Direct relationship between osmotic and ionic conforming behavior and tissue water regulatory capacity in echinoids

Ivonete A. Santos, Giovanna C. Castellano, Carolina A. Freire *

Departamento de Fisiologia, Setor de Ciências Biológicas, Centro Politécnico, Universidade Federal do Paraná, Curitiba, Paraná, CEP 81531–990, Brazil

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A B S T R A C T

Echinoderms are considered marine osmoconforming invertebrates. However, many are intertidal or live next to estuaries, tolerating salinity changes and showing extracellular gradients to dilute seawater. Three species of echinoids – Lytechinus variegatus, which can occur next to estuarine areas, the rocky intertidal Echinometra lucunter, and the mostly subtidal Arbacia lixula – were submitted to a protocol of stepwise (rate of 2–3 psu/h) dilution, down to 15 psu, or concentration, up to 45 psu, of control seawater (35 psu). Coelomic fluid samples were obtained every hour. The seawater dilution experiment lasted 8 h, while the seawater concentration experiment lasted 6 h. Significant gradients (40–90% above value in 15 psu seawater) for osmolality, sodium, magnesium, and potassium were shown by L. variegatus and E. lucunter. A. lixula showed the smallest gradients, displaying the strongest conforming behavior. The esophagus of the three species was challenged in vitro with 20 and 50% osmotic shocks (hypo- and hyperosmotic). A. lixula, the most “conforming” species, showed the highest capacity to avoid swelling of its tissues upon the — 50% hyposmotic shock, and was also the species less affected by salinity changes concerning the observation of spines and ambulacral feet movement in the whole-animal experiments. Thus, the most conforming species (A. lixula) displayed the highest capacity to regulate tissue water/volume, and was also the most euryhaline among the three studied species. In addition, tissues from all three species swelled much more than they shrank under osmotic shocks of same magnitude. This distinct trend to gain water, despite the capacity to hold some gradients upon seawater dilution, helps to explain why echinoderms cannot be fully estuarine, or ever enter fresh water.

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1. Introduction

Echinoderms are exclusively marine; they have spread and diverged down to abyssal depths, but when close to continents, remained subtidal in shallow depths or in the interface to terrestrial habitats, frequently occupying intertidal rocky coasts. However, they are generally absent from estuaries, the boundaries between seawater and fresh water. Interestingly, ophiuroids are the most frequent echinoderms in estuarine brackish waters (Binyon, 1966). Definitely, echinoderms are never found in fresh water (Pell, 1966; Turner and Meyer, 1980). This pattern has been long recognized, and is associated to an osmoconforming strategy, allied to relative stenohalinity (e.g., Sabourin and Stickle, 1981; Diehl, 1986; Stickle and Diehl, 1987; Vidolin et al., 2007). The stenohalinity derives from high permeabilities of the body wall and ambulacral system feet, and other epithelia, as well as from the lack of an excretory system able to perform active vectorial salt transport (e.g., Hyman, 1955; Binyon, 1966; Cavey and Märkel, 1994; Warnau et al., 1998; Santos-Gouveia and Freire, 2007).

High water and ion permeability and a fresh water existence in fact do not fit together. Animals can be highly permeable in estuaries, if they have some escape strategies to employ during low tides (e.g., barnacles or bivalve mollusks), or if they have a remarkable cell volume regulatory capacity, as for instance seen in polychaetes (e.g., Kirschner, 1991; Willner et al., 2005). Echinoderms seem to lack both capabilities. Thus, they exhibit low tolerance to salinity reduction, i.e., mortality, (e.g., Lawrence, 1975; Junqueira et al., 1997; Freire et al., 2011; Meng et al., 2011), or else disturbance of several functions. For example, low salinity disturbs larval survival and proper development (Echinometra lucunter in Metaxas, 1998), reproduction (genera Echinometra and Diadema in Lessios, 1981), the immune response (Asterias rubens in Coteur et al., 2004), regeneration (Ophiopharmacus filograneus, in Talbot and Lawrence, 2002), locomotion or the righting response (Luidia clathrata in Ellington and Lawrence, 1974, and Patiriella mortenseni in Barker and Russell, 2008).

However, despite being considered stenohaline osmoconformers, echinoderms frequently withstand wide variations in salinity either in their environments (especially intertidal), or experimentally in the laboratory (e.g., Binyon, 1966; Stickle and Ahokas, 1974; Stickle and Denoux, 1976; Shumway, 1977; Diehl and Lawrence, 1984; Barker and Russell, 2008; Freire et al., 2011). When tolerating salinity variations, they show some transient gradients between their coelomic
2. Materials and methods

2.1. Species habitats and sampling sites

The species chosen for this study were 1) the green sea urchin _L. variegatus_, which is a soft substrate shallow subtidal species, but reported to occur near estuaries (Drifmeyer, 1981; Ernesto and Blake, 1981; Junqueira et al., 1997); 2) the rock-horning urchin _E. lucunter_, a typically intertidal species on rocky coasts which is also found down to depth of 45 m (Griinbaum et al., 1978; Castro et al., 1995; Hendler et al., 1995; Sánchez-Jérez et al., 2001); and 3) the black sea urchin _A. lixula_, an essentially subtidal urchin, occurring down to a depth of 50 m (Chelazzi et al., 1997; Benedetti-C.ceci et al., 1998), usually reported not to occur exposed during low tides (Castro et al., 1995; Hendler et al., 1995; Bulleri et al., 1999).

_L. variegatus_ (Lamarck, 1816) (total n=66; largest test diameter of 111.4±2.6 mm, 10 measured individuals) were collected through snorkelling or scuba diving in the city of Bombinhas, Santa Catarina State, Brazil (27° 08′–28° 48′ S, 48° 28′–52° 45′ W), never from intertidal areas, only submerged, distant from intertidal coasts. _E. lucunter_ (Linnaneus, 1758) (total n=72, largest test diameter 139.2±4.7 mm, 12 measured individuals), and _A. lixula_ (Linnaneus, 1758) (total n=69, largest test diameter 84.4±1.9 mm, 10 measured individuals) were collected during low tide from rocky coasts, also in Bombinhas, or else from the city of Penha (26° 46′–27° 5 S, 48° 36′–02° W), also in Santa Catarina State, Brazil, from 2006 to 2012. On the rocky coasts of the studied sites, _E. lucunter_ was abundantly observed in intertidal areas, exposed to the air during low tides, sometimes in rock crevices (Santos-Gouvea and Freire, 2007; Freire et al., 2011). _A. lixula_ was collected along the rocky coasts, but always in subtidal, non-exposed areas. On only one occasion four individuals were observed exposed to the air (Castellano, pers. obs.).

2.2. Transport and acclimation to laboratory conditions

All urchins were transported in styrofoam boxes, wrapped by green kelp to retain moisture, for up to 3 h, to the laboratory in Curitiba, State of Paraná. In the laboratory they were acclimated for approximately 5–7 days in a stock tank (160 L) containing seawater (salinity 33–35 psu at 22 ± 2 °C, pH 7.5–8.0), under biological filtration and constant aeration, and natural photoperiod (12 h light:12 h dark). _L. variegatus_ and _E. lucunter_ were fed every two days with large fragments of _Ulva_ sp., while _A. lixula_ specimens were fed with small pieces of fish meat. All procedures described with the urchins were approved by the Committee of Ethics on Animal Experimentation of the Federal University of Paraná: certificates number 254 and 359, issued respectively on August 28, 2007 and April 7, 2009.

2.3. Experiments of stepwise dilution or concentration of seawater

Each urchin used for the seawater (SW) dilution experiment was individually placed inside a 1.5 L aquarium. It was initially exposed to control full-strength SW of 35 psu. After 1 h, the water was replaced with slightly diluted SW of 33 psu. Every hour, water was removed and replaced by further diluted SW: 30, 27, 24, 21, 18, and finally 15 psu, for 8 h. The same procedure was adopted using other species, for the SW concentration experiment: initial control in salinity 3 psu, then 37, 39, 41, 43, and finally 45 psu, taking a total time of 6 h for the whole SW concentration experiment. Ten sea urchins in total were used for the SW dilution and 10 for the SW concentration experiment, for each species. Dilution of SW was achieved through the addition of appropriate volumes of filtered tap water (activated charcoal and cellulose filters; Aqualar, Brazil); salinity was raised in concentrated SW through the addition of commercially available marine salt to full-strength SW. Salinities were always verified using a refractometer (Shibuya S28, Japan). At the end of every hour, before changing the water, a sample of perivisceral coelomic fluid (~500 µL) was withdrawn through puncture of the peristomial membrane using an hypodermic insulin syringe. A sample of the aquarium water was also taken at this moment. Both coelomic fluid and water samples were frozen at −20 °C and maintained frozen until assayed for osmolality and ions.

2.4. Behavioral observations

The “Righting-Time Response” test was employed in order to verify the wellbeing of the urchins during the progressive dilution or concentration of SW. The test was always performed 30 min after changing the experimental water. The urchin was then manually turned upside-down, with its aboral side facing the substrate. The total time taken by the urchin to return with its oral side to the substrate (bottom of the glass aquarium) was recorded. Normal behavior was considered when animals righted themselves within 30 min. At this time the stiffness and degree of movement of the spines and ambulacral feet were also recorded, as well as the response of the urchin to a stimulus: gently touching the urchin with tweezers, and observing the movement of spines and ambulacral feet in the direction of the tweezers. A qualitative arbitrary scale of intensity of the response was set in order to detect the effects of changing salinities on the behaviour of the urchins. Responses are indicated in an arbitrary scale, ranging from the absence of a behaviour or response (−) to its highest intensity, typically as manifested in control full-strength seawater (+ + +); ± and + are intermediary, subjective levels of the response. All those observations were indicative of the general wellbeing of the urchins.

2.5. Assays of osmolality and ions in the coelomic fluid and water of the _aquaaria_

Osmolality was assayed in undiluted coelomic fluid or water samples using a vapor pressure micro-osmometer (VAPRO Wescor 5520). Chloride and magnesium ions were assayed in duplicates in samples diluted in deionized water, using Labtest colorimetric kits, and absorbance read respectively at 470 and 505 nm (Ultraspac 2100 PRO Amersham Pharmacia Biotech, Sweden). Ions sodium and potassium were assayed through flame photometry (B462 Micronal, Brazil), also in samples diluted in deionized water. Osmolality and ions measured in the aquaria water samples are provided in Table 1, where they have been compared to expected values calculated from standard SW values in Prosser (1973). The general agreement between calculated
and measured values for any salinity (the only difference was in sodium, salinity 21 psu — Table 1) allowed the use of calculated values in the x-axes of Figs. 1–3 (osmolality and ions sodium, chloride, potassium, and magnesium) in order to show variability only for coelomic fluid values in y axes.

2.6. Controls

Control urchins (n = 6 of L. variegatus, n = 12 of E. lucunter, and n = 9 of A. lixula) were kept for 6–8 h in full-strength SW (35 psu), changing the water every hour, and sampling coelomic fluid every hour, simulating the handling of the experimental urchins. These controls have been conducted in order to verify whether handling and puncturing the animals every hour would interfere with their behaviour, or on their coelomic fluid concentrations (osmolality, chloride, and magnesium). These control urchins kept in full-strength SW (35 psu) displayed healthy behavior and quite variable righting responses. Coelomic fluid osmolalities, chloride or magnesium concentrations were very stable along the 6–8 h of observation. The only exception was an effect of time on A. lixula chloride concentrations (Table 2). These results showed that sequentially disturbing the animals — turning them upside down or changing the water of the aquarium, and puncturing their peristomial membrane for coelomic fluid sampling — did not affect their behaviour, or their coelomic fluid concentrations.

2.7. Tissue water regulation

In this experiment 8 urchins of each species were retrieved from the stock aquarium (salinity 35 psu) and crio-anesthesized for 15 min. They were then dissected and the esophagus was withdrawn. Tissues were immediately transferred to a 10 mL beaker containing an isosmotic saline solution (Table 3). The esophagus from each urchin was then gently blotted dry on filter paper and weighed (initial weight, analytical balance Bioprecisa FA2104N, Brazil, precision of 0.1 mg), and transferred to another beaker containing either control or one of the experimental salines. Experimental salines were either hyposmotic or hyperosmotic by 20 or 50%, always with respect to the isosmotic saline (Table 3). The weight of the fragments was followed for 75 min, with weight readings every 15 min. Weight changes were interpreted as essentially movement of water. Weight changes of smaller magnitude than that of the osmotic shock offered by the experimental saline were interpreted as capacity of tissue water regulation (Amado et al., 2006; Freire et al., 2008).

2.8. Statistical analysis

Linear regressions were fitted to each set of coelomic fluid data from the experiments of stepwise dilution or concentration of SW. As an exception, a second order regression was fitted to magnesium data of E. lucunter in the SW dilution experiment, given the clear non-linear arrangement of the data. Independent regressions were fitted to the coelomic fluid data of the SW dilution experiment and the SW concentration experiments. Confidence intervals (95%) were calculated and plotted for each regression (Figs. 1–3), in order to reveal whether the regression lines would include or not the isosmotic/iso-ionic line, and thus decide about the existence of significant extracellular gradients (Sigma Plot software v11). When the range delimited by the 95% confidence interval of the regression line did not include the iso-osmotic or iso-ionic line, a significant gradient was implied. In addition, the slopes of the linear regressions (b1) were interpreted as indicative of the degree of buffering of extracellular concentrations, upon the stepwise salinity challenges: the closest to 0, the higher the buffering capacity of the extracellular coelomic fluid, and the capacity to maintain a gradient. In contrast, high slopes close to 1.0 would mean regression lines parallel to the isosmotic/iso-ionic lines, thus implying a lower capacity to stabilize extracellular concentrations upon external change, and a greater conformer character. The value of the intercept (b0) also helped to confirm the suggestion of a certain regulatory signal, versus a conformation signal: lower intercept values implying a greater conformer character. These comparisons were subjective, given that all regressions were fitted to the means.

Coelomic fluid values at the extreme salinities of 15 and 45 psu were compared among the three species using one-way ANOVAs. Confidence intervals (95%) were employed in order to verify whether calculated water osmolalities and ionic levels were different from measured values (Table 1). This was done to support our use of calculated expected osmotic and ionic values in Figs. 1–3, allowing smoother linear regressions to be drawn than if using real and variable measured values of the water. A one-way ANOVA was used to assess whether puncturing the peristomial membrane every one hour to obtain coelomic fluid samples, turning urchins upside-down, and changing the water of the aquarium — although keeping salinity constant (full-strength seawater, control) — would affect coelomic fluid concentrations (osmolality,
of normality and equality of variances. All analyses were conducted using Sigma Plot v 11.0. Level of significance was always of 0.05.

3. Results

3.1. Behavioural observations

The righting behavior was displayed by *L. variegatus* and *A. lixula*, but was not shown by *E. lucunter* in the salinity challenge experiments. However, it was displayed by some control individuals of *E. lucunter* maintained in full strength seawater (SW) salinity (Tables 2 and 4). *L. variegatus* displayed normal righting times (i.e., as fast as urchins in full-strength control SW) in salinities ranging from 30 to 39 psu. Movements of spines, ambulacral feet and response to stimuli were shown in a wider range of salinities, being hindered only when SW dilution reached 21 psu. *L. variegatus* was the only urchin displaying mortality in the lowest salinity tested, 15 psu, when all animals died; in the SW concentration experiment, 2 out of the 10 urchins tested died when salinity reached 41 psu (Table 4).

*E. lucunter* displayed some hindering in the behavioural responses, especially ambulacral feet movement, also in the SW dilution experiment, in salinities lower than 21–18 psu; in the SW concentration experiment, in salinity 41 psu onwards (Table 4). The range of salinities in which *A. lixula* displayed a fast righting behavior was narrower than that shown by *L. variegatus*, it was only in salinities 37 and 39 psu. However, the other behavioural indicators were only hampered in *A. lixula* when salinities reached 18 psu in the SW dilution experiment (Table 4).

3.2. Coelomic fluid concentrations upon stepwise seawater dilution or concentration

3.2.1. Osmolarity

The 95% confidence interval lines were close to the isosmotic line only in salinities around full-strength SW, for all 3 species and in both experiments (Fig. 1). *A. lixula* displayed slopes close to 1 in both experiments, and low intercepts, when compared to the other two species. *L. variegatus* was significantly hyperosmotic with respect to external SW in the SW dilution experiment, with a gradient of 273 mOsm/kg H2O in SW of 15 psu (coelomic fluid value 61% higher than water value at 15 psu SW), at the end of the experiment. Likewise, in the SW concentration experiment it remained hyperosmotic, reaching a gradient of −218 mOsm/kg H2O (16% below 45 psu SW, Fig. 1A). *E. lucunter* displayed a pattern very similar to *L. variegatus*, hyperosmotic in dilute media, with a gradient of 214 mOsm/kg H2O in 15 psu (48% above 15 psu SW), and hyposmotic in concentrated media, with a gradient of −156 mOsm/kg H2O in 45 psu (12% below 45 psu SW) (Fig. 1B). *A. lixula* displayed smaller osmotic gradients, with a gradient of 117 mOsm/kg H2O in 15 psu (26% above 15 psu SW). In concentrated SW, the hypsomotic gradient was of −118 mOsm/kg H2O (9% below 45 psu SW) (Fig. 1C). Comparing coelomic fluid osmolalities among species at the extreme salinities, the result was: *L. variegatus* > *E. lucunter* > *A. lixula* in 15 psu, and *L. variegatus* = *E. lucunter* < *A. lixula* in 45 psu (Fig. 1).

3.2.2. Sodium

The pattern noted with coelomic fluid sodium concentrations followed closely what was described above for osmolarity (Fig. 2A, B, C). Again, *A. lixula* displayed slopes close to 1 in both experiments, and low intercepts, when compared to the other two species. *L. variegatus* had final gradients of 142 mM (69% above 15 psu SW) and −94 mM (15% below 45 psu SW, Fig. 2A), respectively. For *E. lucunter* the final gradients were of respectively 160 mM (78% above 15 psu SW) and −81 mM (13% below 45 psu SW, Fig. 2B). The gradients of *A. lixula* were: 81 mM in 15 psu (39% above 15 psu SW) and −73 mM (12% below 45 psu SW, Fig. 2C). Comparing coelomic fluid sodium among species at the extreme salinities, the result was: *L. variegatus* = *E. lucunter* > *A. lixula*.
**E. lucunter > A. lixula** in 15 psu, and **L. variegatus = E. lucunter = A. lixula** in 45 psu (Fig. 2A, B, C).

### 3.2.3. Chloride

The regression lines of **L. variegatus** for chloride deviated from the iso-ionic line, as indicated by the 95% confidence intervals (Fig. 2D). Differently, in **E. lucunter** regressions were much closer to the iso-ionic line, as also happened in **A. lixula** in the SW dilution experiment (Fig. 2D, E, F). Thus, for chloride a pattern different from osmolality and sodium emerged, with gradients maintained only by **L. variegatus** in both experiments, and **A. lixula** in concentrated SW. In fact, slopes of the chloride data were of 0.62 and 0.89 for **L. variegatus**, 0.89 and 0.70 for **E. lucunter**, 0.95 and 0.98 for **A. lixula**. In comparison, the highest slopes for sodium were those of **A. lixula**: 0.59 and 0.69 (Fig. 2). **L. variegatus** was hyperosmotic by 119 mM (50% above 15 psu SW), and hyposmotic by −86 mM (12% below 45 psu SW, remaining below but parallel to the iso-ionic line) (Fig. 2D). **E. lucunter** displayed iso-conformation in both experiments and **A. lixula** iso-conformed in dilute SW (Fig. 2E, F). Despite the high slope (close to 1) in concentrated SW, a gradient was maintained by **A. lixula**: −66 mM (9% below 45 psu SW), as the 95% confidence interval lines did not include the iso-ionic line (Fig. 2F). Comparing coelomic fluid chloride among species at the extreme salinities, the result was: **L. variegatus > E. lucunter > A. lixula** in 15 psu, and **L. variegatus = A. lixula = E. lucunter** in 45 psu (Fig. 2D, E, F).

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**Fig. 2.** Coelomic fluid sodium and chloride concentrations (mean ± SEM, mM) of the urchins **L. variegatus** (A, D), **E. lucunter** (B, E), and **A. lixula** (C, F) as related to seawater sodium or chloride concentrations. The iso-ionic line is indicated by the dashed line. Regression lines and the respective 95% confidence intervals were separately fitted to the data of the seawater dilution experiment (black circles), and the seawater concentration experiment (white circles). Seawater sodium concentrations in the seawater dilution experiment ranged from 479 mM (salinity 35 psu) down to 205 mM (15 psu); in the seawater concentration experiment from 479 mM up to 616 mM (45 psu). Seawater chloride concentrations in the seawater dilution experiment ranged from 559 mM (salinity 35 psu) down to 239 mM (15 psu); in the seawater concentration experiment from 559 mM up to 718 mM (45 psu). The control value (in 35 psu) is the leftmost white circle. Number of urchins was of 10 for each experiment, and each species. Linear regression coefficients are written next to the lines. Seawater sodium or chloride values were calculated from standard seawater ionic values of Prosser (1973). When error bars do not appear, they are smaller than the symbols.
3.2.4. Potassium

Coelomic fluid potassium was maintained above respective water values in *L. variegatus* and *E. lucunter*; in *A. lixula* the 95% confidence intervals for the regressions run closely parallel to the iso-ionic line (Fig. 3A, B, C). *L. variegatus* displayed a gradient of 3.6 mM in the SW dilution experiment (82% above 15 psu SW), with the lowest slope of all species and conditions. In concentrated SW the gradient was of 0.9 mM (7% above 45 psu SW) (Fig. 3A). *E. lucunter* displayed a gradient of 3 mM (68% above 15 psu SW); in concentrated SW, the gradient was of 1.4 mM (11% above 45 psu SW) (Fig. 3B). *A. lixula* was essentially iso-ionic for potassium (Fig. 3C). Comparing coelomic fluid potassium among species at the extreme salinities, the result was: *L. variegatus* > *A. lixula* in 15 psu (E. lucunter was not different from the other two species), and *E. lucunter* > *L. variegatus* (*A. lixula* was not different from the other two species) in 45 psu (Fig. 3A, B, C).

3.2.5. Magnesium

*L. variegatus* and *E. lucunter* displayed a clear trend of hyper-hypo-ionic gradients for magnesium, with regression lines with low slopes, or a trend to be horizontal, and even the better fit of a second order regression for *E. lucunter* data in the SW dilution experiment (Fig. 3D, E). *A. lixula*, on the contrary, displayed a clear ion-conformation result, with very subtle deviation from the iso-ionic line (Fig. 3F). The gradients in *L. variegatus* were of 22 mM (96% above 15 psu SW) and −16 mM (23% below 45 psu SW, Fig. 3D). Likewise, in *E. lucunter* gradients were of 15 mM (65% above 15 psu SW) and −14 mM (20% below the other two species), and *E. lucunter* > *L. variegatus* (*A. lixula* was not different from the other two species) in 45 psu (Fig. 3A, B, C).
Table 2
Coelomic fluid concentrations (osmolality in mOsm/kg H2O, chloride and magnesium in mM) and behavioural observations in control urchins kept in full-strength SW (salinity 35 psu) for 6–8 h, changing the water every 1 h, without changing the salinity of the water.

<table>
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<th>Species</th>
<th>Time (h) in full strength seawater (35 psu)</th>
<th>Parameter</th>
<th>1</th>
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<th>3</th>
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</table>

Mean ± SEM, n = 5–6 for L. variegatus, 7–12 for E. lucunter, and 4–9 for A. liva, for osmolality determinations. For all the other parameters, n = 4–5 for both E. lucunter and A. liva. One way ANOVA or Kruskal-Wallis ANOVA on ranks (time) revealed that handling the urchins and coelomic fluid sampling did not modify coelomic fluid concentrations, with urchins kept in control full-strength seawater, for all three species and osmolality, chloride (for E. lucunter), and magnesium (P > 0.05). The single exception was chloride for A. liva; P = 0.018. However, the Holm-Sidak post-hoc test did not localize the differences. ¥ RTR = righting time response: percentage of urchins that have righted themselves in less than 30 min, with total n of 5 urchins; nd = no data available, as some controls were run for only 6 h; ++ = urchins were totally healthy, displaying erect spines, normal movement of ambulacral feet, and movement of spines and feet in the direction of a touching stimulus using tweezers.

45 psu SW, Fig. 3E). A. liva was essentially iso-ionic under all conditions, except for the extreme salinities of 15 and 45 psu, but the confidence intervals of the regression lines reached the iso-ionic line (Fig. 3F). Comparing coelomic fluid magnesium among species at the extreme salinities, the result was: L. variegatus = E. lucunter > A. liva in 15 psu, and L. variegatus = E. lucunter > A. liva in 45 psu (Fig. 3D, E, F).

3.3. Tissue water regulation

When exposed to a −50% hyposmotic shock, tissues (esophagus) from L. variegatus and E. lucunter gained weight and stabilized around 130% of the initial weight; A. liva gained distinctly less weight upon this hyposmotic shock of 50% (Fig. 4C). Tissues from L. variegatus gained weight faster than those of E. lucunter (Fig. 4A, B). Upon 20% hyposmotic shock, tissues from all 3 species swell approximately to a same degree. In all species and both hyposmotic conditions, tissues gained approximately 10% less weight than would be expected from absolute lack of any regulatory mechanism (150 or 120%).

Tissues of L. variegatus and A. liva lost weight upon both hyperosmotic shocks, except for A. liva after 60 min of exposure.

Table 3
Composition of control (isosmotic) and experimental (hypo and hyperosmotic by ±20 and ±50% with respect to isosmotic control) salines used for the in vitro tissue (esophagus) water control experiments.

<table>
<thead>
<tr>
<th>Components</th>
<th>NaCl (mM)</th>
<th>KCl (mM)</th>
<th>MgCl2 (mM)</th>
<th>CaCl2 (mM)</th>
<th>Measured osmolality (mOsm/kg H2O)</th>
<th>Percent of isosmotic saline (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isosmotic</td>
<td>470</td>
<td>10</td>
<td>54</td>
<td>10</td>
<td>945</td>
<td>100</td>
</tr>
<tr>
<td>Hyposmotic</td>
<td>376</td>
<td>8</td>
<td>43.2</td>
<td>8</td>
<td>752</td>
<td>80</td>
</tr>
<tr>
<td>(−20%)</td>
<td>235</td>
<td>5</td>
<td>27</td>
<td>5</td>
<td>461</td>
<td>49</td>
</tr>
<tr>
<td>Hyposmotic</td>
<td>564</td>
<td>12</td>
<td>64.8</td>
<td>12</td>
<td>1160</td>
<td>123</td>
</tr>
<tr>
<td>(+20%)</td>
<td>705</td>
<td>15</td>
<td>81</td>
<td>15</td>
<td>1466</td>
<td>155</td>
</tr>
</tbody>
</table>

Additional components, of constant concentration in all salines: D-glucose (5 mM), glycine (5 mM), HEPES acid (5 mM), and NaHCO3 (2 mM), pH 8.2. Osmalalities were measured in 1 or 2 samples of the prepared salines.

Esophagus of E. lucunter did not lose weight upon both hyperosmotic shocks. For all species, tissues exposed to the +50% hyper-osmotic shock shrank much less than expected from absolute lack of any regulatory mechanism, remaining with their final weight around 80% of the initial value (Fig. 4).

4. Discussion

4.1. Extracellular gradients with respect to external seawater: abrupt x gradual salinity changes, habitat and size effect

The maintenance of some osmotic and ionic gradients with respect to dilute or concentrated seawater (SW) was here demonstrated, especially for L. variegatus and E. lucunter. A. liva clearly showed lower capacity to hold extracellular gradients. These results confirmed previous studies on these same species (Vidolin et al., 2007; Freire et al., 2011). In these previous studies, protocols of steep abrupt salinity challenges have been employed, instead of stepwise, gradual and progressive changes in salinity such as was done here. L. variegatus was already shown to be hyper-osmotic and hyper-ionic for sodium and potassium upon abrupt (6 h) exposure to salinities 30 and 25 psu (Vidolin et al., 2007). The large intertidal E. lucunter, under a similar protocol, 6 h of abrupt transfer, basically behaved as the small subtidal A. liva, which displayed less pronounced gradients than L. variegatus (Vidolin et al., 2007), showing only a small gradient for potassium (Freire et al., 2011). Abrupt transfer to different salinities for 6 h does not properly represent real conditions found in intertidal habitats by echinoderms. Providing a more realistic simulation of its natural habitat, it is interesting to note that E. lucunter displayed some buffering of its extracellular concentrations. Thus: L. variegatus displayed significant gradients both upon abrupt (Vidolin et al., 2007) and stepwise (this study) protocols; E. lucunter displayed no gradients upon large abrupt changes in salinity (Freire et al., 2011), but showed gradients upon a stepwise protocol (this study): A. liva displayed essentially no gradients under both abrupt (Vidolin et al., 2007) and stepwise (this study) protocols.

Thus, a relationship to the habitat occupied by the species becomes apparent: upon gradual transfer to different salinities, extracellular gradients are more conspicuous for the (sometimes estuarine) L. variegatus, somewhat less for the intertidal E. lucunter, but not for the subtidal A. liva. L. variegatus, which can be found next to estuaries...
Table 4

<table>
<thead>
<tr>
<th>Behavioural responses</th>
<th>Seawater dilution</th>
<th>Seawater concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Salinity (psu)</td>
<td>Salinity (psu)</td>
</tr>
<tr>
<td>L. variegatus</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Righting response</td>
<td>♦ 0 40 20 100</td>
<td>100 70 20 0</td>
</tr>
<tr>
<td>Spines</td>
<td>♦ - + ++ + + + +</td>
<td></td>
</tr>
<tr>
<td>Ambulacral feet</td>
<td>♦ - ± ++ ++ ++ +</td>
<td></td>
</tr>
<tr>
<td>Response to stimuli</td>
<td>♦ - + ++ ++ ++ +</td>
<td></td>
</tr>
<tr>
<td>E. lucunter</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Righting response</td>
<td>0 0 0 0 0</td>
<td>0 nd 0 0 0 0</td>
</tr>
<tr>
<td>Spines</td>
<td>- - ++ ++ ++ + +</td>
<td></td>
</tr>
<tr>
<td>Ambulacral feet</td>
<td>- - ++ ++ ++ + +</td>
<td></td>
</tr>
<tr>
<td>Response to stimuli</td>
<td>- - ++ ++ ++ + +</td>
<td></td>
</tr>
<tr>
<td>A. lixula</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Righting response</td>
<td>0 0 0 30 60</td>
<td>30 nd 100 100 60 0</td>
</tr>
<tr>
<td>Spines</td>
<td>- - ++ ++ ++ + +</td>
<td></td>
</tr>
<tr>
<td>Ambulacral feet</td>
<td>- - ++ ++ ++ + +</td>
<td></td>
</tr>
<tr>
<td>Response to stimuli</td>
<td>- - ++ ++ ++ + +</td>
<td></td>
</tr>
</tbody>
</table>

Responses are indicated in an arbitrary scale, ranging from the absence of a behaviour or response (–) to its highest intensity, typically as manifested in healthy urchins in control full-strength seawater (+ ++); ± and + are intermediary levels of the response. For the righting response results, the number indicates how many animals that righted themselves within 30 min of observation, in percentage (out of the 10 urchins evaluated). In this case, the remaining urchins did not right themselves within 30 min, and water was then changed. The symbol ♦ indicates that all animals died at this point of the experiment. In addition, 2 (out of 10) L. variegatus died in salinity 41 psu; nd = no data available.

4.2. Ionic differences

Besides species, habitat, or size differences, distinct responses to different ions are also apparent. Magnesium was here the most stable extracellular ion measured, followed by potassium and sodium. The slopes of the regression lines were the lowest in the magnesium data, yielding the most “horizontal” lines among all ions and osmolarity, and even the fit of a second order regression in E. lucunter. Abrupt salinity challenge failed to reveal magnesium gradients (Vidolin et al., 2007; Freire et al., 2011), as they appeared here, for L. variegatus and E. lucunter. The urchin S. droebachiensis displayed even stronger buffering of extracellular magnesium when exposed to a simulation of the tidal cycle (Stickle and Ahokas, 1974). Potassium was distinctly kept above the iso-ionic line in both experiments especially in L. variegatus and E. lucunter. This is very consistent with the literature (Binyon, 1962; Lange, 1964; Stickle and Ahokas, 1974; Stickle and Denoux, 1976; Prusch, 1977; Pagett, 1980; Diehl, 1986; Vidolin et al., 2007), pointing to some potassium transport capacity, yielding some incipient but significant potassium hyper-regulation in echinoderms in general (Freire et al., 2011). Active secretion of potassium by ambulacral feet epithelia in starfish has long been proposed (Robertson, 1949; Prusch and Whoriskey, 1976; Prusch, 1977). It is rather likely that magnesium and potassium (and calcium, not measured here) are specifically transported (even if at very low rates) or at least have their permeabilities somewhat controlled, given their roles in cellular physiology in general (Grubbs et al., 1989; García-Franco, 1992; Frederich et al., 2001) and, in particular, on the stiffness of the mutable connective tissue of echinoderms (e.g., Eylers, 1982).

4.3. Differences in permeability?

The different pattern of response by E. lucunter under the two different protocols discussed above, and the differences in gradient maintenance displayed by a same species to different ions supports the idea that of inter-specific differences in permeability. Urchins could have some epithelial permeability control, or else some specific transport of certain ions. The development of more significant gradients by E. lucunter under the gradual protocol used here could be due to its periodic (every hour) handling, puncturing, or disturbing. This repeated
handling procedure could affect body wall permeability, perhaps by interfering on the rigidity of the connective tissue. This is plain speculation, but in fact, the stiffness of the echinoderm mutable connective tissue responds to mechanical stimulation (Motokawa and Tsuchi, 2003). And furthermore, it is dependent on the concentrations of cations (e.g., Eylers, 1982; Motokawa, 1994; Trotter et al., 1995; Motokawa and Tsuchi, 2003). Thus, a certain capacity for ionic regulation in echinoderms may be related to the regulation of the stiffness of their mutable connective tissues. Specific experiments directed to the detection of ion transport systems or to the effect of the stiffness of the mutable connective tissue on body wall permeability should be conducted. In summary, there is a clear buffering of extracellular ionic concentrations in these urchins, and this phenomenon is not entirely explained by surface/volume relations. Elucidation of mechanisms involved should be pursued in the future.

4.4. Seawater dilution × seawater concentration challenges

Larger extracellular gradients (as percentages of SW values) were more apparent in the SW dilution experiment than in the SW concentration experiment. This relates to the fact that salinity has been reduced down to 15 psu in the SW dilution experiment (reduction of 20 psu), but has been increased up to 45 psu in the SW concentration experiment (increase of 10 psu). So, given that most data fitted well into regression lines, data points indeed deviate more from the iso-osmotic and iso-ionic lines in the SW dilution experiment. If a same magnitude of change is examined in both directions, data seem very symmetrical. However, when extreme hypo- and hyper-osmotic challenges of a same magnitude are presented to echinoderms, it seems a rule that increases in salinity are more deleterious than decreases in salinity (Diehl, 1986; Santos-Gouvea and Freire, 2007; Freire et al., 2011).

The harm caused by a challenging salinity change can be evaluated in sea urchins through the righting behaviour, allied to the response to mechanical stimuli or general position and pattern of movement of spines and ambulacral feet. The urchin normally returns to its normal position within minutes after being turned upside down. A significant delay in this response can be interpreted as meaning some physiological disturbance, such as resulting from salinity challenges (e.g., Lawrence, 1975; Barker and Russell, 2008). Interestingly, E. lucunter did not show this behavior at all during the salinity challenge experiments, but some controls have righted themselves, in full-strength SW for 6 h. This finding may relate to the fact that in its habitat, it occurs tightly fixed inside rock crevices, often with negative slopes. In addition, this species has relatively short ambulacral feet when compared to the length of their spines (McPherson, 1969; Lewis and Storey, 1984; Sánchez-Jérez et al., 2001; Gondim et al., 2008). A. lixula and L. variegatus have shown reduced capacity for righting at the extreme high and low salinities. Salinity decreases or increases by – 10 psu from full-strength SW (35 down to 24 psu or 35 up to 45 psu) actually resulted in a symmetrical trend in both directions, for all 3 species. The righting behavior is facilitated by well developed adhesive disks in the aboral ambulacral feet of L. variegatus (Hill and Lawrence, 2003). Differently, both E. lucunter and A. lixula adhere strongly to rocks, using their feet and spines. A. lixula is normally found in hard substrates under at least moderate wave action, also needing strong adhesion force by its ambulacral feet (Bulleri et al., 1999; Sánchez-Jérez et al., 2001; Hill and Lawrence, 2003; Santos and Flammang, 2005). But actually, this parameter was not entirely conclusive in itself, as urchins have shown great individual variation in the time taken to right themselves, within 30 min of observation: between 2 and 30 minutes. In summary, considering all the behavioural indicators of wellbeing of these urchins, A. lixula has shown the widest tolerance to salinity change, with preserved movement of spines and ambulacral feet, and response to stimuli between 21 and 45 psu.

4.5. Regulation of tissue water

Survival/tolerance, or wellbeing in fluctuating or dilute/concentrated aquatic environments is based on extracellular and intracellular homeostasis. Extracellular concentrations display some stability especially in L. variegatus and E. lucunter, with a clear relationship between this slight capacity and the size of the urchin and perhaps the habitat occupied by the species. If there are species differences in the maintenance of extracellular fluid homeostasis, what can be said of intracellular water/volume control or regulation?

Osmoconformers, when living in areas subject to salinity fluctuations, must be able to control tissue water/volume, if they are to withstand these variations (Pierce, 1982; Diehl and Lawrence, 1984; Diehl, 1986; Mongin and Orlov, 2001; Willmer et al., 2005; Freire et al., 2008; Foster et al., 2010). Cell volume regulation involves the control of the intracellular concentration of inorganic and organic osmolytes: transport of inorganic ions across the cell membrane, and of synthesis/oxidation and transport of aminoacids (Pierce, 1982; Diehl and Lawrence, 1985; Diehl, 1986; Hoffmann and Dunham, 1995; Wehner et al., 2003). Some studies reported on the movements of water to the whole animal, detecting regulation of body mass/volume. For example, in the sea

Fig. 4. Time course of tissue (esophagus) hydration (mean ± SEM, n = 8) of L. variegatus (A), E. lucunter (B), and A. lixula (C) under both hyposmotic (black symbols) shocks of 20% (squares) and 50% (circles) of reduction with respect to isosmotic control saline, and hyper-osmotic (white symbols) shocks of 20% (squares) and 50% (circles) of increase with respect to isosmotic saline. The thicker solid line shows data of tissue fragments of E. lucunter (B) and A. lixula (C) under both hyposmotic (black symbols) shocks of 20% (squares) and 50% (circles) of increase with respect to isosmotic control saline, and hyper-osmotic (white symbols) shocks of 20% (squares) and 50% (circles) of reduction with respect to isosmotic saline. *=significant difference between experimental value and control value at the same time of exposure. # = esophagus of L. variegatus and E. lucunter swell more than that of A. lixula, for the same time of exposure (– 50% hyposmotic saline). Horizontal dotted lines at 150, 120, 100, 80 and 50% are references to indicate the relative osmotic challenges presented to the tissues.
In the *in vitro* experiments performed to evaluate tissue water regulatory capacity in those three urchins, rather remarkably, a higher stability in tissue wet weight was noted upon hyper-osmotic than upon hyposmotic shock offered to the isolated tissues (esophagus). The same *in vitro* challenges were offered in both directions and all species clearly showed less shrinking in the hyper-osmotic salines than swelling in the hyposmotic salines. Although no clear pattern of regulatory volume change has been detected using this method, it is apparent that tissues have prevented volume loss in the +50% hyper-osmotic saline and, to a lesser degree, volume gain in the –50% hyposmotic saline. These results are in agreement with those of Diehl and Lawrence (1984) for *L. clathrata*, reporting higher tissue volume stability upon hyper- than hypo-osmotic shock. However, they diverge from those that concluded that echinoderms in general cannot regulate tissue water upon hyper-osmotic shock (Diehl, 1986). They also diverge from the results of Foglietta and Herrera (1996) reporting a higher capacity to hold tissue water in *Isostichopus badionotus* exposed to reduced than to increased osmolalities. It could be argued that the techniques employed here to evaluate tissue water movements do not properly consider the extracellular space (Amado et al., 2006; Freire et al., 2008). However, our goal here was to compare the 3 species, not to precisely account for absolute measures of cell water regulation. Tissues of echinoderms offer additional challenges; they are rather spongy and/or gelatinous, as already pointed out by Robertson (1980). And further, they seem to be permeable toulin, making it difficult to evaluate the contribution of the extracellular space (Robertson, 1980). In summary, both hyposmotic shocks offered here put into evidence that a stronger shock (–50%) surpassed the capacities of *L. variegatus* and *E. lucunter* to prevent tissue swelling. The subtidal *A. ilixa* was the species whose esophagus clearly displayed the less intense degree of swelling in the –50% hyposmotic saline. In contrast, both hyperosmotic shocks produced basically the same result, the tissues shrank much less than expected, even in the +50% hyperosmotic shock. Thus, 1) tissues of these urchins swell much more than they shrink, when facing osmotic challenges of the same magnitude in both directions, and 2) the species with the strongest conforming behavior, *A. ilixa*, is the one that shows less swelling under the strongest hyposmotic shock Foster et al. (2010).

5. Conclusions

In conclusion, urchins show some signs of extracellular fluid homeostasis especially when facing gradual salinity changes. The species with the least capacity for this extracellular fluid buffering was distinctly the one with the highest capacity for tissue water regulation, especially preventing swelling, namely the small subtidal *A. ilixa*. Competitively, *A. ilixa* was also the species with the widest tolerance to salinity change, as evaluated by the “wellbeing” tests. Thus, albeit in rudimentary intensity, the signal of a direct relationship between osmoconforming behavior and cell water regulatory capacity was also detected here, with these three species of urchins from different habitats.

Echinoderms in general, and echinoids in particular, can then successfully occupy intertidal habitats. However, true estuarine life, or worse, a freshwater existence is unwarranted for these deuterostomes, given their 1) high epithelial permeabilities, 2) limited vectorial salt transport through their interface epithelia, 3) limited capacity to regulate tissue water, and 4) higher trend of their tissues to swell than to shrink. The genetic bases of these characteristics, and the reason for their under-development in echinoderms, when compared to other groups of originally marine invertebrates, could be interesting subjects of future investigation.

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