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The osmotic response of the Asian freshwater stingray (*Himantura signifer*) to increased salinity: a comparison with marine (*Taeniura lymma*) and Amazonian freshwater (*Potamotrygon motoro*) stingrays

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Summary

The white-edge freshwater whip ray *Himantura signifer* can survive in freshwater (0.7‰) indefinitely or in brackish water (20‰) for at least two weeks in the laboratory. In freshwater, the blood plasma was maintained hyperosmotic to that of the external medium. There was approximately 44 mmol l^{-1} of urea in the plasma, with the rest of the osmolality made up mainly by Na^+ and Cl^-. In freshwater, it was not completely ureotelic, excreting up to 45% of its nitrogenous waste as urea. Unlike the South American freshwater stingray *Potamotrygon motoro*, *H. signifer* has a functional ornithine-urea cycle (OUC) in the liver, with hepatic carbamoylphosphate synthetase III (CPS III) and glutamine synthetase (GS) activities lower than those of the marine blue-spotted fan tail ray *Taeniura lymma*. More importantly, the stomach of *H. signifer* also possesses a functional OUC, the capacity (based on CPS III activity) of which was approximately 70% that in the liver. When *H. signifer* was exposed to a progressive increase in salinity through an 8-day period, there was a continuous decrease in the rate of ammonia excretion. In 20‰ water, urea levels in the muscle, brain and plasma increased significantly. In the plasma, osmolality increased to 571 mosmol kg^{-1}, in which urea contributed 83 mmol l^{-1}. Approximately 59% of the excess urea accumulated in the tissues of the specimens exposed to 20‰ water was equivalent to the deficit in ammonia excretion through the 8-day period, indicating that an increase in the rate of urea synthesis *de novo* at higher salinities would have occurred. Indeed, there was an induction in the activity of CPS III in both the liver and stomach, and activities of GS, ornithine transcarbamoylase and arginase in the liver. Furthermore, there was a significant decrease in the rate of urea excretion during passage through 5‰, 10‰ and 15‰ water. Although the local *T. lymma* in full-strength sea water (30‰) had a much greater plasma urea concentration (380 mmol l^{-1}), its urea excretion rate (4.7 μmol day^{-1} g^{-1}) was comparable with that of *H. signifier* in 20‰ water. Therefore, *H. signifer* appears to have reduced its capacity to retain urea in order to survive in the freshwater environment and, consequently, it could not survive well in full-strength seawater.

Key words: ammonia, amino acid, elasmobranch, freshwater stingray, *Himantura signifer*, ornithine-urea cycle, osmoregulation, *Potamotrygon motoro*, stingray, *Taeniura lymma*, urea, urea transporter.

Introduction

Marine elasmobranchs (sharks, skates and rays) are common in tropical waters. They exhibit osmoconforming hypoionic regulation (Yancey, 2001) with body fluid osmolalities equal to or slightly higher than the environment. Their extracellular fluids are actively regulated to have considerably lower salt concentrations than the environment, with the osmotic difference balanced by extracellular (as well as intracellular) nitrogenous organic osmolytes. Hence, unlike most teleost fishes, marine elasmobranchs are ureosmotic. They have an active ornithine-urea cycle (OUC; Anderson, 1980), synthesizing urea through carbamoylphosphate synthetase III (CPS III; Campbell and Anderson, 1991; Anderson, 1980, 1991, 1995, 2001) primarily for osmoregulation (Perlman and Goldstein, 1988; Ballantyne, 1997; Anderson, 2001). Marine elasmobranchs are also ureotelic, excreting the majority of their nitrogen wastes as urea *via* the gills (Perlman and Goldstein, 1988; Shuttleworth, 1988; Wood, 1993; Wood et al., 1995a,b). However, in order to be able to retain urea at high

concentrations (300–600 mmol l^{-1}) in the tissues, there is an effective decrease in permeability to urea resulting from the presence of specific secondarily active (Na^+-coupled) urea transporters in gills and kidney and from the modification of lipid composition in the gills (Fines et al., 2001; Walsh and Smith, 2001).

In tropical waters in Southeast Asia (Thailand, Indonesia and Papua New Guinea) and South America (Amazon River basin), a number of elasmobranch species occur in low salinity waters. For the Amazon freshwater stingrays, a reduction in tissue urea concentration occurs as the result of reduced synthesis (Forster and Goldstein, 1976) and/or a higher renal clearance rate (Goldstein and Forster, 1971). *Potamotrygon* spp. is a stenohaline Amazonian stingray permanently adapted to freshwater. Although it has low levels of some of the enzymes related to urea synthesis (Anderson, 1980), it retains virtually no urea or trimethylamine oxide (TMAO) *in situ*, cannot accumulate urea in laboratory salinity stress (Thorson et al., 1967; Gerst and Thorson, 1977) and, like other teleosts, is primarily ammonotelic (Goldstein and Forster, 1971; Barcellos et al., 1997). In comparison, there is a dearth of knowledge about the freshwater elasmobranchs in Southeast Asia.

The river Batang Hari originates from the Barisan Range, flows eastwards through the whole of Jambi, Indonesia and drains into the South China Sea. The white-edge freshwater whip ray *Himantura signifier* (Compagno and Roberts, 1982; Family: Dasyatidae) is a stingray found in the Batang Hari basin in Jambi, Sumatra. It is believed to occur only in freshwater. In the laboratory, *H. signifier* can survive in freshwater (0.7‰) indefinitely or in brackish water (20‰) for at least two weeks. Unlike *Potamotrygon* spp., which have been isolated from the sea for millions of years, *H. signifer* can travel freely along the river and may encounter brackish water during certain periods of the year. Hence, *H. signifer* represents an ideal species to study the effects of salinity changes on the capacity for urea synthesis and retention in a primarily freshwater elasmobranch with euryhaline capability.

This study was undertaken to test the hypothesis that *H. signifer* has retained the capability to synthesize urea *de novo* but has a lower capability to retain urea compared with its marine counterparts. Specimens of *H. signifer* were exposed progressively from 0.7‰ water to 20‰ water through an 8-day period. The rates of ammonia and urea excretion and the concentrations of ammonia, urea and free amino acids (FAAs) in various tissues and organs were measured. At the same time, activities of various enzymes involved in the OUC were determined. For comparison, the rate of urea excretion and the activities of OUC enzymes in the liver of the marine blue-spotted fantail ray *Taeniura lymma*, which is found in Indonesian waters, were determined. Efforts were also made to study the contents of ammonia and urea in various tissues of *Potamotrygon motoro*, a stenohaline freshwater stingray from the Amazon Basin River for direct comparison. *P. motoro* was exposed progressively from 0.7‰ water to only 13‰ water, beyond which it would not survive.

Materials and methods

Maintenance of specimens

Himantura signifer (Compagno and Roberts 1982) (200–500 g body mass), *Taeniura lymma* (Forskål 1775) (200–600 g body mass) and *Potamotrygon motoro* (Müller and Henule 1841) (250–400 g body mass) were purchased from a local fish farm in Singapore. *H. signifer* and *P. motoro* were maintained in plastic aquaria in freshwater (0.7‰ salinity) while *T. lymma* were kept in full-strength seawater (30‰ salinity) at 25°C in the laboratory with the water changed daily. No attempt was made to separate the sexes. Fish were acclimated to laboratory conditions for at least 5 days before the experiments were begun. During this period, *H. signifer* and *P. motoro* were fed with clam meat, while *T. lymma* was fed with squid meat. Food was withdrawn 24 h prior to experiments so that the fish had empty guts. All experiments were performed under a 12 h:12 h dark:light regime.

Exposure of specimens to experimental conditions and collection of water samples and tissues

Specimens were submerged individually in plastic aquaria tanks (60 cm length × 30 cm width × 20 cm height) containing 10 volumes (w/v) of aerated water at 25°C. For *H. signifer*, control specimens were exposed to freshwater at pH 7 for 8 days. Experimental specimens were exposed to daily increases in salinity from 0.7‰ (day 1) to 5‰ (day 2) to 10‰ (day 3) to 15‰ (day 4) to 20‰ (day 5), remaining at 20‰ on days 6–8. For *P. motoro*, control specimens were exposed to freshwater for 4 days. The experimental specimens were exposed to daily salinity changes from 0.7‰ (day 1) to 4‰ (day 2) to 7‰ (day 3) to 13‰ (day 4). Gradual ascent in salinity was necessary to allow for acclimatization and survival. The salinity was capped at 20‰ and 13‰ for *H. signifer* and *P. motoro*, respectively, because a preliminary study revealed that, beyond these salinities, mortality could be high. Water samples (3 ml) were collected daily, acidified with 70 µl of 1 mol l^{-1} HCl and kept at 4°C until analysed. Concentrations of ammonia and urea were determined according to the methods of Bergmeyer and Beutler (1985) and Felskie et al. (1998), respectively. The rates of ammonia and urea excretion were expressed as µmol day^{-1} g^{-1} fish.

Specimens of *H. signifer* were killed on day 8 and those of *P. motoro* on day 4 for tissue collection. The caudal peduncle of the experimental specimen was severed, and blood exuding from the caudal vessels was collected in heparinized capillary tubes. The blood sample was centrifuged at 4000 ***g*** at 4°C for 10 min to obtain the plasma. A portion of the plasma was used for analyses of osmolality and concentrations of Na^+ and Cl^-. Another portion was deproteinized in 2 volumes (v/v) of ice-cold 6% $HClO_4$ and centrifuged at 10 000 ***g*** at 4°C for 15 min. The resulting supernatant fluid was kept at –80°C until analysed. The brain, liver, stomach, intestine and muscle were quickly excised, with the stomach and 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.

Analyses of plasma osmolality and concentrations of Na^+ and Cl^-

Plasma osmolality was analysed using a Wescor 5500 vapour pressure osmometer. Na^+ and Cl^- concentrations were determined by a Corning 410 flame photometer and Corning 925 chloride analyzer, respectively (Corning Ltd, Halstead, Essex, UK.

Analysis of free amino acids (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% trichloroacetic acid using an Ultra-Turrax homogenizer at 24 000 revs min^{-1} for 20 s each with 10-s off intervals. The homogenate was centrifuged at 10 000 ***g*** at 4°C for 15 min to obtain the supernatant for FAA analyses. The plasma was deproteinized in 2 volumes (v/v) of ice-cold 6% trichloroacetic acid and centrifuged at 10 000 ***g*** at 4°C for 15 min.

For the analysis of FAA, the supernatant fluid obtained 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 analyzed using a Shimadzu LC-6A amino acid analysis system (Kyoto, Japan) with a Shim-pack ISC-07/S1504 Li-type column. Results are expressed as μmol g^{-1} wet mass tissue or mmol l^{-1} plasma.

Analyses of ammonia and urea

Samples were homogenized as stated above except in 5 volumes of 6% $HClO_4$. After centrifugation at 10 000 ***g*** for 15 min, the supernatant was decanted and the pH adjusted to 5.5–6.0 with 2 mol l^{-1} $KHCO_3$. The ammonia and urea concentrations were determined as stated above. Results were expressed as μmol g^{-1} wet mass tissue or mmol l^{-1} plasma.

Determination of TMAO

TMAO was assayed by the iron sulphate method of Wekell and Barnett (1991), which has been extensively modified for the analysis of small samples by Raymond (1998). The difference in absorbance obtained from the sample in the presence and absence of Fe-EDTA and heat treatment (reduction step of TMAO to TMA) was used for the estimation of TMAO in the sample. TMAO obtained from Sigma Chemical Co. (St Louis, MO, USA) was used as a standard for comparison.

Determination of activities of OUC enzymes

Preliminary studies revealed that a full complement of OUC enzymes was present in the liver and stomach of both *H. signifer* and *T. lymma*. However, no carbamoylphosphate synthetase III (CPS III) activities were detectable in the muscle, intestine or brain. Subsequently, the liver and the stomach were minced and suspended in 10 volumes (w/v) of ice-cold extraction buffer (285 mmol l^{-1} sucrose, 3 mmol l^{-1} EDTA and 3 mmol l^{-1} Tris-HCl, pH 7.2), homogenized using an Ultra-Turrax homogenizer at 24 000 revs min^{-1} and sonicated three times for 20 s with a 10 s break between each sonication. The homogenate was centrifuged at 10 000 ***g*** at 4°C for 15 min to obtain the supernatant, which was subsequently passed through a 10 ml Bio-Rad P-6DG column (Bio-Rad Laboratories, Hercules, CA, USA) equilibrated with cold suspension buffer. The collected filtrates were used for the subsequent enzyme analyses.

CPS III (E.C. 2.7.2.5) activity was determined as described by Anderson and Walsh (1995). Radioactivity was measured using a Wallac 1414 liquid scintillation counter (Wallac Oy, Turku, Finland). The CPS activity was expressed as μmol [^{14}C]urea formed min^{-1} g^{-1} wet mass.

Ornithine transcarbamoylase (OTC; E.C. 2.1.3.3) activity was determined by combining the methods of Anderson and Walsh (1995) and Xiong and Anderson (1989). Absorbance was measured at 466 nm using a Shimadzu 160 UV VIS recording spectrophotometer. The OTC activity was expressed as μmol citrulline formed min^{-1} g^{-1} wet mass.

Argininosuccinate synthetase (E.C. 6.3.4.5) and lyase (E.C. 4.3.2.1) activities were determined together, assuming that both were present, by measuring the formation of [^{14}C]fumarate from [^{14}C]aspartate using the method of Cao et al. (1991). Radioactivity was measured using a Wallac 1414 liquid scintillation counter. Argininosuccinate synthetase and lyase activity was expressed as μmol [^{14}C]fumarate formed min^{-1} g^{-1} wet mass.

Arginase (E.C. 3.5.3.1) was assayed as described by Felskie et al. (1998). Urea was determined as described above. Arginase activity was expressed as μmol urea formed min^{-1} g^{-1} wet mass.

Glutamine synthetase (GS; E.C. 6.3.1.2) activity was measured according to the method of Shankar and Anderson (1985). The formation of γ-glutamylhydroxymate was determined at 500 nm using a Shimadzu 160 UV VIS recording spectrophotometer. The GS activity was expressed as μmol γ-glutamylhydroxymate formed min^{-1} g^{-1} wet mass.

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 Duncan's procedure was used to evaluate differences between means in groups where appropriate. Differences where $P<0.05$ were regarded as statistically significant.

Results

After the progressive increase in salinity from freshwater to brackish water (20‰), the blood plasma osmolality and concentrations of Na^+ and Cl^- in *H. signifer* increased significantly (Table 1). On day 8 in 20‰ water, the plasma osmolality (571 mosmol kg^{-1}) was slightly hyperosmotic to

Table 1. *Effects of progressive increase in salinity from 0.7‰ to 20‰ on the osmolality and concentrations of Na^+ and Cl^- in the plasma of* H. signifer

	0.7‰ (control)	20‰
Osmolality (mosmol kg^{-1})	416±19.3	571±4.21*
$[Na^+]$ (mmol l^{-1})	167±7.18	231±6.65*
$[Cl^-]$ (mmol l^{-1})	164±10.0	220±4.77*

Results represent means ± S.E.M. (N=6).
*Significantly different from 0.7‰ control condition at P<0.05.

that of the external medium (543 mosmol kg^{-1}). The rate of ammonia excretion showed a decreasing trend through the 8-day period, especially in 20‰ water from day 5 onwards (Fig. 1). The urea excretion rate decreased significantly on day 2 and day 3 when the experimental specimens were exposed to 5‰ and 10‰ water, respectively, but increased back to the control level thereafter (Fig. 2). In comparison, the urea excretion rate of the marine ray *T. lymma* in seawater was 4.24±1.34 μmol day^{-1} g^{-1}.

The ammonia level in the liver of *H. signifer* in 20‰ water was half that of the control specimens, while plasma ammonia was almost 7-fold higher (Table 2). Urea concentration increased significantly in the muscle, brain and plasma but not in the liver of these specimens (Table 2). Unlike *H. signifer*, there was no increase in blood plasma osmolality or concentrations of Na^+ or Cl^- when *P. motoro* was exposed to increasing salinity from 0.7‰ to 13‰ (Table 3). In addition, there was a decrease in the ammonia level in the muscle of *P. motoro* exposed to increasing salinity (Table 4). Although urea levels in the muscle, liver, brain and plasma increased significantly in *P. motoro* exposed to 13‰ water, these values were very much lower than those in the corresponding tissues of *H. signifer* (Table 4).

A full complement of OUC enzymes was detected in the liver of *H. signifer* (Table 5). The CPS activity could be enhanced by the addition of *N*-acetyl-L-glutamate (AGA) but was refractory to uridine triphosphate (UTP) inhibition

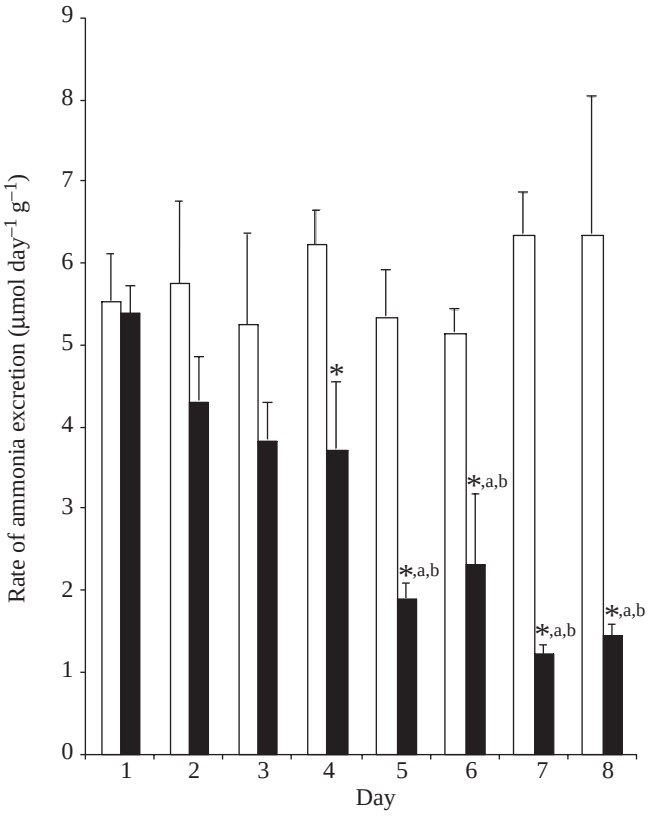

Fig. 1. Changes in the ammonia excretion rate (μmol day^{-1} g^{-1} fish) of *H. signifer* during progressive increase in salinity from 0.7‰ to 20‰ though an 8-day period. Open bars represent constant 0.7‰ salinity for 8 days (control). Filled bars represent increasing salinity from 0.7‰ (day 1) to 5‰ (day 2) to 10‰ (day 3) to 15‰ (day 4) to 20‰ (day 5), where it remained until day 8. Results represent means + S.E.M. (N=3). * Significantly different from 0.7‰ control condition at P<0.05; ^{a,b} significantly different from the corresponding day 1 and 2 values, respectively, at P<0.05.

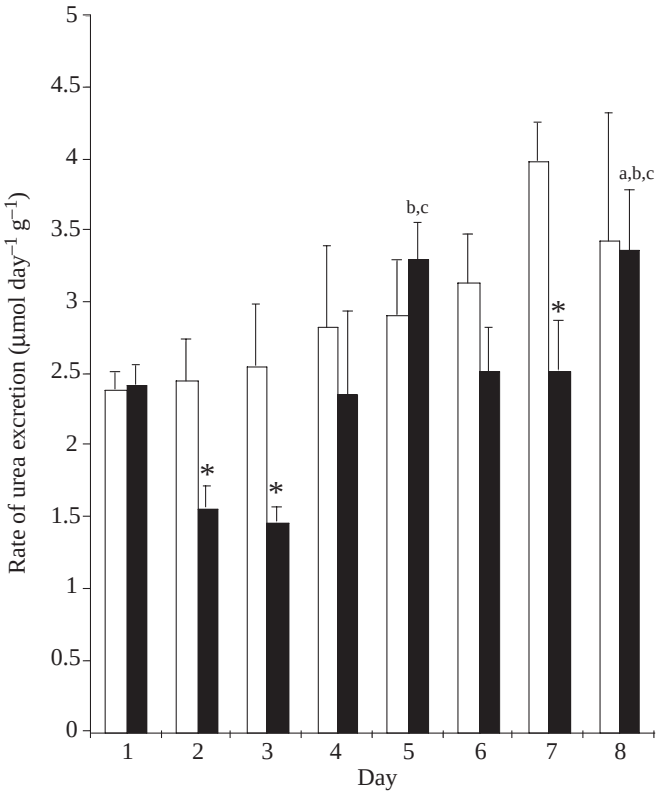

Fig. 2. Changes in the urea excretion rate (μmol day^{-1} g^{-1} fish) of *H. signifer* during progressive increase in salinity from 0.7‰ to 20‰ though an 8-day period. Open bars represent constant 0.7‰ salinity for 8 days. Filled bars represent increasing salinity from 0.7‰ (day 1) to 5‰ (day 2) to 10‰ (day 3) to 15‰ (day 4) to 20‰ (day 5), where it remained until day 8. Results represent means + S.E.M. (N=3). * Significantly different from 0.7‰ control condition at P<0.05; ^{a,b} significantly different from the corresponding day 1 and 2 values, respectively, at P<0.05.

Table 2. *Effects of progressive increase in salinity from 0.7‰ to 20‰ on the ammonia and urea contents in the muscle, liver, brain and plasma of* H. signifer

	[Ammonia]		[Urea]	
Tissues	0.7‰ (control)	20‰	0.7‰ (control)	20‰
Muscle (μmol g^{-1} wet mass)	2.63±0.60	1.98±0.64	70.9±7.73	107±4.47*
Liver (μmol g^{-1} wet mass)	5.06±0.42	2.21±0.20*	49.4±5.22	65.7±12.7
Brain (μmol g^{-1} wet mass)	2.46±0.15	2.89±0.50	59.3±6.37	91.6±1.89*
Plasma (mmol l^{-1})	0.33±0.09	2.25±0.02*	43.8±1.23	82.6±8.40*

Results represent means ± S.E.M. (N=5).
*Significantly different from the corresponding control condition at P<0.05.

Table 3. *Effects of progressive increase in salinity from 0.7‰ to 13‰ on the osmolality and concentrations of Na^+ and Cl^- in the plasma of* P. motoro

	0.7‰ (control)	13‰
Osmolality (mosmol kg^{-1})	349±16	378±9
[Na^+] (mmol l^{-1})	157±16	166±7
[Cl^-] (mmol l^{-1})	163±14	180±5

Results represent means ± S.E.M. (N=4).

(Table 5). The activities of GS, CPS, OTC and arginase were significantly higher in the liver of *H. signifer* exposed to 20‰ water compared with those in 0.7‰ and 10‰, whereas the activities of OUC enzymes in 10‰ water were intermediate between those in 0.7‰ and 20‰ water, except for arginosuccinate synthetase + lyase (ASS+L) (Table 5). A full complement of OUC enzymes was also found in the stomach of *H. signifer* (Table 6). At higher salinities, there was no increment in the enzymatic activities, with the exception of CPS in specimens exposed to 20‰ water (Table 6). The OUC enzymes were absent in the muscle, brain and intestine and hence these data are not presented.

For the marine stingray *T. lymma*, a full complement of OUC enzymes was present in the liver (Table 7). The activities of all the enzymes were higher than that of *H. signifer* in freshwater. A full complement of OUC enzymes was found in the stomach of *T. lymma* as well (Table 7).

In *H. signifer* exposed to 20‰ water, β-alanine and proline accumulated in the muscle (Table 8). The total FAA (TFAA) content was higher in the muscle of specimens exposed to 20‰ water as compared with that of the freshwater control (Table 8). Higher levels of glutamine and proline were found in the liver (Table 8), while β-alanine, glutamate, glutamine and glycine accumulated in the brain (Y. K. Ip and S. F. Chew, unpublished results). No major changes were observed in concentrations of FAAs in the plasma of the experimental or the control fish (Table 8). No TMAO was detected in the muscle or liver of *H. signifier* kept in freshwater or exposed to 20‰ water using the method adopted in this study. For *T. lymma*, 30.6±8.69 μmol g^{-1} and 1.54±0.34 μmol g^{-1} of TMAO were detected in the muscle and liver, respectively, of specimens kept in seawater (30‰).

Discussion

H. signifer *is ureogenic and ureotelic in freshwater*

In the laboratory, *H. signifer* could survive in freshwater (0.7‰) indefinitely or in brackish water (20‰) for at least two weeks. In freshwater, the blood plasma osmolality (416 mosmol kg^{-1}) was maintained hyperosmotic to that of the external medium (38 mosmol kg^{-1}) (Table 1). There was approximately 44 mmol l^{-1} of urea in the plasma (Table 2), with the rest of the osmolality made up mainly by Na^+ (167 mmol l^{-1}) and Cl^- (164 mmol l^{-1}) (Table 1). Judging by these parameters, *H. signifer* appears to adapt more effectively to freshwater than does *Dasyatis sabina*, another freshwater stingray inhabiting the St John River in Florida (Piermarini and Evans, 1998). *D. sabina* also hyperregulates its plasma osmolality (621.4 mosmol kg^{-1}), but to a greater

Table 4. *Effects of progressive increase in salinity from 0.7‰ to 13‰ (and kept at 13‰ for 4 days) on the ammonia and urea levels in the muscle, liver, brain and plasma of* P. motoro

	[Ammonia]		[Urea]	
Tissues	0.7‰ (control)	13‰	0.7‰ (control)	13‰
Muscle (μmol g^{-1} wet mass)	2.06±0.44	0.99±0.13*	0.38±0.08	1.16±0.10*
Liver (μmol g^{-1} wet mass)	1.64±0.28	1.17±0.15	0.04±0.01	0.47±0.06*
Brain (μmol g^{-1} wet mass)	1.49±0.17	1.35±0.13	0.38±0.05	1.29±0.15*
Plasma (mmol l^{-1})	0.25±0.04	0.30±0.03	0.65±0.17	1.28±0.07*

Results represent means ± S.E.M. (N=4).
*Significantly different from the corresponding control condition at P<0.05.

Table 5. *Changes in the units of activities per g tissue of glutamine synthetase (GS), carbamoylphosphate synthetase (CPS), ornithine transcarbamoylase (OTC), arginosuccinate synthetase + lyase (ASS+L) and arginase in the liver of* H. signifer *after progressive increase in salinity from 0.7‰ to 10‰ or 20‰*

		Enzyme activity (μmol min^{-1} g^{-1} wet mass)		
Enzyme	Substrate and effector	0.7‰ (control)	10‰	20‰
GS	–	4.97±1.32	7.30±1.30	14.7±1.31*,†
OTC	–	22.8±5.73	35.1±7.72	48.8±2.80*
ASS+L	–	0.19±0.07	0.44±0.12	0.18±0.04
Arginase	–	29.6±10.2	68.6±12.0	114±9*,†
CPS	Glutamine	0.04±0.01	0.13±0.05	0.10±0.02
	Glutamine + AGA	0.15±0.04	0.24±0.03	0.35±0.05*
	Glutamine + AGA + UTP	0.13±0.04	0.21±0.03	0.31±0.04*

AGA, *N*-acetyl-L-glutamate; UTP, uridine triphosphate.
Results represent means ± S.E.M. (*N*=4).
*Significantly different from 0.7‰ control condition at $P<0.05$.
†Significantly different from 10‰ at $P<0.05$.

extent than does *H. signifer*, with plasma Na^+, Cl^- and urea concentrations of 211.9 mmol l^{-1}, 207.8 mmol l^{-1} and 195.9 mmol l^{-1}, respectively (Piermarini and Evans, 1998). In this regard, *H. signifer* and *D. sabina* are very different from the South American freshwater stingray *P. motoro*, which had a blood osmolality of <350 mosmol kg^{-1} (Table 3) and <1 mmol l^{-1} of urea in the plasma (Table 4). Potamotrygonid rays have apparently lost the capability to synthesize urea and are primarily ammonotelic, probably because they have been isolated in the Amazon River Basin from the ocean for over 65 million years (Thorson et al., 1983; Lovejoy, 1997).

In freshwater, *H. signifer* was not completely ureotelic, excreting up to 45% of its nitrogenous waste as urea (Fig. 2). Similar to marine elasmobranchs, it had a functional OUC with CPS III in the liver (Table 5). CPS III characteristically uses glutamine (preferential to NH_4^+) as a nitrogen donor, is activated allosterically by AGA, is present in mitochondria and is not inhibited by UTP. Although significantly lower than that of the marine *T. lymma* in seawater (0.48 μmol min^{-1} g^{-1}; Table 7), the activity of CPS III in the liver of *H. signifer* in freshwater (0.13 μmol min^{-1} g^{-1}; Table 5) was remarkably high. Hence, it can be concluded that the capacity (maximal activity) to synthesize urea had been downregulated in *H. signifier* in a freshwater environment. However, the remarkable capacity to synthesize urea in freshwater implies that *H. signifer* must excrete urea constantly in order not to create any osmotic problem.

In order to maintain the concentration of urea at a steady state under a certain environmental condition, the rate of urea excretion must be balanced with the rate of urea production. In freshwater, the rate of urea excretion, and hence the rate of urea synthesis, in *H. signifier* was 2.4 μmol day^{-1} g^{-1} (Fig. 2). In a 100 g specimen, there was approximately 50 g muscle, 3 g liver, 0.8 g brain and 2 ml plasma (Y. K. Ip and S. F. Chew, unpublished results). Therefore, for a 100 g fish, the

Table 6. *Changes in the units of activities per g tissueof glutamine synthetase (GS), carbamoylphosphate synthetase (CPS), ornithine transcarbamoylase (OTC), arginosuccinate synthetase + lyase (ASS+L) and arginase in the stomach of* H. signifer *after progressive increase in salinity from 0.7‰ to 10‰ or 20‰*

		Enzyme activity (μmol min^{-1} g^{-1} wet mass)		
Enzyme	Substrate and effector	0.7‰ (control)	10‰	20‰
GS	–	6.03±0.86	10.5±2.90	6.84±0.68
OTC	–	8.40±0.86	6.71±1.92	5.25±0.72
ASS+L	–	0.42±0.09	0.45±0.11	0.46±0.04
Arginase	–	5.57±2.22	15.1±3.26	5.40±0.30
CPS	Glutamine	0.03±0.01	0.01±0.005	0.15±0.07*,†
	Glutamine + AGA	0.10±0.05	0.03±0.01	0.45±0.18*,†
	Glutamine + AGA + UTP	0.09±0.04	0.03±0.01	0.41±0.16*,†

AGA, *N*-acetyl-L-glutamate; UTP, uridine triphosphate.
Results represent means ± S.E.M. (*N*=4).
*Significantly different from 0.7‰ control condition at $P<0.05$.
†Significantly different from 10‰ at $P<0.05$.

Table 7. *Units of activities per g tissue of glutamine synthetase (GS), carbamoylphosphate synthetase (CPS), ornithine transcarbamoylase (OTC), arginosuccinate synthetase + lyase (ASS+L) and arginase in the liver and stomach of* T. lymma *in seawater (30‰) at pH 8*

Enzymes	Substrate and effector	Enzyme activity (μmol min^{-1} g^{-1} wet mass) Liver	Stomach
GS	–	15.6±1.59	22.44±8.09
OTC	–	34.8±3.09	15.27±5.64
ASS+L	–	0.94±0.10	0.30±0.12
Arginase	–	84.8±12.4	32.61±12.37
CPS	Glutamine	0.13±0.02	0.0011±0.0072
	Glutamine + AGA	0.54±0.06	0.0059±0.0026
	Glutamine + AGA + UTP	0.48±0.05	0.0055±0.0025

AGA, *N*-acetyl-L-glutamate; UTP, uridine triphosphate.
Results represent means ± S.E.M. (*N*=4).

rate of urea excretion was 2.4×100=240 μmol day^{-1} or 0.17 μmol min^{-1}. Hence, to sustain the steady-state level of urea in the body of *H. signifer* in freshwater, the liver must be synthesizing urea at a rate of 0.057 μmol min^{-1} g^{-1} (liver). This is much lower than the maximal activity (based on CPS III activity) present in a 3 g liver (0.13 μmol min^{-1} g^{-1} × 3 g=0.39 μmol min^{-1}; Table 5) of a 100 g fish. As for *T. lymma*, the urea excretion rate (or the rate of urea synthesis) in seawater was 4.24±1.34 μmol day^{-1} g^{-1}. In a 100 g fish, this would translate into a urea synthesis rate of 0.098 μmol min^{-1} g^{-1} in the liver, which is approximately 2-fold higher than that in *H. signifer* in freshwater. Hence, besides having a lower maximal capacity of urea synthesis as determined by OUC enzyme activities *in vitro*, freshwater *H. signifier* also had a lower rate of urea synthesis *in situ*.

The above analysis assumes that the liver is the main site of urea formation. Recently, it has been reported that urea synthesis can also take place in the muscle of certain teleost fishes (Anderson, 2001). Here, we report for the first time the presence of a complete OUC in the stomach of elasmobranchs, i.e. both *H. signifer* (Table 6) and *T. lymma* (Table 7), although the activity of CPS III in the stomach of *T. lymma* was relatively low. Going by the activities of CPS III in *H. signifer*, gram by gram, the capacity of OUC in the stomach was approximately 70% that in the liver. It has been reported previously that in mammalian species the only tissue besides liver that has both CPS I and OTC activities is the intestinal mucosa (Jones et al., 1961; Raijman, 1974). However, ASS+L is not present in the intestinal mucosa, and hence a complete OUC is lacking (Meijer et al., 1990). The use of CPS III by most piscine systems requires that GS be intimately involved with the OUC, co-localized to the mitochondria with CPS III (Anderson and Casey, 1984; Walsh and Mommsen, 2001). Incidentally, the GS activity in the stomach of *H. signifer* was high, reaching 124% that in the liver (Table 6). This observation is in agreement with a recent report that the stomach and intestine of the four-eyed sleeper *Bostrichthys sinensis* (teleost) exhibit high GS activity (Anderson et al., 2002). Together, this information indicates that the piscine stomach is an important organ involved in nitrogen metabolism, presumably after feeding rather than simply as a digestive organ (see below). Elasmobranchs are carnivorous and require high protein diets for urea synthesis; an OUC in the stomach would represent the first line of 'defence' against the release of ammonia through protein and amino acid degradation along the digestive tract and to produce a useful osmolyte, i.e. urea, upon feeding.

Urea synthesis is energy expensive; 5 moles of ATP is needed to synthesize one mole of urea *de novo* in fish (except lungfishes, which possess CPS I). Why would *H. signifer* synthesize urea and excrete 47% of its nitrogenous wastes in this form instead of ammonia in freshwater? Unlike potamotrygonid rays living in the Amazon River, *H. signifer* might have invaded the freshwater environment only recently. It is unclear at this moment if *H. signifer* would return to brackish water to reproduce, like the bull shark *Carcharhinus leucas* of Lake Nicarrgua (Thorson, 1976). Although *H. signifer* can be found in Batang Hari as far as 400 km from the South China Sea, there is still the possibility that it may re-enter estuarine and marine environments. Therefore, it would be essential for *H. signifer* to retain the ureosmotic osmoregulatory mechanisms to survive in higher salinities.

Increase in the rate of urea synthesis during exposure to higher salinities

In 20‰, the plasma osmolality in *H. signifier* increased significantly from 416 mosmol kg^{-1} (in freshwater) to 571 mosmol kg^{-1} (Table 1). The increase in plasma Na^+ and Cl^- concentrations could only account for 77% of the difference (155 mosmol kg^{-1}) involved. The rest was apparently made up with urea, the plasma concentration of which increased by 38.8 mmol l^{-1}. These results indicate that *H. signifier* is ureosmotic, albeit with limited capacity in either urea synthesis or urea retention or both (see below).

When *H. signifer* was exposed to a progressive increase in salinity (0.7‰ to 5‰ to 10‰ to 15‰ to 20‰) through an 8-day period, there was a continuous decrease in the rate of ammonia excretion (Fig. 1). By day 8, i.e. 4 days after exposure to 20‰ water, the ammonia excretion rate was only 20% that of the freshwater control. Yet, there was no change in the ammonia contents in the muscle and brain, and the ammonia content in the liver decreased instead (Table 2). This suggests that ammonia was used as a substrate for the synthesis of urea, which was essential for osmoregulation at higher salinities. Indeed, in 20‰ water, urea levels in the muscle, brain and plasma increased significantly (Table 2).

In a 100 g specimen, the reduction in the ammonia excretion rate during the 8-day period can be calculated as [(5.5–5.39) + (5.77–4.32) + (5.27–3.84) + (6.23–3.74) + (5.36–1.91) + (5.16–2.32) + (6.36–1.24) + (6.36–1.45)] × 100=

Table 8. *Effects of progressive increase in salinity from 0.7‰ to 20‰ on the concentrations of various free amino acids (FAAs) and total FAA (TFAA) in the muscle, liver and plasma of* H. signifer

	Concentration (μmol g^{-1} wet mass tissue or mmol l^{-1} plasma)					
	Muscle		Liver		Plasma	
FAAs	0.7‰	20‰	0.7‰	20‰	0.7‰	20‰
Alanine	2.05±0.51	1.80±0.35	0.72±0.16	0.40±0.09	0.12±0.01	0.13±0.009
Arginine	0.28±0.05	0.14±0.05	0.02±0.001	0.06±0.03	0.09±0.01	0.08±0.01
Asparagine	0.13±0.01	0.49±0.07*	0.29±0.03	0.22±0.04	0.009±0.004	0.007±0.0005
Aspartate	0.26±0.10	0.26±0.06	0.05±0.008	0.12±0.03	0.03±0.003	0.04±0.005*
β-alanine	2.84±0.93	9.09±0.64*	0.23±0.10	0.71±0.22	0.02±0.01	0.04±0.005
Glutamine	0.46±0.03	0.96±0.08	1.09±0.09	2.15±0.35*	0.01±0.002	0.02±0.003
Glutamate	0.84±0.26	1.68±0.35	0.10±0.02	0.25±0.08	0.09±0.006	0.16±0.02*
Glycine	5.28±1.66	11.5±3.14	0.36±0.07	0.51±0.07	0.11±0.03	0.11±0.018
Histamine	0.05±0.01	0.03±0.01	0.11±0.004	0.09±0.03	0.03±0.008	0.03±0.002
Isoleucine	0.11±0.02	0.12±0.02	0.08±0.03	0.03±0.008	0.08±0.01	0.07±0.01
Leucine	0.19±0.04	0.23±0.05	0.15±0.04	0.08±0.02	0.17±0.03	0.15±0.03
Lysine	0.04±0.02	0.05±0.01	0.01±0.007	0.006±0.003	0.02±0.003	0.03±0.006
Methionine	0.24±0.02	0.46±0.08*	0.30±0.05	0.32±0.07	0.13±0.02	0.18±0.03
Phenylalanine	0.48±0.17	0.68±0.12	0.41±0.13	0.45±0.08	0.04±0.005	0.02±0.005*
Proline	0.76±0.14	3.90±0.74*	0.56±0.20	1.72±0.12*	0.24±0.02	0.36±0.18
Serine	0.70±0.12	0.46±0.08	0.10±0.02	0.09±0.02	0.07±0.01	0.07±0.007
Taurine	26.0±1.75	26.0±2.46	17.6±2.97	17.5±2.93	1.43±0.48	0.58±0.10
Threonine	0.39±0.16	0.22±0.05	0.26±0.07	0.16±0.02	0.08±0.01	0.06±0.002
Tryptophan	0.06±0.02	0.07±0.007	0.04±0.02	0.01±0.005	0.04±0.009	0.06±0.004
Tyrosine	0.03±0.007	0.09±0.01*	0.04±0.01	0.04±0.03	0.03±0.005	0.06±0.003*
Valine	0.21±0.05	0.20±0.04	0.18±0.07	0.08±0.02	0.15±0.03	0.13±0.03
TFAA	38.7±2.32	56.4±4.97*	22.6±3.15	24.7±3.98	2.97±0.39	2.90±0.35

Results represent means ± S.E.M. (N=4).
*Significantly different from corresponding control condition at P<0.05.

2252 μmoles (calculated from Fig. 1). The increase in ammonia-equivalents stored as urea in the tissue at the end of day 8 in 20‰ water was {[(107–70.9)×50]+[(91.6–59.3)×0.8]+[(82.6–43.8)×2]}×2=3817 μmoles (calculated from Table 2, without liver data which showed no significant changes). Of this, it becomes obvious that 59% could be accounted for simply by the reduction in ammonia excretion, with the rest contributed by a reduction in urea excretion.

These results indirectly indicate that an increase in the rate of urea synthesis *in situ* must have occurred in *H. signifer* during the first 4 days of progressive exposure from freshwater to 20‰ water because unexcreted ammonia was being converted to urea. An evaluation of the activities of OUC enzymes at the end of day 8 (in 20‰) supports this proposition (Table 5). There were significant increases in the total activities (units g^{-1} tissue) of CPS III, OTC, arginase and GS in specimens exposed to 20‰ water. In 10‰ water (day 3), specimens had an OUC capacity intermediate between those of freshwater and 20‰ water. Hence, it can be concluded that there was a gradual upregulation of activities of OUC enzymes in response to increasing salinity.

In freshwater, the rate of urea excretion in a 100 g *H. signifer* was 0.17 μmol min^{-1} (see above), which was one-third of the total activities of CPS III in a 3 g liver (0.39 μmol min^{-1}) determined *in vitro* in the presence of saturating concentrations of substrates and AGA. Assuming the amount of ammonia retained in the body on day 3 was all turned into urea, there must be an increase of (5.27–3.84)×100/(24×60)= 0.10 μmol min^{-1} in the rate of urea synthesis (Fig. 1), bringing up the total urea synthesis rate to 0.27 μmol min^{-1}. This is actually well within the capacity of the CPS III present in the liver (0.39 μmol min^{-1}). Hence, theoretically it would be unnecessary to induce CPS III activity through increasing the concentration of this enzyme. Moreover, by the time the new steady-state concentration of urea was reached in specimens exposed to 20‰ water on day 5, the rate of urea synthesis returned back to the control value, which could be adequately sustained by the OUC capacity in the liver of the specimen in freshwater.

Why then did the activities of CPS III in the liver increase 2.4-fold in specimens exposed to 20‰ water (Table 5)? This could be due to the fact that CPS III activity depends on the presence of AGA, the concentrations of which in the liver and stomach of *H. signifer* are unknown at present. Results obtained with *H. signifer* indicate that hepatic AGA might not reach the level required to derive maximal capacity of CPS III during salinity adaptation, leading to the need to produce greater concentrations of CPS III. Alternatively, CPS III may not function at V_{max} *in vivo* due to the lower than saturation

concentrations of glutamine or ATP. Exposure to higher salinity also led to an increase in the activities of CPS III in the stomach of *H. signifer* (Table 6), which supports the above proposition that the stomach could be involved in producing urea for osmoregulation in addition to its digestive function.

H. signifer *has limited capacity to retain urea in brackish water of high salinity*

Working with the marine little skate *Raja erinacea*, Goldstein and Forster (1971) reported that the plasma urea concentration decreased from 390 mmol l^{-1} to 240 mmol l^{-1} when the skate was adapted progressively from 100% to 50% seawater. By contrast, the urea concentration in the plasma of *H. signifier* in 20‰ (67%) seawater was only 82.6 mmol l^{-1}. Despite the much lower concentration of urea in the plasma, the rate of urea excretion in *H. signifer* (2.4±0.42 μmol day^{-1} g^{-1}; Fig. 2) in freshwater was obviously high, although not as high as that of the marine *T. lymma* (4.24±0.80 μmol day^{-1} g^{-1}), which has a much higher plasma urea concentration (383±11.1 mmol l^{-1}), in seawater. This suggests that *H. signifer* reduced its capability to retain urea, in addition to reducing its capability to synthesize urea, in order to survive in a freshwater environment. There was a significant decrease in the rate of urea excretion in *H. signifer* during passage through 5‰, 10‰ and 15‰ water (Fig. 2). However, the rate of urea excretion increased back to the control value (3.2 μmol day^{-1} g^{-1}) when the specimens reached 20‰ water on day 5, presumably resulting from the steeper urea gradient built up between the blood plasma (83 mmol l^{-1}) (Table 2) and the external medium (0 mmol l^{-1}).

The rate of urea loss from the body can be calculated according to the formula $R=[\text{urea}]_{\text{plasma}}\times k$, where R is the rate of urea excretion in μmol day^{-1} g^{-1}, $[\text{urea}]_{\text{plasma}}$ is plasma urea concentration in mmol l^{-1}, and k is the rate constant in ml g^{-1} day^{-1}. Assuming the urea concentration in the external medium to be zero, the value for k in specimens exposed to freshwater can be calculated as 2.4/(44–0)=0.055 (Table 2; Fig. 2). This value is 5-fold greater than that of *T. lymma*, which had a plasma urea concentration of 380 mmol l^{-1} and a k value of 4.24/(380–0)=0.011. This could mean that either the permeability of the branchial epithelial and skin surfaces to urea was increased or the uptake of urea through the gills and kidney was reduced in *H. signifer* in freshwater in comparison with the marine *T. lymma* in seawater. *H. signifer* could apparently alter the k value for urea when exposed to higher salinities. On day 5 (first day in 20‰ water), the k value for *H. signifer* decreased by 29% to 3.2/(83–0)=0.039 (Table 2; Fig. 2), which is still 3-fold higher than the value of 0.011 for *T. lymma*. Since *H. signifer* could upregulate its urea synthetic capacity (as indicated by the activity of CPS III) to that of *T. lymma*, the reason for its inability to survive well in waters of >20‰ appears to be its limited capacity to retain urea as a consequence of adapting to live in a freshwater environment.

FAAs in tissues remain relatively unchanged during salinity adaptation

Exposure to 20‰ water did not have much effect on the concentrations of FAAs in the tissues of *H. signifer* and it can be concluded that FAAs were not involved in cell volume regulation in brackish water. At high concentration, urea alters many macromolecular structures and functions; for example, assembly of collagen and microtubules and inhibition of enzymes (Yancey, 2001). Some osmolytes, especially the methylamines, stabilize macromolecular structure and have a counteracting effect to that of urea. TMAO is usually a better stabilizer than other known osmolytes, including betaine (Yancey, 2001), perhaps explaining why it is the dominant non-urea osmolyte in most ureosmotic fishes. However, no TMAO was detected in any of the tissues and organs of specimens exposed to freshwater or 20‰ water. On the other hand, glycine, sarcosine and β-alanine can also act as counteracting osmolytes (Yancey and Somero, 1980). The presence of two of these amino acids, glycine and β-alanine, in significantly higher levels in the muscle and liver of *H. signifer* exposed to 20‰ water suggests that they might have a counteracting function when urea built up in these tissues (Table 8). A 1.5-fold build up of urea in the muscle and liver (Table 2) was counter-balanced by at least a 3-fold increment in β-alanine and a 1.5-fold increment in glycine. Taurine is usually found in moderate levels in tissues of marine elasmobranchs (skates; King and Goldstein, 1983). By contrast, the levels of taurine in the muscle and liver of *H. signifier* were high, reaching >20 μmol g^{-1} tissue. It is possible that taurine can also act as a counteracting osmolyte despite the fact that its concentration remained unchanged during salinity adaptation.

The rate of nitrogen metabolism was higher in freshwater than in 20‰ water

In freshwater, the rate of nitrogenous waste (ammonia + urea) excretion in *H. signifer* was 11.2 μmol N day^{-1} g^{-1} (calculated from Figs 1 and 2), which was higher than that in 20‰ water on day 5 (8.50 μmol N day^{-1} g^{-1}). In fact, from day 2 onwards, the total nitrogen excreted was consistently higher in *H. signifer* exposed to freshwater than those exposed to increased salinity. It is logical to deduce that a higher rate of ammonia production, and hence amino acid catabolism, occurred in specimens exposed to freshwater. Since the specimens were not fed during the experiment, presumably the carbon chains released from amino acid catabolism were channelled into ATP production. Hence, these results suggest that the energy demand in specimens surviving in freshwater was higher than that in specimens surviving in brackish water (20‰). *H. signifer* maintained its blood hyperosmotic to the external medium, with the difference in osmolality between the blood and the external medium being much greater in freshwater (416–39=377 mosmol kg^{-1}; Table 1) than in 20‰ (571–543=28 mosmol kg^{-1}; Table 1). As a result, the energy demand with respect to osmoregulation alone was higher in 0.7‰ than in 20‰, because energy was required to drive the various ionic transporters to minimize the loss and/or to increase the uptake of Na^+ and Cl^-. The energetic regulation of putative urea transporters might be involved as well.

Conclusion

Unlike *P. motoro*, *H. signifer* is ureogenic and ureotelic in freshwater. The hyperosmoticity of its blood is greater than other freshwater teleost fishes because of the presence of urea (>40 mmol l^{-1}). Hence, it faces higher energetic demands of osmoregulation due to the steep ionic and urea gradients between the blood and the environment. To survive in freshwater, it reduces the OUC capacity and decreases urea retention as compared with the marine ray *T. lymma*. In 20‰ water, *H. signifer* increases the rate of urea synthesis by upregulating the OUC enzymes, which is accompanied by a reduction in the ammonia excretion rate. However, its capacity to retain urea at higher salinity is limited and consequently it cannot survive well in 20‰ water or beyond.

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