The Journal of Experimental Biology 206, 3625-3633

doi:10.1242/jeb.00612

A comparison of the effects of environmental ammonia exposure on the Asian freshwater stingray *Himantura signifer* and the Amazonian freshwater stingray *Potamotrygon motoro*

Yuen K. Ip[1,*], Wai L. Tam[1], Wai P. Wong[1], Ai M. Loong[1], Kum C. Hiong[1], James S. Ballantyne[2] and Shit F. Chew[3]

[1]*Department of Biological Science, National University of Singapore, Kent Ridge, Singapore 117543, Republic of Singapore,* [2]*Department of Zoology, University of Guelph, Guelph, Ontario, Canada NIG 2W1* and [3]*Natural Sciences, National Institute of Education, Nanyang Technological University, 1 Nanyang Walk, Singapore 637616, Republic of Singapore*

*Author for correspondence (e-mail: dbsipyk@nus.edu.sg)

Accepted 15 July 2003

Summary

The white-edge whip tail ray *Himantura signifer* inhabits a freshwater environment but has retained the capability to synthesize urea *de novo* through the arginine–ornithine–urea cycle (OUC). The present study aimed to elucidate whether the capacity of urea synthesis in *H. signifer* could be upregulated in response to environmental ammonia exposure. When *H. signifer* was exposed to environmental ammonia, fairly high concentrations of ammonia were accumulated in the plasma and other tissues. This would subsequently reduce the net influx of exogenous ammonia by reducing the NH_3 partial pressure gradient across the branchial and body surfaces. There was also an increase in the OUC capacity in the liver. Since the ammonia produced endogenously could not be excreted effectively in the presence of environmental ammonia, it was detoxified into urea through the OUC. In comparison, the South American freshwater stingray *Potamotrygon motoro*, which has lost the capability to synthesize urea *de novo*, was unable to detoxify ammonia to urea during ammonia loading. No increase in glutamine was observed in the various tissues of *H. signifer* exposed to environmental ammonia despite a significant increase in the hepatic glutamine synthetase activity. These results indicate that the excess glutamine formed was channelled completely into urea formation through carbamoyl phosphate synthetase III. It has been reported elsewhere that both urea synthesis and urea retention were upregulated in *H. signifer* exposed to 20‰ water for osmoregulatory purposes. By contrast, for *H. signifer* exposed to environmental ammonia in freshwater, the excess urea formed was excreted to the external medium instead. This suggests that the effectiveness of urea synthesis *de novo* as a strategy to detoxify ammonia is determined not simply by an increase in the capacity of urea synthesis but, more importantly, by the ability of the animal to control the direction (i.e. absorption or excretion) and rate of urea transport. Our results suggest that such a strategy began to develop in those elasmobranchs, e.g. *H. signifer*, that migrate into a freshwater environment from the sea but not in those permanently adapted to a freshwater environment.

Key words: ammonia, ammonia detoxification, ammonia excretion, amino acid, carbamoyl phosphate synthetase, elasmobranch, *Himantura signifer*, nitrogen metabolism, ornithine–urea cycle, osmoregulation, stingray, urea, urea excretion.

Introduction

The synthesis of excretory urea in certain land planaria (Campbell, 1965), earthworms (Bishop and Campbell, 1963, 1965) and snails (Campbell and Bishop, 1970; Campbell and Speeg, 1968; Tramell and Campbell, 1972) *via* the ornithine–urea cycle (OUC) indicates that the usurpation of the basic nutritional pathway of arginine synthesis for ammonia detoxification first took place in invertebrate animals (Campbell, 1973). Osmotic water retention may have been a primary factor in the selection for a high rate of urea synthesis in terrestrial gastropods. As for lower vertebrates, it seems probable that the selective pressures for the integration of the components of OUC for an increased rate of urea synthesis first came about in the marine environment (Campbell, 1973). However, whether this was originally directed towards ammonia detoxification, the synthesis of urea as an osmolyte or both is a moot point. The capacity to synthesize urea during periods of restricted water availability, as seen in the ammonotelic and ureogenic lungfishes, might have pre-adapted the early vertebrates for their transition to the land. The ammonotelic–ureotelic transition made by

metamorphosing anurans (Amphibia) is assumed to be somewhat similar to that made by the first vertebrates – possibly labyrinthodont relatives of primitive dipnoans – to invade the land. In mammals, OUC became the predominant mechanism in detoxification of ammonia released through the catabolism of amino acids and adenylates.

While synthesizing urea for both water retention (osmoregulation) and ammonia detoxification prescribes a high rate of urea synthesis *de novo*, the former dictates the majority of the urea synthesized to be retained within the body while the latter requires urea to be excreted instead. Hence, during evolution, there must be a dichotomy in the development of control for urea transport to either facilitate the retention of urea for osmoregulation or to remove urea for nitrogenous excretion. To date, at least five urea transporters are known to be present in the mammalian kidney, facilitating the excretion of urea (see review by Sands et al., 1997). This capability can be traced back to amphibians, whose kidneys can excrete urea actively (Foster, 1954; Balinsky, 1970). However, fully aquatic amphibians lack the power of active urea secretion (Balinsky 1970), while marine elasmobranchs actively re-absorb urea instead (Fines et al., 2001; Smith and Wright, 1999).

Marine elasmobranchs are ureosmotic, synthesizing urea through the OUC with carbamoyl phosphate synthetase III (CPS III; Anderson, 1980, 1991, 1995, 2001; Campbell and Anderson, 1991), primarily for osmoregulation (Anderson, 2001; Ballantyne, 1997; Perlman and Goldstein, 1998). Urea is retained at high concentrations (300–600 mmol l^{-1}) in the tissues. This is accomplished by the low permeability of the gills to urea (Fines et al., 2001) and by the re-absorption of urea *via* secondary active (Na^+-coupled) urea transporters in the gills (Smith and Wright, 1999) and kidney (see review by Walsh and Smith, 2001). Despite the decrease in effective urea permeabilities (Fines et al., 2001), marine elasmobranchs (sharks, skates and rays) are ureotelic, excreting the majority of their nitrogenous wastes as urea *via* the gills (Perlman and Goldstein, 1998; Shuttleworth, 1988; Wood, 1993; Wood et al., 1995). For elasmobranchs living in freshwater, they must evolve mechanisms to suppress urea production, urea retention (including active urea reabsorption) or both. Hence, they are ideal models for the unravelling of the intricate relationship between urea synthesis *de novo* and urea excretion in response to high concentrations of environmental ammonia.

In tropical waters in Southeast Asia (Thailand, Indonesia and Papua New Guinea) and South America (Amazon River basin), a number of elasmobranch species migrate into low salinity waters where they reduce plasma salt, urea and trimethylamine oxide (TMAO) levels. The stenohaline Amazonian stingray *Potamotrygon* spp., being permanently adapted to freshwater, is primarily ammonotelic like other teleosts (Barcellos et al., 1997; Goldstein and Forster, 1971) and cannot accumulate urea in laboratory salinity stress (Gerst and Thorson, 1977; Thorson et al., 1967). In the present study, an attempt was made to use environmental ammonia as a probe to elucidate whether ammonia exposure (10 μmol ml^{-1} NH_4Cl in freshwater at pH 7.0) would induce the synthesis and accumulation of urea in *Potamotrygon motoro* because no such information is available. Results obtained subsequently revealed that it was unable to do so and therefore *P. motoro* was not an appropriate organism to evaluate the evolutionary role of urea, i.e. whether it was designed for osmoregulatory or ammonia detoxification purposes.

By contrast, the freshwater white-edge whip ray *Himantura signifer* (Family: Dasyatidae), a stingray found in the Batang Hari Basin in Jambi, Sumatra, retains the capability for urea synthesis *de novo* but has a reduced capacity to retain urea in freshwater (Tam et al., 2003). Unlike potamotrygonid rays living in the Amazon River, *H. signifer* might have invaded the freshwater environment only recently. It is currently unclear whether *H. signifer* returns to brackish water to reproduce, as does the bull shark *Carcharhinus leucas* of Lake Nicaragua (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 (Tam et al., 2003). It is because of this that *H. signifer* represents an ideal species for studies on the effects of ammonia loading on the capacity of urea synthesis and capacity of urea retention in a primarily freshwater elasmobranch. In the present study, we aimed to elucidate whether the capacity of *H. signifer* to synthesize urea *de novo* could be upregulated and, more importantly, whether urea excretion would be enhanced during exposure to environmental ammonia in freshwater. We hypothesized that it was capable of doing so. The rate of urea excretion in specimens being exposed to 10 mmol l^{-1} NH_4Cl in freshwater at pH 7.0 was determined. The contents of ammonia, urea and free amino acids (FAAs) in various tissues and organs of the specimens were measured. In addition, activities of OUC enzymes were assayed.

We also aimed to evaluate whether urea synthesis alone would be an effective measure to defend against environmental ammonia toxicity. The formation of urea in fishes is highly energy dependent. A total of 5 moles of ATP are hydrolyzed to ADP for each mole of urea synthesized, corresponding to 2.5 moles of ATP used for each mole of nitrogen assimilated. This may be the major reason why the majority of tropical teleosts studied so far do not adopt ureogenesis as a major strategy to detoxify exogenous and endogenous ammonia during ammonia loading (Ip et al., 2001). However, if the animal had high ammonia tolerance at the cellular and subcellular levels, it could allow ammonia to build up in its tissues and plasma during the early phase of exposure to environmental ammonia. In effect, this would reduce or impede the net influx of exogenous ammonia, and urea synthesis *de novo* could be reserved to detoxify the endogenously produced ammonia, maintaining the newly established steady-state concentration of ammonia in the body. Therefore, we hypothesized that an increase in ammonia contents in the body of *H. signifer* would occur during environmental ammonia exposure, despite its being ureogenic and ureotelic (Tam et al., 2003).

Materials and methods

Specimens

Himantura signifer (Compagno and Roberts, 1982; 200–500 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 at least 20 volumes (w/v) of freshwater (0.7‰ salinity) at 25°C in the laboratory with the water changed daily. No attempt was made to separate the sexes. Specimens were acclimated to laboratory conditions for at least 5 days before the experiments began. During this period, they were fed with clam 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

H. signifer and *P. motoro* were exposed to freshwater (0.7‰) containing 10 mmol l^{-1} NH_4Cl at pH 7 for 4 days. Water samples (3 ml) were collected daily, acidified with 70 µl of 1 mol l^{-1} HCl and kept at 4°C until analysed. Urea was determined according to the method of Felskie et al. (1998). No ammonia assays were performed on these water samples. Control specimens were exposed to ordinary freshwater (0.7‰). In order to calculate the deficit in ammonia excretion in *H. signifer* exposed to NH_4Cl, water samples were collected daily for the control specimen, and ammonia was determined according to the methods of Anderson and Little (1986), as modified by Jow et al. (1999). The rates of urea and ammonia excretion were expressed as µmol day^{-1} g^{-1} fish.

Anaesthetized fish (0.12% ethyl-3-aminobenzoate methanesulfonate) were rinsed thoroughly several times with freshwater to avoid environmental contamination. The caudal peduncle of the experimental specimen was severed, and blood was collected from the caudal vessels into 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 was kept at –80°C until analysed. The liver, stomach and muscle were quickly excised, with the stomach 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.

Determinations of plasma osmolality, concentrations of Na^+ and Cl^- and blood pH

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

Analysis of free FAAs

The frozen muscle and liver samples were weighed, ground to 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 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 of muscle and liver 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 pH of the plasma sample was also adjusted to 5.5–6.0 after deprotenization. The ammonia and urea contents were determined according to the methods of Bergmeyer and Beutler (1985) and Felskie et al. (1998), respectively. Results were expressed as µmol g^{-1} wet mass tissue or mmol l^{-1} plasma.

Determination of activities of OUC enzymes

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) (ASS + L) activities were determined together,

Table 1. *Effects of 4 days of exposure to environmental ammonia (10 mmol l^{-1} NH_4Cl in 0.7‰ water at pH 7) on the ammonia and urea levels in the muscle, liver and plasma of* Potamotrygon motoro

	[Ammonia]		[Urea]	
	Control	NH_4Cl	Control	NH_4Cl
Muscle (μmol g^{-1} wet mass)	2.06±0.44	7.40±0.80*	0.38±0.08	0.77±0.13*
Liver (μmol g^{-1} wet mass)	1.64±0.28	11.1±1.51*	0.04±0.01	0.37±0.07*
Plasma (mmol l^{-1})	0.25±0.04	1.57±0.14*	0.65±0.17	0.65±0.11

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

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. ASS + L 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.

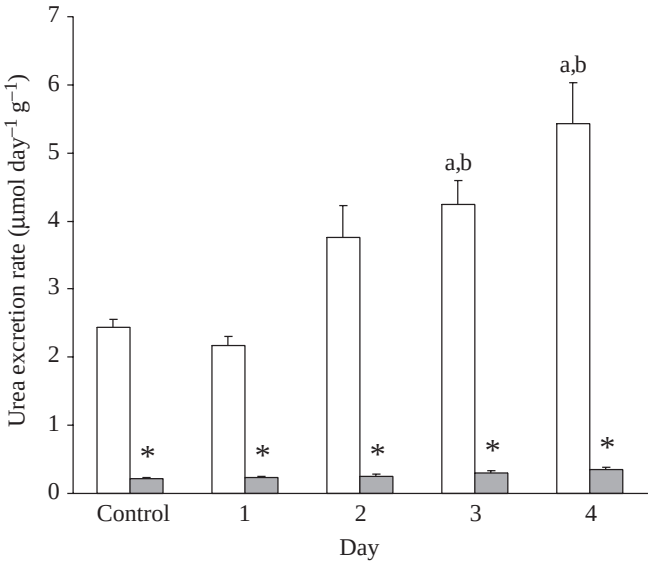

Fig. 1. Changes in the urea excretion rate (μmol day^{-1} g^{-1} fish) of *Himantura signifer* (open bars; N=10) or *Potamotrygon motoro* (filled bars; N=5) during 4 days of exposure to environmental ammonia (10 mmol l^{-1} NH_4Cl). Results represent means ± S.E.M. [a]Significantly different from *H. signifer* control values (P<0.05). [b]Significantly different from *H. signifer* day 1 values (P<0.05). *Significantly different from the corresponding *H. signifer* value.

Statistical analyses

Results are presented as means ± S.E.M. Two-tail 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 of P<0.05 were regarded as statistically significant.

Results

When *P. motoro* was exposed to 10 mmol l^{-1} NH_4Cl at pH 7, ammonia levels in the muscle, liver and plasma increased significantly (Table 1). Urea also accumulated in the muscle and liver, but the quantities involved were minor compared with those of ammonia (Table 1). The rate of urea excretion remained low and unchanged throughout the 4 days of ammonia exposure (Fig. 1). There was no change in the plasma osmolality and plasma Na^+ and Cl^- concentrations in *P. motoro* exposed to environmental ammonia (Table 2). In addition, the urea content in tissues of *P. motoro* (Table 1) was much lower (<1 μmol g^{-1} or <1 μmol ml^{-1}) than that of *H. signifer* (Table 3).

The ammonia excretion rates of control *H. signifer* on days 1, 2, 3 and 4 were 5.5±0.8 μmol day^{-1} g^{-1}, 5.8±1.2 μmol day^{-1} g^{-1}, 5.3±0.7 μmol day^{-1} g^{-1} and 6.1±1.2 μmol day^{-1} g^{-1}, respectively. The rate of urea excretion in specimens exposed to ammonia for the first 2 days was comparable with that of the control (Fig. 1). However, on days 3 and 4, there was a significant increase in the rate of urea excretion in the experimental specimens (Fig. 1). Ammonia

Table 2. *Effects of 4 days of exposure to environmental ammonia (10 mmol l^{-1} NH_4Cl in 0.7‰ water at pH 7) on the osmolality and concentrations of Na^+ and Cl^- in the plasma of* Potamotrygon motoro

	Control	NH_4Cl
Osmolality (mosmol kg^{-1})	349±16	357±10
[Na^+] (mmol l^{-1})	157±16	147±9
[Cl^-] (mmol l^{-1})	163±14	172±10

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

Table 3. *Effects of 4 days of exposure to environmental ammonia (10 mmol l^{-1} NH_4Cl in 0.7‰ water at pH 7) on the ammonia and urea contents in the muscle, liver and plasma of* Himantura signifer

	[Ammonia]		[Urea]	
	Control	NH_4Cl	Control	NH_4Cl
Muscle (μmol g^{-1} wet mass)	2.63±0.60	5.46±0.65*	70.9±7.73	69.8±4.83
Liver (μmol g^{-1} wet mass)	5.06±0.42	6.20±0.97	49.4±5.22	25.5±2.16
Plasma (mmol l^{-1})	0.33±0.09	2.15±0.29*	43.8±1.23	71.6±5.92*

Results represent means ± S.E.M. (N=5).
*Significantly different from 0.7‰ control condition (P<0.05).

Table 4. *Effects of 4 days of exposure to environmental ammonia (10 mmol l^{-1} NH_4Cl in 0.7 ‰ water at pH 7) on the osmolality and concentrations of Na^+ and Cl^- in the plasma of* Himantura signifer

	Control	NH_4Cl
Osmolality (mosmol kg^{-1})	416±19.3	442±8.85
$[Na^+]$ (mmol l^{-1})	167±7.18	155±5.19
$[Cl^-]$ (mmol l^{-1})	164±10.0	155±3.69

Results represent means ± S.E.M. (N=6).

accumulated in the muscle and plasma, but not in the liver, of *H. signifer* exposed to 10 mmol l^{-1} NH_4Cl at pH 7 (Table 3). However, there was no change in the urea content of the muscle or liver. A significant increase in urea level occurred only in the plasma (Table 3). In addition, there was no change in plasma osmolality and plasma Na^+ and Cl^- concentrations in *H. signifer* exposed to ammonia (Table 4).

Ammonia exposure induced a higher OUC capacity in the liver of *H. signifer*. The activities of hepatic GS, CPS III, OTC, ASS + L and arginase increased approximately 3-, 4-, 3-, 4- and 7-fold, respectively (Table 5). A complete OUC was detected in the stomach (Table 6). However, there was no significant increase in the activities of any OUC enzymes in the stomach during exposure to environmental ammonia (Table 6).

In general, the concentrations of various FAAs and total FAA (TFAA) of *H. signifer* exposed to ammonia were comparable with those of the control (Table 7). In particular, there was no accumulation of glutamine during ammonia loading.

Discussion

P. motoro *does not detoxify ammonia to urea during ammonia loading*

P. motoro was purely ammonotelic in freshwater (Thorson et al., 1967; Tam et al., 2003); the urea excretion rate was low and unaffected by exposure to 10 mmol l^{-1} NH_4Cl at pH 7.0 for 4 days. Despite significant increases in ammonia content in all its tissues, there was no significant increase in urea concentration in the plasma and only very minor increases in urea content in the muscle in the specimens exposed to ammonia. Since the ammonia content in the liver of *P. motoro* increased 7-fold with only a very small amount of accumulated urea after 4 days of ammonia exposure, it is logical to conclude that its liver did not possess a functioning OUC. Thus, since *P. motoro* has been isolated in the Amazon River Basin away from the ocean for over 65 million years (Lovejoy, 1997; Thorson et al., 1983), it has lost the capacity for urea synthesis

Table 5. *Changes in activities of glutamine synthetase (GS), carbamoyl phosphate synthetase (CPS), ornithine transcarbamoylase (OTC), arginosuccinate synthetase + lyase (ASS + L) and arginase in the liver of* Himantura signifer *during 4 days of exposure to environmental ammonia (10 mmol l^{-1} NH_4Cl in 0.7‰ water at pH 7)*

		Enzyme activity (μmol min^{-1} g^{-1} wet mass)	
Enzymes	Substrate and effector present	Control	NH_4Cl
GS	–	4.97±1.32	16.1±3.56*
OTC	–	22.8±5.73	60.9±8.46*
ASS + L	–	0.19±0.07	0.83±0.10*
Arginase	–	29.6±10.2	203±5*
CPS	Glutamine	0.04±0.01	0.19±0.04*
	Glutamine + AGA	0.15±0.04	0.63±0.07*
	Glutamine + AGA + UTP	0.13±0.04	0.58±0.08*

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

Table 6. *Changes in activities of glutamine synthetase (GS), carbamoyl phosphate synthetase (CPS), ornithine transcarbamoylase (OTC), arginosuccinate synthetase + lyase (ASS + L) and arginase in the stomach of* Himantura signifer *during 4 days of exposure to environmental ammonia (10 mmol l^{-1} NH_4Cl in 0.7‰ water at pH 7)*

Enzymes	Substrate and effector present	Enzyme activity (μmol min^{-1} g^{-1} wet mass)	
		Control	NH_4Cl
GS	–	6.03±0.86	12.6±3.75
OTC	–	8.40±0.86	7.73±1.54
ASS + L	–	0.42±0.09	0.55±0.15
Arginase	–	5.57±2.22	10.3±2.62
CPS	Glutamine	0.03±0.01	0.009±0.003
	Glutamine + AGA	0.10±0.05	0.02±0.008
	Glutamine + AGA + UTP	0.09±0.04	0.02±0.008

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

for salinity adaptation with the result that the capacity for ammonia detoxification by this pathway has also been lost.

H. signifer *upregulates the capacity to synthesize urea* de novo *when exposed to environmental ammonia*

Similar to marine elasmobranchs, freshwater *H. signifer* is ureogenic and has a functional OUC with CPS III in the liver. In addition, a full complement of OUC enzymes was detected in the stomach of *H. signifer*. 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

Table 7. *Effects of 4 days of exposure to environmental ammonia (10 mmol l^{-1} NH_4Cl in 0.7‰ water) on the contents (μmol g^{-1} wet mass or mmol l^{-1} plasma) of various free amino acids (FAAs) and total FAA (TFAA) in the muscle, liver and plasma of* Himantura signifer

FAAs	Muscle		Liver		Plasma	
	Control	NH_4Cl	Control	NH_4Cl	Control	NH_4Cl
Alanine	2.05±0.51	0.82±0.11	0.72±0.16	0.28±0.12	0.12±0.01	0.24±0.01*
Arginine	0.28±0.05	0.29±0.05	0.02±0.001	0.02±0.01	0.09±0.01	0.12±0.01
Asparagine	0.13±0.01	0.21±0.06*	0.29±0.03	0.10±0.008*	0.009±0.004	0.008±0.002
Aspartate	0.26±0.10	0.09±0.01	0.05±0.008	0.04±0.005	0.03±0.003	0.04±0.004*
β-Alanine	2.84±0.93	0.51±0.07	0.23±0.10	0.09±0.02	0.02±0.01	0.01±0.004
Glutamine	0.46±0.03	1.08±0.33	1.09±0.09	1.03±0.03	0.01±0.002	0.02±0.007
Glutamate	0.84±0.26	0.56±0.08	0.10±0.02	0.10±0.02	0.09±0.006	0.20±0.02*
Glycine	5.28±1.66	3.16±0.89	0.36±0.07	0.48±0.14	0.11±0.03	0.18±0.02
Histamine	0.05±0.01	0.07±0.01	0.11±0.004	0.04±0.001*	0.03±0.008	0.04±0.003
Isoleucine	0.11±0.02	0.21±0.009*	0.08±0.03	0.04±0.005	0.08±0.01	0.15±0.01*
Leucine	0.19±0.04	0.39±0.02*	0.15±0.04	0.10±0.01	0.17±0.03	0.31±0.02*
Lysine	0.04±0.02	0.05±0.01	0.01±0.007	0.02±0.007	0.02±0.003	0.04±0.004
Methionine	0.24±0.02	0.18±0.02	0.30±0.05	0.12±0.02	0.13±0.02	0.12±0.01
Phenylalanine	0.48±0.17	0.14±0.01	0.41±0.13	0.05±0.01	0.04±0.005	0.04±0.005
Proline	0.76±0.14	0.50±0.34	0.56±0.20	0.10±0.07	0.24±0.02	0.57±0.07
Serine	0.70±0.12	0.24±0.02*	0.10±0.02	0.13±0.03	0.07±0.01	0.11±0.006*
Taurine	26.0±1.75	25.4±2.70	17.6±2.97	8.86±0.41*	1.43±0.48	0.74±0.08
Threonine	0.39±0.16	0.19±0.03	0.26±0.07	0.11±0.02	0.08±0.01	0.10±0.01
Tryptophan	0.06±0.02	0.07±0.003	0.04±0.02	0.001±0.001	0.04±0.009	0.06±0.003
Tyrosine	0.03±0.007	0.06±0.002	0.04±0.01	0.009±0.003	0.03±0.005	0.04±0.002
Valine	0.21±0.05	0.31±0.01	0.18±0.07	0.09±0.009	0.15±0.03	0.26±0.02*
TFAA	38.7±2.32	35.8±4.48	22.6±3.15	11.8±0.51*	2.97±0.39	3.51±0.19*

Results represent means ± S.E.M. (*N*=4).
*Significantly different from 0.7‰ control condition (P<0.05).

rate of urea excretion was 2.2 μmol day^{-1}. In a 100 g specimen, there was approximately 50 g muscle, 3 g liver and 2 ml plasma (Y.K.I. and S.F.C., unpublished results). Therefore, for a 100 g fish, the rate of urea excretion was 2.2 μmol day^{-1}×100 g=220 μmol day^{-1} or 0.15 μmol min^{-1}. This implies that the liver must be synthesizing urea at a rate of 0.05 μmol min^{-1} g^{-1} (in a 3 g liver) to sustain the steady-state level of urea in the body of *H. signifer* in freshwater. This is much lower than the maximal capacity of OUC based on CPS activity present in the liver (0.13 μmol min^{-1} g^{-1}).

Unlike *P. motoro*, *H. signifer* increased urea excretion after exposure to 10 mmol l^{-1} NH_4Cl for 3 days, confirming its capability to upregulate urea synthesis in response to environmental ammonia. By day 4, the urea excretion rate increased 2.6-fold to 5.4 μmol day^{-1} g^{-1}. Since this was not accompanied by an increase in urea content in the muscle, which is the bulk of the animal, the rate of urea production in the liver of the experimental animal can be estimated to be 0.125 μmol min^{-1} g^{-1}, which is almost equivalent to the limit of synthetic capacity in the liver of a control specimen (maximal CPS III activities of 0.13 μmol min^{-1} g^{-1}). This could be the reason why inductions of higher activities of CPS III and related enzymes (GS, OTC, ASS + L and arginase) were necessary in specimens being exposed to environmental ammonia.

Exposure to brackish (20‰) water led to an increase in the activity of CPS III in the stomach of *H. signifer*, based on which Tam et al. (2003) concluded that the stomach of *H. signifer* (and another marine stingray, *Taeniura lymma*) could be involved in producing urea for osmoregulation in addition to its digestive function. Results from this study revealed that the CPS III activity in the stomach of *H. signifer* could not be induced by exposure to environmental ammonia, indicating that osmotic stress was the primary inductive factor. How the stomach OUC and hepatic OUC in *H. signifer* respond differentially to two different environmental stresses is unclear at present.

Increase in urea excretion in H. signifer *in response to ammonia loading*

When being exposed to environmental ammonia, *H. signifer* was able to release the excess urea without creating a problem for osmoregulation. The traditional view of urea transport is that urea crosses all cell membranes by lipid-phase permeation. To date, there is evidence for active or facilitated urea transport across various tissues in a number of vertebrates. Five urea transporters have been identified so far (Sands et al., 1997), and an Na^+-dependent urea transporter (for urea re-absorption) has been found in the gills of marine elasmobranchs (Fines et al., 2001). In higher salinities (20‰), *H. signifer* was able to retain urea for osmoregulation, albeit with limited capacity (Tam et al., 2003). By contrast, during exposure to environmental ammonia in freshwater, the excess urea synthesized was not retained but instead excreted to the external medium. Contrary to those exposed to 20‰ water (Tam et al., 2003), experimental animals in the present study showed no change in plasma osmolality and tissue (except plasma) urea content despite similar increases in the rate of urea synthesis and the capacity of the OUC. These results imply that there must be a further reduction in urea re-absorption to facilitate the excretion of the excess urea formed during exposure to environmental ammonia in a freshwater environment. Hence, *H. signifer* is an ideal organism for future studies on the regulation of urea transport in response to osmotic or ammonia stress. In future studies, it would be meaningful to find out if urea excretion was facilitated or impeded in specimens exposed to environmental ammonia in brackish water instead of freshwater.

Both H. signifer *and* P. motoro *can tolerate high levels of ammonia in the body*

Despite being ureogenic, ammonia accumulated in the body of *H. signifer* during environmental exposure, albeit at a lower level than that accumulated in the ammonotelic *P. motoro.* It had been suggested much earlier that NH_4^+ could substitute for K^+ and affect the membrane potential in the squid giant axon (Binstock and Lecar, 1969). In addition, Beaumont et al. (2000) reported depolarisation of muscle fibres in trout with elevated levels of ammonia in their tissues (from –87 mV to –52 mV) that matched the effect predicted on the basis of the measured gradient for NH_4^+ across the cell membranes. How the cells and tissues, especially those in the brain and the heart, of *H. signifer* and *P. motoro* tolerate these high ammonia levels awaits future study.

Since *H. signifer* was ureogenic, and there was an upregulation of the capacity of urea synthesis through OUC, what would be the advantage of allowing the ammonia levels in its tissues to build up when confronted with environmental ammonia? Apparently, the development of the capability to tolerate ammonia at the cellular and subcellular levels facilitates the development of a very effective strategy in defending toxicity of ammonia of exogenous origin: a relatively higher concentration of ammonia can be accommodated in the plasma, which would decrease the NH_3 partial pressure gradient across the branchial and body surfaces and would reduce the net influx of NH_3 during ammonia loading.

Increase in plasma ammonia concentration would reduce the net influx of ammonia in H. signifer *(and* P. motoro*) during ammonia loading*

Detoxification of the accumulating ammonia did not occur in *H. signifer*, at least in the first day of exposure to environmental ammonia. This was reflected by the unaltered urea excretion rate (2.2 μmol day^{-1} g^{-1}) in specimens exposed to ammonia for the first day, during which ammonia excretion (5.5 μmol day^{-1} g^{-1}) was presumably impeded totally. At pH 7.0 and a total ammonia concentration of 10 mmol l^{-1}, the concentration of NH_3 in the external medium was calculated as 0.042 mmol l^{-1} according to the Henderson–Hasselbalch equation (pK_{amm}=9.18; Boutilier et al., 1984). At the beginning of the experiment, the plasma of *H. signifer* contained 0.33 mmol l^{-1} total ammonia and had a pH of 7.521 (Y.K.I. and S.F.C., unpublished results), producing an NH_3 concentration of 0.0073 mmol l^{-1} (pK_{amm}=9.34; Boutilier et

al., 1984). This was much lower than that in the external medium, resulting in a steep NH_3 gradient impeding ammonia excretion and driving NH_3 inwards to the specimen. During this period, both exogenous and endogenous ammonia contributed to the increase in ammonia concentration in the body of the experimental specimens. By the time the plasma ammonia concentration increased to 2.15 mmol l^{-1} in the experimental specimen, which had a blood pH of 7.498 (Y.K.I. and S.F.C., unpublished results) on day 4, the NH_3 concentration increased to 0.047 mmol l^{-1}, which was more than adequate to oppose any net influx of NH_3 from the external medium. This implies that, despite the exchange of endogenous and exogenous ammonia during ammonia loading, the specimens were, in effect, detoxifying endogenous ammonia to urea because the net influx of ammonia (exogenous) would be very small (or closed to zero) after the build-up of the plasma ammonia concentration.

Synthesis of urea *de novo* in fish is energetically intensive. The detoxification of any net influx of exogenous ammonia to urea and excreting it would result in a high expenditure of energy and the maintenance of an inwardly driven NH_3 partial pressure gradient. It is probably because of this that ureogenesis is not commonly adopted by teleosts confronted with high environmental ammonia concentrations (Ip et al., 2001).

For *P. motoro* exposed to environmental ammonia, ammonia also accumulated in the plasma, increasing by 6.3-fold. Basically, this would slow down the influx of exogenous ammonia. However, since *P. motoro* was unable to detoxify ammonia into urea, the primary strategy adopted was to simply tolerate ammonia at the cellular and subcellular levels.

Reduction in rates of proteolysis and/or amino acid catabolism in H. signifer *during ammonia loading*

Ignoring momentarily the net influx of exogenous ammonia, which presumably occurred mainly at the beginning of the experiment, the deficit in ammonia excretion due to its being totally impeded in a 100 g specimen is estimated to be (5.5+5.8+5.3+6.1) μmoles×100 g=2270 μmoles (based on results from the present study). The excess amount of ammonia accumulated in the muscle, liver and plasma during such a period was 148.6 μmoles (calculated from Table 3). The excess amount of nitrogen excreted as urea during this 4-day period amounted to only {[0+(3.7–2.2)+(4.3–2.2)+(5.4–2.2)]×100}×2=1360 μmoles N (calculated from Fig. 1). Hence, the sum of ammonia equivalents accumulated and excreted (as urea) was only 148.6+1360=1508.6 μmoles. The deficit of 2270–1508.6 μmoles (761.4 μmoles) suggests indirectly that there was a decrease in endogenous ammonia production in specimens during the 4 days of exposure to NH_4Cl, which was essential to maintaining the newly established internal ammonia level.

In addition, there was a significant decrease in the content of TFAA in the liver. This implies that both proteolysis and amino acid catabolism decreased in the liver, but the decrease in the former was greater than in the latter. This might be a strategy that marine elasmobranchs cannot afford; being ureosmotic, marine elasmobranchs are committed to carnivory or high rates of proteolysis and amino acid catabolism during fasting because large amounts of nitrogen are needed to synthesize urea to maintain the internal steady-state concentration of urea. Taken together, these results indirectly indicate that the net influx of ammonia into the experimental animal during the 4 days of ammonia exposure was unlikely to be great and that the bulk of the ammonia detoxified to urea was mainly produced endogenously.

There was no significant increase in the TFAA in the muscle of specimens exposed to 10 mmol l^{-1} NH_4Cl. This implies that the process of urea synthesis in *H. signifer* was so effective that it did not have to resort to 'fixing' the endogenous or exogenous ammonia as FAAs, as suggested elsewhere for teleosts (Iwata, 1988). More importantly, the glutamine levels in the muscle, liver and plasma remained relatively unchanged in specimens exposed to 10 mmol l^{-1} NH_4Cl despite the 3-fold increase in hepatic GS activity. Hence, the excess glutamine formed in the experimental specimens must have been channelled completely into urea formation.

Conclusion

Although both *P. motoro* and *H. signifer* are freshwater stingrays, only the latter possesses a functional OUC in the liver and stomach. The capacity of urea synthesis through OUC in the liver of *H. signifer* could be upregulated by exposure to environmental ammonia. Unlike specimens exposed to brackish water, the excess urea produced by *H. signifer* exposed to environmental ammonia is excreted to the external medium instead of being retained in the body. These results suggest that urea has the dual functions of osmoregulation and ammonia detoxification in elasmobranchs living in a freshwater environment, the success of which depends primarily on the regulation of the direction and rate of urea transport. Hence, it can be concluded that the freshwater *H. signifer* is an ideal species for future studies on signals and mechanisms involved in regulating the rate of urea synthesis (in response to salinity changes or ammonia loading) and in controlling the direction and rate of urea transport (for urea retention or urea excretion).

References

Anderson, P. M. (1980). Glutamine- and N-acetylglutamate-dependent carbamoyl phosphate synthetase in elasmobranchs. *Science* **208**, 291-293.

Anderson, P. M. (1991). Glutamine-dependent urea synthesis in elasmobranch fishes. *Biochem. Cell Biol.* **69**, 317-319.

Anderson, P. M. (1995). Urea cycle in fish: molecular and mitochondrial studies. In *Fish Physiology*, vol. 14, *Ionoregulation: Cellular and Molecular Approaches to Fish Ionic Regulation* (ed. C. M. Wood and T. J. Shuttleworth), pp. 57-83. New York: Academic Press.

Anderson, P. M. (2001). Urea and glutamine synthesis: environmental influences on nitrogen excretion. In *Fish Physiology*, vol. 20, *Nitrogen Excretion* (ed. P. A. Wright and P. M. Anderson), pp. 239-277. San Diego: Academic Press.

Anderson, P. M. and Little, R. M. (1986). Kinetic properties of cyanase. *Biochemistry* **25**, 1621-1626.

Anderson, P. M. and Walsh, P. J. (1995). Subcellular localization and biochemical properties of the enzyme carbamoyl phosphate synthetase and

urea synthesis in the batrachoidid fishes, *Opsanus beta*, *Opsanus tau* and *Porichthys notatus*. *J. Exp. Biol.* **198**, 755-766.

Balinsky, J. B. (1970). Nitrogen metabolism in amphibians. In *Comparative Biochemistry of Nitrogen Metabolism* (ed. J. W. Campbell), pp. 519-637. London: Academic Press.

Ballantyne, J. S. (1997). Jaws: the inside story. The metabolism of elasmobranch fishes. *Comp. Biochem. Physiol. B* **118**, 703-742.

Barcellos, J. F. M., Wood, C. M. and Val, A. L. (1997). Ammonia and urea fluxes in *Potamotrygon* sp., a freshwater stingray of the Amazon. In *The Physiology of Tropical Fish, Synposium Proceedings* (ed. A. L. Val, D. J. Randall and D. MacKinlay), pp. 33-37. San Francisco: American Fisheries Society.

Beaumont, M. W., Taylor, E. W. and Butler, P. J. (2000). The resting membrane potential of white muscle from brown trout (*Salmo trutta*) exposed to copper in soft, acidic water. *J. Exp. Biol.* **203**, 2229-2236.

Bergmeyer, H. U. and Beutler, H. O. (1985). Ammonia. In *Methods of Enzymatic Analysis*, vol. 8 (ed. H. U. Bergmeyer, J. Bergmeyer and M. Graßl), pp. 454-461. New York: Academic Press.

Binstock, L. and Lecar, H. (1969). Ammonium ion currents in the squid giant axon. *J. Gen. Physiol.* **53**, 342-361.

Bishop, S. H. and Campbell, J. W. (1963). Carbamyl phosphate synthesis in the earthworm *Lumbricus terrestris*. *Science* **142**, 1583-1585.

Bishop, S. H. and Campbell, J. W. (1965). Arginine and urea biosynthesis in the earthworm *Lumbricus terrestris*. *Comp. Biochem. Physiol.* **15**, 51-71.

Boutilier R. G., Heming, T. A. and Iwama, G. K. (1984). Appendix: physiochemical parameters for use in fish respiratory physiology. In *Fish Physiology*, vol. 10, *Gills, Part A, Anatomy, Gas Transfer, and Acid-Base Regulation* (ed. W. S. Hoar and D. J. Randall), pp. 403-430. New York: Academic Press.

Campbell, J. W. (1965). Arginine and urea biosynthesis in the land planarian: its significance in biochemical evolution. *Nature* **208**, 1299-1301.

Campbell, J. W. (1973). Nitrogen excretion. In *Comparative Animal Physiology*. Third edition (ed. S. L. Prosser), pp. 279-316. Philadelphia: Saunders College Publishing.

Campbell, J. W. and Anderson, P. M. (1991). Evolution of mitochondrial enzyme systems in fish: the mitochondrial synthesis of glutamine and citrulline. In *Biochemistry and Molecular Biology of Fishes. I. Phylogenetic and Biochemical Perspectives* (ed. P. W. Hochachka and T. P. Mommsen), pp. 43-75. Amsterdam: Elsevier Science.

Campbell, J. W. and Bishop, S. H. (1970). Nitrogen metabolism in molluscs. In *Comparative Biochemistry of Nitrogen Metabolism* (ed. J. W. Campbell), pp. 103-206. London: Academic Press.

Campbell, J. W. and Speeg, K. V., Jr (1968). Arginine biosynthesis and metabolism in terrestrial snails. *Comp. Biochem. Physiol.* **25**, 3-32.

Cao, X. Y., Kemp, J. R. and Anderson, P. M. (1991). Subcellular localization of two glutamine-dependent carbamoyl-phosphate synthetases and related enzymes in liver of *Micropterus salmoides* (largemouth bass) and properties of isolated liver mitochondria: comparative relationships with elasmobranchs. *J. Exp. Zool.* **258**, 24-33.

Compagno, L. J. V. and Roberts, T. R. (1982). Freshwater stingrays (Dasyatidae) of Southeast Asia and New Guinea, with description of a new species of *Himantura* and reports of unidentified species. *Environ. Biol. Fishes* **7**, 321-339.

Felskie, A. K., Anderson, P. M. and Wright, P. A. (1998). Expression and activity of carbamoyl phosphate synthetase III and ornithine urea cycle enzymes in various tissues of four fish species. *Comp. Biochem. Physiol. B* **119**, 355-364.

Fines, G. A., Ballantyne, J. S. and Wright, P. A. (2001). Active urea transport and an unusual basolateral membrane composition in the gills of a marine elasmobranch. *Am. J. Physiol.* **280**, R16-R24.

Foster, R. P. (1954). Active cellular transport of urea by frog renal tubules. *Am. J. Physiol*. **179**, 372-377.

Gerst, J. W. and Thorson, T. B. (1977). Effects of saline acclimation on plasma electrolytes, urea excretion, and hepatic urea biosynthesis in a freshwater stingray, *Potamotrygon* sp. Garman, 1877. *Comp. Biochem. Physiol. A* **56**, 87-93.

Goldstein, L. and Forster, R. P. (1971). Osmoregulation and urea metabolism in the little skate *Raja erinacea*. *Am. J. Physiol*. **220**, 742-746.

Ip, Y. K., Chew, S. F. and Randall, D. J. (2001). Ammonia toxicity, tolerance, and excretion. In *Fish Physiology*, vol. 20, *Nitrogen Excretion* (ed. P. A. Wright and P. M. Anderson), pp. 109-148. San Diego: Academic Press.

Iwata, K. (1988). Nitrogen metabolism in the mudskipper, *Periophthalmus cantonensis*: changes in free amino acids and related compounds in carious tissues under conditions of ammonia loading with reference to its high ammonia tolerance. *Comp. Biochem. Physiol. A* **91**, 499-508.

Jow, L. Y., Chew, S. F., Lim, C. B., Anderson, P. M. and Ip, Y. K. (1999). The marble goby *Oxyeleotris marmoratus* activates hepatic glutamine synthetase and detoxifies ammonia to glutamine during air exposure. *J. Exp. Biol.* **202**, 237-245.

Lovejoy, N. R. (1997). Stingrays, parasites, and neotropical biogeography: a closer look at Brooks et al.'s hypotheses concerning the origins of neotropical freshwater rays (Potamotrygonidae). *Syst. Biol.* **46**, 218-230.

Perlman, D. F. and Goldstein, L. (1998). Nitrogen metabolism. In *Physiology of Elasmobranch Fishes* (ed. T. J. Shuttleworth), pp. 253-275. Berlin: Springer-Verlag.

Sands, J. M., Timmer, R. T. and Gunn, R. B. (1997). Urea transporters in kidney and erythrocytes. *Am. J. Physiol*. **273**, F321-F339.

Shankar, R. A. and Anderson, P. M. (1985). Purification and properties of glutamine synthetase from the liver of *Squalus acanthias*. *Arch. Biochem. Biophys.* **239**, 248-259.

Shuttleworth, T. J. (1988). Salt and water balance – extrarenal mechanisms. In *Physiology of Elasmobranch Fishes* (ed. T. J. Shuttleworth), pp. 171-200. Berlin: Springer-Verlag.

Smith, C. P. and Wright, P. A. (1999). Molecular characterization of an elasmobranch urea transporter. *Am. J. Physiol.* **276**, R622-R626.

Tam., W. L., Wong, W. P., Loong, A. M., Hiong, K. C., Chew, S. F., Ballantyne, J. S. and Ip, Y. K. (2003). The osmotic response of the Asian freshwater stingray (*Himantura signifer*) to increased salinity: a comparison to a marine (*Taeniura lymma*) and Amazonian freshwater (*Potamotrygon motoro*) stingrays. *J. Exp. Biol*. **206**, 2931-2940.

Thorson, T. B. (1976). The status of the Lake Nicaragua shark: an updated appraisal. In *Investigations of the Ichthyofauna of Nicaraguan Lakes* (ed. T. B. Thorson), pp. 561-574. Lincoln: University of Nebraska.

Thorson, T. B., Cowan, C. M. and Watson, D. E. (1967). *Potamotrygon* spp.: elasmobranchs with low urea content. *Science* **158**, 375-377.

Thorson, T. B., Brooks, D. R. and Mayes, M. A. (1983). The evolution of freshwater adaptation in stingrays. *Natl. Geogr. Res. Rep.* **15**, 663-694.

Tramell, P. R. and Campbell, J. W. (1972). Arginine and urea metabolism in the South American land snail, *Strophocheilus obloingus*. *Comp. Biochem. Physiol. B* **42**, 439-449.

Walsh, P. J. and Smith, C. P. (2001). Urea transport. In *Fish Physiology*, vol. 20, *Nitrogen Excretion* (ed. P. A. Wright and P. M. Anderson), pp. 279-307. San Diego: Academic Press.

Wood, C. M., Hopkins, T. E., Hogstrand, C. and Walsh, P. J. (1995). Pulsatile urea excretion in the ureagenic toadfish *Opsanus beta*: an analysis of rates and routes. *J. Exp. Biol.* **198**, 1729-1741.

Wood, C. M., Part, P. and Wright, P. A. (1995). Ammonia and urea metabolism in relation to gill function and acid–base balance in a marine elasmobranch, the spiny dogfish (*Squalus acanthias*). *J. Exp. Biol.* **198**, 1545-1558.

Xiong, X. and Anderson, P. M. (1989). Purification and properties of ornithine carbamoyl transferase from the liver of *Squalus acanthias*. *Arch. Biochem. Biophys.* **270**, 198-207.