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RESEARCH ARTICLE

The Chinese soft-shelled turtle, *Pelodiscus sinensis*, decreases nitrogenous excretion, reduces urea synthesis and suppresses ammonia production during emersion

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SUMMARY

The objective of this study was to examine the effects of 6 days of emersion on nitrogen metabolism and excretion in the Chinese soft-shelled turtle, *Pelodiscus sinensis*. Despite having a soft shell with a cutaneous surface that is known to be water permeable, *P. sinensis* lost only ~2% of body mass and was able to maintain its hematocrit and plasma osmolality, [Na^+] and [Cl^-] during 6 days of emersion. During emersion, it ameliorated water loss by reducing urine output, which led to a reduction (by 29–76%) in ammonia excretion. In comparison, there was a more prominent reduction (by 82–99%) in urea excretion during emersion due to a lack of water to flush the buccopharyngeal epithelium, which is known to be the major route of urea excretion. Consequently, emersion resulted in an apparent shift from ureotely to ammonotely in *P. sinensis*. Although urea concentration increased in several tissues, the excess urea accumulated could only account for 13–22% of the deficit in urea excretion. Hence, it can be concluded that a decrease (~80%) in urea synthesis occurred in *P. sinensis* during the 6 days of emersion. Indeed, emersion led to significant decreases in the activity of some ornithine–urea cycle enzymes (argininosuccinate synthetase/argininosuccinate lyase and arginase) from the liver of *P. sinensis*. As a decrease in urea synthesis occurred without the accumulation of ammonia and total free amino acids, it can be deduced that ammonia production through amino acid catabolism was suppressed with a proportional reduction in proteolysis in *P. sinensis* during emersion. Indeed, calculated results revealed that there could be a prominent decrease (~88%) in ammonia production in turtles after 6 days of emersion. In summary, despite being ureogenic and ureotelic in water, *P. sinensis* adopted a reduction in ammonia production, instead of increased urea synthesis, as the major strategy to ameliorate ammonia toxicity and problems associated with dehydration during terrestrial exposure.

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INTRODUCTION

The Chinese soft-shelled turtle, *Pelodiscus sinensis* (Wiegmann 1835), previously known as *Trionyx sinensis*, is a member of the Family Trionychidae. It inhabits standing or slow-flowing bodies of water, such as ponds, reservoirs and rivers, in Eastern Asia. Unlike other chelonids, it has a flat carapace, which is devoid of the usual horny laminae. As it is covered with soft, leathery skin, it has the ability to employ cutaneous respiration in addition to buccopharyngeal respiration underwater during forced submergence (Wang et al., 1989). It may be partially or completely exposed to air when the ponds or creeks dry up during hot spells or when it emerges from waters to bask. *Pelodiscus sinensis* is ureogenic and possesses a full complement of ornithine–urea cycle (OUC) enzymes in its liver (Lee et al., 2006; Lee et al., 2007). It is primarily ureotelic, excreting the majority (71%) of the waste nitrogen (N) as urea-N in freshwater. Unlike other reptiles, it excretes urea predominantly through the mouth instead of the kidney during immersion (Ip et al., 2012). When restrained on land, *P. sinensis* occasionally submerges its head in water, during which urea excretion and O_2 uptake occur simultaneously. Its buccopharyngeal epithelium is capable of active urea excretion, and expresses a putative urea transporter (UT-A2), which is absent from the kidney (Ip et al., 2012).

Buccopharyngeal urea excretion might have facilitated *P. sinensis* (Ip et al., 2012) and other members of Trionychidae (Minnich, 1979) to invade the brackish or marine environment. When exposed to brackish water (salinity 15), significant increases in plasma osmolality, [Na^+], [Cl^-] and [urea], which presumably decrease the osmotic loss of water, occur in *P. sinensis* (Lee et al., 2006). Simultaneously, there are significant increases in concentrations of urea, certain free amino acids (FAAs) and water-soluble proteins in various tissues, presumably for cell volume regulation. Proteolysis is apparently enhanced to release FAAs, which act as osmolytes (Lee et al., 2006). Catabolism of certain amino acids is also enhanced to release ammonia for increased urea synthesis. As the estimated rate of urea synthesis in unfed animals demanded only ~1.5% of the maximal capacity of carbamoyl phosphate synthetase I (CPS I) in the liver, Lee and colleagues (Lee et al., 2007) postulated that *P. sinensis* would have a prominent potential for increased urea synthesis when confronted with ammonia toxicity after feeding or

during emersion. Indeed, the rate of urea synthesis increases 7-fold during the 24 h post-feeding, which effectively prevents any postpandial surge in plasma and tissue ammonia concentration (Lee et al., 2006). However, to date, no information is available on the effects of emersion on excretory nitrogen metabolism in *P. sinensis*.

Bentley and Schmidt-Nielsen reported that, in air, the total evaporative water loss through the cutaneous surface of the soft-shelled turtle *Apalone spinifera* (previously *Trionyx spinifer*; 48.0±4.8 mg cm^{-2} day^{-1}) was three times greater than that of the pond slider *Trachemys scripta* (previously *Pseudemys scripta*; 15.8±1.7 mg cm^{-2} day^{-1}) (Bentley and Schmidt-Nielsen, 1970). They, therefore, concluded that cutaneous water loss in *A. spinifera* approached that of some aquatic amphibians [e.g. *Necturus* (Bentley and Schmidt-Nielsen, 1970)]. As *P. sinensis*, similar to *A. spinifera*, does not possess a horny carapace, it would probably experience more severe dehydration stress than hard-shelled turtles during emersion. Furthermore, as soft-shelled turtles are incapable of producing hyperosmotic urine (Shoemaker and Nagy, 1977) or salt gland secretion (Minnich, 1979; Shoemaker and Nagy, 1977), severe water loss through dehydration would theoretically lead to increases in plasma ionic concentrations. Hence, the first objective of this study was to determine whether 6 days of emersion would result in increases in plasma osmolality, $[Na^+]$ and $[Cl^-]$ in *P. sinensis*. The hypothesis tested was that *P. sinensis* could effectively conserve water through a reduction in urine production during this period. As reduced urine production could theoretically impede waste-N excretion, the second objective of this study was to determine whether 6 days of emersion would result in decreases in the rate of ammonia and/or urea excretion. We hypothesized that the normal ammonia excretion rate would be maintained during emersion because ammonia could be excreted through the highly vascularized ventral carapace, which was in constant contact with a film of water during emersion. Because *P. sinensis* excreted urea mainly through the buccopharyngeal cavity (Ip et al., 2012), and as no water was available to rinse the mouth during emersion, we further hypothesized that emersion would lead to a greater reduction in urea excretion than in ammonia excretion.

Traditionally, studies on ammonia defense in animals focused on the detoxification of ammonia to less toxic compounds like urea, uric acid and/or glutamine (Campbell, 1973; Campbell, 1991; Campbell, 1995). Because *P. sinensis* is ureogenic, the third objective of this study was to examine whether urea accumulation and increased urea synthesis would occur in *P. sinensis* during 6 days of emersion. As ammonia production can be suppressed through a reduction in amino acid catabolism (Jow et al., 1999; Lim et al., 2001, Ip et al., 2001a; Chew et al., 2001; Chew et al., 2003b; Chew et al., 2004; Tay et al., 2003; Walsh et al., 2004; Loong et al., 2005; Ip et al., 2005b; Ip et al., 2005c) or through the partial catabolism of certain amino acids to alanine (Ip et al., 2001c; Chew et al., 2001; Chew et al., 2003a), a reduction in ammonia production can be an important adaptation to ameliorate ammonia toxicity in some aquatic animals during emersion (Ip et al., 2001b; Ip et al., 2004; Chew et al., 2005). Therefore, the fourth objective of this study was to examine changes in tissue ammonia and FAA concentrations in *P. sinensis* during 6 days of emersion, and to evaluate indirectly whether a reduction in amino acid catabolism and/or protein degradation would have occurred. We hypothesized that *P. sinensis* could effectively reduce ammonia production during emersion, which would manifest itself as a deficit between decreases in excretion of nitrogenous waste and increases in accumulation of nitrogenous compounds. If indeed a profound suppression of ammonia production occurred after 6 days of emersion, we speculated that there would be a reduction in the rate of urea synthesis and perhaps even decreases in the activity of some OUC enzymes in the liver of *P. sinensis* exposed to terrestrial conditions, which is contrary to the traditional notion that ureogenic aquatic animals would always increase urea synthesis in response to emersion.

MATERIALS AND METHODS

Animals

Specimens of *P. sinensis* (200–400 g body mass) were purchased from a turtle farm in Malaysia and transferred to the National University of Singapore. They were maintained in plastic aquaria in freshwater (salinity 1) at 25°C in the laboratory, and the water was changed daily. Salinity was monitored using a YSI Model 30/10 FT salinometer (Yellow Springs Instrument Co. Inc., Yellow Springs, OH, USA). No attempt was made to separate the sexes. The turtles were acclimated to laboratory conditions for at least a week. During the acclimation period, they were fed prawn meat. Food was withdrawn 5 days prior to experiments so that urine could be collected without contamination with fecal material. As fasting would naturally affect excretory nitrogen metabolism, it was essential to have parallel controls (kept in water) to compare with the experimental turtles (exposed to terrestrial conditions) at specific time points during the 6 day period. All experiments were performed under a 12 h:12 h dark:light regime. Procedures adopted in this study were approved by the Institutional Animal Care and Use Committee of the National University of Singapore (IACUC 033/12).

Exposure of *P. sinensis* to terrestrial conditions, collection of water and urine samples and determination of urine volume

Body mass of individual turtles was recorded with a Shimadzu animal balance (Shimadzu Co., Kyoto, Japan) to the nearest 0.1 g. Following a previously published procedure (Ip et al., 2012), flexible latex tubing (length 18 cm, radius 0.7 cm) was attached externally around the tail anterior to the cloaca of *P. sinensis* using 3M Vetbond tissue adhesive (3M, St Paul, MN, USA). An opening made at the very tip of the tube was held closed by a dialysis clip. Turtles immersed in 10 volumes (w/v) of freshwater (salinity 1) were regarded as controls. Experimental turtles were exposed to terrestrial conditions at 25°C in plastic aquaria tanks (L44 cm×W29 cm×H11 cm) for 6 days with 50 ml of freshwater (salinity 1), which formed a thin film (<0.4 mm in depth) at the bottom of the tank. Under such conditions, the turtle's body, except perhaps the skin of the ventral carapace, was completely exposed to air and no part of the body was immersed in water. The average relative humidity of the room in which animals were kept was 84%. Water samples (3 ml) were collected daily and acidified with 70 µl of 1 mol l^{-1} HCl to retain NH_4^+, and kept at 4°C until analysis for ammonia and urea, which was performed within 48 h. Urine was collected daily by emptying the contents of the tubing through the opening into a 5 or 10 ml measuring cylinder. Deionized water was introduced into the tubing through the opening to rinse the inside surface before resealing with the dialysis clip. Urine samples were acidified with 1 mol l^{-1} HCl, with the ratio of acid to urine being 7:300, and kept at 4°C until analysis Analysis was performed within 48 h, and preliminary analysis confirmed that no degradation of urea and no loss of ammonia occurred during the 2 day period.

Exposure of *P. sinensis* to terrestrial conditions and collection of water and tissue samples

Turtles were exposed to the control and terrestrial conditions as mentioned above. Both control and experimental turtles could urinate into the container at liberty. Body mass of individual turtles was recorded during 6 days of immersion or 6 days of emersion. Water

samples (3 ml) were collected daily and acidified as mentioned previously, and kept at 4°C until analysis for ammonia and urea.

On days 3 and 6, turtles were killed by a strong blow to the head. The muscle, liver and brain were quickly excised. The excised tissue and organ samples (<1 g) were immediately freeze-clamped in liquid nitrogen with pre-cooled tongs (Faupel et al., 1972). Frozen samples were kept at −80°C until analysis.

Blood was obtained by cardiac puncture. Hematocrit was determined by collecting blood in heparinized capillary tubes, which were centrifuged at 3000 r.p.m. for 7 min. The hematocrit was expressed as percentage packed cell volume against the total volume of blood. In addition, blood samples were collected into heparinized syringes, and centrifuged at 5000 ***g*** and 4°C for 5 min to obtain the plasma. A portion of the plasma was used for analyses of osmolality and concentrations of Na^+ and Cl^-. The rest of the plasma was deproteinized by the addition of an equal volume (v/v) of ice-cold 6% trichloroacetic acid (TCA) and centrifuged at 10,000 ***g*** and 4°C for 15 min. The resulting supernatant was kept at −25°C until analysis.

The frozen samples were weighed, ground to a powder in liquid nitrogen, and homogenized three times in 5 volumes (w/v) of ice-cold 6% TCA at 24,000 r.p.m. for 20 s each using an Ultra-Turrax homogenizer (Janke and Kundel, Stanfeni, Germany) with intervals of 10 s between each homogenization. The homogenate was centrifuged at 10,000 ***g*** and 4°C for 30 min, and the supernatant obtained was kept at −25°C until analysis.

Determination of ammonia and urea concentrations in water and urine samples

Ammonia and urea concentrations in water and urine samples were determined as described previously (Koops et al., 1975; Jow et al., 1999). The pH of the sample was adjusted to 6.0–6.5 with 1 mol l^{-1} KOH. Two reagents were involved in the colorimetric ammonia assay. Reagent A contained 1.44 mol l^{-1} NaCl and 5.5 mol l^{-1} sodium nitroprusside, while reagent B contained 0.031 mol l^{-1} sodium dichloroisocyanurate and 2.7 mol l^{-1} KOH. To 1 ml of the neutralized sample, 0.1 ml of reagent A was added rapidly followed by 0.1 ml of reagent B with immediate mixing. The reaction mixture was incubated at 25°C for 30 min and the absorbance recorded at 660 nm. The NH_4Cl obtained (Merck Chemical Co., Darmstadt, Germany) was used as a standard for comparison. Urea was analyzed colorimetrically by the addition of 0.2 ml of water followed by 0.5 ml of a 2:1 mixture of reagent A, containing 0.6 mmol l^{-1} $FeCl_3$ in 8.5% H_3PO_4 and 30% KH_2PO_4, and regent B, containing 247 mmol l^{-1} 2,3-butanedione monoxime and 5 mmol l^{-1} thiosemicarbazide, to 0.2 ml of neutralized sample. The mixture was incubated at 100°C for 10 min, then cooled on ice, and the absorbance recorded at 525 nm. With another 0.2 ml of the same sample, a urea assay was performed after incubation with 0.2 ml of 20 mmol l^{-1} imidazole buffer (pH 7.2) containing 2 IU urease for 15 min at 30°C but without the addition of 0.2 ml water. The difference in absorbance between the samples with and without urease treatment was used to calculate the urea concentration. Urea obtained from Sigma Chemical Co. (St Louis, MO, USA) was used as a standard for comparison. The rates of ammonia or urea excretion were expressed as μmol N day^{-1} g^{-1} turtle.

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

Plasma osmolality was analyzed using a Wescor 5500 vapor pressure osmometer (Wescor, UT, USA). Concentrations of Na^+ and Cl^- were determined by a Corning 410 flame photometer, and a Corning 925 chloride analyzer, respectively (Corning, Halstead, UK).

Determination of activity of OUC enzymes, glutamine synthetase and glutamate dehydrogenase

Preliminary results indicated that OUC enzymes were present only in the liver of *P. sinensis*. The liver was excised quickly and homogenized three times (20 s each with 10 s intervals) in 5 volumes (w/v) of ice-cold extraction buffer containing 50 mmol l^{-1} Hepes (pH 7.6), 50 mmol l^{-1} KCl, 0.5 mmol l^{-1} EDTA, 1 mmol l^{-1} dithiothreitol and 0.5 mmol l^{-1} phenylmethylsulfonyl fluoride (PMSF) using an Ultra-Turrax homogenizer. The homogenate was sonicated (110 W, 20 kHz; Misonix Incorporated, Farmingdale, NY, USA) three times for 20 s each, with a 10 s break between each sonication. The sonicated sample was centrifuged at 10,000 ***g*** and 4°C for 15 min. After centrifugation, the supernatant was passed through a BioRad P-6DG column (BioRad Laboratories, Hercules, CA, USA) equilibrated with the extraction buffer without EDTA and PMSF. The filtrate obtained was used directly for enzyme assays. The activity of CPS, ornithine transcarbamylase (OTC), argininosuccinate synthetase together with arginiosuccinate lyase (ASS + ASL), arginase and glutamine synthetase (GS) was determined as described previously (Lee et al., 2006). Glutamate dehydrogenase activity in the amination direction was assayed as described elsewhere (Ip et al., 1993). Enzyme activities were expressed as μmol substrate used/product formed min^{-1} g^{-1} tissue.

Determination of ammonia, urea and FAA content of tissue samples

The pH of the deproteinized sample was adjusted to between 6.0 and 6.5 with 2 mol l^{-1} $KHCO_3$. The ammonia and urea content were determined using previously published methods (Bergmeyer and Beutler, 1985; Jow et al., 1999). For FAAs analysis, 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-10 A amino acid analysis system (Shimadzu, Kyoto, Japan) with a Shim-pack ISC-07/S1504 Li-type column. Despite a complete FAA analysis being performed for each sample, only alanine, glutamate, glutamine, total FAA (TFAA) and total essential FAA (TEFAA) content are presented in this report. The TFAA content was calculated by the summation of all FAAs, and that of TEFAA was calculated as the sum of histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine. Results are expressed as μmol g^{-1} tissue or μmol ml^{-1} plasma.

Statistical analyses

The results are presented as means ± s.e.m. The results in Fig. 1 were analyzed using 2-way repeated-measures ANOVA followed by least-square means (LSMEANS) to evaluate differences between means using SAS/STAT. Arcsine transformation was applied to all percentage data before statistical analysis. Results in all other tables and figures, and those for hematocrit, masses, $[Na^+]$ and $[Cl^-]$ were evaluated using independent *t*-tests. Differences with $P<0.05$ were regarded as statistically significant.

RESULTS

Body mass, hematocrit, plasma osmolality, and plasma $[Na^+]$ and $[Cl^-]$

After 3 and 6 days of emersion, the body mass of *P. sinensis* (N=5) decreased by 2.0±0.2% and 2.3±0.5%, respectively. However, the hematocrit value for turtles exposed to terrestrial conditions for 6 days (30±1%, N=5) was not significantly different from that of the controls immersed for 6 days (28±3%, N=4). Similarly, the plasma osmolality, $[Na^+]$ and $[Cl^-]$ for turtles exposed to terrestrial

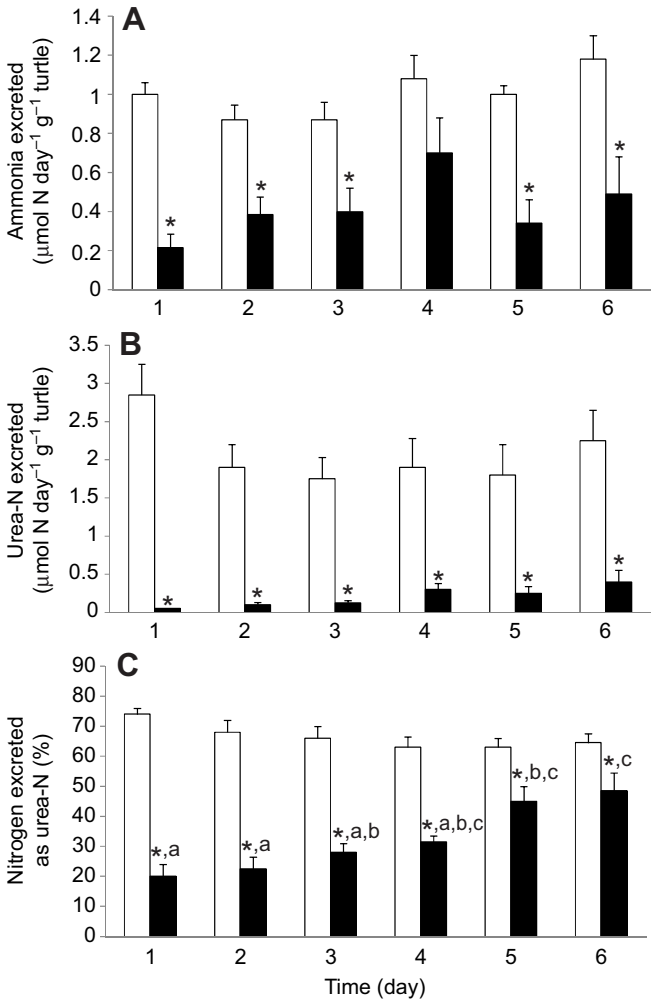

Fig. 1. The rate of excretion of ammonia (A) and urea (B), and the percentage of total-N excreted as urea-N (C) by *Pelodiscus sinensis* over 6 days of emersion, during which turtles could urinate into the container at liberty. White bars represent immersed controls. Black bars represent turtles exposed to terrestrial conditions. Values are means + s.e.m. (N=4). *Significantly different from the corresponding control value, P<0.05. Means with different letters are significantly different, P<0.05.

conditions for 6 days (288±3 mosmol kg^{-1}, 128±1 mmol l^{-1} and 77±4 mmol l^{-1}, respectively, N=5) were not significantly different from those of the controls immersed for 6 days (285±2 mosmol kg^{-1}, 129±2 mmol l^{-1} and 84±2 mmol l^{-1}, respectively, N=4).

Ammonia and urea excretion rates during emersion

On day 1, the rates of ammonia (Fig. 1A) and urea-N (Fig. 1B) excretion (measured from urine collected in the container) in immersed turtles (control) were 1.0 and 2.9 μmol N day^{-1} g^{-1}, respectively. So, *P. sinensis* immersed in water was primarily ureotelic, excreting ~74% of waste-N as urea-N. Surprisingly, turtles apparently switched from ureotely to ammonotely during emersion, and the percentage of waste-N excreted as urea-N decreased from 74% to 20% on day 1 and increased gradually to 47% by day 6 (Fig. 1C). This decrease in the rate of urea excretion was of a much greater magnitude (decreased by 81–99%) than that of ammonia excretion (decreased by 34–77%) over the 6 day period.

Urine volume and rates of ammonia and urea excretion through the urine

By collecting urine into the latex tubing, it was discovered that turtles immersed in water produced 7.3–16 ml of urine daily, but those undergoing 5 days of emersion had a daily urine production of only 2.0–3.4 ml (supplementary material Table S1). On day 6, anuria occurred in 5 out of 6 turtles, with one turtle producing 6.7 ml of urine (supplementary material Table S1).

For control turtles immersed in water, the daily rates of ammonia excretion through the extra-renal and the renal routes were comparable: 31–80% of the ammonia-N excreted during the 6 day period originated from the urine (Table 1). In contrast, renal urea excretion could account for only 0.45–15% of the total urea excreted throughout the 6 days of immersion (Table 2). Emersion resulted in significant decreases in ammonia excretion through the extra-renal (days 1, 3 and 6) and the renal (days 2 and 4) routes (Table 1). It also resulted in a significant decrease in the rate of urea excretion through the extra-renal route on days 1, 2, 3, 4 and 6, but had no significant effects on the rate of renal urea excretion (Table 2).

Effect of emersion on hepatic OUC enzyme activity

There were no significant changes in activity of OUC enzymes from the liver of turtles after 3 days of emersion. However, 6 days of emersion led to significant decreases in the activity of ASS+ASL and arginase, by 36% and 28%, respectively (Table 3).

Effect of emersion on tissue ammonia and urea content

There were no significant changes in the ammonia content of muscle and liver throughout the 6 days of emersion, but the ammonia content of plasma and brain increased significantly by 1.4- and 1.6-fold, respectively, on day 6 (Table 4). In contrast, there were significant increases in the urea content of all these tissues and organs. On day 3, the urea content of the brain, liver, muscle and plasma increased by 2.5-, 2.3-, 2.7- and 2.9-fold, respectively, and leveled off thereafter (Table 4).

Effect of emersion on tissue FAA content

In general, 6 days of emersion had no significant effect on FAA, TFAA and TEFAA content of the liver, muscle and plasma of *P. sinensis* (Table 5). However, there was a significant increase in the glutamine content in the brain on days 3 (1.6-fold) and 6 (2.1-fold). On day 6, there was also a significant decrease in the TEFAA content of the brain (Table 5).

An estimate of the overall N balance for a 300 g *P. sinensis*

Based on previous results (Lee et al., 2006), liver and muscle mass for a 300 g turtle were taken to be 9.4 g and 74 g, respectively. The plasma volume in a 300 g turtle was taken as 15 ml (Lee et al., 2006). The brain was not taken into the calculation because of its light mass. Of note, the carapace and the endoskeleton of a 300 g turtle weighed 150 g and 67 g, respectively, which implies that, similar to animals without a carapace, the liver and muscle together would constitute ~56% of the body mass excluding the carapace but inclusive of the endoskeleton. The construction of a balance sheet (Table 6) for the excretion and retention of N during emersion for a 300 g turtle reveals that emersion indeed resulted in a decrease in nitrogenous waste excretion. The reduction in N-waste excretion amounted to 2417 and 4395 μmol N on days 3 and 6, respectively, but the respective excess N accumulated was only 395 and 472 μmol N (Table 6), which indicates the occurrence of a prominent reduction in ammonia production.

Table 1. Rate of renal or extra-renal ammonia excretion, and the percentage of ammonia-N excreted through the renal route, in *Pelodiscus sinensis* during 6 days of immersion (control) or emersion

	Control			Emersion		
	Rate of ammonia excretion			Rate of ammonia excretion		
Day	Extra-renal	Renal	% Renal ammonia-N	Extra-renal	Renal	% Renal ammonia-N
1	0.21±0.06 (4)	0.26±0.09 (4)	52±5 (4)	0.057±0.011* (7)	0.18±0.10 (7)	46±16 (7)
2	0.45±0.25 (4)	1.0±0.3 (4)	68±17 (4)	0.070±0.028 (7)	0.12±0.05* (7)	40±15 (7)
3	0.23±0.04 (4)	0.65±0.60 (4)	31±22 (4)	0.068±0.015* (7)	0.41±0.11 (7)	81±8 (7)
4	0.17±0.03 (4)	0.75±0.25 (4)	80±3 (4)	0.19±0.08 (7)	0.19±0.08* (7)	34±12 (7)
5	0.34±0.05 (4)	0.36±0.12 (4)	48±12 (4)	0.16±0.05 (5)	0.22±0.11 (5)	43±15 (5)
6	0.36±0.11 (4)	0.78±0.32 (4)	63±12 (4)	0.024±0.011* (6)	1.3 (1)†	98 (1)†

Urine was collected into flexible latex tubing attached to the tail.
Values represent means ± s.e.m., with number of determinations in parentheses. Rate of ammonia excretion is given as μmol N day^{-1} g^{-1} turtle.
*Significantly different from the corresponding control condition.
†Total *N*=6, but no urine production in 5 out of the 6 turtles.

DISCUSSION

Osmotic stress of emersion

In the past, the rates of evaporative water loss in turtles were determined in the laboratory under non-physiological conditions; the external surfaces of the animals were completely dried and dry air was flushed over animals during measurements (e.g. Bentley and Schmidt-Nielsen, 1970). In a more recent study, Peterson and Greenshields exposed *T. scripta* to 10 days of dehydration at a relative humidity of 40% at 25°C (Peterson and Greenshields, 2001). At the end of the 10 day period, the reduction in body mass of *T. scripta* amounted to 19–32%, and the plasma osmolality increased from 275 to 402 mosmol l^{-1} (Peterson and Greenshields, 2001). The hatchlings of the leatherback sea turtle, *Dermochelys coriacea*, also dehydrated rapidly when denied access to seawater; the hematocrit increased significantly from 30±1% to 39±1% and the plasma [Na^+] increased significantly from 138±3 to 166±11 mmol l^{-1} within a 12 h period (Reina et al., 2002). In contrast, *P. sinensis* exhibited only a slight change (2–2.3%) in body mass after 3 or 6 days of terrestrial exposure. Thus, it was not surprising that 6 days of terrestrial exposure had no significant effect on its hematocrit, plasma osmolality, and [Na^+] and [Cl^-]. Taken together, these results indicate that *P. sinensis* can regulate water loss during terrestrial exposure and is well adapted to emersion in its natural habitat.

Decreases in urine production and urea excretion during emersion

Some reptiles exhibit special adaptations to minimize water loss during emersion. When *T. scripta* is exposed to terrestrial conditions, dehydration decreases the glomerular filtration rate and increases tubular reabsorption of water, causing anuria in more severe cases (Dantzler and Schmidt-Nielsen, 1966). Anuria also occurs in *Chelodina longicollis* after 20 days of dehydration, during which the volume of urine in the bladder of *C. longicollis* decreases from a mean of 32 ml to 2 ml, showing that water is reabsorbed from the bladder (Rogers, 1966). In the case of *P. sinensis*, the daily rate of urine excretion decreased from 7–15 ml day^{-1} (control) to 2–3 ml day^{-1} during 5 days of emersion. By day 6, 5 out of the 6 experimental animals exhibited anuria.

During 6 days of emersion, there was a significant decrease in the daily rate of nitrogenous waste (ammonia-N+urea-N) excretion. As hypothesized, the magnitude of the decrease in the rate of urea excretion was much greater than that of ammonia excretion throughout the 6 day period. This could be due to two factors acting separately or in combination. Firstly, urea synthesis through the hepatic OUC was suppressed during emersion. Secondly, different routes were involved in ammonia and urea excretion, and emersion hindered urea excretion more than ammonia excretion. As urea was excreted mainly through the buccopharyngeal cavity (Ip et al., 2012) and as water was not available to flush the buccopharyngeal epithelium during emersion, urea excretion was logically impeded to a greater extent than ammonia excretion.

An explanation for the apparent change from ureotely to ammonotely during emersion

Although the urea excretion rate decreased drastically to 1.5–18.2% of the control value, the ammonia concentration in the urine remained

Table 2. Rate of renal or extra-renal urea excretion, and the percentage of urea-N excreted through the renal route, in *P. sinensis* during 6 days of immersion (control) or emersion

	Control			Emersion		
	Rate of urea excretion			Rate of urea excretion		
Day	Extra-renal	Renal	% Renal urea-N	Extra-renal	Renal	% Renal urea-N
1	1.2±0.2 (4)	0.19±0.12 (4)	11±6 (4)	0.064±0.036* (7)	0.0030±0.0018 (7)	8.3±4.7 (7)
2	0.80±0.20 (4)	0.0043±0.0026 (4)	0.45±0.12 (4)	0.062±0.046* (7)	0.076±0.047 (7)	27±14 (7)
3	0.71±0.15 (4)	0.071±0.070 (4)	4.2±4.0 (4)	0.042±0.023* (7)	0.18±0.08 (7)	49±18 (7)
4	0.72±0.12 (4)	0.044±0.035 (4)	4.2±2.7 (4)	0.058±0.035* (7)	0.076±0.041 (7)	38±16 (7)
5	0.57±0.19 (4)	0.11±0.09 (4)	15±11 (4)	0.14±0.07 (5)	0.14±0.06 (5)	49±21 (5)
6	1.4±0.3 (4)	0.0074±0.0037 (4)	0.65±0.37 (4)	0.023±0.004* (6)	0.0030 (1)†	15 (1)†

Urine was collected into flexible latex tubing attached to the tail.
Values are means ± s.e.m., with number of determinations in parentheses. Rate of urea excretion is given as μmol N day^{-1} g^{-1} turtle.
*Significantly different from the corresponding control condition.
†Total *N*=6, but no urine production in 5 out of the 6 turtles.

Table 3. Activity of OUC enzymes, glutamine synthetase and glutamate dehydrogenase in the amination direction from the liver of *P. sinensis* exposed to 3 or 6 days of immersion (control) or emersion

	Day 3		Day 6	
	Control	Emersion	Control	Emersion
CPS I				
NH_4Cl	n.d.	n.d.	n.d.	n.d.
NH_4Cl + AGA	0.45±0.05	0.56±0.04	0.26±0.04	0.25±0.04
NH_4Cl + AGA+UTP	0.38±0.04	0.48±0.04	0.21±0.04	0.22±0.04
OTC	98±9	100±9	97±15	81±9
ASS+ASL	0.41±0.07	0.43±0.05	0.36±0.04	0.23±0.02*
Arginase	171±12	205±14	208±5	149±12*
GS	1.7±0.7	1.2±0.2	1.0±0.2	0.96±0.16
GDH	90±7	87±11	85±34	77±15

OUC, ornithine–urea cycle; CPS 1, carbamoyl phosphate synthetase I; AGA, *N*-acetyl L-glutamate; UTP, uridine triphosphate; OTC, ornithine transcarbamylase; ASS+ASL, argininosuccinate synthetase + lyase; GS, glutamine synthetase; GDH, glutamate dehydrogenase; and n.d., not detectable.

Activity (mean ± s.e.m.) is given as $\mu mol\,min^{-1}\,g^{-1}$ liver, *N*=4.

*Significantly different from the corresponding control condition.

Table 5. Free amino acid (FAA), total FAA (TFAA) and total essential FAA (TEFAA) content of the brain, liver, muscle and plasma of *P. sinensis* exposed to 3 or 6 days of immersion (control) or emersion

	Day 3		Day 6	
	Control	Emersion	Control	Emersion
Brain				
Alanine	0.28±0.03	0.31±0.09	0.19±0.04	0.22±0.02
Glutamate	4.8±0.9	5.7±0.4	4.8±0.5	5.8±0.3
Glutamine	1.9±0.2	3.0±0.3*	1.5±0.2	3.1±0.6*
TFAA	12±1	12±1	10±1	13±1
TEFAA	0.91±0.13	0.62±0.04	0.68±0.03	0.51±0.02*
Liver				
Alanine	0.14±0.03	0.090±0.036	0.084±0.017	0.055±0.008
Glutamate	0.90±0.07	1.0±0.3	0.71±0.11	0.76±0.14
Glutamine	0.25±0.13	1.0±0.7	0.096±0.052	0.070±0.013
TFAA	10±1	9.2±1.6	8.3±1.1	7.6±1.6
TEFAA	1.0±0.2	0.64±0.09	0.85±0.13	0.56±0.14
Muscle				
Alanine	0.24±0.04	0.27±0.02	0.23±0.04	0.22±0.02
Glutamate	0.79±0.37	0.54±0.16	1.0±0.3	0.59±0.1
Glutamine	0.66±0.09	0.94±0.14	0.50±0.1	0.81±0.16
TFAA	12±2	11±1	11±1	10±1
TEFAA	1.8±0.2	1.6±0.2	2.0±0.2	1.4±0.3
Plasma				
Alanine	0.083±0.031	0.087±0.013	0.095±0.020	0.068±0.008
Glutamate	0.092±0.029	0.058±0.004	0.060±0.021	0.067±0.017
Glutamine	0.13±0.04	0.15±0.03	0.11±0.02	0.13±0.03
TFAA	2.0±0.7	1.6±0.3	2.2±0.4	1.7±0.3
TEFAA	1.0±0.4	0.79±0.21	1.2±0.3	0.86±0.23

FAA content (means ± s.e.m.) is gvien as $\mu mol\,g^{-1}$ tissue, *N*=4.

*Significantly different from the corresponding control condition.

relatively unchanged and the ammonia excretion rate decreased by 34–77% in *P. sinensis* during 6 days of exposure to terrestrial conditions. Consequently, there was an apparent shift from ureotely during immersion to ammonotely during emersion. It is probable that the reduction in ammonia excretion through the urine and buccopharyngeal route during emersion was partially compensated for by an increase in ammonia excretion through the skin of the ventral carapace, which was in constant contact with water. The compensation was gradual and took 2–3 days, indicating that the transition between the renal route and the cutaneous route could be time dependent (Fig. 1). Indeed, the skin of soft-shelled turtles is known to be permeable to respiratory gases (Girgis, 1961; Wang et al., 1989) and therefore it is logical that it also constitutes an appropriate route for ammonia (NH_3) excretion. By contrast, urea transport is known to be facilitated by a urea transporter, and the putative UT-A2 urea transporter obtained from the buccopharyngeal cavity of *P. sinensis* is apparently not expressed in the skin (Y.K.I., unpublished results).

Decrease in urea synthesis during emersion

Six days of emersion led to significant increases in the urea content of all tissues and organs studied. However, the excess urea-N accumulated in a 300 g turtle was only ~414 μmol N on day 3 and ~432 μmol N on day 6, while the respective decreases in urea-N excretion amounted to ~1894 and ~3364 μmol N (Table 6). If urea had been produced at a constant rate, a proportional amount of urea should theoretically be accumulated in the turtle. As the excess urea accumulated could only account for 22% and 13% of the deficit in urea excretion on day 3 and day 6, respectively, it is logical to deduce that there was a decrease in the rate of urea synthesis in *P. sinensis* during 6 days of emersion.

The urea production rate of *P. sinensis* immersed in freshwater can be estimated as $2.1\,\mu mol\,N\,day^{-1}\,g^{-1}$ from the urea excretion rate, because tissue urea content is maintained at a steady state by a balance between urea production and urea excretion during immersion. On day 6 of emersion, the averaged daily urea production rate for a 300 g turtle (Table 6) can be calculated from the urea excreted during the 6 day period (366 μmol N) and excess urea accumulated in various tissues on day 6, i.e. (366+298+29+87) μmol N/(6 days×300 g), or $0.43\,\mu mol\,N\,day^{-1}\,g^{-1}$. That means the averaged daily urea production rate decreased by [(2.1–0.43)/2.1]×100=79.5% during 6 days of emersion.

Judging by enzyme activities determined in the presence of saturating concentrations of substrates, which were close to V_{max}

Table 4. Ammonia and urea content of various tissues of *P. sinensis* exposed to 3 or 6 days of immersion (control) or emersion

	Ammonia ($\mu mol\,g^{-1}$ tissue)				Urea ($\mu mol\,g^{-1}$ tissue)			
	Day 3		Day 6		Day 3		Day 6	
	Control	Emersion	Control	Emersion	Control	Emersion	Control	Emersion
Brain	0.80±0.13	0.69±0.10	0.41±0.07	0.66±0.06*	1.2±0.5	3.0±0.4*	1.1±0.2	3.0±0.3*
Liver	3.0±0.6	1.6±0.2	1.3±0.2	1.6±0.2	1.3±0.4	3.0±0.4*	1.2±0.3	2.8±0.3*
Muscle	0.48±0.11	0.66±0.08	0.37±0.05	0.43±0.06	1.2±0.4	3.2±0.4*	1.4±0.3	3.4±0.2*
Plasma	0.37±0.05	0.46±0.01	0.32±0.05	0.44±0.03*	1.2±0.4	3.5±0.4*	1.4±0.3	4.3±0.5*

Values are means ± s.e.m., *N*=6.

*Significantly different from the corresponding control condition.

Table 6. Estimate of the reduction in nitrogenous excretion and increase in nitrogenous accumulation in a hypothetical 300 g *P. sinensis* exposed to 3 or 6 days of immersion (control) or emersion

	Day 3			Day 6		
	Control	Emersion	Difference	Control	Emersion	Difference
Excreted from *P. sinensis*						
Ammonia-N	825	302	–523	1795	764	–1031
Urea-N	1968	74	–1894	3730	366	–3364
Reduction in N-excretion			**–2417**			**–4395**
Accumulated in muscle (74 g)						
Ammonia-N	36	49	+13	27	32	+5
Urea-N	179	474	+295	208	506	+298
Accumulated in liver (9.4 g)						
Ammonia-N	27	14	–13	11	15	+4
Urea-N	24	54	+30	21	50	+29
Accumulated in plasma (15 ml)						
Ammonia-N	6	7	+1	5	7	+2
Urea-N	37	106	+69	43	130	+87
Increase in N-accumulation			**+395**			**+427**
N-excretion reduction + N-accumulation increase			**–2022**			**–3968**

Nitrogen values (μmol N) were calculated taking into account the muscle, liver and plasma.
The mass of the carapace and endoskeleton of a 300 g *P. sinensis* is 150 g and 67 g, respectively; hence, similar to animals without a carapace, the liver and muscle together would constitute ~56% of the body mass excluding the carapace but inclusive of the endoskeleton.

levels, both CPS I and ASS+ASL could be rate limiting in the hepatic OUC in *P. sinensis*. The decreases in ASS+ASL activity (by 40%) and arginase activity (by 30%) supports the proposition that there was a decrease in the rate of urea synthesis during emersion. Thus, the general notion that increased urea synthesis would occur to ameliorate ammonia toxicity in urogenic aquatic animals during emersion is not applicable to *P. sinensis*. Between increased urea synthesis and decreased ammonia production, the latter would appear to be much more effective for animals that remain quiescent during air exposure.

Possible decrease in ammonia production during emersion

CPS I utilizes ammonia as one of the substrates to produce urea. Hence, there should be increases in the ammonia content of various tissues and organs if decreased urea synthesis occurred concurrently with an unchanged or decreased rate of ammonia excretion during emersion. However, significant increases in ammonia content were observed only in the plasma and brain on day 6, and the accumulated ammonia was inadequate to account for the decrease in urea synthesis. Therefore, it can be deduced that there was also a reduction in ammonia production (~88%; Table 6), which occurred mainly through amino acid catabolism under fasting conditions. As TFAA and TEFAA content remained unchanged in various tissues and organs, it is logical to deduce that the release of amino acids through proteolysis was also proportionally reduced in *P. sinensis* during emersion. It is probable that decreased urea synthesis and ammonia production could contribute to an overall reduction in metabolic activity in response to terrestrial exposure. However, our results indicate that partial catabolism of certain amino acids to alanine (Ip et al., 2001a; Chew et al., 2001; Chew et al., 2003a) was not involved in reducing ammonia production in *P. sinensis*, because there was no accumulation of alanine during emersion. While 6 days of emersion did not result in extraordinary increases in glutamine synthesis in extra-cranial tissues in *P. sinensis* as in some tropical air-breathing fishes exposed to air (Jow et al., 1999; Ip et al., 2001b; Chew et al., 2001; Tay et al., 2003), its brain was capable of detoxifying ammonia to glutamine as in many other vertebrates (Cooper and Plum, 1987; Peng et al., 1998; Ip et al., 2005a).

Conclusions

During 6 days of emersion, *P. sinensis* reduced water loss through a reduction in urine production, which could have contributed to the relatively stable hematocrit and plasma osmolality, $[Na^+]$ and $[Cl^-]$. Despite a decrease in urine production, *P. sinensis* was able to maintain ammonia excretion rates at 23–66% of the immersed control values. However, urea excretion was drastically reduced. Although tissue urea content increased significantly, the rate of urea synthesis decreased, with significant decreases in the activity of certain hepatic OUC enzymes. An analysis of the decrease in nitrogenous excretion and the increase in nitrogenous accumulation reveals that 6 days of emersion resulted in a drastic suppression in ammonia production. As the decrease in urea synthesis occurred without the accumulation of ammonia, TFAA or TEFAA, it can be deduced that suppression in ammonia production was achieved through decreases in amino acid catabolism and protein degradation. In summary, *P. sinensis* adopted a reduction in ammonia production, instead of an increase in urea synthesis, as the major strategy to ameliorate ammonia toxicity and water loss during terrestrial exposure.

AUTHOR CONTRIBUTIONS

Y.K.I. and S.F.C. conceived and directed the project. S.M.L.L. drafted the manuscript and performed the experiments. Y.K.I. edited the manuscript. W.P.W. worked with the animals.

COMPETING INTERESTS

No competing interests declared.

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