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# Metabolic substrates are not mobilized from the osmoregulatory organs (gills and kidney) of the estuarine pufferfishes *Sphoeroides greeleyi* and *S. testudineus* upon short-term salinity reduction

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The marine-estuarine species of pufferfishes *Sphoeroides testudineus* and *S. greeleyi* are very efficient osmoregulators. However, they differ with respect to their tolerance of salinity reduction. During low tide *S. testudineus* remains in diluted estuarine waters, whereas *S. greeleyi* returns to seawater (SW). The hypothesis tested here was that the short-term mobilization of metabolic substrates stored in their main osmoregulatory organs would correlate with this differential tolerance. Fishes exposed to 5‰ (for 6 h) were compared to those kept in 35‰. Branchial and renal contents of triglycerides, protein and glycogen were evaluated, and total ATPase activity accounted for the tissues' metabolism. Plasma osmolality, chloride and glucose, hematocrit, and muscle water content were also measured. Total triacylglycerol content was higher in *S. greeleyi* than in *S. testudineus* in both salinities and in both organs. Kidney glycogen contents were higher in *S. greeleyi* than in *S. testudineus* in 5 and 35‰. Total ATPase activity was reduced in 5‰ when compared to 35‰ in the kidney of *S. greeleyi*, and was higher in the gills of *S. greeleyi* than in those of *S. testudineus*, in both salinities. Upon exposure to dilute SW, both species displayed a similar osmoregulatory pattern: plasma osmolality and chloride were reduced. Again in both species, stability in muscle water content indicated cellular water content control. Although the metabolic substrates stored in the osmoregulatory organs of both species were not mobilized during these short-term sea water dilution events, some differences could be revealed between the two species. *S. greeleyi* showed more metabolic reserves (essentially triacylglycerols) in these organs, and its gills showed higher total ATPase activity than those *S. testudineus*.

Os baiacus marinhos *Sphoeroides testudineus* e *S. greeleyi* são freqüentadores de estuários e eficientes osmorreguladores. No entanto, eles diferem quanto à sua capacidade de tolerar a redução de salinidade. Durante a descida de maré *S. testudineus* permanece no estuário com salinidade reduzida e *S. greeleyi* retorna para a água do mar. A hipótese testada neste estudo foi a de que a mobilização em curto prazo dos substatos metabólicos armazenados nos órgãos osmorregulatórios poderia explicar esta tolerância diferencial a redução de salinidade. Peixes expostos a 5‰ (por 6 h) foram comparados com os mantidos em 35‰ . O conteúdo branquial e renal de triacilglicerol, proteína e glicogênio foram avaliados, e a atividade ATPásica total foi quantificada para representar o metabolismo do tecido. Osmolalidade, cloreto e glicose plasmáticos, o hematócrito e o conteúdo de água no músculo também foram mensurados. O conteúdo total de triacilglicerol foi maior em *S. greeleyi* do que em *S. testudineus* em ambas as salinidades e em ambos os órgãos. O conteúdo renal de glicogênio foi maior em *S. greeleyi* quando comparado a *S. testudineus* na duas salinidades. A atividade ATPásica total foi reduzida em 5‰ quando comparada a 35‰ no rim de *S. greeleyi*, e foi maior nas brânquias de *S. greeleyi* quando comparada aos valores para *S. testudineus* em ambas as salinidades. Após a exposição a água do mar diluída, ambas as espécies apresentaram padrão osmorregulatório similar: osmolalidade e cloreto plasmáticos foram reduzidos. Em ambas as espécies a estabilidade do conteúdo de água no músculo indicou capacidade de regular o conteúdo de água intracelular. Os substratos metabólicos estocados nos órgãos osmorregulatórios de ambas as espécies não foram mobilizados durante a exposição de curto-prazo a diluição da água do mar, porém algumas diferenças foram reveladas entre as duas espécies. *S. greeleyi* apresentou maior reserva metabólica (essencialmente triacilglicerol) em seus órgãos, e suas brânquias apresentaram maior atividade ATPásica total quando comparado a *S. testudineus*.

**Key words:** Fish gill, Fish kidney, Glycogen, Metabolism, Osmoregulation, Tetraodontidae.

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## Introduction

Marine teleosts are hypo-osmotic to seawater. Their gills act to maintain plasma homeostasis by secreting the excess of salt taken up as they drink seawater (Jobling, 1995; Evans *et al*., 1999, 2005). The kidney is not as relevant as are the gills for osmotic and ionic regulation because renal tubules essentially secrete multivalent ions ($Mg^{+2}$ and $SO_4^{-2}$) (Beyenbach & Baustian, 1989; Beyenbach *et al*., 1993; Hentschel & Zierold, 1994; Jobling, 1995). Estuarine teleosts, while facing fast salinity changes, maintain the homeostasis of their extracellular fluid activating energy-consuming osmoregulatory mechanisms (mainly in the gills) in order to switch from salt secretion to salt absorption mechanisms or vice-versa (Mancera & McCormick, 2000; Prodocimo & Freire, 2001; 2004; Scott *et al*., 2004; Evans *et al*., 2005; Sangiao-Alvarellos *et al*., 2005; Prodocimo *et al*., 2007). Vectorial salt transport against electrochemical gradients is ultimately ATP-driven (*e.g.*, Stahl & Baskin, 1990; Evans *et al*., 2005). In fact, the major ATPase involved with the energization of salt transport in gills of euryhaline fishes is the Na,K-ATPase (reviewed in Evans *et al*., 1999, 2005). In the osmoregulatory organs, the Na,K-ATPase is the major contributor to total ATPase activity (Venturini *et al*., 1992). Thus, in those organs, ATPase activity changes can indeed at least partially represent changes in the organs' energy budget, and can be related to the Na,K-ATPase activity, in fishes submitted to osmotic stress (Perry & Walsh, 1989; Venturini *et al*., 1992; Díaz *et al*., 1998). Both hypo-regulation in high salinity and hyper-regulation in low salinity demand energy consumption, for gradient maintenance. Although fish metabolism measured by oxygen consumption as a function of salinity displays a variable response, it is often reported as an "U-shaped" curve: higher metabolism in very low (frequently fresh water) or high salinities (often hypersaline), *versus* lower metabolism in intermediary, more isosmotic salinities (*e.g.*, Jensen *et al*., 1998; Kelly *et al*., 1999; Imsland *et al*., 2003; Laiz-Carrión *et al*., 2005).

The energy requirement of the gills and kidney is partially maintained by using the metabolic substrates stored in these organs (Tseng *et al*., 2007). However, there is scant information about the mobilization of their metabolic substrates according to the salinity of acclimation in fish (Hansen *et al*., 1999; Laiz-Carrión *et al*., 2002, 2005; Sangiao-Alvarellos *et al*., 2003, 2005). Still, metabolic changes in branchial and renal tissues have been essentially associated to long periods (days) of exposure to different salinities (Hansen *et al*., 1999; Laiz-Carrión *et al*., 2005; Sangiao-Alvarellos *et al*., 2003, 2005). The liver has been the major focus of studies on metabolism *versus* salinity (Woo & Fung, 1981; Woo & Wu, 1982; Nakano *et al*., 1998). It is proposed that the liver is the main site of glycogen storage and it provides plasma with glucose, which is then taken up for energy generation in the gills (*e.g.*, Vijayan *et al*., 1996; Sangiao-Alvarellos *et al*., 2005). However, marine teleosts such as the sea bream *Chrysophrys major* (Woo & Fung, 1981), the red grouper (*Epinephelus akaara*), and also the black sea bream (*Mylio macrocephalus*) (Woo & Wu, 1982) presented no changes in their hepatic glycogen content and protein after long periods (10-14 days) of exposure to dilute seawater (~ 5‰).

The effects of short-term (hours) salinity changes on metabolic reserves of fish have been poorly investigated (Woo & Wu, 1982; Nakano *et al*., 1998; Tseng *et al*., 2007). In the freshwater euryhaline tilapia (*Oreochromis mossambicus*) liver glycogen content increased after 6 hours in 23‰ (Nakano *et al*., 1998), but rapid mobilization of the local reserves of branchial glycogen has been observed to fuel ion transport mechanisms in ionocytes of the gills of tilapia upon short-term (3 hours) increase in salinity (25‰) (Tseng *et al*., 2007). In the marine teleosts red grouper (*Epinephelus akaara*) and black sea bream (*Mylio macrocephalus*), short-term (6 hours) exposure to 7‰ salinity (from 30‰) did not induce consumption of liver glycogen content (Woo & Wu, 1982). We are not aware of any study using marine/estuarine teleosts in a short-term protocol of salinity reduction with further evaluation of branchial and renal metabolic substrate store, in a protocol somewhat related to their natural habitat.

The pufferfish *Sphoeroides testudineus* (Linnaeus, 1758) is an abundant teleost in bays and estuaries along the Brazilian coast, in places with salinities ranging from 34‰ to 0‰ (Figueiredo & Menezes, 2000). *Sphoeroides greeleyi* Gilbert, 1900 is also common along the Brazilian coast, but is found in areas of higher salinity (Figueiredo & Menezes, 2000; Vendel *et al*., 2002), and has been observed to be more sensitive to salinity reduction either in the laboratory (Prodocimo & Freire, 2001) or in the field (Prodocimo & Freire, 2004), when compared to *S. testudineus*. The hypothesis tested here was that the two species of pufferfishes, with distinct tolerances of seawater dilution, might differ in their short-term use of metabolic substrates stored in their main osmoregulatory organs, when challenged with salinity alteration. The disturbance in the osmotic homeostasis of these fish would impose a metabolic demand of the energetic substrates stored in their osmoregulatory organs, thus potentially more readily available (Tseng *et al*., 2007). This hypothesis is supported by previous reports that rapid alterations in transport mechanisms (including changes in $Na^+$, $K^+$-ATPase activity) ensue when estuarine fishes endure salinity changes in their environment (Mancera & McCormick, 2000; Sakamoto *et al*., 2000; Marshall, 2003; Wood & Laurent, 2003; Prodocimo *et al*., 2007).

## Material and Methods

Adults, male or female of *S. testudineus* (~ 15 cm body length, ~ 40 g body mass, deposited at the Museum of Natural History Capão da Imbuia, Curitiba, MHNCI 11700) and *S. greeleyi* (~ 10 cm, ~ 20 g, MHNCI 11701) were obtained from Baguaçu river tidal creek (25°33'06.33''S, 48°23'41.63''W), at the south margin of Paranaguá Bay, State of Paraná, Brazil, in May and August, 2003, May and June 2004, June 2006, and finally in February, 2007. Fish were obtained during flow tide using a fyke type net installed across the creek channel. The net has remained in place for 6 hours, until the high tide peak,

when the net was retrieved and the animals were transferred to plastic gallons with aerated water from the collection site. Water salinity was verified (29-30‰) immediately after fishing the animals. Fishes were then transferred to the laboratory, where they were acclimated for ~7 days in a 160 L tank with 30‰, temperature of 21±1°C, constant aeration and biological filtration, under natural photoperiod. Fish were daily fed *ad libitum* with fresh prawns and earthworms.

*Sphoeroides testudineus* and *S. greeleyi* were exposed for 6 hours to full-strength seawater 35‰ or to diluted seawater 5‰. Experiments were performed in 30-liter aquaria with constant aeration and temperature of 21±1°C, and a maximum number of 3 fish per experimental aquarium. Seawater 35‰ was prepared appropriately by mixing 30‰ seawater with the first thaw of frozen seawater. Experimental fishes submitted to 35‰ were directly transferred from the stock tank of 30‰ to the experimental aquarium 35‰. Seawater was diluted with filtered dechlorinated tap water from ~30‰ down to ~10-12‰ along 4 hours (flux of 0.7 ml/seg) to avoid osmotic shock to fishes exposed to 5‰. The fish then remained 10-12 hours in salinity 10-12‰ overnight, and were then transferred to 5‰, where they remained for 6 hours. Those fish submitted to salinity reduction had this protocol of transfer because of the large difference in salinity.

Fishes were then anaesthesized with benzocaine (in ethanol, 80 mg/l of aquarium water), were opened by ventral incision, and a blood sample was withdrawn through cardiac puncture using an heparinized insulin syringe. Blood samples were centrifuged for 10 min (2100×g) and plasma was immediately frozen at −20°C. Blood was also collected into glass capillaries for immediate hematocrit reading. All gill arches and the whole kidney were removed for the assays of total ATPase activity, triacylglycerol and glycogen contents, and total protein concentration. A dorsal axial muscle sample (~0.40 g) was removed for tissue water content determination. These procedures have been approved by the Committee on Ethics in Animal Experimentation (CEEA) of the Institute of Biological Sciences of the Universidade Federal do Paraná (UFPR), certificate number 072, issued on May 3rd, 2004.

Plasma osmolality (vapor pressure micro-osmometer Vapro 5520, Wescor, USA), chloride (commercial kit, Labtest, Brazil), and glucose (commercial glucose oxidase kit, Laborclin, Brazil) were assayed in thawed samples. Hematocrit was determined after 12,000 rpm centrifugation (10 min, Presvac centrifuge, Argentina). For muscle water content determination, muscle samples were weighed on an analytical balance, dried at 100°C for 24h, then weighed again, and total water content was expressed as a percentage of the wet mass.

Gills and kidneys were homogenized (Potter-S, in ice) in imidazole buffer 10 mM pH 7.5, and total ATPase activity was quantified in the supernatant (1000xg, 10 min) as the appearance of inorganic phosphate (Fiske & Subbarow, 1925), using Tris-ATP 3 mM as substrate, at 20 °C, as described in detail previously (Freire *et al*., 1995; Romão *et al*., 2001). These same samples had their total protein and triacylglycerol content assayed, using commercially available kits (Labtest, Brazil). Proteins were read according to the Biuret method, and triacylglycerols using an enzymatic method: lipoprotein lipase, glycerol kinase, glycerol-3-phosphate oxidase, and peroxidase. Tissue glycogen content was determined as routinely in the literature, according to Keppler & Decker (1984), using approximately 0.07 g of gills or kidneys. Total protein concentration of these tissue samples was measured according to Bradford (1976).

Two-way ANOVA was performed to identify the effect of salinity (5‰ and 35‰) or species (*S. testudineus* and *S. greeleyi*), on all parameters, followed by Tukey post hoc test. Statistical significance was indicate by $p < 0.05$.

## Results

Plasma osmolality and chloride levels in fishes of both species were lower in 5‰ than in 35‰. On the contrary, the hematocrit of both species was higher in 5‰ than in fishes exposed to 35‰. Further, the hematocrit of *S. greeleyi* was higher than that of *S. testudineus* in both salinities. Muscle water content and plasma glucose of *S. testudineus* and *S. greeleyi* were both unaffected by salinity reduction (5‰) (Table 1).

**Table 1.** Plasma osmolality (mOsm.kg$H_2O^{-1}$), chloride (mM), plasma glucose (mg.$dL^{-1}$), hematocrit (%), and muscle water content (%) of *Sphoeroides testudineus* and *S. greeleyi* after short-term exposure to seawater of 5‰ or 35‰ salinity, mean ± standard error. * = salinity effect (5‰ ≠ 35‰), $p<0.05$; & = species effect (*S. testudineus* ≠ *S. greeleyi*), $p<0.05$.

| | Salinity | *S. testudineus* | *S. greeleyi* |
|---|---|---|---|
| Plasma Osmolality | 5‰ | 308.5±2.4 (n=11)* | 304.6±7.1 (n=11)* |
| | 35‰ | 343.0±9.4 (n=11) | 341.7±4.0 (n=11) |
| Plasma Chloride | 5‰ | 144.3±2.5 (n=11)* | 148.9±5.1 (n=11)* |
| | 35‰ | 155.2±2.9 (n=11) | 161.6±2.4 (n=11) |
| Hematocrit | 5‰ | 27.9±1.1 (n=9)* | 30.5±1.2 (n=11)*& |
| | 35‰ | 25.7±1.1 (n=11) | 27.4±1.2 (n=11)& |
| Muscle Water Content | 5‰ | 80.7±2.0 (n=6) | 81.0±0.3 (n=11) |
| | 35‰ | 82.7±0.9 (n=6) | 81.3±0.8 (n=11) |
| Plasma Glucose | 5‰ | 22.9±2.8 (n=11) | 19.0±1.7 (n=6) |
| | 35‰ | 24.8±2.9 (n=13) | 23.7±2.9 (n=5) |

Branchial total ATPase activity (Table 2) in both species did not change upon salinity reduction ($p>0.05$). Renal total ATPase activity (Table 2) in *S. greeleyi* decreased upon exposure to dilute seawater ($p<0.05$) and the activity in seawater

of 35‰ was higher in the kidney of *S. greeleyi* than in that of *S. testudineus* ($p<0.05$). Glycogen content of gills and kidney of both species was not modified after exposure to 5‰ (Table 2). Kidney glycogen content in the *S. greeleyi* was higher when compared to *S. testudineus*, in both salinities ($p<0.05$). *S. greeleyi* presented higher levels (~twofold) of triacylglycerol (Table 2) both in branchial and renal tissue, and in both salinities, than its congener *S. testudineus* ($p<0.05$). Finally, total protein concentration of both tissues and in both species was not affected by salinity reduction (Table 2).

**Table 2.** Total ATPase activity (μmolPi.mgProt/$h^{-1}$), triacylglycerol (mg.Triac/mg.Prot) and glycogen content (μmol glycosyl units/g), and protein concentration (mg/mL) of branchial and renal tissues of *S. testudineus* and *S. greeleyi* after short-term exposure to seawater of 5‰ or 35‰ salinity, mean ± standard error. * = salinity effect (5‰ ≠ 35‰), $p<0.05$; & = species effect (*S. greeleyi* ≠ *S. testudineus*), $p<0.05$.

| | | Gill | | Kidney | |
|---|---|---|---|---|---|
| | Salinity | *S. testudineus* | *S. greeleyi* | *S. testudineus* | *S. greeleyi* |
| Total ATPase activity | 5‰ | 1.2±0.1 (n=8) | 1.7±0.2 (n=8)& | 1.7±0.2 (n=7) | 1.4±0.2 (n=8)* |
| | 35‰ | 1.3±0.04 (n=7) | 1.7±0.1 (n=7)& | 1.4±0.2 (n=9) | 2.1±0.3 (n=8)& |
| Glycogen content | 5‰ | 23.7±0.4 (n=8) | 23.6±1.1 (n=6) | 17.9±0.7 (n=8) | 23.1±1.38 (n=4)& |
| | 35‰ | 24.8±0.8 (n=8) | 23.3±0.5 (n=5) | 19.9±1.0 (n=8) | 21.2±1.0 (n=3)& |
| Triacylglycerol concentration | 5‰ | 0.082±0.004 (n=6) | 0.152±0.018 (n=6)& | 0.065±0.009 (n=6) | 0.130±0.018 (n=6)& |
| | 35‰ | 0.081±0.005 (n=6) | 0.147±0.012 (n=6)& | 0.068±0.006 (n=6) | 0.137±0.027 (n=6)& |
| Protein concentration | 5‰ | 5.7±0.3 (n=8) | 6.1±0.7 (n=6) | 8.3±0.2 (n=7) | 8.1±0.7 (n=3) |
| | 35‰ | 6.4±0.4 (n=8) | 6.1±0.3 (n=5) | 8.7±0.3 (n=7) | 8.3±0.7 (n=4) |

## Discussion

Plasma osmolality and chloride concentration found here in those two species of pufferfishes of the genus *Sphoeroides* were in agreement with previous studies using the same species (respectively ~300 mOsm.kg$H_2O^{-1}$, and ~150 mM) (Prodocimo & Freire, 2001, 2004, 2006). The results also fell within the range reported for other marine/estuarine teleosts (*e.g.*, Maren *et al*., 1992; Claiborne *et al*., 1994; Zadunaisky *et al*., 1995; Zadunaisky, 1996; Jensen *et al*., 1998; Marshall *et al*., 1999). When facing salinity changes, plasma osmolality and chloride concentrations directly reflect the fishes capacity of extracellular fluid homeostasis. Alterations in these concentrations indicate disturbances in this function. Although both species are euryhaline and efficient osmoregulators, as expected for estuarine fishes, a decrease in plasma concentrations was noted upon short-term exposure of the fish to diluted seawater, confirming previous studies (Prodocimo & Freire, 2004, 2006).

If there is extracellular fluid dilution, tissues endure osmotic challenge, and water influx is expected. Hematocrit and muscle tissue water are simple and useful techniques frequently employed to access osmotic challenges to the intracellular compartment (Plaut, 1998; Marshall *et al*., 1999; Claireaux & Audet, 2000; Brown *et al*., 2001; Martinez-Álvarez *et al*., 2002; Prodocimo & Freire, 2006). Hematocrit results obtained for *S. testudineus* and *S. greeleyi* were similar to those reported for other teleosts, of ~25-30% (Plaut, 1998; Claireaux & Audet, 2000; Brown *et al*., 2001; Martinez-Álvarez *et al*., 2002). Salinity reduction (5‰) lead to increased hematocrit in both pufferfishes, a result which may reflect red cell swelling, and the results in the *S. greeleyi* were higher than those of *S. testudineus*. As *S. testudineus* apparently has a higher capacity for tissue water content regulation regulation than *S. greeleyi* (Prodocimo & Freire, 2006), its hematocrit increased, but not as much as in *S. greeleyi*. The opposite salinity challenge can lead to the same response. There are several reports of increased hematocrit upon exposure/acclimation of fishes to salinity increase. In this case, loss of extracellular water to the external medium would be the primary cause, although partially compensated by red cell shrinkage. However, eventually, regulatory volume increase of the red cells, coupled to an increase in red cell counts (long-term change) in order to cope with increasing demand for oxygen delivery following the increased metabolism would at the end lead to increased hematocrit (Plaut, 1998; Claireaux & Audet, 2000; Brown *et al*., 2001; Martinez-Álvarez *et al*., 2002). In fact, in either direction, the response is variable, depending on the time of exposure, magnitude of the salinity challenge imposed, degree of change in plasma osmolality, and volume regulatory capacity of the red blood cells. Despite of the changes in hematocrit seen in the pufferfishes, their muscle cells apparently have a greater or faster capacity for tissue water regulation (as also in Prodocimo & Freire, 2006), as in both species no differences in muscle tissue water content were noted, compatible with a previous report on the red grouper *Epinephelus akaara* and the black sea bream *Mylio macrocephalus* also submitted to dilute seawater (Woo & Wu, 1982). The values of muscle water content (~ 80%) for both pufferfishes were similar to previous values reported for other euryhaline species (Feldmeth & Waggoner, 1972; Plaut, 1998; Kelly & Woo, 1999; Claireaux & Audet, 2000; Brown *et al*., 2001; Martinez-Álvarez *et al*., 2002), as well as for the same species (Prodocimo & Freire, 2006).

No mobilization of the metabolic substrates stored in the gills or kidney or in plasma glucose have been detected in *S. testudineus* and *S. greeleyi* upon this short-term exposure to salinity reduction. These results, coupled to the observation of no change in the total ATPase activity in both organs, means that branchial and renal metabolism remain stable upon this salinity challenge of short duration, a situation somewhat similar to what that these fishes could endure during ebb tide.

The lack of change in branchial total ATPase activity in both species when facing seawater dilution may either mean that this parameter does not adequately reflect the intensity of intermediary metabolism supplying ATP for this organ, or

that the challenge imposed really does not mean a significant change in the energy budget of the gills. Actually it is possible that either to hypo-regulate in full-strength seawater, or to hyper-regulate in dilute seawater both require increased levels of branchial ATP supply ("U-shaped" curves, *e.g.* Stuenkel & Hillyard, 1980; Jensen *et al*., 1998; Kelly *et al*., 1999; Imsland *et al*., 2003; Lin *et al*., 2004; Laiz-Carrión *et al*., 2005). Another possibility is that structural changes such as covering the apical pits of chloride cells turns off salt secretion, as reported for estuarine fish facing reduced salinities (Sakamoto *et al*., 2000; Daborn *et al*., 2001; Marshall, 2003), thus preventing the need to increase ATPase consumption and the activation of energy-consuming processes. In the kidney of *S. greeleyi*, total ATPase activity was reduced in dilute seawater when compared to full-strength seawater. It may well be that divalent ion secretion, one of the main responsibilities of the renal tubule when the fish is hypo-regulating in seawater, is turned off in dilute seawater (Jobling, 1995; Dantzler, 2003). This result is in agreement with that obtained in *Sparus aurata*, in which a direct relationship has been detected between renal $Na^+$,$K^+$-ATPase activity and salinity (Sangiao-Alvarellos *et al*., 2003). It should be pointed out that the marine euryhaline *Dicentrarchus labrax* displayed an opposite response, with increased renal ATPase activity when the fish were exposed to diluted seawater (3‰) and FW for 24 hours, due to the role of the renal tubules in salt absorption contributing to plasma hyper-regulation (Venturini *et al*., 1992). Still a different response may occur, in which the renal $Na^+$,$K^+$-ATPase is unaffected by salinity (McCormick *et al*., 1989). This issue is worth of additional investigation, also clarifying the quantitative role played by the $Na^+$,$K^+$-ATPase with respect to total ATPase activity and tissue metabolism in fish gills and kidneys. Except for the renal activity in low salinity, *S. greeleyi* in general displayed higher total ATPase activities than *S. testudineus*. This finding may indicate that *S. greeleyi*, which prefers higher salinities (Prodocimo & Freire, 2001, 2004, 2006), uses more ATP mainly in its gills either to hypo-regulate in full-strength seawater, or to hyper-regulate in dilute seawater.

Glycogen stored in branchial and renal tissues of the pufferfishes was very stable and unchanged due to exposure to reduced salinity. This may indicate that indeed fuel (glucose) may come from the main glycogen storage organ (the liver), being taken up by the gills, as previously suggested (Soengas *et al*., 1992; Vijayan *et al*., 1996; Laiz-Carrión *et al*., 2002; Sangiao-Alvarellos *et al*., 2003, 2005). Alternatively, the gills of the pufferfishes may provide the glucose they need, since they have glycogen levels ~20-100-fold higher than glycogen contents of the gills of *Sparus auratus* (Sangiao-Alvarellos *et al*., 2003, 2005; Laíz-Carrión *et al*., 2002, 2005), measured using the same methodology (Keppler & Decker, 1974). Indeed, this mobilization of branchial glycogen has been recently demonstrated in the gills of the Mozambique tilapia (Tseng *et al*., 2007).

Plasma glucose was also maintained constant, and unchanged upon short-term exposure to dilute seawater. Interestingly, glycemia in the pufferfishes was very much lower (~20 mg/dL, or ~1mM) than values reported either for freshwater or marine teleosts. This is consistent with the observation that pufferfish gills store lots of glycogen, thus not requiring the mobilization of liver glycogen, and blood transport of glucose. The freshwater rainbow trout displayed values in the range ~36-144 mg/dL (Soengas *et al*., 1992; Figueroa *et al*., 2000), and the tilapia *Oreochromis mossambicus* ~50-120 mg/dL (Nakano *et al*., 1998) or 36-108 mg/dL (Vijayan *et al*., 1996). Other marine teleosts such as the sea bass *Dicentrarchus labrax* (~80 mg/dL, Roche *et al*., 1989), the sea breams *Sparus auratus* (80-90 mg/dL, Mancera *et al*., 1993; 72-90 mg/dL, Sangiao-Alvarellos *et al*., 2005), *Sparus sarba* (~60-75 mg/dL, Kelly & Woo, 1999), and *Chrysophrys major* (~30-45 mg/dL, Woo & Fung, 1981), the angelfish *Pomacanthus imperator* (~10-50 mg/dL, Woo & Chung, 1995), and the red grouper *Epinephelus akaara* (~30-50 mg/dL, Woo & Wu, 1982) had also values well above those measured for the pufferfishes. The marine teleosts *Sparus sarba* and *S. aurata*, submitted to salinities of 6-7‰ for 6-24 hours displayed increased plasma glucose levels (Mancera *et al*., 1993; Kelly & Woo, 1999; Sangiao-Alvarellos *et al*., 2005). It may be that, being estuarine and more euryhaline, reduced salinities for a short period are not as stressful and energy demanding for the pufferfishes as it apparently is for less euryhaline marine teleosts.

Besides higher ATPase activity levels, *S. greeleyi* also displayed higher levels of triacylglycerol in its gills and kidney than *S. testudineus*, confirming a species difference in metabolism. The meaning of this difference cannot be explained by the present results; we cannot ascertain whether the rate of synthesis and storage is higher or the rate of degradation is lower in *S. greeleyi* than in *S. testudineus*. We cannot in fact infer that triacylglycerol stored in the gills and kidney would be a source of energy for the osmoregulation of these pufferfishes, as *S. greeleyi* displayed higher branchial ATPase activity than *S. testudineus* in diluted seawater. Plasma triacylglycerol have been shown to increase during osmoregulatory challenges imposed on *Sparus auratus* submitted to salinities of 6‰ or 55‰ for 14 days, thus suggesting the use of these lipids as energy source for osmoregulation (Sangiao-Alvarellos *et al*., 2005). In accordance, the same species submitted to the isosmotic salinity of 12‰ also for 14 days displayed in no change in plasma triacylglycerol, in agreement with expected metabolism for osmoregulatory function (Sangiao-Alvarellos *et al*., 2003; Laiz-Carrión *et al*., 2005). Lipids as substrates to power ion transport in osmoregulatory organs should be the subject of future investigations.

The third metabolic substrate evaluated, protein, was also unchanged upon the salinity challenge imposed. This is an expected outcome, since the preferred metabolic substrates glycogen and lipids were also unchanged (Black & Love, 1986). Protein concentration was assayed in the plasma and was also reported as stable upon salinity reduction in *Sparus auratus* (Laiz-Carrión *et al*., 2005; Sangiao-Alvarellos *et al*., 2003, 2005).

In conclusion, besides the ecological difference in salinity

preference/tolerance, both species have confirmed previous results of an almost identical short-term osmoregulatory behaviour when facing salinity reduction, exception only for the higher hematocrit in *S. greeleyi*. In addition, they both did not display any clear sign of rapid metabolic substrate mobilization from stores in the osmoregulatory organs (gills and kidney). Euryhaline estuarine fish, when facing fast salinity changes, have been reported to rapidly activate several mechanisms to counteract changing gradients (Jacob & Taylor, 1983; Marshall, 2003). Among these actions, they rapidly activate the $Na^+$,$K^+$-ATPase (Mancera & McCormick, 2000), promote covering of the apical pits of the chloride cells (Sakamoto *et al*., 2000), and end up modulating unidirectional fluxes (Wood & Laurent, 2003; Prodocimo *et al*., 2007). This fast pattern is strikingly in contrast with the slow responses observed in euryhaline migrating fish such as anguilids and salmonids (Madsen *et al*., 1996; Wilson *et al*., 2004), and apparently do not demand fast and significant depletion of metabolic stores in osmoregulatory organs.

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