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Glutamine accumulation and up-regulation of glutamine synthetase activity in the swamp eel, *Monopterus albus* (Zuiew), exposed to brackish water

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SUMMARY

The swamp eel, *Monopterus albus*, is an air-breathing teleost which typically lives in freshwater but can also be found in estuaries, where it has to deal with ambient salinity fluctuations. Unlike other teleosts, its gills are highly degenerate. Hence, it may have uncommon osmoregulatory adaptations, but no information is available on its osmoregulatory capacity and mechanisms at present. In this study *M. albus* was exposed to a 5 day progressive increase in salinity from freshwater (1‰) to brackish water (25‰) and subsequently kept in 25‰ water for a total of 4 days. The results indicate that *M. albus* switched from hyperosmotic hyperionic regulation in freshwater to a combination of osmoconforming and hypoosmotic hypoionic regulation in 25‰ water. Exposure to 25‰ water resulted in relatively large increases in plasma osmolality, [Na^+] and [Cl^-]. Consequently, fish exposed to 25‰ water had to undergo cell volume regulation through accumulation of organic osmolytes and inorganic ions. Increases in tissue free amino acid content were apparently the result of increased protein degradation, decreased amino acid catabolism, and increased synthesis of certain non-essential amino acids. Here we report for the first time that glutamine is the major organic osmolyte in *M. albus*. Glutamine content increased to a phenomenal level of >12 μmol g^{-1} and >30 μmol g^{-1} in the muscle and liver, respectively, of fish exposed to 25‰ water. There were significant increases in glutamine synthetase (GS) activity in muscle and liver of these fish. In addition, exposure to 25‰ water for 4 days led to significant increases in GS protein abundance in both muscle and liver, indicating that increases in the expression of GS mRNA could have occurred.

Key words: ammonia, glutamine, glutamine synthetase, *Monopterus albus*, nitrogen metabolism, osmoregulation, swamp eel.

INTRODUCTION

Gills are important osmoregulatory organs in freshwater and marine teleosts. Freshwater fishes are typically exposed to a hypoosmotic medium, and they do not drink in order to prevent dilution of their extracellular fluid. They also actively absorb salt from the environment through their gills to counter the passive loss of ions to the environment (Randall et al., 2002). By contrast, marine fishes are exposed to a hyperosmotic medium and are therefore confronted with the problem of dehydration. Thus, they drink to replenish the water in their body, resulting in a huge influx of salt. The excess salt load must be excreted back into the external medium, which occurs mainly through the gills (Evans et al., 2005). Euryhaline fishes can survive large fluctuations in ambient salinity, and the consequential osmotic stresses are mainly overcome by osmoregulatory acclimation involving morphological and biochemical changes in their gills (McCormick and Saunders, 1987; Evans et al., 2005). Most of the information available on euryhaline fishes has been collected from water-breathing species (for reviews, see Evans et al., 2005; Marshall and Grosell, 2006).

Some teleosts living in estuaries or in the intertidal zone have special adaptations for air breathing. Recently, Chang and colleagues (Chang et al., 2007) studied salinity adaptation in the air-breathing freshwater climbing perch, *Anabas testudineus*, which possesses accessory breathing organs that facilitate the utilization of atmospheric oxygen (Graham, 1997). Its gills have relatively short primary lamellae of small diameter, and their secondary lamellae are small with a large diffusion distance (Graham, 1997). The objective of the study (Chang et al., 2007) was to elucidate whether the gills of *A. testudineus*, which are not well adapted for water breathing, would undergo acclimation and modification to play a major role in osmoregulation in seawater. Chang and colleagues discovered that *A. testudineus* could acclimate through a progressive increase in salinity to seawater. It was able to use free amino acids (FAAs) as major osmolytes to counteract minor perturbations of plasma osmolality, up-regulate gill Na^+/K^+-ATPase activity to facilitate effective ionoregulation and depend more on water breathing during seawater acclimation (Chang et al., 2007). At present, there is no information on how euryhaline fishes without functional gills would osmoregulate when exposed to salinity stress, and hence this study focused on the swamp eel, *Monopterus albus* (Zuiew 1783), which is an obligatory air breather with highly degenerate gills that are non-functional for water breathing (Graham, 1997).

M. albus is a tropical teleost that belongs to Family Synbranchidae and Order Synbranchiformes. Its unusually degenerate gills are reduced to a fold of skin within the opercular chamber (Graham, 1997). During either immersion or emersion, it respires both by holding air in the buccopharyngeal cavity and through the skin. Its natural habitats include swamps, rice fields, muddy ponds and canals (Rainboth, 1996), where it may occasionally encounter hyperosmotic stress either during high tides or when the external medium dries up during drought. At present, no information is available on how *M. albus* osmoregulates in a hyperosmotic environment. Its renowned ability to survive long periods of emersion has drawn

much attention to phenomena related to air breathing (for a review, see Graham, 1997) and ammonia tolerance (Tay et al., 2003; Ip et al., 2004c; Chew et al., 2005), resulting in a great need for knowledge on its osmoregulatory capacity and related mechanisms.

Therefore, this study was undertaken to elucidate the osmoregulatory adaptations in *M. albus* exposed to a progressive increase in ambient salinity from freshwater (1‰) to brackish water (25‰) followed by exposure to 25‰ water for 3 more days. Experiments were performed to examine the plasma osmolality, $[Na^+]$ and $[Cl^-]$, and tissue osmolyte concentrations. The hypotheses tested were that (1) unlike other teleosts, *M. albus* is unable to effectively regulate the ionic, and hence osmotic, concentrations in the plasma due to the lack of functional gills and (2) organic osmolytes such as FAAs and perhaps inorganic osmolytes are retained in tissues for cell volume regulation.

Increased glutamine synthesis is a defence mechanism commonly found in the brains of vertebrates (Cooper and Plum, 1987), including fish (Ip et al., 2001a; Ip et al., 2004a; Ip et al., 2004b; Chew et al., 2006), which contain high levels of glutamine synthetase (GS). Several tropical air-breathing fishes, however, are exceptional because of their ability to up-regulate GS activity in extra-cranial tissues to detoxify ammonia during emersion and/or ammonia loading. These include *M. albus* (Tay et al., 2003; Ip et al., 2004c; Lim et al., 2004; Chew et al., 2005), the marble goby, *Oxyeleotris marmorata* (Jow et al., 1999), the four-eyed sleeper, *Bostrychus sinensis* (Ip et al., 2001b; Anderson et al., 2002) and the weatherloach, *Misgurnus anguillicaudatus* (Chew et al., 2001). However, to date, no information is available on whether *M. albus*, or any other fishes, could increase glutamine synthesis and accumulate glutamine in tissues in response to hyperosmotic stress. Thus, we also aimed to test the hypothesis that (3) glutamine could act as a major osmolyte in *M. albus* exposed to 25‰ water for 4 days. In addition, efforts were made to examine whether exposure to 25‰ water for 4 days would lead to increases in the activity and expression of GS from the muscle and liver.

MATERIALS AND METHODS

Fish

M. albus (150–500 g body mass) were purchased locally from a fish farm in Singapore. They were maintained in plastic aquaria in freshwater (1‰) at 25°C under a 12 h:12 h dark:light regime in the laboratory and water was changed completely every day. No attempt was made to separate the sexes. Fish were acclimated to laboratory conditions for at least 1 week before experimentation, during which they were fed live guppy. Food was withdrawn 48 h prior to experiments, which gave sufficient time for the gut to be emptied of all food and waste products.

Exposure of fish to experimental conditions and collection of tissue and water samples

Fish were immersed individually in plastic aquaria (30 cm length×15 cm width×20 cm height) containing 10 volumes (w/v) of water adjusted to pH 7 at 25°C, had free access to air and were maintained under a 12 h:12 h dark:light regime. Control fish were exposed to freshwater (1‰) for 8 days. Experimental specimens were exposed to daily increases in salinity from freshwater (1‰; day 0) to 5‰ (day 1), 10‰ (day 2), 15‰ (day 3), 20‰ (day 4) and 25‰ (day 5), and then remained in 25‰ for 3 more days (days 6–8). Water samples (3.6 ml) were collected twice daily, once right after the change of water and once after 24 h of exposure. The water samples were acidified with 40 µl of 2 mol l^{-1} HCl and kept at 4°C until analysed.

Fish were killed on day 8 by a strong blow to the head. For the collection of plasma, the caudal peduncle of the fish was severed, and blood from the caudal artery was collected in ammonium heparin-coated capillary tubes for osmolality studies. The plasma obtained after centrifugation at 5000 ***g*** and 4°C for 5 min was used for the determination of plasma osmolality, $[Na^+]$ and $[Cl^-]$. Some blood was also collected in sodium heparin-coated capillary tubes, centrifuged at 5000 ***g*** and 4°C for 5 min to obtain the plasma. The plasma was deproteinized by adding an equal volume (v/v) of ice-cold 6% trichloroacetic acid (TCA) and centrifuged at 10,000 ***g*** at 4°C for 10 min. The resulting supernatant was kept at –20°C for analysis of ammonia, urea and FAAs. Samples of the liver and lateral muscle were excised and immediately freeze-clamped with tongs pre-cooled in liquid nitrogen. Samples were stored at –80°C until analysis.

Determination of ammonia and urea concentration in water samples

Ammonia concentration was determined colorimetrically according to the method of Anderson and Little (Anderson and Little, 1986). Urea was assayed according to the method of Jow and colleagues (Jow et al., 1999). The rates of ammonia and urea excretion are expressed as µmol day^{-1} g^{-1} fish.

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

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

Analysis of water content in muscle and liver

Frozen muscle and liver samples were completely thawed under air-tight conditions and weighed to determine the wet mass to the nearest 0.0001 g using a Sartorius CP224S mass balance (Sartorius, NY, USA). The thawed samples were then dehydrated in an oven at 100°C for 48 h. The dehydrated samples were quickly wrapped in pre-weighed aluminium foil to eliminate contact with air and kept for another 2 h in a desiccator before being weighed to determine the dry mass. The tissue water content was then calculated and expressed as a percentage of the wet tissue mass.

Determination of $[Na^+]$, $[K^+]$ and $[Cl^-]$ in muscle

Na^+, K^+ and Cl^- were extracted from the muscle samples according to the method of Gilles-Baillien (Gilles-Baillien, 1973). The $[Na^+]$ and $[Cl^-]$ in the extracts were determined as described above. $[K^+]$ was determined using an Inductively Coupled Plasma Optical Emission Spectrometer (ICP-OES; Optima 2000DV, PerkinElmer, CT, USA). $[Na^+]$, $[K^+]$ and $[Cl^-]$ are expressed as µmol ml^{-1} tissue water (Fig. 2).

In Table 6, $[Na^+]$ and $[Cl^-]$ (µmol ml^{-1}) were converted to µosmol ml^{-1} or mosmol l^{-1} using an osmotic coefficient of 0.933 (25°C, 100 kPa) (Pitzer et al., 1984). The osmotic coefficient used for $[K^+]$ was 0.926 (25°C, 100 kPa) (Archer, 1999).

Determination of tissue ammonia, urea and FAAs

The frozen muscle and liver samples were weighed, ground to a powder in liquid nitrogen and homogenized three times in 5 volumes (w/v) of 6% TCA using an Ultra-Turrax T25 homogenizer (Ika®-Labortechnik, Staufen, Germany) at 24,000 r.p.m. for 20 s each with 10 s intervals. The homogenate was centrifuged at 10,000 ***g*** at

4°C for 15 min to obtain the supernatant, which was then stored at –20°C for subsequent analysis.

For ammonia analysis, the pH of the deproteinized sample was adjusted to between 6.0 and 6.5 with 2 mol l^{-1} $KHCO_3$. The ammonia content was determined using the method of Bergmeyer and Beutler (Bergmeyer and Beutler, 1985). Freshly prepared NH_4Cl solution was used as the standard for comparison. Urea concentration in the deproteinized sample was analysed colorimetrically according to the method of Jow and colleagues (Jow et al., 1999). Urea (Sigma-Aldrich, MO, USA) was used as a standard. Results are expressed as μmol g^{-1} wet tissue mass or μmol ml^{-1} plasma.

For analysis of FAAs, deproteinized muscle, liver and plasma samples were diluted with an equal volume of 2 mol l^{-1} lithium citrate buffer and adjusted to pH 2.2 with 4 mol l^{-1} LiOH. The samples were then analysed for FAA concentration using a Shimadzu LC-10ATVP amino acid analysis system with a Shim-pack ISC-07/Amino Li-type column (Shimadzu, Kyoto, Japan). Results are expressed as μmol g^{-1} wet mass for muscle and liver samples and as μmol ml^{-1} for plasma samples. The total FAA (TFAA) concentration was expressed as the sum of the concentrations of FAAs, while the total essential free amino acid (TEFAA) content was calculated as the sum of the histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine content.

In Table 6, the muscle TFAA content (μmol g^{-1} or mmol kg^{-1}) was converted to TFAA concentration (mmol l^{-1} and mosmol l^{-1}) based on the muscle water content, assuming that all FAAs were in solution and had an osmotic coefficient of 1.

Determination of enzyme activities from muscle and liver

Frozen muscle or liver sample was weighed and homogenized three times in 5 volumes (w/v) of ice-cold extraction buffer containing 50 mmol l^{-1} imidazole (pH 7.0), 1 mmol l^{-1} EDTA, 1 mmol l^{-1} EGTA, 25 mmol l^{-1} NaF and 0.1 mmol l^{-1} PMSF. The homogenate was sonicated for 10 s, and then centrifuged at 10,000 ***g*** and 4°C for 20 min. The supernatant obtained was passed through a Bio-Rad P-6DG column (Bio-Rad Laboratories, CA, USA) equilibrated with elution buffer containing 50 mmol l^{-1} imidazole (pH 7.0) and 1 mmol l^{-1} EDTA. The resulting eluate was used for enzymatic analysis. Enzyme activities were recorded with a Shimadzu UV-1601 UV-VIS recording spectrophotometer at 25°C. All chemicals and coupling enzymes were purchased from Sigma-Aldrich (MO, USA).

GS activity was determined colorimetrically according to the method of Shankar and Anderson (Shankar and Anderson, 1985). GS activity was expressed as μmol γ-glutamyl hydroxamate formed min^{-1} g^{-1} wet mass. Freshly prepared glutamic acid monohydroxamate solution was used as a standard for comparison. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activity was determined in the direction of alanine and aspartate degradation according to Peng and colleagues (Peng et al., 1994). Glutamate dehydrogenase activity in the amination direction (GDH-a) and deamination direction (GDH-d) were assayed according to Ip and colleagues (Ip et al., 1993). ALT, AST and GDH-a activity was monitored at 340 nm and expressed as μmol NADH utilized min^{-1} g^{-1} wet tissue mass. GDH-d activity was monitored at 492 nm and expressed as μmol formazan formed min^{-1} g^{-1} wet tissue mass.

Western blotting for GS

Frozen muscle and liver samples were processed as described above for the determination of enzyme activity. The protein content in the supernatant obtained was determined according to the method of Bradford (Bradford, 1976). Bovine γ-globulin dissolved in 25% glycerol was used as a standard for comparison. The supernatant of muscle samples was subsequently diluted to 2.5 μg protein $μl^{-1}$ while that of the liver samples was diluted to 1.25 μg protein $μl^{-1}$ in Laemmli buffer (Laemmli, 1970).

Proteins were separated by polyacrylamide gel electrophoresis (PAGE) under denaturing conditions following the method described by Laemmli (Laemmli, 1970), using a vertical mini-slab apparatus (Bio-Rad). The resolved proteins were transferred to Immun-blot™ PVDF membrane (Bio-Rad) using a semi-dry transfer apparatus (Bio-Rad). Blots were then blocked for 1 h or overnight 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).

GS was immunolocalized 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 (MA, USA) and kept at –20°C before use. Actin (pan Ab-5, Cat. #MS-1295-P1) was purchased from Thermo Fisher Scientific (CA, USA) and used as the housekeeping control with a dilution of 1:5000. Blots were incubated with primary antiserum for 1 h at room temperature or 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, CA, USA). Bands were visualized by chemiluminescence (Western Lightning™, Chemiluminescence Reagent Plus, PerkinElmer Life Sciences, MA, USA) and with exposure to Konika-Minolta film processed using a Kodak X-OMAT 3000 RA processor (Kodak, Tokyo, Japan). Band intensity was quantified using SigmaScan Pro 5 (Hearne Scientific Software Pty Ltd, Melbourne, Australia).

Statistical analyses

Results are presented as means ± standard error of the mean (s.e.m.). Data in the tables and figures were analysed using independent *t*-tests. Fig. 1 was also analysed using one-way analysis of variance (ANOVA) followed by Bonferroni's *post-hoc* test to evaluate differences between means. Differences with $P<0.05$ were regarded as statistically significant.

RESULTS

Changes in plasma osmolality, $[Na^+]$ and $[Cl^-]$

There was a 1.5-fold increase in plasma osmolality, with significant increases in plasma $[Na^+]$ and $[Cl^-]$ in fish exposed to 25‰ water for 4 days as compared with those in the freshwater control (Table 1).

Changes in ammonia and urea excretion rates

Exposure of *M. albus* to a 5 day progressive increase in salinity from freshwater to 25‰ water (day 5) followed by 3 more days of

Table 1. Plasma osmolality and Na^+ and Cl^- concentration in *Monopterus albus* exposed to 25‰ water for 4 days after a progressive increase in salinity from freshwater

	Control	25‰ water (4 days)
Osmolality	299±4	451±12*
$[Na^+]$	157±3	228±8*
$[Cl^-]$	111±3	198±9*

Osmolality, mosmol kg^{-1}; concentration, mmol l^{-1}.
Results represent means ± s.e.m. (N=4).
*Significantly different from the corresponding control value, $P<0.05$.

exposure to 25‰ water (day 6 to day 8) resulted in significant decreases in the rate of ammonia excretion on days 4 (20‰), 5 (25‰) and 6 (25‰; Fig. 1A) and in the rate of urea excretion on days 4 (20‰), 6 (25‰) and 8 (25‰) (Fig. 1B).

Tissue ammonia and urea content

Short-term (1 day) exposure to 25‰ water had no significant effects on the ammonia content of the muscle, liver and plasma, but led to a significant increase in the urea content of the liver of *M. albus* (Table 2). In comparison, exposure to 25‰ water for 4 days resulted in significant increases in the urea content of the muscle, liver and plasma (Table 2).

Changes in tissue FAA, TFAA and TEFAA content

There were significant increases in proline, tryptophan and TEFAA content in the muscle of *M. albus* exposed to 25‰ water for 1 day (Table 3). After exposure to 25‰ water for 4 days, the muscle glutamine content increased significantly by 6-fold. There were also significant increases in alanine, arginine, asparagine, lysine, proline, serine, threonine and valine content in the muscle, resulting in significant increases in the muscle TEFAA and TFAA content (Table 3).

Short-term (1 day) exposure to 25‰ water led to significant increases in arginine, asparagine, β-alanine, isoleucine, leucine, tyrosine and valine content, and hence also the TFAA content, of the liver of *M. albus* (Table 4). Surprisingly, the glutamine content increased 18-fold to an extraordinarily high level of ~37 μmol g^{-1} in the liver of fish exposed to 25‰ water for 4 days. There were also significant increases in the arginine, aspartate, asparagine, β-alanine, lysine, proline, phenylalanine, serine and threonine content, resulting in significant increases in TEFAA and TFAA content of the liver (Table 4).

As for the plasma, there were significant increases in the concentration of arginine, proline and TEFAA in fish exposed to 25‰ water for 1 day (Table 5). Compared with freshwater controls, the plasma of fish exposed to 25‰ water for 4 days had significant increases in the concentration of alanine, arginine, aspartate, asparagine, glutamine, glutamate, glycine, histidine, lysine, proline, serine, threonine, tyrosine, valine, TEFAA and TFAA (Table 5).

Changes in tissue water content and muscle Na^+, K^+ and Cl^- concentration

The muscle water content (% wet tissue mass) of fish exposed to 25‰ water for 4 days (76.8±0.4%, N=4) decreased significantly by ~4% compared with controls kept in freshwater (73.0±0.3%, N=4), but there was no significant change in the liver water content. There were also significant increases in the concentration of Na^+ (2-fold; Fig. 2A), K^+ (1.5-fold; Fig. 2B) and Cl^- (4-fold; Fig. 2C) in the muscle of the experimental fish.

Magnitude of change in plasma osmolality in comparison with that in muscle osmolyte concentration

Based on the results obtained, a balance sheet (Table 6) was constructed to examine the contribution of inorganic and organic

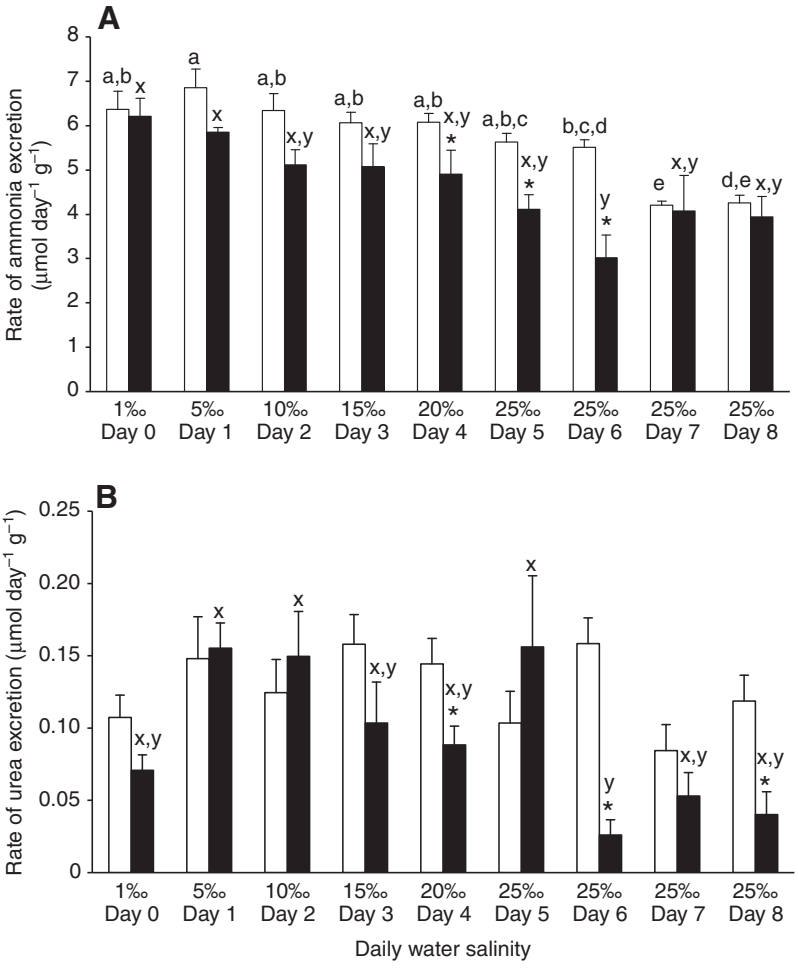

Fig. 1. Rates (μmol day^{-1} g^{-1} fish) of (A) ammonia and (B) urea excretion in *Monopterus albus* kept in freshwater (control; open bars) for 8 days or exposed to a progressive increase in salinity (filled bars) from freshwater (1‰) to 25‰ water followed by exposure to 25‰ water for a total of 4 days. Results represent means ± s.e.m. (N=6 for control, N=4 for experimental fish). *Significantly different from the corresponding control value, P<0.05; means of rates within the control group (open bars) or within the experimental fish group (filled bars) with different letters are significantly different, P<0.05.

Table 2. Ammonia and urea content in muscle and liver and concentration in plasma of *M. albus* kept in freshwater (control) or exposed to 25‰ water for 1 or 4 days after a progressive increase in salinity from freshwater

	Ammonia				Urea			
	Freshwater (1 day control)	25‰ water (1 day)	Freshwater (4 days control)	25‰ water (4 days)	Freshwater (1 day control)	25‰ water (1 day)	Freshwater (4 days control)	25‰ water (4 days)
Muscle	2.39±0.28	2.86±0.60	1.87±0.38	2.34±0.25	1.49±0.18	2.19±0.25	1.26±0.18	2.47±0.33*
Liver	6.01±2.13	6.84±1.56	2.64±0.99	4.52±0.41	1.34±0.14	2.07±0.24*	1.29±0.14	2.80±0.48*
Plasma	0.32±0.09	0.86±0.27	0.13±0.03	0.14±0.02	1.88±0.11	2.87±0.47	1.87±0.24	4.14±0.69*

Content and concentration are presented as μmol g^{-1} wet tissue mass and μmol ml^{-1} plasma, respectively.
Results represent means ± s.e.m. (*N*=5).
*Significantly different from the corresponding control value, *P*<0.05.

osmolytes to cell volume regulation in the muscle of *M. albus*. Changes in muscle Na^+, K^+, Cl^- and TFAA osmolarity were able to account for 86% of the increase required to counteract the change in plasma osmolality, assuming that the 'osmol gap' between osmolality and osmolarity was zero (Erstad, 2003). The change in glutamine content accounted for 50% of the increase in TFAA osmolarity, confirming its role as a major organic osmolyte. Muscle water-soluble protein was not included in the calculation because preliminary results obtained indicated that its concentration remained statistically unchanged after exposure to 25‰ water.

Changes in enzyme activities from muscle and liver

Fish exposed to 25‰ water for 4 days showed a 4.7-fold and 3.2-fold increase in GS activity from muscle and liver, respectively (Table 7). Furthermore, there was a significant increase in ALT activity (1.8-fold) and in AST activity (2.2-fold) from the muscle. Liver ALT and AST activity remained unchanged in fish exposed to 25‰ water for 4 days (Table 7). Exposure to 25‰ water for 4 days had no significant effect on the GDH amination and deamination activities from the liver, but led to a significant increase in the GDH amination activity from the muscle (Table 7).

GS protein abundance in liver and muscle

Western blotting (Fig. 3A) revealed that exposure to 25‰ water for 4 days resulted in an increase in the GS protein abundance in the muscle (Fig. 3B) and liver (Fig. 3C).

DISCUSSION

Fish have to adopt either an osmoregulating or an osmoconforming strategy to survive in external media that range from freshwater to seawater. Some marine fishes (e.g. hagfish, sharks, skates and rays) are osmoconformers, maintaining the osmolality of their extracellular fluid equal to that of the surrounding seawater at about 1000 mosmol kg^{-1} (Yancey, 2001). On the other hand, most

Table 3. Free amino acid (FAA), total FAA (TFAA) and total essential FAA (TEFAA) content of the muscle of *M. albus* kept in freshwater (control) or exposed to 25‰ water for 1 or 4 days after a progressive increase in salinity from freshwater

	1 day		4 days	
	Control	25‰ water	Control	25‰ water
FAA				
Alanine	0.70±0.24	1.13±0.22	0.86±0.21	2.65±0.39*
Arginine	0.04±0.01	0.06±0.01	0.04±0.00	0.07±0.01*
Aspartate	0.15±0.07	0.16±0.02	0.11±0.03	0.28±0.08
Asparagine	0.28±0.11	0.29±0.06	0.38±0.09	0.90±0.19*
β-Alanine	0.23±0.02	0.32±0.12	0.22±0.05	0.39±0.08
Glutamate	0.86±0.43	0.34±0.07	0.83±0.22	0.98±0.34
Glutamine	2.18±0.77	4.11±1.12	2.73±0.82	12.47±1.30*
Glycine	3.68±1.16	4.58±0.49	5.33±1.75	7.95±2.17
Histidine	0.42±0.16	0.45±0.07	0.51±0.13	0.63±0.07
Isoleucine	0.14±0.01	0.22±0.06	0.14±0.04	0.20±0.05
Leucine	0.29±0.02	0.43±0.15	0.21±0.02	0.32±0.09
Lysine	1.32±0.07	1.90±0.28	1.04±0.07	1.89±0.18*
Proline	0.12±0.03	0.29±0.06*	0.10±0.02	0.79±0.12*
Phenylalanine	0.09±0.03	0.06±0.02	0.06±0.01	0.10±0.03
Serine	0.31±0.08	0.31±0.07	0.37±0.07	0.84±0.16*
Taurine	9.71±0.61	10.70±2.34	9.64±1.96	9.34±1.77
Threonine	0.38±0.12	0.60±0.17	0.46±0.10	1.41±0.24*
Tryptophan	n.d.	0.33±0.09*	0.21±0.01	0.19±0.01
Tyrosine	0.07±0.01	0.06±0.02	0.07±0.01	0.09±0.01
Valine	0.28±0.01	0.39±0.10	0.19±0.01	0.36±0.04*
TEFAA	2.97±0.24	4.43±0.47*	2.85±0.28	5.16±0.44*
TFAA	21.25±2.78	26.73±2.39	23.49±2.27	41.84±2.25*

Content is presented as μmol g^{-1} wet tissue mass.
Results represent means ± s.e.m. (*N*=4).
n.d., not detectable (detection limit=0.01 μmol g^{-1}).
*Significantly different from the corresponding control value, *P*<0.05.

Table 4. FAA, TFAA and TEFAA content of the liver of *M. albus* kept in freshwater (control) or exposed to 25‰ water for 1 or 4 days after a progressive increase in salinity from freshwater

	1 day		4 days	
	Control	25‰ water	Control	25‰ water
FAA				
Alanine	0.28±0.07	1.58±0.98	0.30±0.06	1.97±1.10
Arginine	0.05±0.01	0.09±0.01*	0.04±0.01	0.06±0.01*
Aspartate	0.18±0.03	0.50±0.14	0.15±0.01	0.45±0.06*
Asparagine	0.12±0.05	0.51±0.06*	n.d.	0.96±0.14*
β-Alanine	0.08±0.02	0.20±0.02*	0.11±0.01	0.22±0.03*
Glutamate	1.93±0.33	3.17±0.72	2.18±0.17	3.22±1.20
Glutamine	1.85±0.87	9.66±3.96	2.12±0.32	36.73±8.15*
Glycine	1.62±0.67	2.83±0.39	0.91±0.05	3.23±1.90
Histidine	0.11±0.01	0.12±0.01	0.10±0.00	0.11±0.03
Isoleucine	0.13±0.02	0.25±0.04*	0.07±0.01	0.21±0.06
Leucine	0.24±0.03	0.59±0.10*	0.18±0.02	0.56±0.12
Lysine	3.57±1.32	4.23±0.94	2.31±0.46	5.31±0.62*
Proline	0.04±0.00	0.25±0.09	0.02±0.00	0.32±0.09*
Phenylalanine	0.08±0.02	0.11±0.01	0.06±0.01	0.15±0.02*
Serine	0.12±0.03	0.14±0.02	0.06±0.01	0.22±0.06*
Taurine	2.67±0.76	5.16±1.26	2.40±0.50	3.35±0.36
Threonine	0.18±0.02	0.91±0.30	0.27±0.04	1.91±0.57*
Tryptophan	n.d.	n.d.	0.16±0.05	0.19±0.01
Tyrosine	0.10±0.02	0.20±0.02*	0.13±0.03	0.16±0.05
Valine	0.27±0.02	0.71±0.10*	0.18±0.01	0.53±0.11
TEFAA	4.71±1.36	6.99±0.66	3.37±0.39	8.99±1.07*
TFAA	13.68±1.22	31.19±1.86*	11.79±0.74	59.81±7.38*

Content is presented as $\mu mol\,g^{-1}$ wet tissue mass.
Results represent means ± s.e.m. (*N*=4).
n.d., not detectable (detection limit=0.01 $\mu mol\,g^{-1}$).
*Significantly different from the corresponding control value, $P<0.05$.

Table 5. FAA, TFAA and TEFAA concentration in the plasma of *M. albus* kept in freshwater (control) or exposed to 25‰ water for 1 or 4 days after a progressive increase in salinity from freshwater

	1 day		4 days	
	Control	25‰ water	Control	25‰ water
FAA				
Alanine	0.07±0.01	0.16±0.04	0.09±0.01	0.58±0.14*
Arginine	0.01±0.00	0.03±0.01*	0.01±0.00	0.03±0.00*
Aspartate	n.d.	0.01±0.00	0.01±0.00	0.03±0.01*
Asparagine	0.04±0.01	0.08±0.04	0.06±0.00	0.15±0.01*
β-Alanine	0.01±0.00	0.01±0.00	n.d.	0.02±0.01
Glutamate	0.05±0.04	0.05±0.02	0.03±0.00	0.15±0.04*
Glutamine	0.29±0.09	0.89±0.32	0.29±0.03	2.41±0.22*
Glycine	0.17±0.02	0.26±0.05	0.21±0.01	0.59±0.11*
Histidine	0.04±0.00	0.05±0.01	0.04±0.00	0.07±0.01*
Isoleucine	0.12±0.02	0.21±0.08	0.07±0.01	0.15±0.03
Leucine	0.32±0.07	0.56±0.19	0.20±0.02	0.50±0.10
Lysine	0.35±0.01	0.71±0.18	0.27±0.02	0.87±0.17*
Proline	0.01±0.00	0.04±0.01*	0.01±0.00	0.12±0.03*
Phenylalanine	0.07±0.03	0.06±0.01	0.04±0.01	0.07±0.02
Serine	0.05±0.01	0.05±0.02	0.05±0.01	0.15±0.03*
Taurine	0.17±0.06	0.08±0.03	0.17±0.06	0.13±0.04
Threonine	0.09±0.01	0.19±0.06	0.08±0.00	0.45±0.03*
Tryptophan	0.32±0.01	0.42±0.05	0.28±0.04	0.28±0.04
Tyrosine	0.03±0.00	0.04±0.01	0.03±0.00	0.06±0.01*
Valine	0.31±0.06	0.49±0.13	0.19±0.02	0.46±0.09*
TEFAA	1.63±0.14	2.70±0.40*	1.20±0.08	2.89±0.23*
TFAA	2.53±0.16	4.36±0.74	2.15±0.05	7.26±0.52*

Concentration is presented as $\mu mol\,ml^{-1}$.
Results represent means ± s.e.m. (*N*=4).
n.d., not detectable (detection limit=0.01 $\mu mol\,ml^{-1}$).
*Significantly different from the corresponding control value, $P<0.05$.

Table 6. Balance sheet showing the increase in plasma osmolality and the concurrent changes in concentration and osmolarity of inorganic osmolytes and TFAA in the muscle of *M. albus* exposed to 25‰ water for 4 days after a progressive increase in salinity from freshwater, in comparison with fish kept in freshwater (control)

	Control	25‰ water (4 days)	Difference
Plasma osmolality	299	451	+152
Muscle ionic concentration (and osmolarity)			
Na^+	37.2 (34.6)	78.6 (73.1)	+41.4 (+38.5)
K^+	108 (100.0)	143 (132.4)	+35 (+32.4)
Cl^-	11.4 (10.6)	47.2 (43.9)	+35.8 (+33.3)
Muscle TFAA content (and osmolarity)	23.5 (33.3)	41.8 (59.2)	+18.3 (+25.9)
Total increase in osmolarity in muscle			(+130.1)

Osmolality, mosmol kg^{-1}; concentration, mmol l^{-1}; content, mmol kg^{-1}; osmolarity (in parentheses), mosmol l^{-1}.

freshwater and marine teleosts are osmoregulators; maintaining their extracellular fluid at approximately 300 mosmol kg^{-1} regardless of the osmolality of the environment they are exposed to (Jobling, 1995; Yancey, 2001). Many euryhaline teleosts, e.g. the freshwater *Oreochromis mossambicus* and the marine *Fundulus heteroclitus*, can acclimate to water of different salinities, but they maintain the plasma osmolality relatively constant (Marshall and Grosell, 2006).

A combination of osmoconforming and hypoosmotic hypoionic regulation in *M. albus* acclimated to 25‰ water

M. albus is unique because its gills are highly degenerate and non-functional. Hence, it is logical to deduce that it would not be able to regulate its ionic content as effectively as other teleosts, and would have to tolerate a relatively large fluctuation in plasma osmolality. Indeed, for fish exposed to 25‰ water for 4 days, the plasma osmolality increased significantly from 299 to 451 mosmol kg^{-1}, with significant increases in plasma [Na^+] and [Cl^-]. Hence, it can be concluded that *M. albus* had partially adopted an osmoconforming strategy to handle salinity stress. However, the increased plasma osmolality was equivalent to only 68% of that of the external medium (800 mosmol kg^{-1}). Thus, *M. albus* must possess mechanisms that can alleviate dehydration in a hyperosmotic environment, the elucidation of which awaits future studies.

More importantly, the increased plasma [Na^+] (228 mmol l^{-1}) and [Cl^-] (198 mmol l^{-1}) were still lower than those (350 and 375 mmol l^{-1}, respectively) in the 25‰ water. Therefore, it can be concluded that *M. albus* also exhibited hypoosomotic hypoionic regulation in 25‰ water. Both osmoconforming (ureosmotic) hypoionic and hypoosmotic hypoionic regulation might have evolved in estuarine or freshwater environments (Griffith, 1991). It has been suggested that hypoosmotic hypoionic regulation is more metabolically costly than osmoconforming hypoionic regulation for those fish that reinvaded the oceans (Griffith and Pang, 1979). Hence, it could be advantageous for *M. albus* to uniquely adopt a combination of partially osmoconforming and hypoosmotic hypoionic regulations to deal with salinity stress.

High extracellular concentrations of inorganic ions have been implicated in the perturbation of membrane potentials (Yancey et al., 1982), the destabilization of proteins and stunted cell growth (Somero and Yancey, 1997; Smith et al., 1999). *M. albus* was apparently capable of regulating the plasma/extracellular ionic concentrations at tolerable levels to avoid detrimental effects, although the site of ionic regulation is unknown at present. Efforts should be made in the future to examine the possible osmoregulatory roles of its skin, intestine, buccal epithelium and kidney, which are known to be minor osmoregulatory organs in other fish species (Marshall, 1977; Marshall and Nishioka, 1980; Marshall and Grosell, 2006; Yokota et al., 2004; Nonnotte et al., 1979; Degnan and Zadunaisky, 1980).

Accumulation of significant amounts of inorganic ions in the muscle

Muscle makes up the major bulk of the fish and thus it was essential to examine the mechanism of cell volume regulation in this tissue. From Table 6 and Fig. 2, it can be concluded for the first time that *M. albus* exposed to 25‰ water accumulates significant quantities of inorganic ions (Na^+, K^+ and Cl^-) as osmolytes. The increases in [K^+], [Na^+] and [Cl^-] in the muscle of *M. albus* that experienced a large increase in plasma osmolality (152 mosmol kg^{-1}) were 35, 41.4 and 35.8 μmol ml^{-1}, respectively (Table 6). It is an unusual phenomenon because animals that carry out cell volume regulation through the uptake or release of inorganic ions usually do so over a limited range of ambient osmolality fluctuations (Lang et al., 1998), but the experimental fish in this study was confronted with a huge

Table 7. Glutamine synthetase (GS), alanine aminotransferase (ALT), aspartate aminotransferase (AST) and glutamate dehydrogenase activity in the amination direction (GDH-a) and in the deamination direction (GDH-d) from the muscle and liver of *M. albus* kept in freshwater (control) or exposed to 25‰ water for 4 days after a progressive increase in salinity from freshwater

	Muscle		Liver	
	Control	25‰ water (4 days)	Control	25‰ water (4 days)
GS	0.16±0.04	0.76±0.10*	1.51±0.36	4.87±0.83*
ALT	0.32±0.03	0.56±0.06*	3.88±0.44	3.45±0.30
AST	3.71±0.39	8.10±0.68*	56.6±7.6	65.3±2.5
GDH-a	0.14±0.02	0.28±0.04*	40.1±4.9	28.1±2.4
GDH-d	n.d.	n.d.	1.32±0.20	1.03±0.11

Enzyme activity is presented as μmol min^{-1} g^{-1} wet tissue mass.
Results represent means ± s.e.m. (*N*=5).
n.d., not detectable.
*Significantly different from the corresponding control value, $P<0.05$.

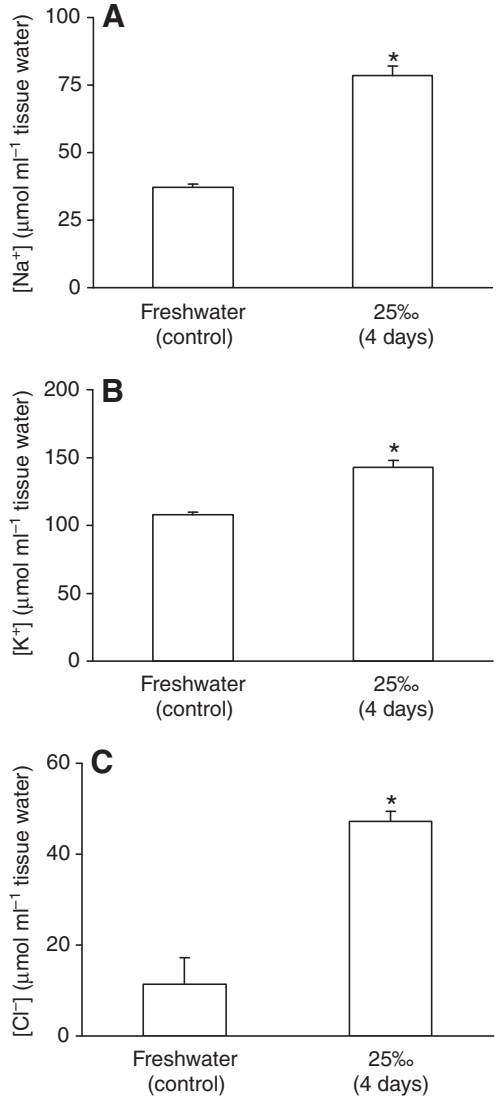

Fig. 2. Concentration (μmol ml^{-1} tissue water) of (A) Na^+, (B) K^+ and (C) Cl^- in the muscle of *M. albus* kept in freshwater (control) or exposed to 25‰ water for 4 days after a progressive increase in salinity from freshwater. Results represent means ± s.e.m. (N=5). *Significantly different from the corresponding control value, P<0.05.

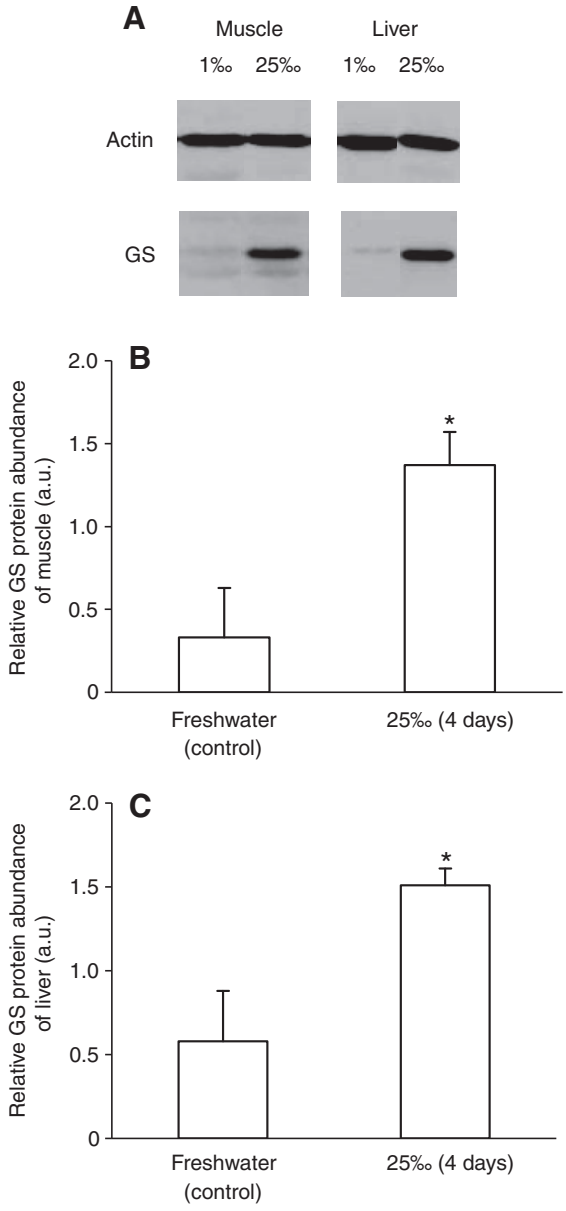

Fig. 3. (A) Representative immunoblots of glutamine synthetase (GS) from muscle and liver of *M. albus* kept in freshwater (control) or exposed to 25‰ water for 4 days after a progressive increase in salinity from freshwater, (B) relative abundance of immunoreactive bands of GS from muscle (N=3) and (C) relative abundance of immunoreactive bands of GS from liver (N=4). Results represent means ± s.e.m. *Significantly different from the corresponding control value, P<0.05.

change (~800 mosmol kg^{-1}) in ambient osmolality. Animals usually do not depend on ions as major intracellular osmolytes because large changes in intracellular ionic concentrations would affect membrane potentials and have disruptive effects on macromolecular function (Brown and Simpson, 1972; Wyn Jones et al., 1977; Yancey et al., 1982).

FAAs as organic osmolytes

In cells capable of surviving long-term or large-scale dehydration, organic osmolytes eventually replace ions for volume regulation, because the former would not disrupt macromolecular function (Brown and Simpson, 1972; Yancey, 2001). For *M. albus* exposed to salinity stress, there were significant decreases in the rate of ammonia and urea excretion, specifically during the phase of acclimation to 20‰ and 25‰ water. Theoretically, this should lead to increases in ammonia and urea content in the muscle and liver, but the results obtained indicate otherwise. Therefore, the logical explanation is that a reduction in amino acid catabolism had occurred in fish exposed to brackish water. The accumulation of several essential FAAs supports the view that their catabolic rates were suppressed. In addition, there could be an increase in protein degradation in fish acclimated to 25‰ water, releasing FAAs for cell volume regulation.

As FAAs can act as osmolytes, it can be concluded that *M. albus* had the capacity to regulate nitrogen metabolism and excretion for osmoregulatory purposes. Indeed, there were increases in various FAA and TFAA content of tissues of fish exposed to 25‰ water. Specifically, there were increases in some non-essential FAAs, which could be synthesized by the fish, and the most prominent one

was glutamine. Thus, the decrease in ammonia excretion by fish acclimated to salinity changes was the result not only of a decrease in amino acid catabolism but also of an increase in amino acid synthesis. In this case, the synthesis of glutamine and other non-essential amino acids did not occur simply to detoxify ammonia but acted as a source of organic osmolytes for cell volume regulation.

Glutamine as the major organic osmolyte

The increase in tissue amino acid levels in response to elevated salinity also occurs in many teleosts (Assem and Hanke, 1983), including the rainbow trout (Kaushik and Luquet, 1979), *Oreochromis mossambica* (Venkatachari, 1974; Fiess et al., 2007), *Anguilla anguilla* (Huggins and Colley, 1971), *Pleuronectes flesus* (Lange and Fugelli, 1965) and *A. testudineus* (Chang et al., 2007). Even stenohaline species such as carp (*Cyprinus carpio*) display an elevated liver alanine, glutamate and taurine content, and muscle taurine, glycine, alanine and histidine content, but not glutamine, upon transfer to 1.5% seawater (Hegab and Hanke, 1983). Taurine plays a particularly important role as an intracellular osmolyte, and the level of this amino acid has been shown to rise with environmental salinity in flounder erythrocytes (Fugelli and Zachariassen, 1976) and heart (Vislie and Fugelli, 1975), and in rainbow trout intestinal mucosa (Auerswald et al., 1997). However, glutamine has never been reported to act as an important osmolyte in these fishes, although Fiess and colleagues (Fiess et al., 2007) reported recently a possible increase in the muscle glutamine (and alanine) content of *O. mossambica* exposed to seawater. As a result, glutamine is not regarded as a compatible organic osmolyte involved in osmoregulation in fish (Yancey, 2001), although it is known to be a compatible osmolyte in bacteria (Lai et al., 1991; Frings et al., 1993).

In contrast, glutamine is known to accumulate in vertebrate brains in defence against ammonia toxicity and ammonia is detoxified to glutamine in preference to other amino acids (Cooper and Plum, 1987). In mammals, the accumulation of glutamine would result in the swelling of astrocytes, which is one of the leading explanations of hepatic encephalopathy (Brusilow, 2002). Although a similar explanation of ammonia toxicity may not be applicable to fish (Ip et al., 2005; Wee et al., 2007), fish brains possess high GS activity and detoxify ammonia to glutamine, resulting in glutamine accumulation, under certain conditions (Peng et al., 1998) (see review by Chew et al., 2006). Hence, for fishes like *M. albus* which exhibit high GS activity in extra-cranial tissues, it is probable that they can increase the synthesis of glutamine in these tissues not only for ammonia detoxification during emersion or ammonia exposure but also for cell volume regulation during exposure to water of high salinity.

Indeed, glutamine was the major organic osmolyte, which accumulated to the phenomenal level of 12 and 37 μmol g^{-1} in muscle and liver, respectively, of *M. albus* exposed to 25‰ water. This is a novel finding because such a physiological/osmoregulatory role of glutamine has never been reported before for fish or any other animal. Given the various physiological roles of glutamine, it may be advantageous for *M. albus* to accumulate glutamine as a major organic osmolyte. The first advantage is that accumulated glutamine could act as a precursor for the synthesis of physiologically important molecules such as purines, pyrimidines and mucopolysaccharides upon returning to freshwater. Studies have found that glutamine may be utilized by some teleosts as an oxidative substrate for red muscle mitochondria (Chamberlin et al., 1991; Chamberlin and Ballantyne, 1992; Mommsen et al., 2003; Ballantyne, 2004). Therefore, another possible advantage is that the accumulated glutamine may also serve as an alternative energy source for *M. albus* upon returning to favourable ambient conditions. In mammals, glutamine also serves to partially counter the effects of metabolic acidosis through renal catabolic activities (Taylor and Curthoys, 2004). Additionally, glutamine accumulation in human muscle cells has been implicated in aiding trauma recovery through improving nitrogen balance, triggering wound matrix formation, reducing whole-body protein degradation, improving immune response and inducing anabolism (Buffington, 1992; Sacks, 1999; Flaring et al., 2003; Peng et al., 2005).

Up-regulation of GS activity and protein abundance in response to salinity stress

The involvement of GS in the defence against ammonia toxicity is common in vertebrate brains (Cooper and Plum, 1987), but increases in GS activity in non-cerebral tissues have been reported in *M. albus* (Tay et al., 2003; Ip et al., 2004c; Chew et al., 2005), *B. sinensis* (Ip et al., 2001b; Anderson et al., 2002a), *O. marmorata* (Jow et al., 1999) and *M. anguillicaudatus* (Chew et al., 2001) in response to emersion and/or ammonia exposure. Working on the marsh clam, *Polymesoda expansa*, Hiong and colleagues (Hiong et al., 2004) have concluded that the increased glutamine synthesis is probably an adaptation evolved primarily for ammonia detoxification instead of osmoregulation among invertebrates. As for fish, exposure of the euryhaline brackish water *B. sinensis* (Peh, 2008) and the euryhaline freshwater *O. marmorata* (Tng, 2008) to seawater led to either no significant change or very minor increases in glutamine content in various tissues.

Here, we report for the first time that GS activity was up-regulated in the muscle and liver of *M. albus* after 4 days of exposure to 25‰ water. The activity of GS from liver was higher than that from muscle, but muscle makes up the bulk of the fish and thus it also contributed significantly to the increased synthesis of glutamine as an osmolyte. At present, it is unclear whether GS regulation in *M. albus* involved post-translational covalent modification. However, it is known that GS tyrosine nitration occurs in rat brains in response to hyperammonaemia and hepatic encephalopathy, in cultured astrocytes exposed to ammonia, and in livers of septic rats (Schliess et al., 2002; Gorg et al., 2003; Gorg et al., 2005; Gorg et al., 2006). The mechanism of protein tyrosine nitration can be mediated by reactive nitrogen species such as peroxynitrite and nitrogen dioxide (Radi, 2004). In addition, phosphorylated GS has also been detected in hepatic carcinoma tissues, in which GS could be phosphorylated at Ser 320 and Ser 322 (Kuramitsu et al., 2006).

Western blotting revealed significant increases in the GS protein abundance in muscle and liver of fish exposed to 25‰ water for 4 days. Hence the increase in activity could be the result of an increase in GS protein abundance. This is a novel finding because the induction of elevated GS protein abundance in fish muscle and liver by increased salinity has never been reported before, and further studies will be required to establish whether these increases were mediated by increases in GS mRNA expression. In addition, the possibility of differential mRNA expression of GS isozymes in tissues of *M. albus* during salinity adaptation cannot be ignored, because four GS genes (*Onmy-GS01*, *Onmy-GS02*, *Onmy-GS03* and *Onmy-GS04*) have been identified in the rainbow trout *O. mykiss*, and they exhibit differential expression in different tissues (Murray et al., 2003), with those in the brain being inducible by hyperammonaemia (Wright et al., 2007). Furthermore, two different GS isoforms are present in the liver (Walsh et al., 1999; Wood et al., 2003), with another GS isoform specific to the gills (Walsh et al., 2003) of the toadfish, *Opsanus beta*.

Changes in GDH, ALT and AST activity

Glutamine synthesis requires glutamate as a substrate, but no significant decreases in tissue glutamate content were observed in *M. albus* exposed to salinity stress. Glutamate can be synthesized through the GDH amination reaction which requires α-ketoglutarate and NH_4^+ as substrates. Indeed, there was a significant increase in the GDH amination activity in the muscle of fish exposed to 25‰ water for 4 days, which indicates once again the important role of the muscle in amino acid metabolism for osmoregulatory purposes. Although the GDH amination activity from the muscle was lower than that from the liver, it is probable that the up-regulation of GDH amination activity in the muscle led to an increase in glutamate formation to sustain increased glutamine synthesis therein.

In addition, glutamate can be derived from certain amino acids, e.g. alanine and aspartate, through transamination reactions catalysed by various aminotransferases. Indeed, there was a significant increase in ALT and AST activity, in the direction of alanine and aspartate degradation, respectively, from the muscle of *M. albus* exposed to 25‰ water for 4 days. Hence, it can be deduced that alanine and aspartate released through proteolysis in the muscle were channelled into transamination reactions to support increases in the formation of glutamate and subsequently glutamine. As a result only relatively minor changes in alanine and aspartate content were observed in the muscle, liver and plasma of fish exposed to brackish water.

Conclusion

M. albus was a hyperosomotic hyperionic regulator in freshwater, but exhibited hypoosomotic hypoionic regulation with a certain degree of osmoconforming capacity when exposed to a hyperosmotic external medium. There were significant increases in plasma osmolality and ionic concentrations in fish exposed to 25‰ water for 4 days, and cell volume regulation was achieved through accumulation of inorganic and organic osmolytes. The tissue FAA content increased as a result of decreased amino acid catabolism, increased protein degradation, and increased synthesis of certain non-essential FAAs. The most exciting finding in this study is that glutamine is the major osmolyte in *M. albus*, reaching more than 30 μmol g^{-1} in the liver of fish exposed to 25‰ water for 4 days. In addition, we report for the first time that exposure to 25‰ water for 4 days led to an up-regulation of GS activity and protein abundance in muscle and liver. The extraordinary capacity of *M. albus* to increase glutamine synthesis and accumulation for cell volume regulation is probably a consequence of the lack of functional gills (Graham, 1997). This could have developed as an extension of its ability to increase glutamine synthesis to detoxify ammonia during emersion (Tay et al., 2003), aestivation in mud (Chew et al., 2005) or exposure to environmental ammonia (Ip et al., 2004c).

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