°C until assayed for osmolality and ionic concentrations. The muscle sample was then obtained, for the determination of its degree of hydration (fresh tissue) and level of ninhydrin-positive substances (NPS, after freezing at −20°C); in shrimps, abdominal muscle was sampled, while in crabs, the choice was the penniform muscle of the chelipods.

Hemolymph Osmolality and Ionic Concentrations

Hemolymph osmolality was measured using a vapor pressure micro-osmometer (Wescor VAPRO 5520, Logan), in undiluted samples. Sodium concentration was determined through flame photometry (Analiser-900, Brazil) in samples appropriately diluted with ultrapure water. Chloride, magnesium, and calcium were determined through colorimetric methods using commercial kits (Labtest, Brazil) in samples appropriately diluted in ultrapure water, and absorbance read in a spectrophotometer (Ultrospec 2100 PRO Amersham Pharmacia Biotech, Uppsala, Sweden).

Muscle Water and NPS

Muscle slices were weighed fresh, immediately after dissection, and were weighed again after 24–26 hr at 60°C. Weight loss in the oven was expressed as a percentage of the initial wet weight of the slice (muscle water content). The content of NPS in muscle samples was determined using a colorimetric method adapted from Clark ('68), as already used for the red crab *Dilocarcinus pagei* (Amado et al., 2006). Briefly, muscle tissue was homogenized in ultrapure water using a Potter-Elvehjem Teflon-glass homogenizer. An aliquot of 500 µL of the homogenate was transferred to another tube, to which 1.25 mL of 95% ethanol was added. The sample was centrifuged (1 min at 2,100*g*), and NPS was detected in the supernatant, in citrate buffer, in the presence of the ninhydrin reagent. Ninhydrin reagent contained ninhydrin saturated in ethylene-glycol and ascorbic acid. After boiling and cooling, 60% ethanol was added. Absorbance was read at 570 nm. A standard curve was performed for each set of analyzed samples using the amino acid glycin (10–50 µg/mL), one of the

Table 1. Composition of control (Cont) and experimental (Exp) salines (40% change in NaCl with respect to the respective control saline, so that final change in osmolality would approach 30% of change) used for the in vitro assessment of water content regulation in muscle slices of the marine crab *Hepatus pudibundus*, the estuarine shrimp *Palaemon pandaliformis*, the freshwater shrimp *Macrobrachium acanthurus* and the freshwater crab *Dilocarcinus pagei*

	NaCl (mM)		KCl (mM)		$MgCl_2$ (mM)		$CaCl_2$ (mM)		Measured osmolality (mOsm/kg H_2O)	
Species	Cont	Exp	Cont	Exp	Cont	Exp	Cont	Exp	Cont	Exp (% change)
Marine: Hp (hyposmotic shock)	475	285	11	11	60	60	18	18	1096	753 (31% ⇓)
Estuarine and Freshwater: Pp, Ma, and Dp (hyper-osmotic shock)	190	266	5	5	3	3	10	10	416	555 (33% ⇑)
Estuarine: Pp (hyposmotic shock)	190	114	5	5	3	3	10	10	416	268 (36% ⇓)

Additional components, of constant concentration in all salines: glucose (5 mM), $NaHCO_3$ (2 mM), HEPES acid (5 mM), glycin (5 mM), pH 7.6.

most abundant amino acids in crustaceans (Gilles, '79; Burton and Feldman, '82; Che Mat and Potts, '85; Barbe and Sevilla, '87; Lang '87; Dalla Via, '89; Dooley et al., 2000; McNamara et al., 2004; Soria et al., 2006; Augusto et al., 2007a,b).

In Vitro Assay of Capacity of Muscle Water Regulation

Composition of saline was the same as that previously employed in our laboratory (Freire et al., 2008b), for this same experimental procedure. Experimental salines only differed from respective controls in NaCl concentration, as indicated in Table 1. A hyposmotic challenge was presented to the muscle of the marine crab *H. pudibundus* and the estuarine shrimp *P. pandaliformis*, while a hyperosmotic challenge was presented to the muscle of the estuarine shrimp, the freshwater shrimp *M. acanthurus*, and the freshwater crab *D. pagei* (Table 1). Muscle slices were obtained from specimens maintained under control conditions. The abdomen of the shrimp *M. acanthurus* was sliced in two pieces, and the chelipod muscles from both chelipods were dissected from the crabs; one slice was submitted to the control saline, and the other to the experimental saline. The muscle slices (the whole abdomen) of the small *P. pandaliformis* were all independent, obtained from different individuals. All muscle slices were at first incubated for ~1 hr in their respective control saline, were then carefully blotted on filter paper, weighed, and then individually immersed into vials (10 mL), under room temperature (21–22°C) either in control or in experimental saline (Table 1). Each tissue slice was weighed every 15 min, until 120 min (Balance Bioprecisa FA2104N, Brazil, precision 0.1 mg), and its initial weight (time zero) was used as reference (100%) for all subsequent values obtained for this same slice (Freire et al., 2008b). This whole procedure was repeated 7–8 times, for each species. An additional control was performed using the shrimp *M. acanthurus*. To further assure that the muscle slices would be in physiological conditions during the course of the experiment, muscle slices were submitted to the experimental saline for 45 min, and were returned to the control isosmotic saline for another 45 min: their weight immediately returned to respective initial values ($n = 4$, data not shown).

Statistical Analysis

The evaluated parameters are displayed in graphs as mean ± standard error of the mean. One-way ANOVAs have been run to evaluate the effect of time of exposure to the experimental salinity on each parameter. Tukey post hoc test allowed to localize differences between groups. When data did not meet the assumptions of normality and homogeneity of variances, Dunn's test was performed (Sigma Stat, SPSS v. 2.0). In the in vitro muscle weight assay, Student's *t*-tests (either paired or unpaired, as appropriate) were performed to compare control and experimental data of weight change, at a same time of exposure. The limit of significance considered was always of 0.05.

RESULTS

Hemolymph Concentrations and Muscle Water Content

Hepatus pudibundus. Hemolymph sodium and chloride in the marine osmoconformer crab *H. pudibundus* were of respectively 426 and 414 mM in controls. Despite a similar appearance in the behavior of both ions along time, no differences were detected in chloride values until the end of the experiment (Fig. 1A). Sodium values fluctuated until 2 hr of exposure to dilute sea water, and then stabilized between 6 and 16 hr of exposure (Fig. 1A). Magnesium (62 mM in controls) has shown a steady trend for reduction until 2 hr, also attaining some stability between 6 and 16 hr (Fig. 1A); basically, the same pattern was observed for calcium, with 18 mM in controls (Fig. 1A).

Hemolymph osmolality, following the ions, has shown a pattern of progressive reduction from controls (962 mOsm/kg H_2O) until 2–6 hr, reaching 684 mOsm/kg H_2O after 6 hr, and then gaining stability between 6 and 16 hr. However, muscle water

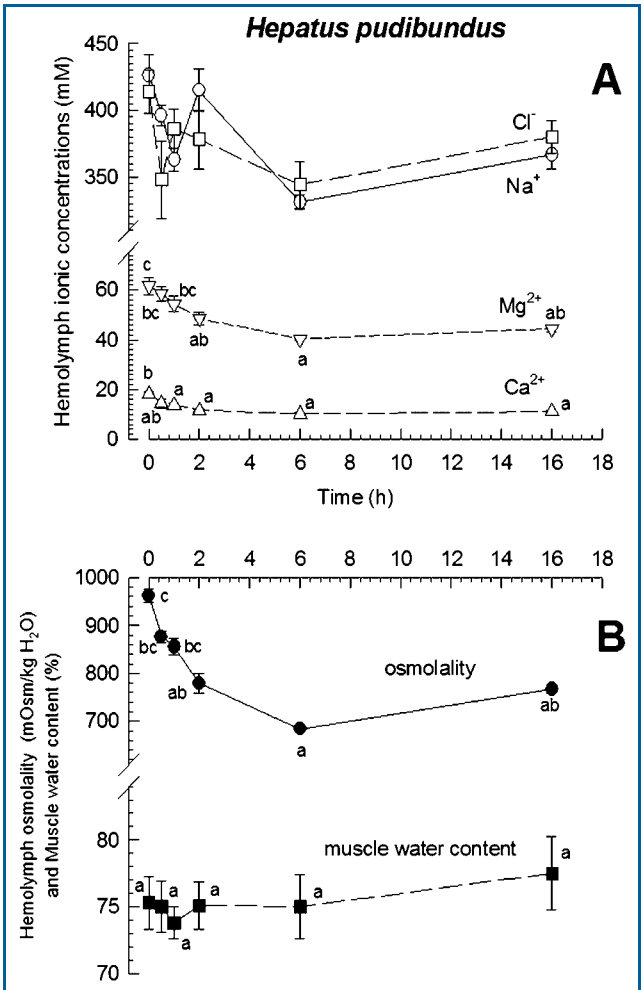

Figure 1. Time course of variation in hemolymph concentrations and muscle water content in the marine osmoconformer crab *Hepatus pudibundus* submitted to diluted sea water of salinity 25‰ ($6 < n < 8$ for all parameters). **A**: hemolymph ionic concentrations (mM); **B**: hemolymph osmolality (mOsm/kg H_2O) and muscle water content (%). Within a same line/parameter, symbols that share a same letter represent groups that are not significantly different. To avoid crowding in the figure, statistical results for Na^+ and Cl^- were not placed next to symbols. For Cl^-: for all time groups "a"; for Na^+: time 0 "d," time 0.5 hr "bcd", time 1 hr "ab," time 2 hr "cd," time 6 hr "a," time 16 hr "abc."

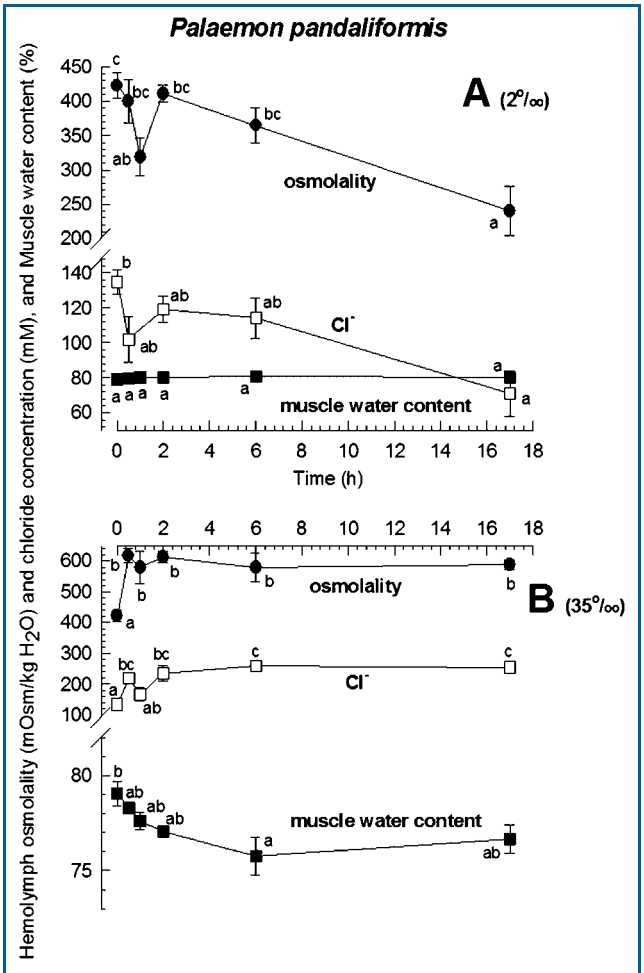

Figure 2. Time course of variation in hemolymph osmolality (mOsm/kg H_2O) and chloride (mM), and muscle water content (%) in the estuarine resident osmoregulator shrimp *Palaemon pandaliformis* submitted to **A**: very diluted sea water of salinity 2‰ ($4 < n < 7$ for chloride, and $5 < n < 10$ for the other two parameters), or **B**: full-strength sea water of salinity 35‰ ($4 < n < 6$ for chloride, and $n = 5$–6 for the other two parameters). Within a same line/parameter, symbols that share a same letter represent groups that are not significantly different.

content remained unchanged until 16 hr of exposure of the crab to dilute sea water, being of 75% in controls (Fig. 1B).

Palaemon pandaliformis. Hemolymph osmolality of *P. pandaliformis* was 423 mOsm/kg H_2O in shrimps kept in control salinity (12‰) (Fig. 2). In shrimps challenged with salinity reduction down to 2‰, a decrease followed by a recovery within the first 2 hr was noted, then followed by a progressive decrease until the 17th hour of exposure: 241 mOsm/kg H_2O (Fig. 2A). The same pattern was observed for chloride, with hemolymph levels decreasing from 136 mM in controls to 71 mM after 17 hr in the dilute salinity (Fig. 2A). Muscle water content of these shrimps remained unchanged along the whole period of exposure (~80%) (Fig. 2A).

Hemolymph of *P. pandaliformis* submitted to full-strength sea water (35‰) increased immediately, already after 0.5 hr in this salinity, from 423 in controls to 618 mOsm/kg H_2O, remaining elevated until the 17 hr of exposure (Fig. 2B). Chloride concentration followed the same pattern, increasing from 136

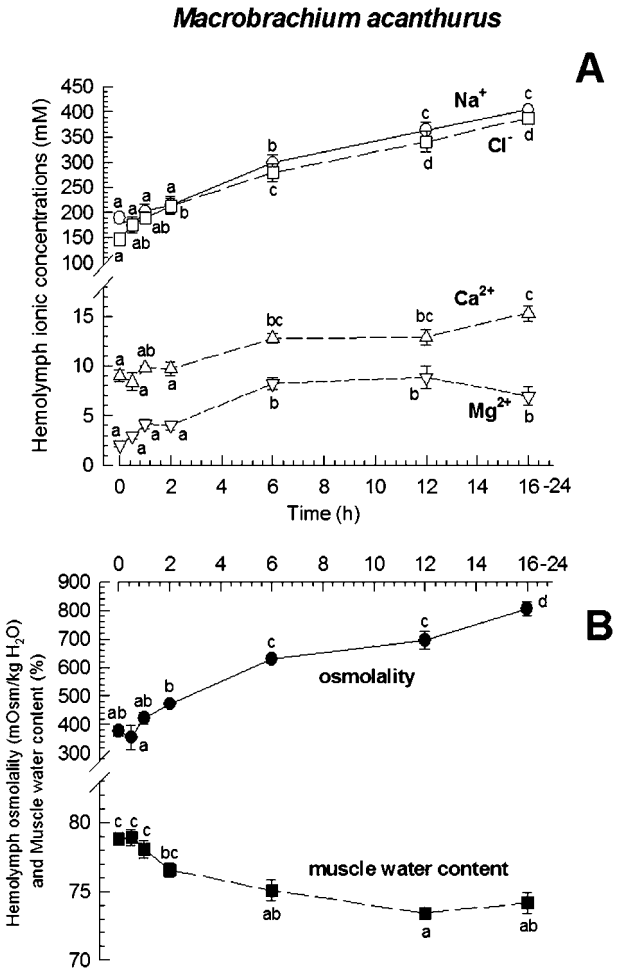

Figure 3. Time course of variation in hemolymph concentrations and muscle water content in the freshwater diadromous osmoregulator shrimp *Macrobrachium acanthurus* submitted to sea water of salinity 30‰. A: hemolymph ionic concentrations, $6<n<9$ for calcium, and $6<n<8$ for the other ions (mM); B: hemolymph osmolality, $6<n<9$ (mOsm/kg H_2O) and muscle water content, $6<n<9$ (%). Within a same line/parameter, symbols that share a same letter represent groups that are not significantly different.

in controls to 260 mM after 6 hr (Fig. 2B). Muscle water content fell from 79% in controls down to 76% after 6 hr (Fig. 2B).

Macrobrachium acanthurus. Hemolymph sodium and chloride increased progressively upon exposure of the diadromous freshwater shrimp *Macrobrachium acanthurus* to saline water of salinity 30‰. Sodium increased from 190 mM in freshwater controls to 405 mM (an increase of 113%) after 16 hr in 30‰ (Fig. 3A). Chloride concentration increased from 148 mM in freshwater controls to 388 mM (an increase of 163%) in those shrimps exposed for more than 16 hr to 30‰ (Fig. 3A). Calcium and magnesium were held relatively stable in the first hours of exposure, but later increased, after ~6 hr. Hemolymph magnesium increased from 2 mM in freshwater controls, reaching 9 mM after 12 hr (Fig. 3A). Hemolymph calcium increased from 9 mM in freshwater controls to 15 mM after 16 hr in the hyper-saline medium (Fig. 3A).

Hemolymph osmolality of *M. acanthurus* was of 378 mOsm/kg H_2O in fresh water, increasing to 806 mOsm/kg H_2O (an increase of 113%) in shrimps exposed for more than 16 hr to salinity 30‰ (Fig. 3B). With the increase in extracellular osmolality, muscle water content decreased from 79% in controls in fresh water to 73% after 12 hr of exposure to the hyper-saline medium (Fig. 3B).

Dilocarcinus pagei. Except for some fluctuation in the hemolymph magnesium (3.8–4.8 mM) of freshwater trichodactylid red crab *D. pagei*, during the first hour of exposure to dilute sea water of 7‰, all other hemolymph concentrations remained unchanged during the whole time course of the in vivo study (Fig. 4). In freshwater controls, hemolymph sodium and chloride were respectively 158 and 126 mM; calcium and magnesium were respectively 7.7 and 3.8 mM (Fig. 4A), and hemolymph osmolality was 328 mOsm/kg H_2O (Fig. 4B). As hemolymph concentrations did not change, in accordance, muscle water content also remained constant, at ~80% (Fig. 4B).

NPS in Muscle

Muscle NPS measured in the four crustaceans submitted to their respective experimental salinities displayed a pattern of decreased levels in the marine species, a pattern of increase in the freshwater species, and some oscillation in the estuarine species. Muscle of the marine *H. pudibundus* displayed a decrease until 6 hr of exposure, from 24.4 µg/mg wet weight in sea water controls to 15.4 after 6 hr in 25‰, remaining unchanged after 16 hr of exposure: 20.3 (Fig. 5A). The muscle of the estuarine *P. pandaliformis*, exposed to 2 and 35‰, showed the same behavior in both extreme salinities, of oscillating NPS values (range: 10.9–20.8), though without differences (Fig. 5B). Muscle taken from the two freshwater species submitted to increased salinity showed a similar behavior. In *M. acanthurus* muscle, NPS values increased from 16.9 in freshwater controls to 24.2–27.3 after 12–24 hr in 30‰ salinity (Fig. 5C). In *D. pagei* muscle, there was an increase already after 2 hr of exposure (from 9.8 in controls to 15.6), and remained high until the end of the experiment (Fig. 5D).

Water Regulation in Isolated Muscle Slices

Muscle slices were dissected from control animals and submitted in vitro to a 30% osmotic challenge. This value was chosen, as it represented the in vivo change detected in the hemolymph of the osmoconformer *H. pudibundus*, as studied here (Fig. 1B). Muscle of *H. pudibundus* showed regulation of its wet weight in the

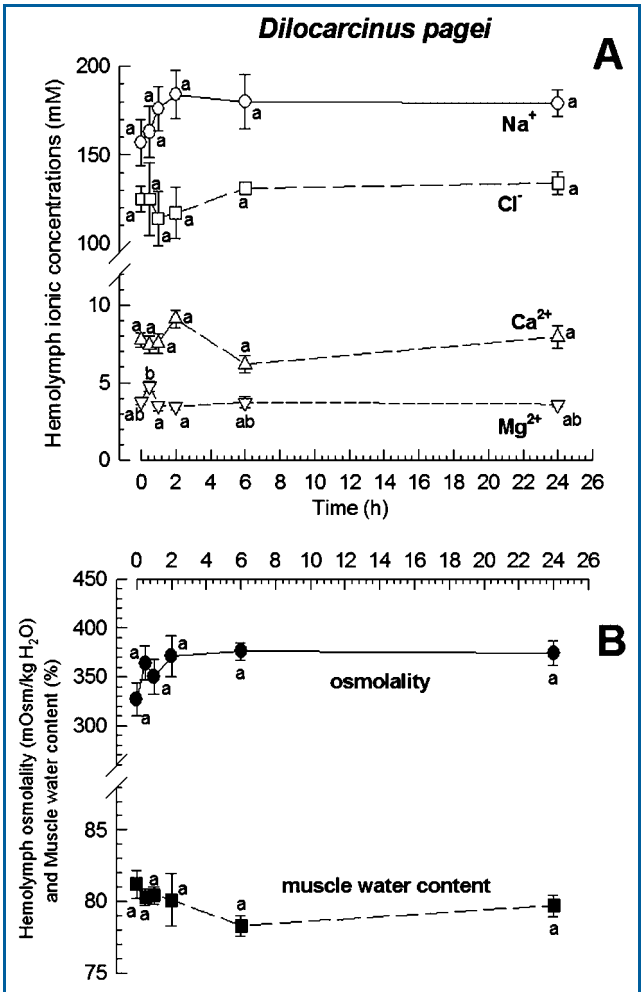

Figure 4. Time course of variation in hemolymph concentrations and muscle water content in the freshwater hololimnetic osmoregulator red crab *Dilocarcinus pagei* submitted to diluted sea water of salinity 7‰ ($6 < n < 10$ for all parameters). **A**: hemolymph ionic concentrations (mM); **B**: hemolymph osmolality, (mOsm/kg H_2O) and muscle water content (%). Within a same line/parameter, symbols that share a same letter represent groups that are not significantly different.

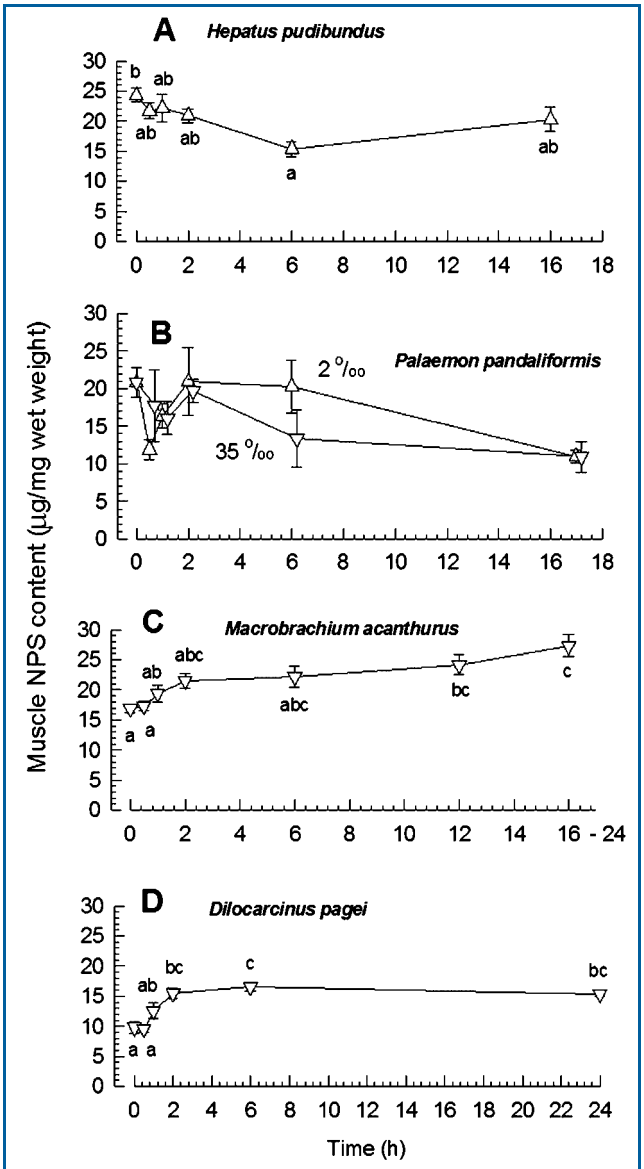

Figure 5. Time course of variation in muscle ninhydrin positive substances (NPS, µg/g muscle wet weight) for the four species of crustaceans submitted to their respective experimental salinities. **A**: *Hepatus pudibundus* in 25‰ ($5 < n < 7$); **B**: *Palaemon pandaliformis* in 2‰ ($n = 5$–6), and in 35‰ ($5 < n < 8$); **C**: *Macrobrachium acanthurus* in 30‰ ($6 < n < 10$); **D**: *Dilocarcinus pagei* in 7‰ ($5 < n < 7$). Within a same line/parameter, symbols that share a same letter represent groups that are not significantly different. For *P. pandaliformis* (B), in order to avoid crowding in the figure, statistical results were not placed next to symbols, but for both salinities, all time groups had an "a."

experimental saline (30% hyperosmotic), behaving as the muscle slices in the control saline. However, in the experimental time series, there was a certain swelling of the tissue followed by a recovery between 30 and 75 min (Fig. 6A). Muscle of *P. pandaliformis* submitted to the 30% hyposmotic saline showed gain in weight already after 15 min of exposure, remaining stable with that weight until the end of the 2 hr of data collection (~104%) (Fig. 6B). Muscle slices of *P. pandaliformis* submitted to the 30% hyperosmotic challenge displayed weight loss when compared with respective controls, for all time points, reaching 89.3% of the initial weight after 120 min (Fig. 6C). Muscle slices of *M. acanthurus* and *D. pagei* showed a behavior similar to *P. pandaliformis* submitted to the hyperosmotic saline, reaching 91.2% of the initial muscle slice weight

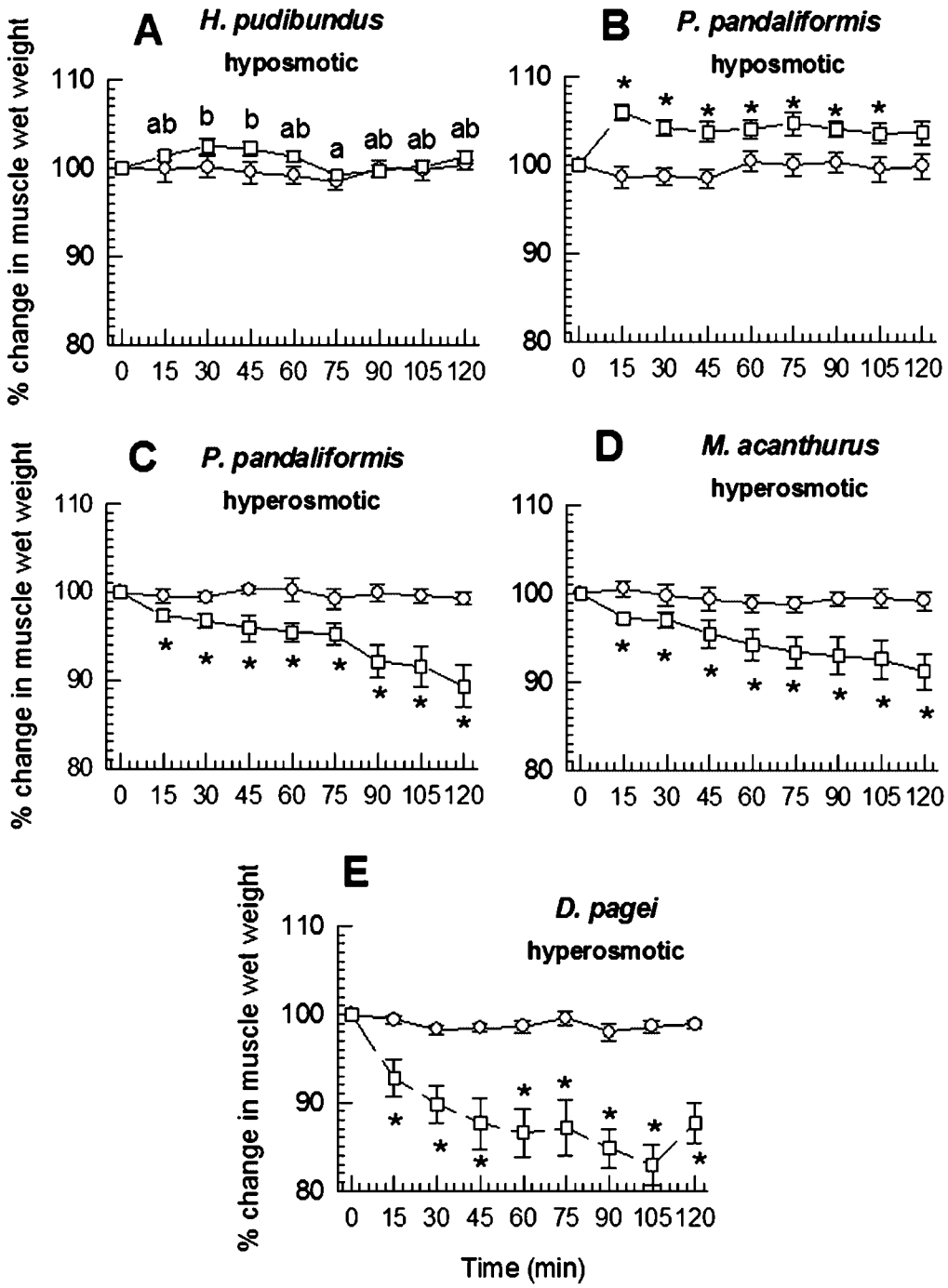

Figure 6. Time course of change in muscle wet weight (as % of initial weight) upon a 30% osmotic challenge in vitro. **A**: *Hepatus pudibundus*, hyposmotic shock ($n = 7$); **B**: *Palaemon pandaliformis*, hyposmotic shock ($n = 8$); **C**: *P. pandaliformis*, hyperosmotic shock ($n = 8$); **D**: *Macrobrachium acanthurus*, hyperosmotic shock ($n = 8$); **E**: *Dilocarcinus pagei*, hyperosmotic shock ($n = 7$). Muscle slices were incubated in vitro in either control (–○–) or experimental (–□–) saline. For all species, same *n* for control and experimental conditions. Two muscle fragments from the same individual were exposed to control and experimental salines, in a paired protocol, for all species, except *P. pandaliformis*. The * indicates significant differences between values in experimental and control saline, for the same time of exposure. In the experimental line of A, symbols that share a same letter represent groups that are not significantly different. For controls A–E, and experimental lines of B–E, all time groups had an "a."

after 120 min (*M. acanthurus*, Fig. 6D), and 82.9% of the initial muscle slice weight after 105 min (*D. pagei*, Fig. 6E).

DISCUSSION

Decapod crustaceans succeeded to occupy a wide range of aquatic, as well as terrestrial habitats, attaining high diversity. This has been accomplished, starting from marine ancestors, through a keen capacity to withstand salinity fluctuations in estuaries or to absorb salt from freshwater, or even to tolerate air exposure, especially in crabs (Kirschner, '91; Péqueux, '95; Freire et al., 2008a,b). Selected osmoregulatory strategies underlying this range of distribution follow a pattern: osmoconformation in sea water, osmoregulation in fresh water, and a variable behavior in estuaries (Kirschner, '91, 2004; Péqueux, '95; Lucu et al., 2000; Freire et al., 2008a,b). It should be noted that this is a general invertebrate pattern, not exclusive to the decapod crustaceans (Kirschner, '91, 2004; Péqueux, '95; Freire et al., 2008a,b), as there is no way out from osmoregulation (AER) in fresh water. Along with differentially expressed AER mechanisms—involving permeability control, gill transport mechanisms, and the production of a dilute urine—survival in a potentially changing/fluctuating or very dilute medium necessitate the complementary capacity for IIR, or cell volume regulation (Kirschner, '91; Péqueux, '95; Freire et al., 2008b). So, invasion of fresh water from sea water, as it happened with crustaceans, involved the selection for AER mechanisms, in a way presumably similar to what we see in extant species: weak regulators in marine/estuarine euryhaline crustaceans, and strong regulatory capacity in fully freshwater species (Kirschner, '91, 2004; Péqueux, '95; Freire et al., 2008a,b). In this process, as AER mechanisms and their regulation were not fully effective, one might suppose that IIR mechanisms were relatively more important for survival and fitness. Thus, in a recent study we discussed that for freshwater invasion in crustaceans, AER capacity would be required, along with IIR capacity, but the latter would be later lost, after "long time" of evolution in this new biotope (Freire et al., 2008b). This would be accompanied by a decrease in the amount of free amino acids in muscle; amino acids participate actively in IIR, and high concentrations in tissues are typical of sea water invertebrates (Kévers et al., '79; Willett and Burton, 2002; Yancey et al., 2002; McNamara et al., 2004; Augusto et al., 2007a,b; Freire et al., 2008b). Obviously, several constraints and pressures apply to this character (IIR capacity), crucial for survival in a changing/unstable or very dilute environment (Kirschner, '91; Péqueux, '95). Clearly, IIR capacity, given its fundamental nature, even due to cell metabolism, can be expected to be preserved; i.e., a certain basic (constitutive) capacity would always be present (Gilles, '79; Hoffmann and Dunham, '95; Strange, 2004; Hoffmann et al., 2009). So, it is not a matter of having or not the capacity, but the degree of display of that capacity. Marine osmoconformers, which do not keep their ECF significantly different from the external sea water, are not able to hold it stable when challenged with sea water dilution, as they cannot rely on AER for extracellular homeostasis. So, for marine osmoconformers, degree of euryhalinity is tightly coupled to IIR capacity. This coupling is presumably less evident in osmoregulators, which have mechanisms to "buffer" ECF osmolality change. So, IIR capacity may be at least partly related to habitat change, in particular fresh water invasion, and the time of evolution in this new habitat, a relationship that deserves further and deeper testing. However, it was found interesting to specifically test the relationship between IIR and AER capacities in crustaceans, the invertebrates with the highest success in the occupation of the freshwater biotope (Péqueux, '95; Freire et al., 2008b).

The osmoregulatory behavior of the four species studied here was entirely compatible with previous studies using these species, and with what would be expected according to the habitats they occupy (Moreira et al., '83; Melo, '96; Brailovsky and Galera, '97; Teixeira and Sá, '98; Onken and McNamara, 2002; Freire et al., 2003, 2008b; Amado et al., 2006). The marine crab *Hepatus pudibundus*, facing a 24% reduction in sea water salinity, has shown concomitant hemolymph dilution, in typical osmoconforming behavior (Péqueux, '95; Lucu et al., 2000; Freire et al., 2008b). Despite a 30% reduction in hemolymph osmolality, from 962 mOsm/kg H_2O in full-strength 33‰ sea water to 684 mOsm/kg H_2O in 25‰, muscle water content of those very same crabs remained unchanged. Osmolality of sea water of 25‰ salinity is estimated at 750 mOsm/kg H_2O, from the relationship: 1‰ = ~30 mOsm/kg H_2O. Water osmolality and ionic levels have not been measured; whenever they are mentioned, they have been estimated based on standard sea water values (salinity 34.33‰) found in Prosser ('73).

The estuarine shrimp *P. pandaliformis* experienced hemolymph osmotic dilution when in 2‰ (19%, from 423 to 341 mOsm/kg H_2O), and hemolymph concentration when in 35‰ (46%, from 423 to 618 mOsm/kg H_2O), with chloride values confirming osmolality values. *P. pandaliformis* was still hyperosmotic in 2‰ and hyposmotic in 35‰, when compared with presumed water osmolalities: ~60 (2‰) and ~1,050 mOsm/kg H_2O (35‰). However, while muscle water content was stable upon salinity reduction (2‰), it decreased from 79 to 76% upon osmotic concentration (in 35‰). *P. pandaliformis* has been previously shown to hyper-regulate in salinities below 6‰, but still undergoing some hemolymph dilution, and to be unable to survive more than 3 hr in fresh water (Freire et al., 2003). Although some regulatory capacity has been detected within the time frame of the experiment, the increase in hemolymph osmolality paralleled by the loss of muscle water in 35‰ demonstrates that a physiological limit has been reached. In low salinity, muscle water was maintained, reflecting the shrimp's higher tolerance of low salinity than of high salinities, which makes plain sense for a species that lives in the estuary, where salinity will not reach full-strength sea water levels, and, in broader terms, this reflects the evolutionary movement taken by

the Crustacea, invading dilute environments (Gilles, '73; Péqueux, '95; Taylor and Seneviratna, 2005; Freire et al., 2008b).

The diadromous palaemonid shrimp *M. acanthurus* is very euryhaline, even occurs in estuaries, and is a strong hyper-regulator in low salinities (Moreira et al., '83; Brailovsky and Galera, '97; Teixeira and Sá, '98; Freire et al., 2008b). However, the salinity challenge imposed here (30‰) approached the physiological limit for this species, and was beyond its osmoregulatory capacity. After ~17 hr in this salinity, the shrimp has shown morbidity, and measured hemolymph osmolality (806 mOsm/kg H_2O) approached external water calculated values (~900 mOsm/kg H_2O). In consequence, muscle water was not maintained, and decreased. A decrease of 5.4% was measured (after 12 hr), the largest value among the four decapods. Thus, the shrimp, after 16–24 hr, was not able to hold a gradient anymore, being unable to hypo-regulate in this high salinity, showing conformation, as already shown (Moreira et al., '83; Brailovsky and Galera, '97). The same response was shown by *M. rosenbergii* as well, exposed to sea water of 980 mOsm/kg H_2O, which was unable to survive 24 hr in this salinity (Wilder et al., '98). It is important to notice that the maintenance of a gradient, as in this case of *M. acanthurus*, for some hours, does not mean the gills are able to secrete salt, it may simply be a matter of apparent permeability control, but mechanisms have not been investigated here at all. Taken together, these results highlight the fact that the difference between osmoregulators and osmoconformers is a difference "of kind," as regulators express transporters while conformers do not (Péqueux, '95; Freire et al., 2008a). However, osmoregulators may show differences in the "degree" of expression or kinetic characteristics, or regulation of transporters, which of course will have effects on their euryhalinity (Piller et al., '95; Lucu et al., 2000; Kirschner, 2004; Chung and Lin, 2006; Jayasundara et al., 2007; Santos et al., 2007; Tsai and Lin, 2007; Freire et al., 2008a). And when the salinity challenge represents upper or lower limits of tolerance (in intensity and/or duration), a transition from osmoregulation to osmoconformation is observed (Lang, '87; Kirschner, '91; Péqueux, '95; Freire et al., 2003).

However, the challenge presented to the freshwater hololimnetic trichodactylid red crab *Dilocarcinus pagei* was much milder (7‰) than the challenge imposed on the palaemonid shrimps, so that both hemolymph concentrations and muscle water content were well regulated in the red crab. This species inhabits continental rivers and streams lacking any interference from tides or sea water. Hemolymph osmolality (~330 mOsm/kg H_2O) was a little low when compared with freshwater decapods in general (Kirschner, '91, 2004; Péqueux, '95; Freire et al., 2008b), and to previous data for this species (Onken and McNamara, 2002; Amado et al., 2006), of ~360–390 mOsm/kg H_2O, a slight difference that may be ascribed to seasonal or population differences. *D. pagei* was previously submitted to 15‰, showing an increase in hemolymph osmolality from ~400 to ~520 mOsm/kg H_2O after 24 hr in this salinity (Amado et al., 2006), evidencing at half-strength sea water, the breakdown of its osmoregulatory capacity. It is relevant to notice here that the diadromous *M. acanthurus* has shown a limit of salt tolerance in 30‰, while this much less euryhaline hololimnetic crab has shown its limit in half the salt concentration: 15‰.

In summary, it is clear that the challenges imposed to the four species were not of the same intensity. Clearly, the hyperosmotic stresses imposed on *M. acanthurus* and *P. pandaliformis* were steeper and much more challenging than the hyposmotic stresses imposed on *H. pudibundus* or on *P. pandaliformis*, or the hyperosmotic stress on *D. pagei*. So, a simple index was calculated, in order to standardize the stresses imposed in vivo to all species, and to be able to arrange the species in a sequence of capacity to regulate tissue hydration when facing extracellular osmotic change. The index was the ratio between the maximum change (increase or decrease) in muscle hydration with respect to the control value measured for the species and the maximum change in hemolymph osmolality, not necessarily at the same time point. It is important to notice that the maximum numerical variation with respect to the control value was considered, independent of its statistical significance. In order to produce a value that could be easier to handle, the ratio was multiplied by 1,000. This way, species with higher capacity to regulate muscle water content (reflecting IIR capacity) would have a lower value for this index, as they would have lower variation in muscle hydration (numerator) even when facing large osmolality variations in their hemolymph (denominator). The two species submitted to hyposmotic challenge showed the best results, that is, the lowest values for the index (Table 2). So, arranging the indexes calculated to estimate muscle water regulatory capacity when facing hemolymph osmolality change in increasing sequence (lower value = higher relative capacity to hold muscle water), we find: *H. pudibundus* (8.1% kg H_2O/mOsm $\times 10^3$) > *P. pandaliformis* (2‰, 9.2) > *M. acanthurus* (12.6) > *P. pandaliformis* (35‰, 16.7) > *D. pagei* (60.4). Interestingly, the freshwater hololimnetic crab *D. pagei* displayed small variation in hemolymph osmolality (49 mOsm/kg H_2O), as the hyperosmotic challenge offered was perfectly tolerable. However, this stress was sufficient to evoke a 3% decrease in muscle hydration, and the index calculated was thus the largest of all species studied here. Thus, from the set of data collected here, the hypothesis proposed was confirmed, the osmoconformer decapod is relatively better at muscle water regulation than the regulators. It is apparently easier to perform RVD upon hyposmotic shock than RVI upon hyperosmotic shock, in a variety of organisms and cells (Kévers et al., '79; Gilles and Péqueux, '83; Lang, '87; Dragolovich and Pierce, '92; Garner and Burg, '94; Deaton, '97; Amado et al., 2006). It could be argued that the osmoconformer was challenged with the "easier" RVD than the osmoregulators, but the regulator estuarine species was challenged in both directions. Indeed, it seems that the relationship we are trying to

Table 2. Index of tissue water regulatory capacity

	Species and experimental salinity				
	Hp (25‰)	Pp (2‰)	Pp (35‰)	Ma (30‰)	Dp (7‰)
Δ% muscle water (MW)	2.2	1.7	3.3	5.4	3.0
$\Delta[Osm]_h$ (mOsm/kg H_2O)	278	182	195	428	49
$(\Delta\%\ MW/\Delta[Osm]_h)*1{,}000$	8.1	9.2	16.7	12.6	60.4

Ratio between maximum relative variation (in % of respective control value for the species) in muscle water content and the absolute maximum variation in hemolymph osmolality (difference with respect to control value). Values for the studied species (*Hp*, *Hepatus pudibundus*; *Pp*, *Palaemon pandaliformis*; *Ma*, *Macrobrachium acanthurus*; *Dp*, *Dilocarcinus pagei*), submitted to those experimental salinities.

demonstrate here is robust, as it was apparent even with a mild stress imposed on the strictly freshwater regulator, *D. pagei*. It was submitted to a very small hyperosmotic challenge, and even so displayed the lowest capacity for muscle hydration control. This may be related to its long time of evolution in fresh water, with its consequent reduced levels of tissue amino acids (Augusto et al., 2007b; Freire et al., 2008b).

However, even calculating this index in order to standardize the challenge imposed in vivo, allowing us to draw numerical conclusions with respect to capacity for AER and for IIR, there was the need to complement these data with direct, comparable results, submitting the tissues of these four species to the same stress. This was accomplished with our in vitro results using isolated muscle slices. The unified stress to be imposed in vitro was chosen as the percentual change in osmolality detected in the hemolymph of the osmoconformer *H. pudibundus*, of ~30% (from 962 in controls to 684 mOsm/kg H_2O after 6 hr of exposure). In fact, this was not the largest variation in hemolymph osmolality detected in the in vivo experiments, as *P. pandaliformis* in 35‰ has shown an increase in hemolymph osmolality from 423 to 618 mOsm/kg H_2O after 0.5 hr, that is, a 46% increase, and *M. acanthurus* in 30‰ has shown hemolymph increase from 378 to 806 after 16–24 hr, that is, a 113% increase. And it is worth noting that, under hyperosmotic challenge, considering the in vivo index and the in vitro experiment (30% osmotic challenge), the performance of the estuarine *P. pandaliformis* was poorer than that of the freshwater diadromous *M. acanthurus*, a result compatible with our previous observations using these two species (Freire et al., 2008b), with a 50% osmotic challenge. There was compatibility between our previous results (Freire et al., 2008b) and this study. IIR capacity is presumably needed for freshwater invasion, but is lost later. Thus, it is "still" shown in *Macrobrachium*, as the family Palaemonidae is considered to be in the process of fresh water invasion: *M. acanthurus* in this study, and *M. acanthurus* and *M. potiuna* in Freire et al. (2008b). Representing much older groups in fresh water, in accordance, *Aegla schmitti* (Freire et al., 2008b), and *D. pagei* (this study), show much less IIR capacity. As also discussed previously, *P. pandaliformis*, belonging to a genus associated to more saline waters (not fresh water), is a good osmoregulator in estuarine waters, not "requiring" good IIR capacity (Freire et al., 2008b). A large capacity for IIR is found in the osmoconformer *H. pudibundus*, which has not undergone habitat change or migration. This capacity is an adaptive trait for a marine osmoconformer, as proposed. As many factors influence IIR capacity, and osmoconformers show variable degrees of euryhalinity, we do not mean that all osmoconformers will have greater capacity for IIR than any regulator. The stenohaline conformer *Homarus vulgaris* has shown no RVD capacity (isolated axon), in sharp contrast to the high capacity for RVD shown by the very euryhaline and strong regulator *Eriocheir sinensis* (Gilles, '73).

Besides osmolality and the main extracellular osmolytes sodium and chloride, the other inorganic components of the hemolymph assayed yielded interesting results. *H. pudibundus* was essentially iso-ionic for magnesium under control conditions in full-strength sea water (~60 mM in hemolymph, ~54 mM expected in sea water), and has shown proportional dilution upon exposure to dilute sea water (~42 mM, or 30% reduction), in ionic conformation for this ion. This means absence of magnesium secretion by the antennal gland (Freire et al., 2008a), resulting in lack of hypo-regulatory capacity for magnesium in sea water. This may be related to the low mobility shown by this flecked box crab, locally known as "sleepy" by fishermen. High hemolymph magnesium is associated with low muscular activity in crustaceans (Morritt, '89; Morritt and Spicer, '93; Frederich et al., 2000). The capacity to maintain hemolymph magnesium stable despite wide variations in external salinity in a pattern of tight regulation has been verified for many euryhaline crustaceans (Morritt, '89; Frederich et al., 2000; Freire et al., 2008a), and palaemonids in particular, such as *P. pandaliformis*, *M. potiuna*, and *M. olfersi* (Freire et al., 2003), and was confirmed here for *M. acanthurus* and *D. pagei*. In *D. pagei*, magnesium hyporegulation in water of salinity 7‰ (~3.5 mM in the hemolymph, and ~11 mM estimated for the 7‰ water) in a strictly freshwater crab was in fact surprising, evidencing the physiological relevance of this ion and of its precise extracellular regulation by the antennal gland epithelium in osmoregulators.

Table 3. Relationship between maximum variation in content of ninhydrin-positive substances (NPS) in muscle and percentual variation in muscle water content (Δ% Muscle water, MW; values in first line of Table 2)

	Species and experimental salinity				
Muscle parameters	Hp (25‰)	Pp (2‰)	Pp (35‰)	Ma (30‰)	Dp (7‰)
Δ% Muscle NPS	36.9	47.1	47.6	61.5	69.4
Δ% Muscle water (MW)	2.2	1.7	3.3	5.4	3.0
(Δ%NPS/Δ%MW)	16.8	27.7	14.4	11.4	23.1

Values for the studied species (*Hp*, *Hepatus pudibundus*; *Pp*, *Palaemon pandaliformis*; *Ma*, *Macrobrachium acanthurus*; *Dp*, *Dilocarcinus pagei*), submitted to those experimental salinities.

Hemolymph calcium is always tightly regulated by the antennal gland in crustaceans, given its central role in the molting process and its mobilization for the hardening of the cuticle. So, as already vastly demonstrated and reviewed (Péqueux, '95; Wheatly, '99; Freire et al., 2008a), it was well regulated even by the marine *H. pudibundus*, remaining above external water values: 18–10 mM in the hemolymph and 10–7.4 mM in the external water. In *M. acanthurus*, calcium in the hemolymph was also kept above the water value even when the shrimp was showing breakdown of its osmoregulatory capacity in sea water of salinity 30‰ (estimated water value of ~9 mM). Calcium was also perfectly regulated by *D. pagei*: ranging between 6 and 9 mM.

Besides hemolymph concentrations, and muscle hydration from the specimens submitted to the in vivo experiments, the level of NPS was also assayed in muscle, for a comparative evaluation of their role in IIR, also related to the habitat of the species (Augusto et al., 2007a,b; Freire et al., 2008b). Cells have intracellular stores of free amino acids and other organic solutes used in IIR, and this pool is maintained and regulated according to volume regulation needs, through three basic mechanisms. They are: (1) difference between rates of synthesis and oxidation of these compounds, (2) changes in efflux and uptake through the plasma membrane, and (3) displacement of the equilibrium between synthesis and degradation of proteins (Gilles, '77, '79; Gilles and Péqueux, '81; Goolish and Burton, '88; Neufeld and Wright, '96). Indeed, the typical response of invertebrates to a hyperosmotic shock is a rise in intracellular FAA, and to a hyposmotic shock is a decrease in intracellular FAA (Gilles, '77, '79; Gilles and Péqueux, '81; Goolish and Burton, '88; Dalla Via, '89; Dooley et al., 2000; McNamara et al., 2004).

Upon hyposmotic challenge, *H. pudibundus* maintained muscle hydration constant (2.2% change, but nonsignificant), while showing a 37% decrease in muscle NPS. In order to estimate the real participation of NPS in muscle volume regulation of these species, in a way similar to what has been shown above for the index on muscle hydration and hemolymph osmolality homeostasis, we calculated the ratio between maximum variation in muscle NPS and maximum variation in muscle hydration (Table 3). If NPS change would be due entirely to a change in muscle water content (as NPS are expressed per muscle wet weight), then the percentages of change would be approximately the same, and the ratio would be approximately 1. This was clearly not the case. For all species, the percentage variation in muscle NPS was many-fold higher than the percentage variation in muscle hydration (Table 3). This result points to a real and effective role of NPS on IIR. Although muscle NPS (or free amino acids) are not the sole effectors in IIR, they are widely reported as playing a major role in the process (Tucker and Costlow, '75; Péqueux, '95; Amado et al., 2006). Consistently, *H. pudibundus* and *P. pandaliformis* submitted to hyposmotic shock showed muscle NPS reduction, and there was NPS increase in the freshwater species *M. acanthurus* and *D. pagei* submitted to hyperosmotic challenge, as also previously observed for *D. pagei* (Amado et al., 2006). Interestingly, the estuarine species *P. pandaliformis* displayed a more complex pattern when submitted to salinity changes in both directions, in contrast to *Palaemon elegans*, which showed a 55% increase (whole animal) in free amino acids when submitted for 48 h to salinity 40‰ (Dalla Via, '89). Although we cannot determine by which mechanisms these changes in NPS have been effected, or the quantitative role it played on IIR, the direction of change was very consistent with the literature, strengthening our conclusions on the muscle volume regulation taking place in these experiments (Vincent-Marique and Gilles, '70; Gilles, '77; Farmer and Reeve, '78; Kévers et al., '79; Péqueux et al., '79; Gilles and Péqueux, '81).

In summary, the marine species *H. pudibundus*, osmoconformer, kept the hydration levels of its tissues despite large variations in hemolymph osmolality caused by the in vivo hyposmotic challenge. On the other hand, the osmoregulator species of distinct habits and habitats *P. pandaliformis*, *M. acanthurus*, and *D. pagei*, submitted to different salinity challenges, were not as effective. When the isolated muscle of all species was submitted to the same degree of osmotic challenge in vitro, the result was confirmed, answering the question posed by the title: osmoregulators were not as good as the osmoconformer at muscle water regulation, at least as tested here.

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