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Exposure to brackish water, upon feeding, leads to enhanced conservation of nitrogen and increased urea synthesis and retention in the Asian freshwater stingray *Himantura signifer*

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Summary

The white-edge freshwater whip ray *Himantura signifer* is ammonotelic in freshwater, but retains the capacities of urea synthesis and ureosmotic osmoregulation to survive in brackish water. The first objective of this study was to examine whether exposure to brackish water would lead to increases in food intake, and/or conservation of nitrogen in *H. signifer* upon daily feeding. Results obtained showed that a progressive increase in ambient salinity, from 1‰ to 15‰ over a 10-day period, did not lead to an increase in daily food intake. However, there were significant reductions in daily rates of ammonia and urea excretion in *H. signifer* during salinity changes, especially between day 5 (in 10‰ water) and day 10 (in 15‰ water) when compared to those of the control kept in 1‰ water. Consequently, there was a significant decrease in the percentage of nitrogen (N) from the food being excreted as nitrogenous waste (ammonia-N+urea-N) during this period. On day 10, the tissue urea contents in fish exposed to 15‰ water were significantly greater than those of fish kept in 1‰ water, and the excess urea-N accumulated in the former fish could totally account for the cumulative deficit in excretion of urea-N+ammonia-N during the 10-day period. Thus, it can be concluded that *H. signifer* is N-limited, and conserved more N from food when exposed to brackish water. The conserved N was converted to urea, which was retained in tissues for osmoregulation. The second objective of this study was to elucidate whether the retention of the capacity of N conservation in *H. signifer* would lead to an accumulation of urea in fish exposed to not only 15‰ water, but also 1‰ water, upon feeding. For fish pre-acclimated to 1‰ water or 15‰ water for 10 days and then fasted for 48 h, the rate of ammonia excretion in fish exposed to 15‰ water was consistently lower than that of fish exposed to 1‰ water, throughout the 36-h post-feeding period. In addition, the hourly rate of urea excretion in the former was significantly lower than that of the latter between hours 12 and 36. There were postprandial increases in ammonia contents in the muscle, liver, stomach, intestine, brain and plasma of fish kept in 1‰ water; but postprandial increases in ammonia occurred only in the liver and brain of fish exposed to 15‰ water, and the magnitudes of increases in the latter were smaller than those in the former. Indeed, postprandial increases in tissue urea contents occurred in both groups of fish, but the greatest increase in urea content was observed in the muscle of fish exposed to 15‰ water. Taken together, these results indicate that *H. signifer* in freshwater could be confronted with postprandial osmotic stress because of its capacity of conserving N and increasing urea synthesis upon feeding.

Key word: ammonia, feeding, stingray, *Himantura signifer*, nitrogen metabolism, osmoregulation, urea.

Introduction

Marine elasmobranchs are ureogenic because they possess a functional ornithine–urea cycle (OUC) and synthesize urea through carbamoyl phosphate synthetase III (CPS III; Campbell and Anderson, 1991; Anderson, 2001). 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 (Yancey, 2001). Because urea is retained at high concentrations (300–600 mmol l^{-1}) in the body fluid and tissues for osmotic water retention (Ballantyne, 1997; Perlman and Goldstein, 1998), marine elasmobranchs are described as ureosmotic. Urea retention is accomplished by a low permeability of the branchial epithelium to urea and by re-absorption of urea in the gills (Smith and Wright, 1999) and kidney (Morgan et al., 2003a,b). However, in spite of low urea

permeabilities (Fines et al., 2001), marine elasmobranchs are ureotelic, and the majority of their waste nitrogen (N) is excreted as urea *via* the gills (Shuttleworth, 1988; Wood, 1993; Wood et al., 1995; Perlman and Goldstein, 1998) as a result of the large gradient of urea concentration between their bodies and the ambient seawater.

Urea synthesis is energy intensive; 5 μmol of ATP are required for the formation of every mole of urea. Because urea-N is much more costly to make than ammonia-N, Mommsen and Walsh (1991) postulated that marine elasmobranchs would excrete excess nitrogen, over and above the needs of osmoregulation, in the form of ammonia-N rather than urea-N after feeding. When the dogfish shark was infused with ammonia at a rate of 1500 $\mu mol\ kg^{-1}\ h^{-1}$ for 6 h (Wood et al., 1995), both ammonia-N and urea-N excretion increased by similar extents during infusion, though the former more rapidly, and the entire ammonia-N load (actually 132%) was excreted within 18 h. Based on this, Wood (2001) concluded that the postulate of Mommsen and Walsh (1991) might be partially correct, although he pointed out that NH_4Cl infusion was very different from natural feeding. At the same time, Wood (2001) argued that marine elasmobranchs were N-limited, and suggested that they would avoid the loss of N after feeding by converting as much excess N as possible to urea. Indeed, feeding via a stomach tube results in no increase in urea-N excretion, but only a very small increase in excretion of ammonia in the Pacific spiny dogfish (Wood et al., 2005). So, these results (Wood et al., 2005) are in support of the proposition made by Wood (2001) earlier. Because there was only a small increase in ammonia excretion after feeding, Wood et al. (2005) concluded that their results also supported the postulate of Mommsen and Walsh (1991).

Since the nitrogen-limiting status of a marine elasmobranch is defined by the salinity of the external medium, the most direct approach to test the postulate of Mommsen and Walsh (1991) is to acclimatize marine elasmobranchs to diluted or full-strength seawater before feeding experiments. However, marine elasmobranchs are rarely euryhaline and can usually tolerate no more than 30% change in ambient salinity. The availability of the white-edge freshwater whip ray, *Himantura signifer* Compagno and Roberts 1982 (Family: Dasyatidae), which can survive well in brackish water (Tam et al., 2003) in South East Asia presented us with the opportunity to examine the hypotheses of Mommsen and Walsh (1991) and Wood (2001) using an approach different from that of Wood et al. (2005). *H. signifer* is found in the Batang Hari Basin in Jambi of Sumatra in Indonesia. It retains the ability to synthesize urea but reduces the capacity of retaining it in freshwater (Tam et al., 2003). Although this stingray can be found in Batang Hari, as far as 400 km from the South China Sea, it may re-enter estuarine and marine environments during the breeding season. Indeed, *H. signifer* possesses ureosmotic osmoregulatory mechanisms to survive in brackish water (Tam et al., 2003). However, that means, in freshwater, *H. signifer* has to suppress both urea production and urea retention, including active urea re-absorption (Tam et al., 2003). Hence, *H. signifer* is the most desirable species for studies on the effects of salinity changes on the excretion and retention of food-N after feeding, because it is ureogenic, ureosmotic and euryhaline.

In the first series of experiments, fish were divided into two groups. One group was kept in 1‰ water (control) for 10 days, while the other group was exposed to a progressive increase in salinity, reaching 15‰ on days 9 and 10. Food was provided every day during this 10-day period, and the objective was to examine the effects of salinity changes on the daily food ration of *H. signifer*. The daily excretion rates of ammonia and urea were also determined in order to estimate the percentage of food-N being excreted as ammonia-N+urea-N. On day 10, fish were sacrificed for the collection of tissues for analyses of ammonia and urea. We aimed to answer three important questions in this series of experiment. Would a progressive increase in ambient salinity lead to a greater food intake in *H. signifer*? Would an increase in ambient salinity result in a reduction in nitrogenous waste excretion, and therefore an increase in retention of N, after feeding in *H. signifer*? Would there be a greater rate of urea synthesis and a greater retention of urea in specimens of *H. signifer* exposed to brackish water as compared with those kept in freshwater?

Through the determination of ammonia and urea excretion rates and the examination of the gut content of fish being sacrificed after feeding, we obtained preliminary results which indicated that complete digestion of a meal in *H. signifer* took at least 48 h, which was longer than the time taken by some other fishes (e.g. *Protopterus dolloi*, Lim et al., 2004; *Periophthalmodon schlosseri*, Ip et al., 2004). So, in order to determine the effects of a single food ration on N excretion and retention in *H. signifer*, fish were kept in freshwater (1‰) or exposed to a progressive increase in salinity through a 10-day period as in the first series of experiments. On day 11, both groups of fish were fasted for 48 h; food was then provided on day 13. Water samples were collected during the next 36 h for the determination of hourly ammonia and urea excretion rates. Some fish were sacrificed at various time points for the analyses of tissue ammonia and urea contents. A period of 36 h was chosen because preliminary experiments indicated that ammonia and urea contents in tissues of this fish would reach the highest levels between 24 and 36 h post-feeding. Here, we aimed to answer two other questions. Would feeding lead to an increase in urea content in tissues of *H. signifer* kept in freshwater (1‰ water) because of its capacity of N conservation after a meal? Would fish kept in 15‰ water conserve a greater percentage of the daily food-N as urea than fish kept in 1‰ water?

Materials and methods

Animals

Specimens of *Himantura signifer* (200–500 g body mass) were purchased from a local fish farm in Singapore, and maintained individually in approximately 10 volumes (w/v) of water in plastic aquaria in freshwater (0.7‰) at 25°C in the laboratory. Water was changed daily. No attempt was made to

separate the sexes. Fishes were acclimated to laboratory conditions for at least 1 week before experimentation. During that period, fish were fed with freshwater shrimp. All experiments performed in this study were under a 12 h:12 h light:dark regime.

Feed analysis

The wet masses of samples of freshwater shrimp (approximately 1 g, N=3) were obtained to the nearest milligram. They were then freeze-dried and the dry masses recorded. Subsequently, the samples were analyzed for N and carbon (C) using a Eurovector EA3011 Elemental Analyzer (Milan, Italy) equipped with Callidus software. BBOT ($C_{26}H_{26}N_2O_2S$) standard obtained from Eurovector (Milan, Italy) was used as a standard for comparison. In addition some samples were extracted in 70% ethanol for 24 h to remove non-protein N-compounds before being freeze-dried for N and C analyses. The difference obtained between samples with and without ethanol extraction gives an indication of the combined contribution of ammonia, urea, free amino acids, purines and pyrimidines to the N and C contents of the freshwater shrimp.

Series 1

Fish were divided into two groups. Both groups of fish were fed with live freshwater shrimps at about 2% of their body mass. Feeding was performed *ad libitum* until satiation, which took no more than 2 h, as described previously for the giant mudskipper (Ip et al., 2004) and the slender lungfish (Lim et al., 2004). Excess food was removed when the fish stopped feeding. The actual mass of the feed consumed by the fish was calculated as the difference between the mass of shrimps provided initially and the mass of shrimps left over. Fish were then gently transferred individually, by hand, to new tanks (60 cm×30 cm×20 cm, length×width×height) containing either 2 or 4 l of 1‰ water at 25°C, depending on the size of the fish; this point was considered as hour 0 at the start of the experiment. *H. signifer* usually stays relatively quiescent after feeding, and there would be minimal struggling during the transfer when the fish was lifted transiently out of water with its ventral surface supported by both palms. Water samples (3 ml), acidified with 70 μl of 1 mol^{-1} HCl, were collected 24 h later for ammonia and urea analyses. The above procedure was repeated with 1‰ water for the control group (N=5) for 10 days. For the experimental group (N=5), fish were exposed to daily increases in salinity from 1‰ on days 1 and 2 to 5‰ on days 3 and 4, followed by 10‰ on days 5 and 6, 13‰ on days 7 and 8, and 15‰ on days 9 and 10. A gradual increase in salinity was necessary to allow for acclimatization and survival. Water samples were collected for ammonia and urea assays every 24 h. Concentrations of ammonia and urea in water samples were determined according to the methods of Anderson and Little (1986) and Felskie et al. (1998) as modified by Jow et al. (1999), respectively. Ammonia and urea excretion rates were expressed as μmol day^{-1} g^{-1} fish. Five fish in 1‰ water were killed, by severing the spinal cord, for the collection of tissues on day 0. On day 10, 5 fish in 1‰ water and another 5 in 15‰ water for a second day were killed and their tissues collected.

Series 2

Fish were kept in 1‰ water or taken through a progressive increase in ambient salinity as described above. They were allowed to feed *ad libitum* during this 10-day period. On day 11, fish were fasted for 48 h. A known amount of food was provided on day 13 and the fish was allowed to feed until satiation. They were then transferred to other tanks (60 cm×30 cm×20 cm, length×width×height) containing either 2 or 4 l of 1‰ or 15‰ water, depending on the size of the fish. Water samples were collected at 12 h intervals during the subsequent 36 h period post-feeding. Fish were killed at 0 h (before the provision of food) and 12, 24 and 36 h post-feeding for tissue collection. Fish transferred to tanks without the provision of food served as controls.

Collection of tissues and analyses of ammonia and urea

Fish were killed with a strong blow to the head, the blood samples were collected from the severed caudal peduncle into heparinized capillary tubes. The collected blood was centrifuged at 5000 ***g*** at 4°C for 1 min to obtain the plasma. 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. The resulting supernatant was kept at –25°C until analysed. The muscle, liver, stomach, intestine and brain were quickly excised. The excised tissues and organs were immediately freeze-clamped with aluminium tongs pre-cooled in liquid N_2. Frozen samples were kept at –80°C until analysis. The frozen tissue samples were weighed, ground to a powder in liquid nitrogen and homogenized three times in 5 volumes (w/v) of 6% $HClO_4$ at 24 000 revs min^{-1} for 20 s, using an Ultra-Turrax homogenizer, with intervals of 10 s between each homogenization. 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} $KHCO_3$. Ammonia was determined according to the method of Bergmeyer and Beutler (1985) and urea determined as described above. Results were expressed as μmol g^{-1} wet mass tissue or μmol ml^{-1} plasma.

Statistical analyses

Results are presented as means ± standard error of the mean (s.e.m.). Data in all the figures were analysed using repeated-measures analysis of variance (ANOVA) followed by least-square means (LS-MEANS) to evaluate differences between means. Data in all the tables were assessed using one-way analysis of variance followed by Bonferroni's multiple range test to evaluate differences between means. Differences where P<0.05 were regarded as statistically significant.

Results

Feed analysis

For every 1 g wet mass of shrimp (N=3), there was 0.21 g of freeze-dried materials, of which 10.0±0.1% and 38.2±2.1%

of the dry mass were N and C, respectively. So, there was (1 g×0.21 g g^{-1}×10%)×1000 mmol mol^{-1}/14 g mol^{-1} or 1.5 mmol N g^{-1} shrimp. After ethanol extraction, 9.38±0.17% and 36.5±1.6% of the dry mass was N and C, respectively, indicating that proteins were the major contributor of N. The ammonia and urea contents (μmol g^{-1} wet mass) of shrimp were 2.4±0.4 and 0.91±0.22, respectively.

Series 1

The amounts of food consumed by the fish daily remained relatively constant throughout the 10-day period of exposure to a progressive increase in salinity, and were comparable to those of fish kept in 1‰ water (Fig. 1). Using the values in Fig. 1, the total food intakes during the 10-day period for fish kept in 1‰ and 15‰ water were calculated to be 0.142 and 0.150 g g^{-1} fish, respectively.

During the 10-day experimental period, fish kept in 1‰ water excreted a total of 88.5 μmol N g^{-1} fish of ammonia and 73.4 μmol N g^{-1} fish of urea (calculated from Fig. 2). For fish exposed to a progressive increase in salinity, the total amounts of ammonia and urea excreted during the 10-day period were 56.8 and 52.4 μmol N g^{-1} fish, respectively. The daily ammonia excretion rate of *H. signifer* exposed to a progressive increase in salinity was significantly lower than that of fish kept in 1‰ water during the period of day 3 to day 10 (Fig. 2A). The theoretical total amount of ammonia retained in fish exposed to salinity changes can be estimated from Fig. 2A as (3.2+2.5+3.1+3.5+4.9+5.1+4.2+4.1)=30.6 μmol N g^{-1} fish. In addition, the daily urea excretion rate of the experimental fish exposed to increased salinity was significantly lower than that of the control kept in 1‰ water from day 4 onwards (Fig. 2B). So, the theoretical total amount of urea-N retained can be estimated from Fig. 2B as (1.1+1.9+1.7+1.5+2.1+1.9+1.2)×2= 22.8 μmol N g^{-1} fish. Overall, there was a significant decrease in the percentage of food-N being excreted as ammonia-N+urea-N (total-N) by the fish exposed to a progressive increase in salinity on day 5 (10‰ water) and thereafter.

Ammonia contents in the muscle, liver, stomach, intestine, brain and plasma of control fish in 1‰ water on day 0, fish kept in 1‰ water for 10 days and fish exposed to a progressive increase in salinity through a 10-day period were not significantly different from each other (Table 1). Urea contents in the muscle, liver, stomach, intestine, brain and plasma of fish in 1‰ water on day 0 and those kept in 1‰ water for 10 days were comparable. By contrast, there were significant increases in urea contents in all these tissues and organs of fish exposed to a progressive increase in salinity (Table 1).

Because contents of ammonia and urea in fish kept in 1‰ water for 10 days were not significantly different from those of the day 0 fish in 1‰ water, the rate of urea excretion must be balanced with the rate of urea synthesis. Therefore, the averaged daily rate of urea synthesis during this 10-day period

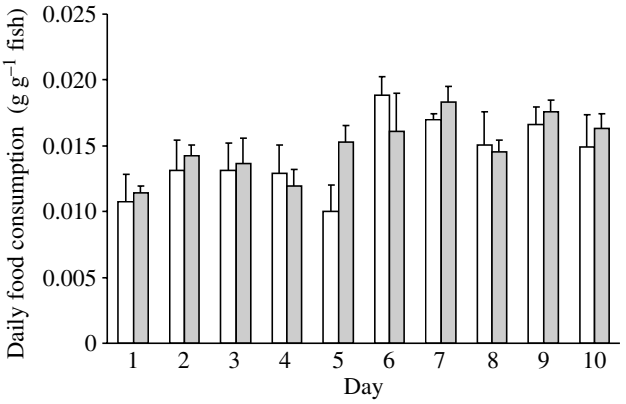

Fig. 1. Daily food consumption (g g^{-1} fish) of *Himantura signifer* during 10 days of exposure to 1‰ water (control; open bars), or 10 days of exposure to a progressive increase in salinity from 1‰ to 15‰ (filled bars): 1‰ (days 1, 2) to 5‰ (days 3, 4) to 10‰ (days 5, 6) to 13‰ (days 7, 8) to 15‰ (days 9, 10). Values are means ± s.e.m. (*N*=5 for control group; *N*=5 for experimental group).

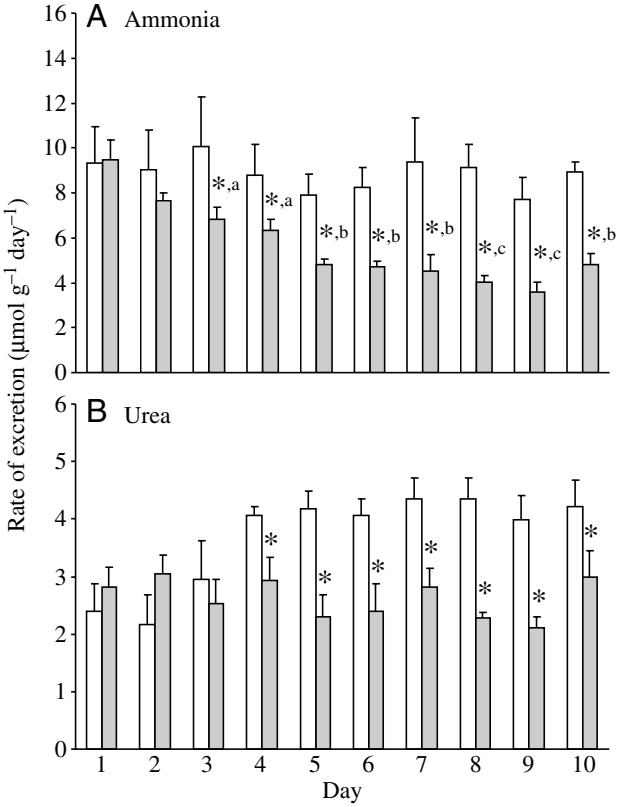

Fig. 2. Daily excretion rates (μmol day^{-1} g^{-1}) of ammonia (A) and urea (B) in *Himantura signifer* during 10 days of exposure to 1‰ water (control; open bars), or 10 days of exposure to a progressive increase in salinity from 1‰ to 15‰ (filled bars): 1‰ (days 1, 2) to 5‰ (days 3, 4) to 10‰ (days 5, 6) to 13‰ (days 7, 8) to 15‰ (days 9, 10). Values are means ± s.e.m. (*N*=5 for control group; *N*=5 for experimental group). *Significantly different from corresponding control value; [a]significantly different from the corresponding day 1 value; [b]significantly different from the corresponding days 1, 2 and 3 values; [c]significantly different from the corresponding days 1, 2, 3 and 4 values.

Table 1. *Ammonia and urea content of plasma and tissues of Himantura signifer in 1‰ water on day 0, after 10 days of exposure to 1‰ water, or a progressive exposure to increased salinity reaching 15‰ for a second day on day 10*

	Ammonia content*			Urea content*		
Tissue	1‰ (day 0)	1‰ (day 10)	15‰ (day 10)	1‰ (day 0)	1‰ (day 10)	15‰ (day 10)
Muscle	2.7±0.2	2.9±0.3	2.3±0.2	69±4	72±2	126±4†,‡
Liver	2.6±0.3	2.5±0.4	1.8±0.2	63±5	60±5	118±3†,‡
Stomach	2.4±0.4	2.3±0.3	1.7±0.3	54±5	57±3	117±4†,‡
Intestine	2.1±0.3	2.6±0.2	2.3±0.2	59±4	63±3	93±4†,‡
Brain	1.6±0.2	1.4±0.3	1.7±0.1	65±3	67±4	105±3†,‡
Plasma	0.22±0.05	0.19±0.03	0.17±0.02	78±2	74±2	153±4†,‡

Values are means ± s.e.m. (N=5).
*μmol g^{-1} tissue or μmol ml^{-1} plasma.
†Significantly different from the corresponding 1‰ (day 0) value; ‡significantly different from the corresponding 1‰ (day 10) value.

can be estimated as (2.4+2.2+3.0+4.1+4.2+4.1+4.4+4.3+4.0+4.2) μmol 10 $days^{-1}$ g^{-1} fish/10=3.7 μmol day^{-1} g^{-1} fish.

For fish exposed to a progressive increase in salinity, the averaged daily urea synthesis rate during the 10-day period is equivalent to the summation of the averaged daily rate of urea excretion and the excess urea accumulated in the fish on day 10 divided by 10 days. The averaged rate of urea excretion is estimated as (2.6+3.0+2.5+2.9+2.3+2.4+2.8+2.3+2.1+3.0) μmol 10 day^{-1} g^{-1} fish/10=2.6 μmol day^{-1} g^{-1} fish. There were 50 g muscle, 3 g liver, 2 g stomach, 3 g intestine, 1 g brain and 2 ml plasma in a 100 g *H. signifer* (Tam et al., 2003). So, the excess amount of urea that would accumulate in the body of a hypothetical 100 g fish exposed to 15‰ water on day 10 can be estimated (from Table 1) as [(126–72) μmol g^{-1}×50 g]+[(118–60) μmol g^{-1}×3 g]+[(117–57) μmol g^{-1}×2 g]+[(93–63) μmol g^{-1}×3 g]+[(105–67) μmol g^{-1}×1 g]+[(153–74) μmol ml^{-1}×2 ml]=3280 μmol (equivalent to 6560 μmol N). This is equivalent to 32.8 μmol urea g^{-1} fish, accumulated during a 10-day period. Hence, the averaged daily rate of urea accumulation amounts to 3.3 μmol day^{-1} g^{-1} fish. This means that the rate of urea synthesis in fish exposed to a progressive increase in salinity was 2.6+3.3=5.9 μmol day^{-1} g^{-1} fish.

Series 2

The ammonia excretion rate of fish fasted for 48 h in 15‰ water was significantly lower than that of the control fasted for 48 h in 1‰ water throughout the 36-h period (0–12 h, 12–24 h and 24–36 h) post-feeding (Fig. 3A). In addition, the urea excretion rate of fish in 15‰ water was significantly lower than that of the control in 1‰ water during the 12–24 h and 24–36 h post-feeding (Fig. 3B).

For *H. signifer* kept in 1‰ water, the liver ammonia content increased significantly at 12, 24 and 36 h post-feeding (Table 2). In addition, there were significant increases in ammonia content in the intestine, brain and plasma at 24 and 36 h (Table 2). For the muscle and the stomach, significant increases in ammonia contents occurred only at 36 h (Table 2). Twenty-four hours and 36 h after feeding, there were significant increases in urea content in the muscle, liver, stomach, intestine, brain and plasma of fish kept in 1‰ water (Table 2).

For *H. signifer* kept in 15‰ water, there was no significant change in ammonia contents in the muscle, stomach, intestine and plasma during the 36-h post-feeding period (Table 3). However, the ammonia content in the liver increased significantly at 24 and 36 h, and that in the brain also

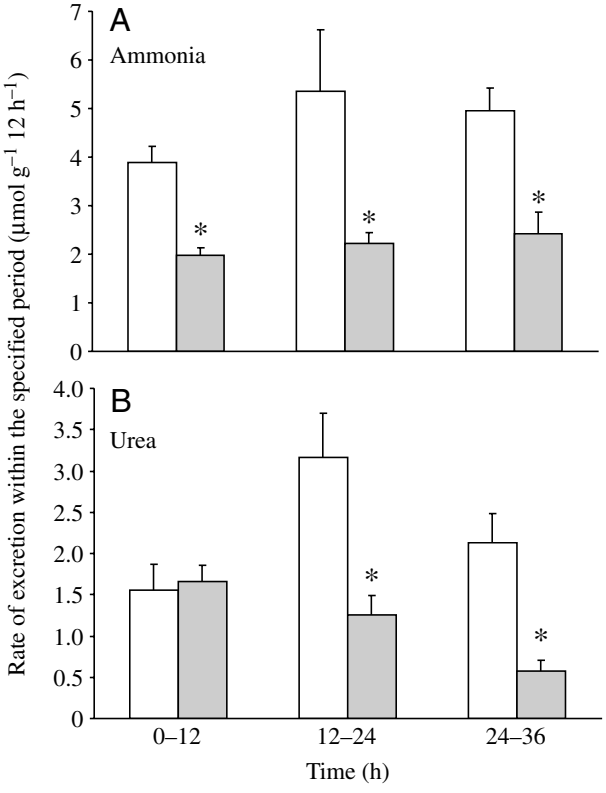

Fig. 3. Rates (μmol 12 h^{-1} g^{-1}) of ammonia (A) and urea (B) excretion in *Himantura signifer* at 12-h intervals during a period of 36 h post-feeding. Open bars represent fish kept in 1‰ water (control; N=5). Filled bars represent fish kept in 15‰ water (N=5). Values are means ± s.e.m. *Significantly different from the corresponding control value.

Table 2. *Ammonia and urea content of plasma and tissues of Himantura signifer kept in 1‰ water (0 h control; no feeding) or fed freshwater shrimp and kept in 1‰ water for 12, 24 or 36 h post-feeding*

	Ammonia content*				Urea content*			
Tissue	0 h	12 h.p.f.	24 h.p.f.	36 h.p.f.	0 h	12 h.p.f.	24 h.p.f.	36 h.p.f.
Muscle	2.7±0.1	1.5±0.1†	3.5±0.2‡	4.8±0.3†,‡,§	69±1	69±7	106±5†,‡	92±6†,‡
Liver	2.4±0.2	6.7±0.9†	5.8±0.6†	8.6±0.7†,§	59±9	61±9	98±4†,‡	88±7†
Stomach	2.2±0.3	2.6±0.2	4.4±0.7	5.1±0.8†	59±1	72±5	94±3†,‡	85±7†
Intestine	2.6±0.2	3.1±0.2	3.8±0.2†	4.7±0.4†,‡	58±1	64±2	92±3†,‡	83±6†,‡
Brain	1.3±0.2	1.8±0.4	3.3±0.2†,‡	3.9±0.2†,‡	65±2	57±2	114±4†,‡	99±7†,‡,§
Plasma	0.18±0.01	0.21±0.04	0.71±0.07†,‡	1.2±0.1†,‡,§	72±1	90±2	132±8†,‡	142±7†,‡

h.p.f., hours post feeding.
Values are means ± s.e.m. (N=5).
*μmol g^{-1} tissue or μmol ml^{-1} plasma.
†Significantly different from 0 h control value; ‡significantly different from the 12 h post-feeding value; §significantly different from the 24 h post-feeding value.

increased significantly at 36 h, post-feeding (Table 3). Twenty-four hours and 36 h after feeding, there was a significant increase in urea content in the muscle of fish kept in 15‰ water (Table 3). In addition, there were significant increases in urea content in the liver, stomach, intestine and brain at 36 h post-feeding (Table 3). However, no significant change in plasma urea concentration was observed throughout the 36-h period.

The excess urea that would accumulate 36 h post-feeding in a hypothetical 100 g fish in 1‰ water can be estimated (from Table 2) to be [(23 μmol g^{-1}×50 g)+(29 μmol g^{-1}×3 g)+(26 μmol g^{-1}×2 g)+(25 μmol g^{-1}×3 g)+(34 μmol g^{-1}×1 g)+(70 μmol ml^{-1}×2 ml)]=1538 μmol (equivalent to 3076 μmol N). A similar calculation reveals that the excess ammonia-N accumulated in these tissues and organs would be only 140 μmol N.

At 36 h, the excess urea that would accumulate in a 100 g fish in 15‰ water after feeding can be estimated (from Table 3) to be [(51 μmol g^{-1}×50 g)+(31 μmol g^{-1}×3 g)+(21 μmol g^{-1}×2 g)+(25 μmol g^{-1}×3 g)+(25 μmol g^{-1}×1 g)]=2485 μmol (equivalent to 4970 μmol N), which is 1.6-fold greater than that (1538 μmol) of fish kept in 1‰ water.

Discussion

Although *H. signifer* is believed to inhabit only freshwater, it may return to brackish water to reproduce (Tam et al., 2003). That could be the reason why it retains the capacity to synthesize and accumulate urea for osmoregulation and can survive well in brackish water. *H. signifer* suppresses urea synthesis and reduces urea retention to survive in freshwater, but in brackish water, it up-regulates urea synthesis and retains urea in its tissues (Tam et al., 2003). It is important to point out that Tam et al. (2003) performed their studies with fish fasted for 48 days before experiments and withheld food throughout the experimental period. Thus, there is a dearth of knowledge on relationships between feeding and osmoregulatory needs in *H. signifer* at present. *H. signifer* exposed to brackish water has to increase the internal urea content for osmoregulation, and so when food is available,

Table 3. *Ammonia and urea content of plasma and tissues of Himantura signifer kept in 15‰ water (0 h control; no feeding) or fed freshwater shrimp and kept in 15‰ water for 12, 24 or 36 h post-feeding*

	Ammonia content*				Urea content*			
Tissue	0 h	12 h.p.f.	24 h.p.f.	36 h.p.f.	0 h	12 h.p.f.	24 h.p.f.	36 h.p.f.
Muscle	2.2±0.1	2.4±0.3	2.1±0.3	2.0±0.2	106±4	110±5	139±6†	151±9†,‡
Liver	1.9±0.1	2.0±0.2	4.2±0.8†,‡	5.7±0.9†,‡	100±3	100±3	115±3	131±8†,‡
Stomach	1.8±0.1	2.0±0.2	1.5±0.1	2.1±0.4	102±3	102±4	108±4	123±5†,‡
Intestine	2.2±0.1	2.2±0.1	2.1±0.2	2.3±0.1	88±2	87±3	99±3	113±4†,‡
Brain	1.6±0.1	1.7±0.1	1.7±0.1	2.1±0.1†,‡,§	101±2	104±3	117±5	126±5†,‡
Plasma	0.16±0.01	0.19±0.01	0.33±0.11	0.28±0.02	151±3	152±4	139±6	147±5

h.p.f., hours post feeding.
Values are means ± s.e.m. (N=5).
*μmol g^{-1} tissue or μmol ml^{-1} plasma.
†Significantly different from 0 h control value; ‡significantly different from the 12 h post-feeding value; §significantly different from the 24 h post-feeding value.

there would theoretically be a greater nitrogenous intake through, and/or a greater conservation of N from, food consumption.

Would a progressive increase in ambient salinity lead to a greater food intake in H. signifer?

Our results reveal that *H. signifer* was able to acclimate to a progressive increase in salinity from 1‰ to 15‰ through a 10-day period without increasing the daily food consumption (Fig. 1). The averaged food rations for fish kept in 1‰ water and fish exposed to a progressive increased in salinity during the 10-day period were 0.014 and 0.015 g g^{-1} fish, respectively. So, increased conservation of nitrogen from food intake appears to be the main adaptation exhibited by *H. signifer* to survive in brackish water.

Would an increase in ambient salinity result in an increase in retention of N after feeding in H. signifer?

The rates of ammonia and urea excretion in *H. signifer* kept in 1‰ water are comparable to those of the little skate, *Raja erinacea* in 100% or 75% seawater (Steele et al., 2005). However, when *H. signifer* was exposed to a progressive increase in salinity, there was a progressive decrease in the rate of ammonia excretion. Yet, despite a reduction in ammonia excretion, there was no significant change in ammonia contents in various tissues of fish exposed to 15‰ water on day 10. Tissue urea contents, in contrast, were significantly higher in these experimental fish than those in fish kept in 1‰ water for 10 days. So, fish exposed to brackish water indeed conserved a greater portion of the N from the relatively constant amount of food intake, and incorporated the N into urea for osmoregulatory purposes. In addition, there was a progressive decrease in the rate of urea excretion in fish exposed to salinity changes, which indicates that *H. signifer* also retained more urea in response to the increasing ambient salinity. Thus, our results are in support of the postulate of Wood (2001) that ureosmotic elasmobranchs are nitrogen-limited and would avoid the loss of nitrogen after feeding by converting as much excess N as possible to urea.

Because preliminary results indicate that it took at least 48 h for *H. signifer* to completely clear its gut of food, it would be inappropriate to calculate the daily percentage food-N being excreted by fish in this series of experiments. However, it would be meaningful to perform the calculation over the 10-day period. Since the total food intake for fish kept in 1‰ water was 0.142 g g^{-1} fish and since each gram of feed contained 1.5 mmol N, the total N intake was 213 μmol N g^{-1} fish. During this 10-day period, the total waste-N (ammonia-N+urea-N) excreted was 88.5+73.4=161.9 μmol N g^{-1} fish. Thus, (161.9×100)/213 or 76% of the food-N was excreted by *H. signifer* in 1‰ water. Alternately, this means only 24% of the food-N was conserved, which is lower than those for the non-ureosmotic fish species such as the African lungfish *Protopterus dolloi* (Lim et al., 2004) and the giant mudskipper *Periophthalmodon schlosseri* (Ip et al., 2004). This could be a result of *H. signifer* maintaining a relatively high level of urea in its plasma, and simultaneously reducing the capacity to retain urea in order to survive in freshwater (Tam et al., 2003; Ip et al., 2004).

Because the total food intake for fish exposed to a progressive increase in salinity was 0.15 g g^{-1} fish, the total N intake was 225 μmol N g^{-1} fish. During this 10-day period, the total N excreted was 56.8+52.4=109.2 μmol N g^{-1} fish. Thus, (109.2×100)/225 or 49% of the food-N was excreted by *H. signifer* exposed to a progressive increase in salinity, which means 51% of the food-N was conserved. From these calculations, it is confirmed that a 2.1-fold (=51%/24%) increase in conservation of N occurred in *H. signifer* exposed to brackish water as compared with those kept in 1‰ water.

Would there be a greater rate of conversion of the retained N to urea in H. signifer exposed to brackish water?

There was a significant decrease in the rate of ammonia excretion in *H. signifer* exposed to a progressive increase in salinity from day 3 to day 10. The total amount of ammonia retained was 30.6 μmol N g^{-1}. In addition, there was a significant decrease in the rate of urea excretion in these experimental fish between day 4 and day 10, amounting to 22.8 μmol N g^{-1}. Together the sum of N conserved during the 10-day period is 30.6+22.8=53.4 μmol N g^{-1}. So, for a 100 g *H. signifer*, a total of 5340 μmol N would have been conserved. On day 10, the excess amount of urea accumulated in the tissues and organs of a 100 g fish exposed to 15‰ water was estimated to be 6560 μmol N, which is even greater than the estimated amount of 5340 μmol N conserved. Hence, it can be concluded that the conserved N was completely converted into urea for osmoregulation.

The estimated rate of urea synthesis in a fish in 1‰ water during the 10-day period was 3.7 μmol day^{-1} g^{-1} fish. For fish exposed to a progressive increase in salinity, the average urea synthesis rate during a similar period is estimated to be 5.9 μmol day^{-1} g^{-1} fish. This means that the rate of urea synthesis in fish exposed to a progressive increase in salinity was up-regulated 1.6-fold (5.9/3.7).

Would feeding lead to an increase in urea contents in tissues of H. signifer kept in 1‰ water?

Mommsen and Walsh (1991) postulated that marine elasmobranchs might excrete excess nitrogen intake, over and above the needs of osmoregulation, in the form of ammonia rather than urea after feeding. Although results obtained from our series 1 experiment confirmed that *H. signifer* was N-limited, these results could not verify whether the postulate made by Mommsen and Walsh (1991) was correct; so, the second series of experiments was performed.

In 1‰ water, *H. signifer* cannot be N-limited, and therefore should excrete a major portion of the food-N as ammonia according to the hypothesis of Mommsen and Walsh (1991). However, our results proved it otherwise. After a single intake of food, the percentage of total waste-N (ammonia-N+urea-N) excreted as ammonia in *H. signifer* fasted for 48 h and kept in 1‰ water during the 0–12, 12–24 and 24–36 h periods was

relatively constant. More importantly, besides increases in ammonia contents, there were postprandial increases in urea contents in all the tissues studied in *H. signifer*. The excess urea-N accumulated at 36 h in a 100 g fish is estimated to be 3076 μmol N. Because the excess ammonia-N accumulated in these tissues and organs was only 140 μmol, that means a major portion of the food-N was converted into urea in spite of *H. signifer* not being nitrogen-limited in 1‰ water. There can be two reasons for this as suggested by Lim et al. (2004) and Ip et al. (2004). Firstly, urea synthesis, being an energy-intensive process, is not a major issue when food-N is concerned, because food intake prescribes the availability of energy resources. Secondly, the majority of excess amino acids from food intake are catabolized by glutamate dehydrogenase, which is present in the matrix of liver mitochondria. Also present in the mitochondrial matrix are glutamine synthetase and CPS III, which convert ammonia to carbamoyl phosphate for subsequent urea synthesis. So, naturally, ammonia produced through catabolism of excess amino acid, unlike that infused/injected into the blood or peritoneal cavity, is readily available for urea synthesis in the liver. Because *H. signifer* retains the capacity of increased urea synthesis and retention, our results indicate that it would be confronted with osmotic stress transiently in 1‰ water after feeding.

Would fish keep in 15‰ water conserve a greater percentage of the food-N than fish kept in 1‰ water?

For *H. signifer* kept in 15‰ water and fasted for 48 h, there was indeed a greater conservation of N after feeding. The postprandial rate of ammonia excretion in fish exposed to 15‰ water was significantly lower than that in fish kept in 1‰ water during the 0–12, 12–24 and 24–36 h periods. Simultaneously, there was a significantly lower rate of urea excretion in the former as compared to the latter, indicating a greater retention of urea in fish kept in 15‰ water.

In contrast to fish kept in 1‰ water, there was no increase in ammonia contents in various tissues and organs, except the liver and the brain, of fish exposed to 15‰ water in spite of the greater conservation of food-N. Once again, this is in support of the conclusion that the conserved food-N was completely converted to urea for osmoregulatory purposes. At 36 h, the excess urea-N accumulated in a 100 g fish in 15‰ water is estimated to be 4970 μmol N, which is 1.6-fold greater than that of fish kept in 1‰ water (3076 μmol N). Thus, these results confirm once again that fish kept in 15‰ water converted a greater percentage of the food-N to urea than fish kept in 1‰ water. They are in support of the proposition that urea synthesis in elasmobranchs is adapted more for osmoregulation than ammonia detoxification as suggested by Ip et al. (2005), although the ornithine–urea cycle of invertebrates may function mainly to detoxify ammonia (Hiong et al., 2005).

Conclusion

H. signifer is N-limited, and conserves more N from food when exposed to brackish water. The conserved N is converted to urea, which is retained in tissues for osmoregulation. However, because of its capacity to conserve N and increase urea synthesis upon feeding, *H. signifer* can be confronted with postprandial osmotic stress in freshwater.

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