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The freshwater Amazonian stingray, *Potamotrygon motoro*, up-regulates glutamine synthetase activity and protein abundance, and accumulates glutamine when exposed to brackish (15‰) water

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SUMMARY

This study aimed to examine whether the stenohaline freshwater stingray, *Potamotrygon motoro*, which lacks a functional ornithine–urea cycle, would up-regulate glutamine synthetase (GS) activity and protein abundance, and accumulate glutamine during a progressive transfer from freshwater to brackish (15‰) water with daily feeding. Our results revealed that, similar to other freshwater teleosts, *P. motoro* performed hyperosmotic regulation, with very low urea concentrations in plasma and tissues, in freshwater. In 15‰ water, it was non-ureotelic and non-ureoosmotic, acting mainly as an osmoconformer with its plasma osmolality, $[Na^+]$ and $[Cl^-]$ comparable to those of the external medium. There were significant increases in the content of several free amino acids (FAAs), including glutamate, glutamine and glycine, in muscle and liver, but not in plasma, indicating that FAAs could contribute in part to cell volume regulation. Furthermore, exposure of *P. motoro* to 15‰ water led to up-regulation of GS activity and protein abundance in both liver and muscle. Thus, our results indicate for the first time that, despite the inability to synthesize urea and the lack of functional carbamoyl phosphate synthetase III (CPS III) which uses glutamine as a substrate, *P. motoro* retained the capacity to up-regulate the activity and protein expression of GS in response to salinity stress. *Potamotrygon motoro* was not nitrogen (N) limited when exposed to 15‰ water with feeding, and there were no significant changes in the amination and deamination activities of hepatic glutamate dehydrogenase. In contrast, *P. motoro* became N limited when exposed to 10‰ water with fasting and could not survive well in 15‰ water without food.

Key words: amino acids, freshwater stingray, glutamate, glutamine, glutamine synthetase, glutamate dehydrogenase, nitrogen metabolism, *Potamotrygon motoro,* urea.

INTRODUCTION

Freshwater fishes undergo hyperosmotic regulation and are confronted with the problem of losing ions and gaining water. By contrast, marine teleosts practice hypoosmotic hypoionic regulation and they are confronted with the problem of gaining ions from and losing water to the external medium. Unlike marine teleosts, marine elasmobranchs are osmoconforming hypoionic regulators. Their extracellular fluids have considerably lower ionic concentrations than seawater, but the plasma osmolalities are maintained slightly hyperosmotic to the external medium by the retention of the organic osmolyte urea (Yancey, 2001). Marine elasmobranchs are ureogenic and contain a full complement of ornithine–urea cycle (OUC) enzymes in the liver (Anderson, 1995) and muscle (Steele et al., 2005), but unlike tetrapods, they possess carbamoyl phosphate synthetase III (CPS III) instead of CPS I. CPS III synthesizes carbamoyl phosphate from glutamine while CPS I utilizes NH_4^+ as a substrate (Anderson, 1980). Hence, glutamine synthetase (GS), the enzyme which synthesizes glutamine from glutamate and NH_4^+, is essential to the supply of nitrogen (N) to the OUC in marine elasmobranchs (Anderson, 1991). Marine elasmobranchs are ureosmotic, and urea synthesized through the OUC is predominantly retained in the body, through re-absorption in the gills (Smith and Wright, 1999) and kidneys (Morgan et al., 2003a; Morgan et al., 2003b), reaching 300–600 mmol l^{-1}. Thus, marine elasmobranchs are N limited and would avoid the loss of N after feeding by converting as much excess N as possible to urea (Wood, 2001; Wood et al., 2005). In spite of this, marine elasmobranchs are ureotelic, with urea-N constituting >50% of the total waste N.

In tropical waters several elasmobranch species (e.g. *Himantura signifer*, *Dasyatis sabina*, *Carcharhinus leucas*) migrate to low salinity water. Urea synthesis and retention are still essential to osmoregulation in these euryhaline species in brackish water and/or seawater (Piermarini and Evans, 1998; Tam et al., 2003; Anderson et al., 2005; Ip et al., 2005). For instance, the activities of GS and CPS III are up-regulated in the liver of the euryhaline Asian freshwater stingray, *Himantura signifer*, acclimated to brackish water (20‰) (Tam et al., 2003), and increased urea retention is achieved through alterations in the permeability of the body surfaces to urea during brackish water acclimation (Tam et al., 2003). Furthermore, exposure to brackish water leads to enhanced conservation of N for increased urea synthesis in *H. signifier* after feeding (Chew et al., 2006a).

In South America, the Amazon basin harbours the most diverse assemblage of freshwater elasmobranchs in the world. Some marine-derived freshwater fish species originated as a by-product of massive movements of marine waters into the upper Amazon region during the Early Miocene epoch, i.e. 15–23 million years ago (Lovejoy et al., 1998). Being permanently adapted to freshwater over millions of years, the stenohaline Potamotrygonid stingrays possess low levels of OUC enzymes (Goldstein and Forster, 1971;

Anderson, 1980; Anderson, 1995; Anderson, 2001), and they osmoregulate in a way similar to freshwater teleosts. Hence, unlike marine elasmobranchs, Potamotrygonid stingrays are ammonotelic, excreting the majority of the waste N as ammonia (Goldstein and Forster, 1971; Gerst and Thorson, 1977; Barcellos et al., 1997). They can survive up to 40% seawater in the laboratory (Thorson, 1967; Gerst and Thorson, 1977), but unlike other euryhaline stingrays (Piermarini and Evans, 1998; Tam et al., 2003; Ip et al., 2005), there is no induction of increased urea synthesis and retention (Thorson, 1970) as the expression of CPS III has been suppressed (Gerst and Thorson, 1977). Interestingly, their plasma [Na^+] and [Cl^-] increase with the ambient salinity (Bittner and Lang, 1980; Gerst and Thorson, 1977). However, to date, no information is available on how tissues of Potamotrygonid stingrays respond to increased plasma ionic concentrations. Furthermore, there is no information on how GS would respond to increases in ambient salinity in these stingrays. Since glutamine has many important physiological functions besides acting as a substrate for CPS III, we surmised that the expression of GS in Potamotrygonid stingrays would not be suppressed through evolution.

Therefore, this study was undertaken to test the hypothesis that, like other euryhaline freshwater teleosts [*Anguilla anguilla* (Huggins and Colley, 1971), *Cyprinus carpio* (Hegab and Hanke, 1983), *Salmo gairdneri* (Kaushik and Luquet, 1979), *Oreochromis mossambicus* (Venkatachari, 1974; Assem and Hanke, 1983; Fiess et al., 2007), *Anabas testudineus* (Chang et al., 2007), *Monopterus albus* (Tok et al., 2009)] the ocellated river stingray, *Potamotrygon motoro*, accumulates free amino acids (FAAs), which act as organic osmolytes for cell volume regulation, in its tissues when exposed to 15‰ water. Fish were fed freshwater shrimp daily in this set of experiments in order to test the hypotheses that feeding would not induce ureotely (i.e. excreting >50% of nitrogenous waste as urea-N), and would not enhance the conservation of nitrogen for increased urea synthesis during acclimation to 15‰ water. The second but more important hypothesis tested in this study was that GS activity and protein abundance are up-regulated in the liver and muscle of *P. motoro* exposed to 15‰ water. In relation to this, it was anticipated that glutamine would be one of the major FAAs accumulated in its liver and muscle as glutamine would no longer be channelled to the OUC for urea synthesis. Since glutamine synthesis through GS requires glutamate as a substrate, we also determined the effects of exposure to 15‰ water on the activity and protein abundance of glutamate dehydrogenase (GDH) in *P. motoro*. Finally, efforts were made to test the third hypothesis that *P. motoro* becomes N limited when exposed to 10‰ water in the absence of a food supply as it needs to conserve FAAs for osmoregulatory purposes, which would lead to a decrease in the production and excretion of endogenous ammonia resulting from a reduction in amino acid catabolism or increased synthesis of certain non-essential amino acids.

MATERIALS AND METHODS

Fish

Potamotrygon motoro Müller and Henle 1841 (104–150 g body mass) were purchased from a local fish farm in Singapore. Fish were kept individually in plastic aquaria tanks (43.5 cm length×29.0 cm width×10.0 cm height) containing 3–4 l of freshwater (0.4‰) at 25°C in the laboratory, and water was changed daily. No attempt was made to identify the sex of the fish. Fish were acclimated to laboratory conditions for 7 days before the experiments were begun. During this period, *P. motoro* were fed freshwater shrimps daily. All experiments were performed under a 12 h:12 h dark:light regime.

Exposure of fish progressively from freshwater to 15‰ water with feeding

Control fish were kept in freshwater for 13 days. Experimental fish were exposed to daily increases in salinity from 0.4‰ (days 1–2) to 5‰ (day 3), then to 10‰ (days 4–7), and finally to 15‰ (days 8–13). A progressive increase in salinity was necessary to allow for acclimation and there was no mortality. Fish were fed freshwater shrimps daily at 09:00 h. Unconsumed shrimps were removed 2 h afterwards, and the water was changed. Water samples (3 ml) were collected at 08:00 h daily before feeding. They were acidified with 40 μl of 1 mol l^{-1} HCl and kept at 4°C for analysis of ammonia and urea, which was performed within 1 week.

Fish were killed at the end of day 13 for tissue collection. The caudal peduncle of the experimental specimen was severed, and blood was collected in heparinized capillary tubes. The blood sample was centrifuged at 4000 ***g*** at 4°C for 10 min to obtain the plasma. The plasma was deproteinized with an equal volume (v/v) of ice-cold 6% trichloroacetic acid (TCA) and centrifuged at 10,000 ***g*** at 4°C for 15 min. The resulting supernatant was kept at –80°C until analysed. The liver, intestine and muscle were quickly excised, and the intestine flushed with ice-cold saline solution (0.9% NaCl). The excised tissues and organs were immediately freeze-clamped with tongs pre-cooled in liquid nitrogen. Samples were kept at –80°C until analysed.

Determination of ammonia and urea concentrations in water samples

Concentrations of ammonia and urea were determined according to the methods described by Anderson and Little (Anderson and Little, 1986) and Jow and colleagues (Jow et al., 1999), respectively. Rates of ammonia and urea excretion are expressed as μmol day^{-1} g^{-1} fish.

Analyses of plasma osmolality, [Na^+] and [Cl^-]

Plasma osmolality was analysed using a Wescor 5500 vapour pressure osmometer (Wescor, Logan, UT, USA). [Na^+] and [Cl^-] were determined by a Corning 410 flame photometer and Corning 925 chloride analyzer, respectively (Corning, Halstead, Essex, UK). Plasma osmolality is expressed as mosmol kg^{-1} while [Na^+] and [Cl^-] are expressed as mmol l^{-1} plasma.

Analyses of ammonia, urea and FAAs in tissues

Frozen tissue samples were ground to a powder, weighed, and homogenized three times for 20 s each, with 10 s intervals, at 24,000 r.p.m. in 5 volumes (w/v) of ice-cold 6% TCA using an Ultra-Turrax T25 homogenizer (Ika-Labortechnik, Staufen, Germany). The homogenates were centrifuged at 10,000 ***g*** and 4°C for 15 min to obtain the supernatant for ammonia, urea and FAA analyses.

For ammonia and urea assays, the pH of the supernatant was adjusted to pH 5.5–6.0 with 2 mol l^{-1} $KHCO_3$. Ammonia content was determined according to the method of Bergmeyer and Beutler (Bergmeyer and Beutler, 1985) while urea content was determined as mentioned above.

For FAA analysis, the pH of the supernatant (N=4) obtained above was adjusted to pH 2.2 with 4 mol l^{-1} lithium hydroxide and diluted appropriately with 0.2 mol l^{-1} lithium citrate buffer (pH 2.2). FAAs were analysed using a Shimadzu LC-10ATVP amino acid analysis system with a Shim-pack –07/Amino Li-type column. The total FAA (TFAA) content was calculated by the summation of all FAAs, while the total essential free amino acid (TEFAA) content was calculated as the sum of arginine (as *P. motoro* is non-ureogenic), histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine content. Results are expressed as μmol g^{-1} wet tissue mass or mmol l^{-1} plasma.

Determination of enzyme activities

Samples of muscle and liver were homogenized three times in 5 volumes (w/v) of ice-cold extraction buffer, containing 50 mmol l^{-1} imidazole, 1 mmol l^{-1} EDTA, 1 mmol l^{-1} EGTA, 25 mmol l^{-1} NaF and 0.1 mmol l^{-1} PMSF, at 24,000 r.p.m. for 20 s each with a 10 s off interval using an Ultra-Turrax homogenizer. The homogenates were centrifuged at 10,000 ***g*** at 4°C for 15 min. The supernatants obtained were passed through a 5 ml Bio-Rad P-6DG column (Bio-Rad Laboratories, Hercules, CA, USA) equilibrated with cold elution buffer which contained 50 mmol l^{-1} imidazole. The filtrates were used for enzymatic assays.

GS transferase activity was determined by the method of Tay and colleagues (Tay et al., 2003), and expressed as nmol γ-glutamylhydroxamate formed min^{-1} mg^{-1} protein. Freshly prepared glutamic acid monohydroxamate solution was used as a standard for comparison. The amination and deamination activities of GDH were determined according to Peng and colleagues (Peng et al., 1994). Amination activity was expressed as nmol NADH oxidised min^{-1} mg^{-1} protein while deamination activity was expressed as nmol formazan formed min^{-1} mg^{-1} protein. Alanine aminotransferase (ALT) in the direction of alanine degradation and aspartate aminotransferase (AST) in the direction of aspartate degradation were determined following the method of Peng and colleagues (Peng et al., 1994). Specific activities of ALT and AST are expressed as μmol NADH oxidized min^{-1} g^{-1} wet mass. Protein was determined according to the method of Bradford (Bradford, 1976), with bovine gamma globulin dissolved in 25% glycerol as a standard for comparison.

Western blotting of GS and GDH

Frozen liver and muscle samples were weighed and homogenized in ice-cold RIPA buffer [50 mmol l^{-1} Tris-HC1 (pH 7.4), 150 mmol l^{-1} NaCl, 1% NP-40, 1% sodium deoxycholate and 1 mmol l^{-1} EDTA] containing 1 mmol l^{-1} PMSF, 1 mmol l^{-1} sodium vanadate, 1 mmol l^{-1} NaF and HALT protease inhibitor cocktail (Pierce Biotechnology, Rockford, IL, USA). The homogenates were sonicated for 10 s, and then centrifuged at 10,000 ***g*** and 4°C for 20 min. Portions of the supernatants were diluted to 5 μg protein μl^{-1} in Laemmli buffer (Laemmli, 1970). Protein concentration was determined according to the method of Bradford (Bradford, 1976).

Proteins were separated by polyacrylamide gel electrophoresis in a vertical mini-slab apparatus (Bio-Rad Laboratories) under denaturing conditions following the method described by Laemmli (Laemmli, 1970). The resolved proteins were transferred to Immunblot™ PVDF membrane (Bio-Rad Laboratories) using a semi-dry transfer apparatus (Bio-Rad Laboratories). Blots were then blocked for 1 h in 10% skimmed milk/TTBS (0.05% Tween 20 in Tris-buffered saline: 2 mmol l^{-1} Tris, 50 mmol l^{-1} NaCl; pH 7.6).

The protein abundance of GS was determined using affinity-purified rabbit polyclonal antibodies raised against the KLH-conjugated highly conserved oligopepetide GS sequence (acetylcysteinyl-CPRSVGQEKKG–YFEDRRPS-amide) (Anderson et al., 2002) at a dilution of 1:5000. The antibody was procured from Quality Controlled Biochemicals (Hopkinton, MA, USA) and kept at –20°C before use. The protein abundance of GDH in the liver sample was determined using rabbit polyclonal anti-GDH antibody (US Biological, cat. no. G4000-50; Swampscott, MA, USA) diluted 1:10,000. Since the muscle exhibited only low activity of GDH, no effort was made to determine the GDH protein abundance therein. Actin (pan Ab-5, cat. no. MS-1295-P1) was purchased from Thermo Fisher Scientific (Fremont, CA, USA) and used as the housekeeping control with a dilution of 1:5000. Blots were incubated with primary antiserum overnight at 4°C. Subsequently, after a series of washes with TTBS, blots were incubated with either goat anti-rabbit or anti-mouse horseradish peroxidase-conjugated antibody (Santa Cruz Biotechonology, Santa Cruz, CA, USA). Bands were visualized by chemiluminescence (Western Lightning™, Chemiluminescence Reagent Plus, PerkinElmer Life Sciences, MA, USA) using Konika-Minolta films which were processed by a Kodak X-OMAT 3000 RA processor (Kodak, Tokyo, Japan). Band intensities were quantified by NIH ImageJ software (version 1.40) and calibration was performed using a step tablet (Stouffer no. R3705-1C Calibrated 37 Step Reflection Scanner Scale, Mishawaka, IN, USA). Relative band intensities were calculated by dividing the band OD values obtained from the program by the respective actin loading control band intensities.

Determination of rates of ammonia and urea excretion and tissue ammonia and urea content in fish acclimated to 10‰ water with fasting

This set of experiments involved two groups of fish that were not fed. Preliminary experiments indicated that the survival rate was low for unfed *P. motoro* exposed to a progressive increase in salinity up to 15‰ water. Therefore, the experimental group was kept in freshwater on day 1 and exposed to 5‰ and 10‰ water on day 2 and day 3, respectively, followed by exposure to 10‰ water for another 6 days. The control group was kept in freshwater (0.4‰) for a total of 9 days. Daily excretion rates of ammonia and urea were determined, as described above, to evaluate whether *P. motoro* would reduce nitrogenous waste during acclimation to brackish water in the absence of food. On day 9, fish were killed and muscle and liver tissues sampled for the determination of ammonia and urea content as stated above.

Statistical analyses

Results are presented as means ± s.e.m. Student's *t*-test or analysis of variance (ANOVA) followed by multiple comparisons of means by Bonferroni's procedure was used to evaluate differences between means in groups where appropriate. Differences where $P<0.05$ were regarded as statistically significant.

RESULTS

Plasma osmolality, $[Na^+]$ and $[Cl^-]$ in fish acclimated to 15‰ water with feeding

The plasma osmolality of *P. motoro* exposed to 15‰ water for 6 days after a progressive increase in salinity increased significantly by 25% compared with that of the freshwater control (Table 1). In addition, the plasma $[Na^+]$ and $[Cl^-]$ increased by 36% and 28%, respectively, in the former compared with the latter (Table 1).

Ammonia, urea and FAA content in tissues of fish acclimated to 15‰ water with feeding

There were no significant changes in ammonia content in muscle, liver, intestine and plasma of *P. motoro* exposed to 15‰ water for 6 days after a progressive increase in salinity compared with control fish kept in freshwater (Table 2). In contrast, urea content increased significantly in muscle (5-fold), liver (1.7-fold) and intestine (6-fold) but remained unchanged in plasma of fish exposed to 15‰ water (Table 2).

The content of various FAAs increased significantly in muscle and liver of fish exposed to 15‰ water for 6 days, and led to significant increases in TEFAA (2.3-fold) and TFAA (2.5-fold) content in the muscle and TFAA (1.7-fold) content in the liver (Table 3). The major contributors to the increase in TFAA in the

Table 1. Osmolality and concentration of Na^+ and Cl^- in the plasma of *Potamotrygon motoro* exposed to freshwater (0.4‰; control) for 13 days or to a progressive increase in salinity from freshwater to 15‰ water over a 7 day period followed by exposure to 15‰ water for another 6 days, with feeding

	Freshwater (control)	15‰ water
Osmolality (mosmol kg^{-1})	339±4	424±2*
$[Na^+]$ (mmol l^{-1})	174±2	236±3*
$[Cl^-]$ (mmol l^{-1})	159±5	203±4*

Results represent means ± s.e.m. (N=4 for 0.4‰ and N=3 for 15‰).
*Significantly different from the control value (P<0.05).

liver were glutamate (29.4%) and glutamine (29.7%). In comparison, increases in the glutamine and glycine content contributed 7.7% and 40.8%, respectively, to the increase in TFAA in the muscle. In contrast, the concentrations of FAAs, and hence TEFAA and TFAA, in the plasma of fish kept in freshwater were relatively low, and they remained relatively unchanged after fish were exposed to 15‰ water for 6 days (Table 3).

GS, GDH, ALT and AST activity from the liver and muscle of fish acclimated to 15‰ water with feeding

The GS activity from the liver and muscle of fish exposed to 15‰ water for 6 days was significantly greater (by 40- and 4-fold, respectively) than that of the freshwater control (Table 4). However, exposure to 15‰ water for 6 days after a progressive increase in salinity had no significant effect on the amination and deamination activity of GDH, or on ALT and AST activity, from the liver and muscle of *P. motoro* (Table 4).

GS and GDH protein abundance in liver and muscle of fish acclimated to 15‰ water with feeding

Exposure to 15‰ water for 6 days after a progressive increase in salinity led to a significant increase in GS protein abundance in the liver of *P. motoro* without any effect on the GDH hepatic protein abundance (Fig. 1). Similarly, GS protein abundance in the muscle of fish exposed to 15‰ water for 6 days was significantly greater than that in control fish kept in freshwater (Fig. 2).

Rates of ammonia and urea excretion in fish acclimated to 15‰ water with feeding

With feeding, the rates of ammonia (Fig. 3A) and urea (Fig. 3B) excretion were comparable between control fish and fish exposed to 15‰ water for 6 days after a progressive increase in salinity. Between day 8 and day 13, the ammonia excreted by the control fish in freshwater and by fish exposed to 15‰ water amounted to 56±7 μmol g^{-1} fish and 68±5 μmol g^{-1} fish, respectively (Fig. 3A). During this 13 day period, both control and experimental fish remained ammonotelic, excreting less than 10% of the total nitrogenous waste (ammonia-N + urea-N) as urea-N. Specifically, urea constituted 8.9% of N waste in fish exposed to 15‰ water between day 8 and day 13.

Rates of ammonia and urea excretion and tissue ammonia and urea content in fish acclimated 10‰ water with fasting

In contrast, with fasting, rates of ammonia (Fig. 4A) and urea (Fig. 4B) excretion in fish exposed to 10‰ water (between day 3 and day 7) were significantly lower than those of the freshwater control. Between day 3 and day 9, the total amount of ammonia excreted by the control fish in freshwater (55±5 μmol g^{-1} fish) was significantly greater than that excreted by fish exposed to 10‰ water (30±5 μmol g^{-1} fish). A similar phenomenon was observed for urea excretion between day 3 and day 9, with the value for the control fish (2.4±0.3 μmol g^{-1} fish) being significantly greater than that for the experimental fish (1.1±0.3 μmol g^{-1} fish).

With fasting, the ammonia content in fish exposed to 10‰ water for 7 days after a progressive increase in salinity decreased by 81% and 43% in muscle and intestine, respectively, compared with the freshwater control (Table 5). However, glutamine content in the muscle and liver of fish exposed to 10‰ water was significantly greater than that of the control fish (Table 5). On the other hand, exposure to 10‰ water had no significant effects on the urea and glutamate content in muscle, liver and intestine of fish undergoing fasting (Table 5).

DISCUSSION

Potamotrygon motoro remained non-ureoosmotic in 15‰ water with feeding

Potamotrygon motoro is non-ureogenic as it does not possess a functional OUC in the liver (Anderson, 1995; Anderson, 2001). Therefore, in contrast to the ureogenic African lungfish *Protopterus dolloi* [dipnoan (Lim et al., 2004)], the Asian freshwater stingray *H. signifier* [elasmobranch (Chew et al., 2006a)] and the marginally ureogenic giant mudskipper *Periophthalmodon schlosseri* (Ip et al., 2004a), daily feeding did not lead to ureotely in *P. motoro* during 13 days of exposure to freshwater or to salinity stress. Even with a continuous supply of dietary protein, the urea content, despite significant increases, remained low in the muscle and liver. The slight but significant increases in tissue urea content might have originated from argininolysis and purine catabolism (Ip et al., 2001b; Chew et al., 2006b), since food would provide a source of arginine and osmotic stress would result in a greater energy demand with a greater turnover of purine nucleotides. Overall, it can be concluded that *P. motoro* is non-ureosmotic, and therefore, unlike other marine

Table 2. Ammonia and urea content in the muscle, liver, intestine and plasma of *P. motoro* exposed to freshwater (0.4‰; control) for 13 days or to a progressive increase in salinity from freshwater to 15‰ water over a 7 day period followed by exposure to 15‰ water for another 6 days, with feeding

	Ammonia		Urea	
	Freshwater (control)	15‰ water	Freshwater (control)	15‰ water
Muscle (μmol g^{-1} wet mass)	1.1±0.4	1.1±0.1	0.63±0.02	3.2±0.9*
Liver (μmol g^{-1} wet mass)	1.3±0.5	2.6±0.6	0.83±0.11	1.4±0.1*
Intestine (μmol g^{-1} wet mass)	3.3±0.6	3.1±0.1	0.43±0.11	2.6±0.4*
Plasma (mmol l^{-1})	0.15±0.03	0.12±0.02	0.43±0.06	1.5±0.1*

Results represents means ± s.e.m. (N=5 for control and N=4 for fish in 15‰ water).
*Significantly different from the control value (P<0.05).

Table 3. Free amino acid (FAA), total FAA (TFAA) and total essential free amino acid (TEFAA) content in the muscle, liver and plasma of *P. motoro* exposed to freshwater (0.4‰; control) for 13 days or to a progressive increase in salinity from freshwater to 15‰ water over a 7 day period followed by exposure to 15‰ water for another 6 days, with feeding

	Muscle (μmol g^{-1} wet mass)		Liver (μmol g^{-1} wet mass)		Plasma (mmol l^{-1})	
	Freshwater (control)	15‰ water	Freshwater (control)	15‰ water	Freshwater (control)	15‰ water
FAAs						
Taurine	17±2	16±1	17±2	19±3	0.75±0.08	0.52±0.06
Aspartate	0.096±0.044	0.47±0.12*	0.37±0.14	0.66±0.31	0.0029±0.0003	0.0042±0.0006
Threonine	0.24±0.13	0.56±0.12	0.12±0.05	0.14±0.05	0.053±0.005	0.073±0.009
Serine	0.34±0.11	0.82±0.23	0.65±0.54	0.19±0.07	0.066±0.013	0.057±0.006
Asparagine	0.14±0.08	0.80±0.11*	0.038±0.013	1.0±0.1*	0.019±0.003	0.032±0.004
Glutamate	0.13±0.05	0.43±0.04*	1.0±0.3	6.0±0.4*	0.0062±0.0003	0.0095±0.0009
Glutamine	0.12±0.04	2.9±0.3*	0.14±0.04	5.2±1.5*	0.092±0.014	0.12±0.02
Proline	0.43±0.26	1.9±0.4*	0.12±0.03	0.44±0.17	0.056±0.012	0.032±0.002
Glycine	2.3±1.5	17±2.4*	0.69±0.24	0.89±0.20	0.15±0.03	0.15±0.02
Alanine	0.58±0.19	5.2±2.3	0.15±0.10	0.94±0.28	0.11±0.01	0.13±0.01
Valine	0.12±0.06	0.45±0.05*	0.16±0.06	0.33±0.08	0.13±0.02	0.15±0.01
Isoleucine	n.d.	0.10±0.02*	0.077±0.012	0.069±0.015	0.057±0.008	0.084±0.007
Leucine	0.094±0.025	0.27±0.04*	0.14±0.06	0.12±0.04	0.12±0.02	0.17±0.02
Tyrosine	0.13±0.03	0.22±0.01	0.15±0.10	0.10±0.04	0.017±0.001	0.023±0.003
Phenylalanine	0.028±0.018	0.14±0.01*	0.044±0.004	0.032±0.011	0.018±0.001	0.022±0.002
β-Alanine	0.75±0.46	10±1*	0.030±0.030	0.23±0.03*	0.020±0.011	0.013±0.002
Tryptophan	0.12±0.12	0.57±0.11	0.15±0.15	0.17±0.17	0.058±0.010	0.056±0.006
Histidine	0.11±0.05	0.18±0.04	0.037±0.003	0.042±0.010	0.015±0.001	0.017±0.001
Lysine	0.81±0.25	1.2±0.1	2.2±0.5	4.2±1.3	0.082±0.013	0.067±0.011
Arginine	0.030±0.002	0.032±0.001	0.031±0.012	0.031±0.006	0.15±0.03	0.21±0.05
TEFAA	1.5±0.4	3.5±0.3*	3.0±0.4	5.2±1.6	0.55±0.11	0.62±0.17
TFAA	24±4	60±6*	23±2	40±5*	2.03±0.11	2.15±0.22

Results represent means ± s.e.m. (*N*=4).
*Significantly different from the control value (*P*<0.05). n.d., not detectable.

(Wood, 2001; Wood et al., 2005) and euryhaline freshwater (Chew et al., 2006a) elasmobranchs, it was not N limited and did not increase the retention of N during acclimation to 15‰ water with feeding.

Potamotrygon motoro behaved as an osmoconformer and accumulated FAAs during acclimation to 15‰ water with feeding

Marine stingrays excrete excess ions through the rectal gland (Conte, 1969; Haywood, 1975), but the rectal gland of *P. motoro* is non-functional (Goldstein and Forster, 1971; Gerst and Thorson, 1977; Thorson et al., 1978; Wood et al., 2002). Since 15‰ water contained 250 mmol l^{-1} Na^+ and 220 mmol l^{-1} Cl^-, and since the respective concentrations of these two ions in the plasma of *P. motoro* were 235 mmol l^{-1} and 210 mmol l^{-1}, it can be concluded that *P. motoro* behaved largely as an osmoconformer in 15‰ water. With significant increases in plasma osmolality in *P. motoro* acclimated to 15‰ water, it is logical to deduce that cell volume regulation must be part of the whole osmoregulatory process. Indeed, there were significant increases in the content of various FAAs and TFAA in muscle and liver of *P. motoro* exposed to 15‰ water. As concentrations of all FAAs and TFAA in the plasma, and hence also in the interstitial fluid, remained low and unchanged in fish acclimated to 15‰ water, it can be concluded that tissue FAAs were mainly retained within cells and acted as intracellular organic osmolytes. Increases in TFAA content might result from a

Table 4. Glutamate dehydrogenase (GDH) activity in the amination and deamination reactions and the ratio of amination to deamination, and asparate aminotransferase (AST) and alanine aminotransferase (ALT) activity from the muscle and liver of *P. motoro* exposed to freshwater (0.4‰; control) for 13 days or to a progressive increase in salinity from freshwater to 15‰ water over a 7 day period followed by exposure to 15‰ water for another 6 days, with feeding

Tissue	Enzyme	Freshwater (control) (μmol min^{-1} g^{-1} tissue)	15‰ water (μmol min^{-1} g^{-1} tissue)
Muscle	GS	0.26±0.21	1.0±0.2*
	GDH (amination)	0.70±0.09	0.96±0.09
	GDH (deamination)	n.d.	n.d.
	AST	6.3±0.9	7.0±0.8
	ALT	0.77±0.11	1.1±0.2
Liver	GS	0.134±0.017	5.4±1.3*
	GDH (amination)	70±6	58±11
	GDH (deamination)	3.24±0.28	2.51±0.44
	GDH (amination/deamination)	22±0.6	23±1
	AST	28±1	24±4
	ALT	10±0.8	8.7±1.5

Results represents means ± s.e.m. (*N*=5 for control and *N*=4 for fish in 15‰ water).
*Significantly different from the control value (*P*<0.05). n.d., not detectable.

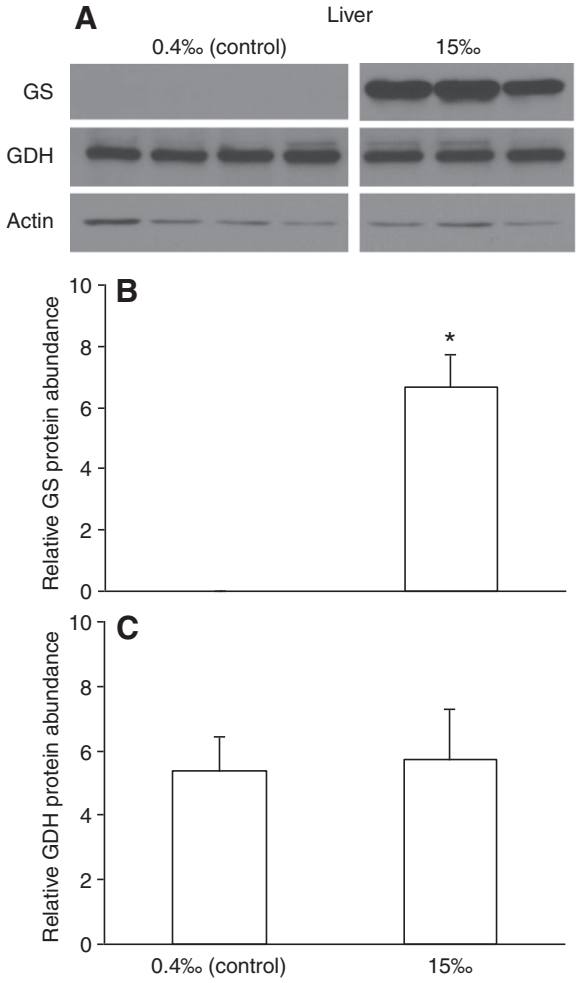

Fig. 1. (A) Western blot analysis of glutamine synthetase (GS) and glutamate dehydrogenase (GDH) from the liver of *Potamotrygon motoro* exposed to freshwater (0.4‰; control; *N*=4) for 13 days or to a progressive increase in salinity from freshwater to 15‰ water (*N*=3) over a 7 day period followed by exposure to 15‰ water for another 6 days, with feeding. (B) Relative GS protein abundance and (C) relative GDH protein abundance normalized against the respective actin loading controls. Results represent means + s.e.m. *Significantly different from the corresponding control value, *P*<0.05.

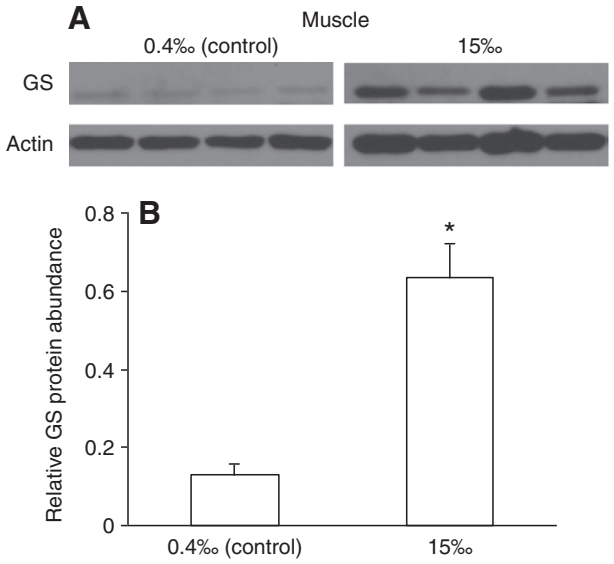

Fig. 2. (A) Western blot analysis of glutamine synthetase (GS) from the muscle of *P. motoro* exposed to freshwater (0.4‰; control; *N*=4) for 13 days or to a progressive increase in salinity from freshwater to 15‰ water (*N*=4) over a 7 day period followed by exposure to 15‰ water for another 6 days, with feeding. (B) Relative GS protein abundance normalized against the actin loading control. Results represent means + s.e.m. *Significantly different from the corresponding control value, *P*<0.05.

suppression of amino acid catabolism in general, an increase in the synthesis of certain non-essential amino acids, and/or a reduction in the utilization of FAAs for protein synthesis. As fish were fed during the experimental period and exposure to salinity stress had no major effects on rates of ammonia and urea excretion, it is unlikely that suppression of amino acid catabolism, which could lead to decreases in waste N excretion, would have occurred. Hence, increases in TFAA content in fish exposed to salinity stress could have resulted from a reduction in the utilization of dietary FAAs for protein synthesis and a simultaneous increase in the synthesis of certain non-essential amino acids, like glutamine. As the magnitude of the increase in plasma osmolality (~85 mosmol kg^{-1}) was greater than the magnitude of the increase in TFAA content in muscle and liver, either there was an ionic component to cell volume regulation, as recently reported for *M. albus* (Tok et al., 2009), or some other non-FAA intracellular osmolytes were involved.

Glutamine and glutamate acted as organic osmolytes in *P. motoro* acclimated to15‰ water

The most prominent FAAs involved in cell volume regulation in *P. motoro* exposed to 15‰ water were glycine, glutamate and glutamine. Out of these three amino acids, glutamine has rarely been reported to act as an important osmolyte in fishes (Yancey et al., 1982; Yancey, 1994; Yancey, 2001), although it is known to be a compatible osmolyte in bacteria (Lai et al., 1991; Frings et al., 1993) and it accumulates in vertebrate brains as a defence against ammonia toxicity (Cooper and Plum, 1987). In mammalian brains intoxicated with ammonia, increased glutamine synthesis and accumulation would result in the swelling of astrocytes, which is one of the leading explanations of hepatic encephalopathy (Brusilow, 2002). As for fish, exposure of the euryhaline brackish water *B. sinensis* (Peh et al., 2009) and the euryhaline freshwater *O. marmorata* (Chew et al., 2009) to seawater led to either no significant changes or very minor increases in glutamine content in various tissues. However, Tok and colleagues (Tok et al., 2009) reported recently that glutamine accumulated to high levels (12 and 37 μmol g^{-1} in muscle and liver, respectively) in *M. albus* exposed to 25‰ water. In the present study, we also demonstrated significant increases in glutamine content in liver and muscle of *P. motoro* exposed to 15‰ water, although the level of glutamine accumulation was lower than that of *M. albus*. The advantages of accumulating glutamine include its capacity to act as a precursor for the synthesis of physiologically important molecules and as an oxidative substrate for red muscle mitochondria (Chamberlin and Ballantyne, 1992) upon returning to freshwater.

GS activity and protein abundance were up-regulated during acclimation to 15‰ water with feeding

Working on the marsh clam, *Polymesoda expansa*, Hiong and colleagues (Hiong et al., 2004) proposed that increased glutamine synthesis functioned primarily for ammonia detoxification rather

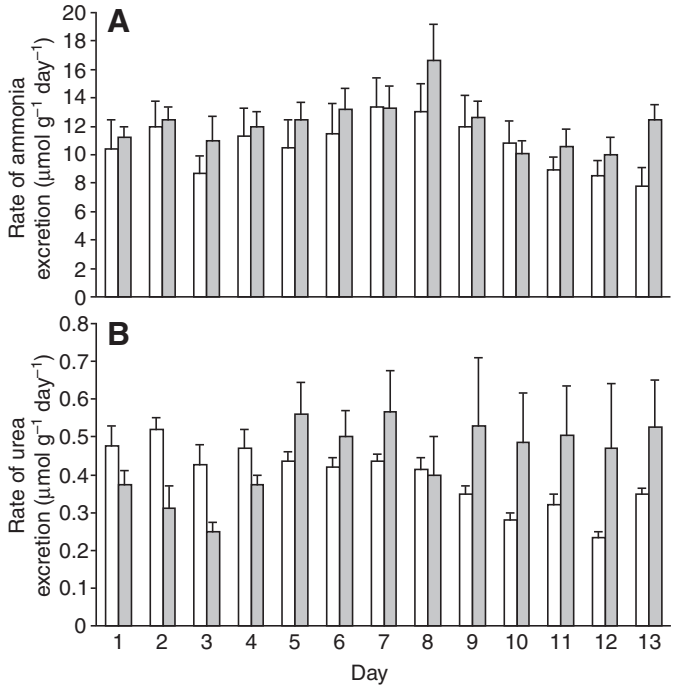

Fig. 3. (A) Ammonia and (B) urea excretion rates in *P. motoro* exposed to freshwater (0.4‰; control) for 13 days or to a progressive increase in salinity from freshwater to 15‰ water over a 7 day period followed by exposure to 15‰ water for another 6 days, with feeding. Open bars represent control fish and filled bars represent experimental fish exposed to salinity changes. Results represent means + s.e.m. (*N*=5 for control group; *N*=6 for experimental group).

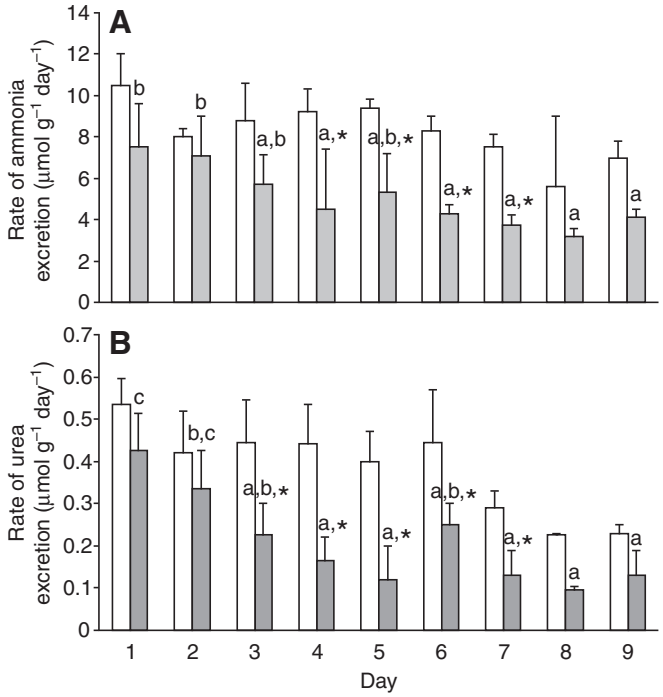

Fig. 4. (A) Ammonia and (B) urea excretion rates in *P. motoro* exposed to freshwater (0.4‰; control) for 9 days or to a progressive increase in salinity from freshwater to 10‰ water over a 3 day period followed by exposure to 10‰ water for another 6 days, without feeding. Open bars represent control fish and filled bars represent experimental fish exposed to salinity changes. Results represent means + s.e.m. (*N*=3). *Significantly different from 0.4‰ water condition ($P<0.05$). Means not sharing the same letter are significantly different from each other ($P<0.05$).

than osmoregulation in invertebrates. Ammonia is also detoxified to glutamine through GS in vertebrate brains (Cooper and Plum, 1987), although increases in glutamine synthesis and GS activity in non-brain tissues are uncommon among vertebrates, except for several air-breathing fishes, which include *M. albus* (Tay et al., 2003; Ip et al., 2004b; Chew et al., 2005), *Bostrychus sinensis* (Ip et al., 2001a; Anderson et al., 2002), *Oxyeleotris marmorata* (Jow et al., 1999) and *Misgurnus anguillicaudatus* (Chew et al., 2001), exposed to terrestrial conditions or environmental ammonia. In contrast, Tok and colleagues (Tok et al., 2009) reported that acclimation to 25‰ water led to significant increases in GS activity and protein abundance in the liver of *M. albus*. In this study, we also demonstrated that GS activity and protein expression were up-regulated in muscle and liver of *P. motoro* exposed to 15‰ water. At present, it is unclear whether GS regulation in *P. motoro* involved post-translational covalent modification through GS tyrosine nitration (Schliess et al., 2002; Gorg et al., 2003; Gorg et al., 2005; Gorg et al., 2006) or phosphorylation (Kuramitsu et al., 2006). Glutamate, the precursor required for the synthesis of glutamine, may be synthesized from α-ketoglutarate and NH_4^+ through the aminating activity of GDH. Since no significant increase in GDH protein abundance was detected in the liver of fish exposed to 15‰ water through western blotting, it is probable that the increased rate of glutamate formation is accommodated by the normal capacity of GDH in the control fish.

Since *P. motoro* is akin to marine elasmobranchs, results obtained in this study may have implications that differ from those reported by Tok and colleagues (Tok et al., 2009) in the teleost, *M. albus*. In euryhaline freshwater or marine elasmobranchs, the OUC is the dominant pathway of urea production and GS is the key initial nitrogen-tapping enzyme for urea production *via* CPS III-based OUC. For the euryhaline freshwater *H. signifier* exposed to brackish water (25‰), there were significant increases in the urea synthesis rate and GS and CPS III activity (Tam et al., 2003). However, in

Table 5. Ammonia, urea, glutamine and glutamate content in the muscle, liver and intestine of *P. motoro* exposed to freshwater (0.4‰; control) for 9 days or to a progressive increase in salinity from freshwater to 10‰ water over a 3 day period followed by exposure to 10‰ water for another 7 days, without feeding

	Ammonia (μmol g^{-1} wet mass)		Urea (μmol g^{-1} wet mass)		Glutamine (μmol g^{-1} wet mass)		Glutamate (μmol g^{-1} wet mass)	
Tissue	Freshwater (control)	10‰ water	Freshwater (control)	10‰ water	Freshwater (control)	10‰ water	Freshwater (control)	10‰ water
Muscle	3.3±0.6	0.63±0.07*	0.79±0.12	1.2±0.2	0.34±0.03	0.80±0.07*	0.37±0.08	0.30±0.10
Liver	3.4±1.2 (3)	0.66±0.13	0.56±0.08	1.5±0.5	0.083±0.018	0.66±0.18*	0.55±0.11	2.3±0.8
Intestine	2.8±0.3	1.6±0.2*	0.69±0.05	1.5±0.4	0.17±0.01	0.26±0.05	0.65±0.40	0.63±0.08

Results represents means ± s.e.m. (*N*=4).
*Significantly different from the control value ($P<0.05$).

spite of an increase in GS activity, there were no significant increases in tissue glutamine content in *H. signifier* exposed to brackish water, indicating that the excess glutamine formed was channelled completely into urea synthesis (Tam et al., 2003). In contrast, *P. motoro* is incapable of synthesizing urea as a result of the suppression of CPS III expression (Gerst & Thorson, 1977), and therefore our results have two important implications: (1) *P. motoro* retains the ability to up-regulate GS activity and protein abundance in response to salinity stress, which represents an evolutionary link to its marine relatives, and (2) the excess glutamine produced through increased GS activity was accumulated in tissues as an organic osmolyte during exposure to 15‰ water.

Potamotrygon motoro became N limited when exposed to 10‰ water with fasting

Unlike marine elasmobranchs (Wood, 2001; Wood et al., 2005) and *H. signifier* (Chew et al., 2006a), the non-ureosmotic *P. motoro* was not N limited during acclimation to 15‰ water with feeding. However, given our results indicated that it was essential for *P. motoro* to retain FAAs for cell volume regulation during brackish water acclimation, the logical question to ask was whether it would become N limited and have to suppress amino acid catabolism and convert N to non-essential amino acids during exposure to brackish water (10‰) without food supply. Our results indicate that a reduction in ammonia production did indeed occur in fish acclimated to 10‰ water with fasting, as evidenced by decreases in the excretion of ammonia and urea, and in the ammonia content in muscle and intestine. During fasting, ammonia was produced mainly through amino acid catabolism, and it is therefore logical to deduce that a reduction in amino acid catabolism had occurred. In addition, ammonia was apparently channelled to the formation of glutamine, the content of which increased significantly in the liver and muscle of fish acclimated to 10‰ water. Taken together, these results confirm that *P. motoro* became N limited when confronted with salinity stress during fasting, and this could have contributed partially to the inability of fasting fish to survive well in 15‰ water.

CONCLUSION

In freshwater, *P. motoro* undergoes hyperosmotic regulation. When *P. motoro* was exposed to 15‰ water with feeding, the plasma osmolality, $[Na^+]$ and $[Cl^-]$ increased significantly to levels close to those of the external medium. *Potamotrygon motoro* was non-ureosmotic, behaved largely as an osmoconformer, and accumulated some FAAs in tissues, which would contribute in part to cell volume regulation, in 15‰ water. Exposure of *P. motoro* to 15‰ water led to significant increases in glutamine content and in GS activity and protein abundance in muscle and liver. Overall, our results indicate that, despite loss of the ability to synthesize urea through CPS III, which uses glutamine as a substrate, *P. motoro* retains the capacity to up-regulate both the activity and the protein abundance of GS in response to salinity stress.

LIST OF ABBREVIATIONS

ALT	alanine aminotransferase
AST	aspartate aminotransferase
CPS III	carbamoyl phosphate synthetase III
FAAs	free amino acids
GDH	glutamate dehydrogenase
GS	glutamine synthetase
OUC	ornithine–urea cycle
TEFAA	total essential free amino acid
TFAA	total free amino acid

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