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RESEARCH ARTICLE

The Chinese soft-shelled turtle, *Pelodiscus sinensis*, excretes urea mainly through the mouth instead of the kidney

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SUMMARY

The Chinese soft-shelled turtle, *Pelodiscus sinensis*, is well adapted to aquatic environments, including brackish swamps and marshes. It is ureotelic, and occasionally submerges its head into puddles of water during emersion, presumably for buccopharyngeal respiration. This study was undertaken to test the hypothesis that the buccophyaryngeal cavity constitutes an important excretory route for urea in *P. sinensis*. Results indicate that a major portion of urea was excreted through the mouth instead of the kidney during immersion. When restrained on land, *P. sinensis* occasionally submerged their head into water (20–100 min), during which urea excretion and oxygen extraction occurred simultaneously. These results indicate for the first time that buccopharyngeal villiform processes (BVP) and rhythmic pharyngeal movements were involved in urea excretion in *P. sinensis*. Urea excretion through the mouth was sensitive to phloretin inhibition, indicating the involvement of urea transporters (UTs). In addition, saliva samples collected from the buccopharyngeal surfaces of *P. sinensis* injected intraperitoneally with saline contained ~36 mmol N l^{-1} urea, significantly higher than that (~2.4 mmol N l^{-1}) in the plasma. After intraperitoneal injection with 20 μmol urea g^{-1} turtle, the concentration of urea in the saliva collected from the BVP increased to an extraordinarily high level of ~614 μmol N ml^{-1}, but the urea concentration (~45 μmol N ml^{-1}) in the plasma was much lower, indicating that the buccopharyngeal epithelium of *P. sinensis* was capable of active urea transport. Subsequently, we obtained from the buccopharyngeal epithelium of *P. sinensis* the full cDNA sequence of a putative *UT*, whose deduced amino acid sequence had ~70% similarity with human and mouse UT-A2. This *UT* was not expressed in the kidney, corroborating the proposition that the kidney had only a minor role in urea excretion in *P. sinensis*. As UT-A2 is known to be a facilitative urea transporter, it is logical to deduce that it was localized in the basolateral membrane of the buccopharyngeal epithelium, and that another type of primary or secondary active urea transporter yet to be identified was present in the apical membrane. The ability to excrete urea through the mouth instead of the kidney might have facilitated the ability of *P. sinensis* and other soft-shelled turtles to successfully invade the brackish and/or marine environment.

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Key words: ammonia, buccopharyngeal respiration, buccopharyngeal villiform processes, nitrogen metabolism, urea excretion, urea transporter, UT-A.

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INTRODUCTION

The Chinese soft-shelled turtle, *Pelodiscus sinensis* Wiegmann 1835, previously known as *Trionyx sinensis*, belongs to the family Trionychidae. It is well adapted to an aquatic environment and inhabits standing or slow-flowing bodies of water, including brackish swamps and marshes. Emersion can occur during basking under the sun or when the ponds or creeks dry up during hot spells.

More than a century ago, Gage and Gage (Gage and Gage, 1886) discovered the presence of numerous minute buccopharyngeal villiform processes (BVP) inside the mouth of soft-shelled turtles and postulated that they have functions similar to those of gills. Because these BVP in *Trionyx* spp. are highly vascularized (Girgis, 1961; Winokur, 1973) and contain mitochondria-rich cells (Yokosuka et al., 2000), it has been suggested that they play a functional role in oxygen uptake during prolonged submergence (Girgis, 1961; Wang et al., 1989) and in salt uptake from the external medium (Dunson and Weymouth, 1965; Dunson, 1967; Yokosuka et al., 2000). Despite being air-breathers with lungs as the principal respiratory organ, *P. sinensis* (and presumably other soft-shelled turtles) can tolerate apnea during prolonged submergence by respiring through the highly vascularized buccopharyngeal and cutaneous surfaces with a reduction in activity (Wang et al., 1989). During submergence, the hyoid apparatus in the pharynx moves rhythmically (14–16 pulses min^{-1}), filling the buccopharyngeal cavity with water and then discharging it back to the external medium (Gage and Gage, 1886; Girgis, 1961). However, to date, the relative contribution of the buccopharyngeal and cutaneous surfaces to respiration in soft-shelled turtles during submergence remains controversial (Dunson, 1960; Girgis, 1961; Wang et al., 1989), probably because BVP have multiple physiological functions, and therefore perform differently in various environmental conditions with different physiological demands.

Pelodiscus sinensis is ureogenic and possesses a full complement of ornithine-urea cycle enzymes in the liver (Lee et

al., 2006; Lee et al., 2007). In water, it is primarily ureotelic, excreting ~70% of waste nitrogen (N) as urea-N. However, the major route of urea excretion in *P. sinensis* has not been established, although the general assumption is that urea excretion occurs through the kidneys of turtles (Dantzler, 1995). Our preliminary observations revealed that *P. sinensis* occasionally submerged its head into puddles of water with rhythmic pharyngeal movement characteristic of buccopharyngeal respiration during emersion, despite the fact that air-breathing through the lungs would meet metabolic demands. Furthermore, there was a decrease in pharyngeal movement in *P. sinensis* during the initial phase of submergence in brackish water (salinity 15) (Y.K.I., unpublished), which occurred in association with an accumulation of urea in the body (Lee et al., 2006). Therefore, this study was undertaken to test the hypothesis that the buccopharyngeal cavity, inclusive of the BVP, constitutes an important route of urea excretion in *P. sinensis*. We also aimed to demonstrate that buccopharyngeal urea excretion and buccopharyngeal respiration occur simultaneously, and that the former is sensitive to phloretin inhibition. Subsequently, attempts were made to determine the urea concentrations in the saliva collected from various parts of the buccopharyngeal cavity and the plasma of *P. sinensis* injected intraperitoneally with saline or exogenous urea in order to elucidate whether the buccopharyngeal epithelium is capable of active urea transport during emersion. Finally, efforts were made to verify that a putative urea transporter gene (*UT*) is expressed in the buccopharyngeal cavity, but not the kidney, of *P. sinensis*, and that the mRNA expression of *UT* in the buccopharyngeal epithelium could be upregulated under certain experimental conditions.

MATERIALS AND METHODS

Animals

Specimens of *P. sinensis* (200–400 g body mass) were purchased from the China Town wet market, Singapore. They were maintained in freshwater (salinity 1; ~15 mmol l^{-1} NaCl) at 25°C in plastic tanks, and water was changed daily. No attempt was made to separate the sexes. Turtles were fed tiger prawn (*Penaeus monodon*) meat daily. Food was withdrawn 5 days prior to experiments. All experiments were performed under a 12 h:12 h light:dark 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).

Determination of concentrations of urea excreted *via* renal and extra-renal routes during immersion

Flexible latex tubing (length 18 cm, radius 0.7 cm) was attached around the tail anterior to the cloaca of *P. sinensis* (*N*=4) using Vetbond tissue adhesive (3M, Maplewood, MN, USA). An opening made at the tip of the tube was held closed by a dialysis clip. Turtles were immersed in 10 volumes (w/v) of freshwater (salinity 1) for 6 days. Water samples (3 ml) were collected daily from the external medium and acidified with 70 µl of 1 mol l^{-1} HCl to trap NH_4^+. 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 and kept at 4°C until analysis. Ammonia and urea concentrations were determined according to the methods of Koops et al. (Koops et al., 1975), and Jow et al. (Jow et al., 1999), respectively. Results are presented as average daily rates (µmol N day^{-1} g^{-1} turtle) of ammonia and urea excretion into the external medium through the extra-renal route or into the urine collected in the latex tubing.

Determination of rates of ammonia or urea excretion through the head and tail (urine) during emersion

Individual turtles (*N*=5) were taken out of water and restrained on a solid platform (18.5×11.5×5.5 cm, length × width × height). A plastic box of water (salinity 1; 700 ml) was placed directly in front of the head so that the experimental turtle could dip its head fully into the water at liberty. The volume of water was determined at the end of 24 h, and water was changed daily. A box of water, to which the turtle had no access, was used as a control to estimate evaporative water loss. The change in volume minus the volume due to evaporation was regarded as the volume of water consumed by the turtle. Another plastic box was placed directly below the tail to collect the urine. Water and urine samples (3 ml) were collected from these two plastic boxes daily, acidified with 70 µl of 1 mol l^{-1} HCl, and kept at 4°C until analyzed. Ammonia and urea concentrations in water samples were determined as mentioned above. Results are presented as daily rates (µmol N day^{-1} g^{-1} turtle) of ammonia or urea excretion during the 6-day period.

Simultaneous determination of buccopharyngeal urea excretion and respiration

Individual turtles (*N*=3) were restrained separately on a solid platform as described above, with a plastic box of water (100 ml) placed directly in front of the head. The partial pressure of O_2 (P_{O_2}) in the water within this plastic box was monitored continuously using an Ocean Optics FOXY Fiber Optics oxygen-sensing system S2000 with a FOXY-R O_2 electrode (Ocean Optics, Dunedin, FL, USA). Water samples were collected at the very beginning and right after the occurrence of a decrease in the P_{O_2} in the water for the determination of urea concentration. The assumption was that the turtle would submerge its head in the water for a certain period and fill the buccopharyngeal cavity with water to facilitate urea excretion. Because such an action, similar to total submergence, would impede pulmonary respiration, leading to apnea, buccopharyngeal respiration could occur simultaneously with O_2 being extracted from the water in the mouth. Therefore, when the turtle discharged the water in the mouth back into the box, there would be a reduction in the overall P_{O_2} in the water therein, accompanied with a simultaneous increase in urea concentration. Urea concentrations in water samples were determined as mentioned above.

Determination of effects of phloretin on buccopharyngeal urea excretion

Phloretin at a concentration of 0.1 mmol l^{-1} was incorporated into the box of water (100 ml) placed in front of the head of the turtle restrained on land as described above. After 24 h, water samples (3 ml) were collected, acidified with 70 µl of 1 mol l^{-1} HCl and kept at 4°C until analyzed. Urea concentrations in water samples were determined as described above. Urea excretion was determined in the same animals to provide a baseline excretion rate before phloretin was added to the box of water.

Determination of urea concentrations in fluid (saliva) samples collected from various regions of the buccopharyngeal cavity and in plasma

Intraperitoneal injection was performed through an area of the skin between the hind leg and the soft carapace without using anesthesia, and the injection took less than 10 s. Specimens were injected intraperitoneally with NH_4Cl [7.5 µmol N g^{-1} turtle; following Ip et

al. (Ip et al., 2008)], urea (20 μmol g^{-1} turtle or 40 μmol N g^{-1} turtle) or an equivalent volume of 0.9% NaCl solution (1 ml per 100 g turtle). At 3 h post-injection, turtles were anesthetized and three different regions of their buccal cavity were swabbed with small pieces of pre-weighed Whatman GF/C glass microfibre filter paper (~2×5 mm). These three regions were the upper jaw (near external nares), upper jaw (near oesophagus) and lower jaw. The amount of fluid (referred to as saliva hereafter) absorbed by the filter paper was determined gravimetrically from the difference between the masses of the dry and wet filter paper, which were measured to the nearest 0.001 g. Swabbing and weighing were performed in less than 1 min in an atmosphere of >85% humidity to avoid evaporative loss of water. The filter paper was then stored in a known aliquot of 0.5 ml of 1 mol l^{-1} HCl at 4°C until analysis. After the first round of collection of saliva samples, the buccal cavity of the turtle was rinsed with water (1‰). At 6 h post-injection, another round of sample collection was performed. The urea concentration in the 0.5 ml of HCl was determined as described above, and the result obtained was used to calculate the urea concentration in the saliva absorbed by the filter paper.

At hour 6, experimental turtles were killed and blood samples were collected into heparinized syringes through cardiac puncture, and centrifuged at 5000 ***g*** and 4°C for 5 min to obtain the plasma. The pH of the deproteinized samples was adjusted to between 6.0 and 6.5 with 2 mol l^{-1} $KHCO_3$, and urea contents were determined using the method of Jow et al. (Jow et al., 1999).

Gene sequencing

Turtles in the control conditions were killed, and tissue samples from the roof of the mouth and pharynx were excised and kept at –80°C until analysis. Total RNA was extracted using the chaotropic extraction protocol of Whitehead and Crawford (Whitehead and Crawford, 2005). The resulting RNA pellet was rinsed twice with 500 μl of 70% ethanol, followed by further purification using the Qiagen RNeasy Mini Kit (Qiagen, Hilden, Germany). RNA quality was checked electrophoretically by running 1 μg of RNA in 1% agarose gel, and RNA quantification was performed spectrophotometrically using a Hellma traycell (Hellma, Müllheim, Germany).

Total RNA (1 μg) was reverse transcribed into cDNA using a RevertAid first strand cDNA synthesis kit (Fermentas, Burlington, ON, Canada) for PCR work. Forward (5′-GTGATGTTYG-TCARCAAYCC-3′) and reverse (5′-CCANGGRTTRTCRC-ANCCRTA-3′) primers were designed based on identifying highly conserved regions from multiple alignment of *UT* mRNA sequences from *Homo sapiens* (L36121.1), *Rattus norvegicus* (NM_177962.3 and AF042167.1), *Rana esculenta* (Y12784.1), *Bufo marinus* (AB212932.1) and *Trachemys scripta elegans* (AB308450.1) available in GenBank. Each PCR was performed in a volume of 25 μl containing 2.5 μl of 10× DreamTaq Buffer, 0.5 μl of dNTPs (10 mol l^{-1}), 0.5 μl of $MgCl_2$ (25 mmol l^{-1}), 0.125 μl of DreamTaq DNA polymerase (5 units μl^{-1}) (Fermentas), 1.25 μ1 of forward primer (10 μmol l^{-1}), 1.25 μl of reverse primer (10 μmol l^{-1}) and 0.5 μl of cDNA template using a Bio-Rad Peltier thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA). The thermocycling program used was 95°C for 3 min, followed by 40 cycles of 94°C for 30 s, 42°C for 30 s, 72°C for 1.5 min and one cycle of final extension of 72°C for 10 min. PCR products were separated electrophoretically in 1% agarose gel containing Gelred (Biotium, Hayward, CA, USA). The band of expected size was excised, and the PCR product was purified using Wizard SV gel and a PCR clean-up system kit (Promega, Madison, WI, USA). Sequencing reaction was performed with a BigDye Terminator cycle sequencing kit (Applied Biosystems, Foster City, CA, USA), and the nucleotide sequence was determined using an ABI Prism 3130XL sequencer (Applied Biosystems).

For rapid amplification of cDNA ends (RACE)-PCR, total RNA (1 μg) isolated from the mucosal membrane of *P. sinensis* was reverse transcribed into 5′-RACE-Ready cDNA and 3′-RACE-Ready cDNA using the SMARTer RACE cDNA Amplification kit (Clontech Laboratories, Mountain View, CA, USA). RACE-PCR was performed using the Advantage 2 PCR kit (Clontech Laboratories) to generate the 5′ and 3′ cDNA fragments, with *UT*-specific primers (5′RACE: 5′-AGAAGCCACCAATAGAAA-TCTCCCT-3′; 3′RACE: 5′-AGGGAGATTTCTATTGGTGG-CTTCT-3′) designed based on the partial sequences obtained from the mucosal membrane of *P. sinensis*. The RACE-PCR thermocycling program used was 25 cycles of 94°C (30 s), 65°C (30 s) and 72°C (4 min). RACE-PCR products were separated using gel electrophoresis. Products of expected sizes were purified and sequenced.

Potential phosphorylation sites at serine, threonine or tyrosine residues were identified using NetPhos 2.0 (Blom et al., 1999). The transmembrane domains were predicted using MEMSATS and MEMSAT-SVA provided by the PSIPRED protein structure prediction server. For comparison, the transmembrane domains of *T. s. elegans* UT was taken from Uchiyama et al. (Uchiyama et al., 2009), whereas the domains of *Triakis scyllium* and *Mus musculus* UT-A2 were taken from Kakumura et al. (Kakumura et al., 2009).

Phylogenetic analysis

The phylogenetic relationship between the deduced amino acid sequences of UT from the buccopharyngeal epithelium of *P. sinensis* and those of other animal species was analyzed using the neighbor-joining method (NEIGHBOR) in the PHYLIP phylogeny package (version 3.67) (Felsentein, 1989), with the inclusion of 100 bootstraps. The phylogenetic trees were generated with CONSENSE using the 50% majority rule, and plotted with the program TREEVIEW. Bootstrap values are indicated at the nodes of the tree branches. The accession numbers for the amino acid sequences of various UTs/Uts (from GenBank or UniProtKB/TrEMBL) used in the analysis are as follows: *Alcolapia grahami* Ut (AAG49891.1), *Anguilla japonica* gill Ut (BAC53976.1), *A. japonica* Ut-C (BAD66672.1), *Balaenoptera acutorostrata* UT-A2 (BAF46914.1), *Balaenoptera borealis* UT-A2 (BAF46916.1), *Balaenoptera brydei* UT-A2 (BAF46917.1), *Homo sapiens* UT-A1 (AAL08485.1), *H. sapiens* UT-A2 (CAA65657.1), *H. sapiens* UT-B1 (CAB60834.1), *Mus musculus* UT-A1 (AAM00357.1), *M. musculus* UT-A2 (AAM21206.1), *M. musculus* UT-A3 (AAG32168.1), *M. musculus* UT-A5 (AAG32167.1), *M. musculus* UT-B (AAL47138.1), *M. musculus* UT-B1 (CAD12807.1), *Oncorhynchus mykiss* Ut (ABV44670.1), *Opsanus beta* Ut (AAD53268.2), *Physeter catodon* UT-A2 (BAF46918.1), *Rana esculenta* ADH-regulated UT (CAA73322.1), *Rana pipiens* UT (partial; AFE48182.1), *Rana septentrionalis* UT (partial; AFE48183.1), *Rana sylvatica* UT (partial; AFE48181.1), *Rattus norvegicus* UT-A3 (AAD23098.1), *R. norvegicus* UT-A4 (AAD23099.1), *Rhinella marina* UT (BAE16706.1), *Takifugu rubripes* Ut (BAD66674.1), *T. rubripes* Ut-C (NP_001033079.1), *Trachemys scripta elegans* UT (BAF76798.1) and *Squalus acanthias* Ut (AAF66072.1; as the outgroup).

Tissue expression

The mRNA expression of *UT* was determined in cDNA samples from anterior roof of the mouth, posterior roof of the mouth, tongue,

Table 1. Rates (μmol N day^{-1} g^{-1} turtle) of ammonia and urea excretion into the external medium through the extra-renal route or into the urine collected by a flexible latex tubing attached to the tail, and the percentage of ammonia-N or urea-N excreted through urine in *Pelodiscus sinensis* during 6 days of immersion

	Rate of ammonia or urea excretion		Ammonia-N or urea-N excreted through urine (%)
	Extra-renal route	Urine	
Ammonia	0.29±0.09	0.63±0.28	57±12
Urea	0.90±0.49	0.071±0.054	5.92±4.03

Values represent means ± s.e.m., *N*=4.

oesophagus, intestine, kidney and bladder of *P. sinensis*. PCR was carried out using a Bio-Rad Peltier thermal cycler (Bio-Rad Laboratories) using DreamTaq DNA polymerase (Fermentas) and primers designed for qPCR (see below). The cycling conditions consisted of 95°C (3 min), followed by 30 cycles of 94°C (30 s), 60°C (30 s), 72°C (30 s) and one cycle of final extension at 72°C (10 min). PCR products were then electrophoresed in 2% agarose gel.

qPCR

RNA from the buccophargneal epithelium obtained from the upper jaw of *P. senensis* was treated with deoxyribonuclease I (Sigma-Aldrich, St Louis, MO, USA) to remove any contaminating genomic DNA. First-strand cDNA was then synthesized from 1 μg of total RNA using random hexamer primer and the RevertAid first stand cDNA synthesis kit (Fermentas).

qPCR was performed in triplicate using a StepOnePlus Real-Time PCR System (Applied Biosystems). The PCR reactions contained 5 μl of 2× Fast SYBR Green Master Mix (Applied Biosystems), 0.3 μmol l^{-1} each of forward and reverse primers, and cDNA (equivalent to 1 ng of RNA) in a total volume of 10 μl. Cycling conditions were 95°C for 20 s (one cycle), followed by 45 cycles of 95°C for 3 s and 60°C for 30 s. Data [threshold cycle (C_T) values] were collected at each elongation step. Runs were followed by melt-curve analysis by increasing from 60 to 95°C in 0.3°C increments to confirm the presence of only a single product. The PCR products were separated in a 2% agarose gel to verify the presence of a single band.

The forward and reverse qPCR primers of the putative *UT-A2* from *P. sinensis* were 5′-ACCGTTGTCTCAACATTTACAG-3′ and 5′-CTATGAGCAGTCCCACCAG-3′, respectively. Actin was chosen as the reference gene, and the forward and reverse qPCR primers were 5′-GAGACCCGACAGACTACC-3′ and 5′-ACGCACAATTTCCCTTTCAG-3′, respectively. The amplification efficiencies of the putative *UT* and actin were 98.3 and 93.5%, respectively. The application of the $2^{-\Delta\Delta CT}$ calculations for the relative quantification of the mRNA expressions of *UT* was validated by plotting the validation curve, ΔC_T ($C_{T,target}$–$C_{T,actin}$) against log cDNA dilution (1000-fold) of the samples obtained from control turtles (Livak and Schmittgen, 2001). The gradient of the slope obtained was 0.021. Freshwater control (day 0) was used as the reference sample for calculation. Results are reported as fold-change, but the fold-change values were transformed into logarithmic values ($\log_2$) before statistical analysis.

Statistics

Results are presented as means ± s.e.m. Student's *t*-test and one-way ANOVA, followed by multiple comparisons of means by the Tukey's test, were used to evaluate differences between means where applicable. Differences were regarded as statistically significant at $P<0.05$.

RESULTS

During immersion, *P. sinensis* produced 7.3–16 ml of urine daily, and 57% (range: 31–80%) of the ammonia-N excreted during the 6-day period was recovered from the urine (Table 1). By contrast, the urea excreted through the renal route accounted for only 5.92% (range=0.45–15%) of the total urea excreted throughout the 6-day period (Table 1).

When turtles were restrained on land with a known volume of water made available to the head, the daily rate of water consumption was 30–41 ml over a 6-day period (Table 2). Throughout this period, the urea excretion rate through the mouth was significantly greater (15- to 49-fold) than that through the cloaca (Table 2). By contrast, the rates of ammonia excretion through the mouth and the cloaca were comparable (Table 2). We confirmed that during forced emersion, turtles occasionally submerged their heads into water positioned in front of them for periods ranging between 20 and 100 min, during which water was held in the mouth and then discharged back to the container with observable pharyngeal movement. An increase in urea concentration in the water inside the plastic container positioned in front of the turtle occurred only

Table 2. Volumes (ml) of water consumed and rates (μmol N day^{-1} g^{-1} turtle) of ammonia and urea excretion through the head or tail region (presumably the mouth and cloaca, respectively) of *Pelodiscus sinensis* during 6 days of restraint on land

		Rate of ammonia excretion			Rate of urea excretion		
No. of days	Water consumed	Through the mouth	Through the cloaca (urine)	Total ammonia-N	Through the mouth	Through the cloaca (urine)	Total urea-N
1	33±5	0.17±0.05	0.37±0.06*	0.54±0.10	0.95±0.07	0.038±0.011*	0.99±0.08
2	35±12	0.32±0.10	0.35±0.08	0.68±0.06	2.0±0.5	0.11±0.05*	2.1±0.5
3	41±15	0.24±0.06	0.42±0.16	0.66±0.13	1.7±0.4	0.11±0.08*	1.8±0.5
4	34±9	0.34±0.17	0.87±0.43	1.2±0.5	1.8±0.2	0.037±0.025*	1.9±0.2
5	32±6	0.37±0.14	0.65±0.29	1.0±0.3	1.6±0.3	0.11±0.07*	1.8±0.3
6	30±7	0.33±0.11	0.61±0.31	0.94±0.37	1.3±0.3	0.036±0.015*	1.4±0.3

Values represent means ± s.e.m., *N*=5.
*Significantly different from the corresponding head region.

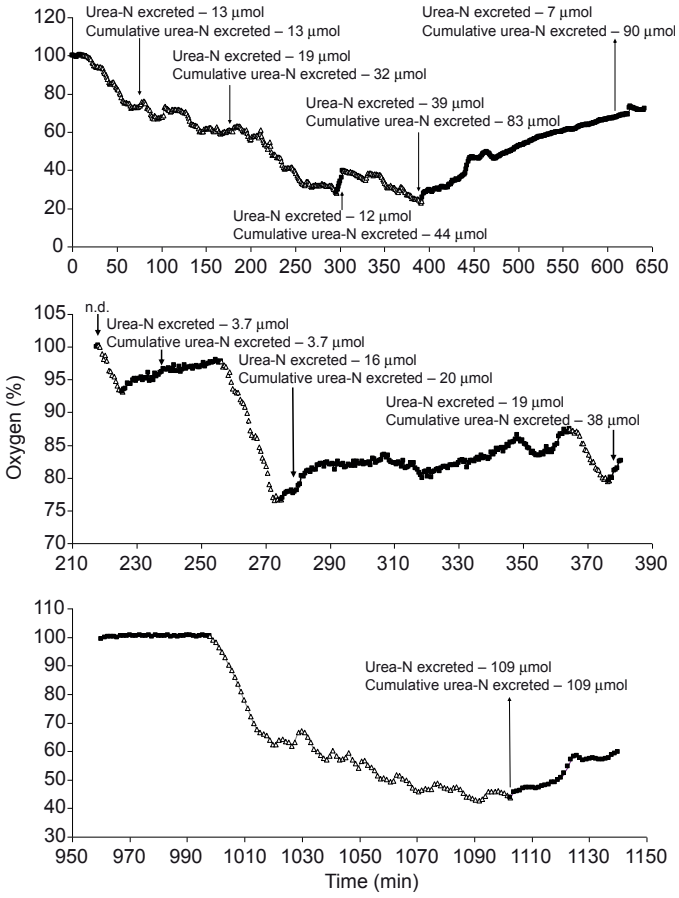

Fig. 1. Quantity of urea excreted (μmol N) immediately after a decrease in oxygen level (%) in 100 ml of freshwater (1‰) made available to three individuals of *Pelodiscus sinensis*, when they submerged their heads into water during three different periods of forced emersion. Solid square markers represent periods when the head was not in the water. Each graph shows the profile for an individual turtle. n.d., not detectable.

after a decrease in the P_{O_2} therein, which coincided with the observed rinsing of the mouth (Fig. 1). When phloretin (0.1 mmol l^{-1}) was included in the water presented to the turtle, the buccopharyngeal urea excretion rate decreased significantly to ~4% of the control value (Fig. 2).

High concentrations (up to 36 μmol N ml^{-1}) of urea were present in the saliva collected from the buccopharyngeal surfaces of turtles injected intraperitoneally with saline (Table 3), but the plasma urea

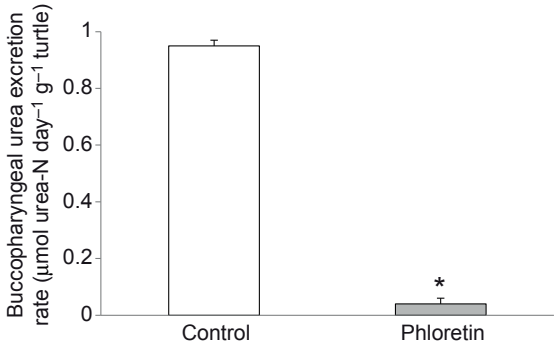

Fig. 2. Buccopharyngeal urea excretion rate (μmol urea-N day^{-1} g^{-1} turtle) of *Pelodiscus sinensis* with (N=3) or without (control; N=5) the presence of phloretin (0.1 mmol l^{-1}) in the water presented to the head during forced emersion. *Significantly different from the control (P<0.05).

concentration collected from turtles 6 h after the intraperitoneal injection of saline was only 2.48±0.82 mmol N l^{-1} (N=4). An intraperitoneal injection with NH_4Cl did not have a significant effect on the concentration of urea in the saliva. By contrast, extraordinarily high concentrations (up to 614 mmol N l^{-1}) of urea were detected in saliva samples collected from the BVP of turtles 6 h after an intraperitoneal injection with urea (Table 3). At hour 6, the saliva urea concentration was approximately 13.5-fold greater than the plasma urea concentration (45.5±9.24 μmol N ml^{-1}; N=4).

The complete coding of the cDNA sequence of 1164 bp for a putative *UT* was obtained from the buccopharyngeal epithelium, including the BVP, of *P. sinensis* (supplementary material Fig. S1) and deposited with GenBank (accession number JN587496). The translated protein consisted of 388 amino acids (Fig. 3) with an estimated molecular mass of 42.5 kDa. It had 10 transmembrane-spanning domains, with a potential *N*-glycosylation site on the third extracellular loop (Fig. 3). There was one potential serine (serine 72) and two potential threonine (threonine 11 and threonine 23) phosphorylation sites on the inner N terminus. In addition, one potential serine (serine 142) phosphorylation site was found on the second outer loop and two (serine 213 and serine 220) on the third outer loop. There was one potential threonine (threonine 190) phosphorylation site in TM5 and another one (threonine 384) was found on the outer COOH terminus of this protein. Furthermore, one potential tyrosine (tyrosine 374) phosphorylation site was found on the outer COOH terminus. A comparison of the deduced amino acid sequence of the putative UT from the buccopharyngeal epithelium of *P. sinensis* with those of other species (Table 4) indicated that it shared the highest

Table 3. Concentrations (mmol N l^{-1}) of urea in saliva samples collected from various regions of the buccal cavity (anterior upper jaw, posterior upper jaw, lower jaw inclusive of the tongue) of *Pelodiscus sinensis* at 3 or 6 h post-injection with saline (0.9% NaCl; 1 ml 100 g^{-1} turtle), NH_4Cl (7.5 μmol N g^{-1} turtle) or urea (20 μmol g^{-1} turtle or 40 μmol N g^{-1} turtle)

Time	Regions of buccopharyngeal epithelium	Urea concentration (mmol N l^{-1})		
		Saline-injected	NH_4Cl-injected	Urea-injected
3 h	Anterior upper jaw	20±11[a]	19±8[a]	154±49[b]
	Posterior upper jaw	23±11[c]	40±4[c]	608±87[d]
	Lower jaw	29±18[e]	32±5[e]	547±82[f]
6 h	Anterior upper jaw	9.4±4.2[g]	10±3[g]	167±83[h]
	Posterior upper jaw	31±16[i]	40±7[i]	614±154[j]
	Lower jaw	36±14[k]	42±7[k]	433±84[l]

Values are means ± s.e.m., N=4.
Values not sharing the same letter for each specific region are significantly different between the three experimental conditions (P<0.05).

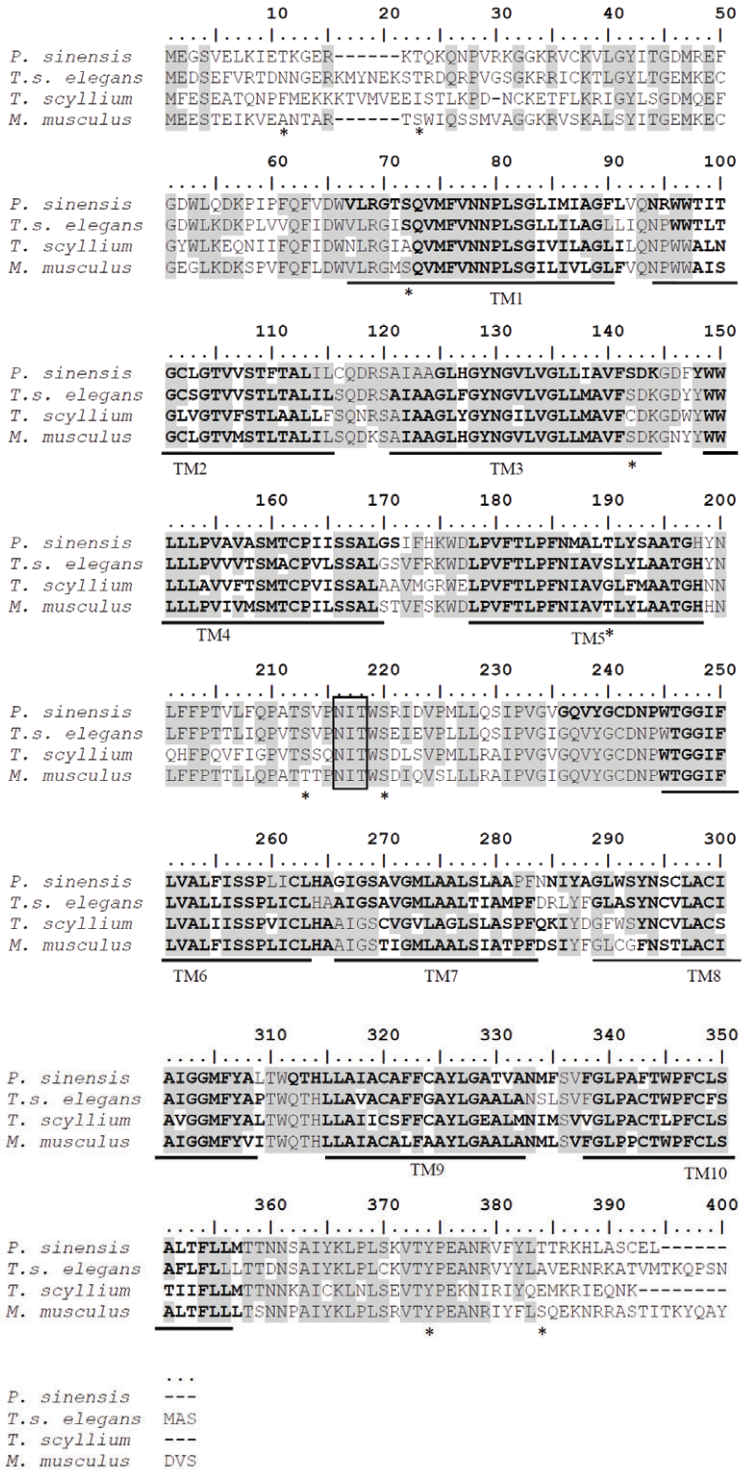

Fig. 3. Multiple amino acid alignment of the putative urea transporter (UT) from the buccopharyngeal epithelium of *Pelodiscus sinensis*, with four other known urea transporters from *Trachemys scripta elegans* (BAF76798.1), *Triakis scyllium* (BAC75980.1) and *Mus musculus* UT-A2 (AAM21206.1). Identical amino acids are indicated by shaded residues. The predicted transmembrane spanning domains (TMs) are underlined and in bold. Potential *N*-glycosylation sites are boxed. Asterisks refer to potential phosphorylation sites.

similarity with the UT from the kidney of the red-eared slider turtle, *T. s. elegans* (71.7%), and UT-A2 from the kidneys of mammals (69.7–71.2%). A phylogenetic analysis grouped the putative UT from *P. sinensis* with that from *T. s. elegans*, and together they were more closely related to UT-A than to UT-B or UT-C (Fig. 4)

This putative *UT* was expressed in the anterior and posterior roof of the buccopharyngeal cavity and tongue, but not in the oesophagus, intestine, kidney or bladder of *P. sinensis* (Fig. 5). The mRNA expression of the putative *UT* in the buccopharyngeal epithelium of *P. sinensis* remained unchanged 6 h after an intraperitoneal injection with urea (40 μmol N g^{-1} turtle) compared with the control injected with saline (results not shown). Similarly, there was no significant change in the mRNA expression of the putative *UT* in the buccopharyngeal epithelium of *P. sinensis* exposed to terrestrial conditions for 24 h, followed by an intraperitoneal injection with saline and then kept in terrestrial conditions for another 24 h. However, significant increases in the mRNA expression of the putative *UT* in the buccopharyngeal epithelium were observed in turtles exposed to terrestrial conditions for 24 h, followed by an

Table 4. The percentage sequence identity of the deduced amino acid sequence of the putative urea transporter (UT) from the buccopharyngeal epithelium of *Pelodiscus sinensis* as compared with those of other UTs obtained from GenBank (accession numbers in brackets)

Classification	Species	Similarity (%)
Reptile	*Trachemys scripta elegans* UT (BAF76798.1)	71.7
Mammal	*Balaenoptera brydei* UT-A2 (BAF46917.1)	71.2
	Balaenoptera borealis UT-A2 (BAF46916.1)	71.2
	Physeter catodon UT-A2 (BAF46918.1)	71.0
	Balaenoptera acutorostrata UT-A2 (BAF46914.1)	70.5
	Mus musculus UT-A2 (AAM21206.1)	70.5
	Homo sapiens UT-A2 (CAA65657.1)	69.7
	Mus musculus UT-B (AAL47138.1)	61.7
	Mus musculus UT-B1 (CAD12807.1)	61.7
	Homo sapiens UT-B1 (CAB60834.1)	61.3
	Mus musculus UT-A5 (AAG32167.1)	59.3
	Rattus norvegicus UT-A4 (AAD23099.1)	57.7
	Rattus norvegicus UT-A3 (AAD23098.1)	56.9
	Mus musculus UT-A3 (AAG32168.1)	56.8
	Mus musculus UT-A4 (AAO65921.1)	36.3
	Homo sapiens UT-A1 (AAL08485.1)	30.1
	Mus musculus UT-A1 (AAM00357.1)	28.1
Amphibian	*Rhinella marina* UT (BAE16706.1)	70.5
	Rana septentrionalis UT (partial; AFE48183.1)	66.1
	Rana sylvatica UT (partial; AFE48181.1)	66.1
	Rana esculenta ADH-regulated UT (CAA73322.1)	66.1
	Rana pipiens UT (partial; AFE48182.1)	65.3
Elasmobranch	*Squalus acanthias* Ut (AAF66072.1)	63.7
Teleost fish	*Danio rerio* Ut2 (NP_001018355.1)	49.8
	Takifugu rubripes Ut (BAD66674.1)	48.3
	Opsanus beta Ut (AAD53268.2)	47.7
	Anguilla japonica gill Ut (BAC53976.1)	47.4
	Alcolapia grahami Ut (AAG49891.1)	46.5
	Anguilla japonica Ut-C (BAD66672.1)	36.8
	Takifugu rubripes Ut-C (NP_001033079.1)	33.5
	Oncorhynchus mykiss Ut (ABV44670.1)	26.0

Sequences are arranged in descending order of similarity.

intraperitoneal injection with NH_4Cl (7.5 μmol N g^{-1} turtle) or urea (40 μmol N g^{-1} turtle) and another 24 h of exposure to terrestrial conditions (Fig. 6).

DISCUSSION

Buccopharyngeal urea excretion in *P. sinensis*

Our results confirmed that during immersion, the kidney of *P. sinensis* played a limited role in urea excretion and a major portion of urea excreted took a non-renal route. During forced emersion with a known volume of water positioned in front of the head, *P. sinensis* consumed water at liberty and therefore produced urine as usual. However, instead of excreting urea in urine, a major portion of the urea was excreted through the buccopharyngeal cavity while the head was submerged in water. Buccopharyngeal urea excretion in *P. sinensis* was highly efficient during emersion because the sum total of nitrogenous (ammonia-N + urea-N) excretion through the mouth and the urine (Table 2) during forced emersion accounted for >90% of the nitrogenous wastes excreted by control turtles immersed in freshwater (Table 1).

Being an air-breather, the period of submergence of the head by *P. sinensis* during emersion was unusually long (20–100 min), which would lead to apnea. During this period, buccopharyngeal respiration and urea excretion occurred simultaneously, confirming that the turtle had held water in the mouth and discharged it back into the container. It has been established that the blood vessels lining the buccopharyngeal epithelium of soft-shelled turtles form a dense network (rete mirabile) and are connected with the common carotid arteries (Girgis, 1961). Hence, the blood supplied to the BVP is fully oxygenated and does not require further oxygenation under normal circumstance (Girgis, 1961). However, buccopharyngeal uptake of O_2 through BVP becomes essential when the whole body of the turtle is submerged in water for a prolonged period, resulting in apnea. Because air is easily available for pulmonary respiration in these experimental turtles restrained on land, there should not be a need for buccopharyngeal respiration. Therefore, it is logical to deduce that the primary function of submerging the head into water during emersion was to excrete urea, and that buccopharyngeal respiration occurred as a secondary phenomenon to compensate for a decrease in the blood P_{O_2} due to the prolonged submergence of the head without access to air. These results are novel and suggest a new functional role of urea excretion for the rhythmic pharyngeal movements of soft-shelled turtles more than a century after their discovery by Gage and Gage (Gage and Gage, 1886).

Buccopharyngeal urea excretion is sensitive to phloretin inhibition

The inclusion of phloretin (0.1 mmol l^{-1}) in the water made available to the head of *P. sinensis* restrained on land resulted in a significant decrease in the rate of buccopharyngeal urea excretion, indicating the involvement of some sort of UT. Members of the UT-A are known to be inhibited by phloretin [UT-A1 (Shayakul et al., 1996); UT-A2 (You et al., 1993); UT-A4 (Karakashian et al., 1999); UTA-5 (Fenton et al., 2000); and UT-A6 (Smith et al., 2004)], and hence certain forms of UT-A could be involved in buccopharyngeal urea

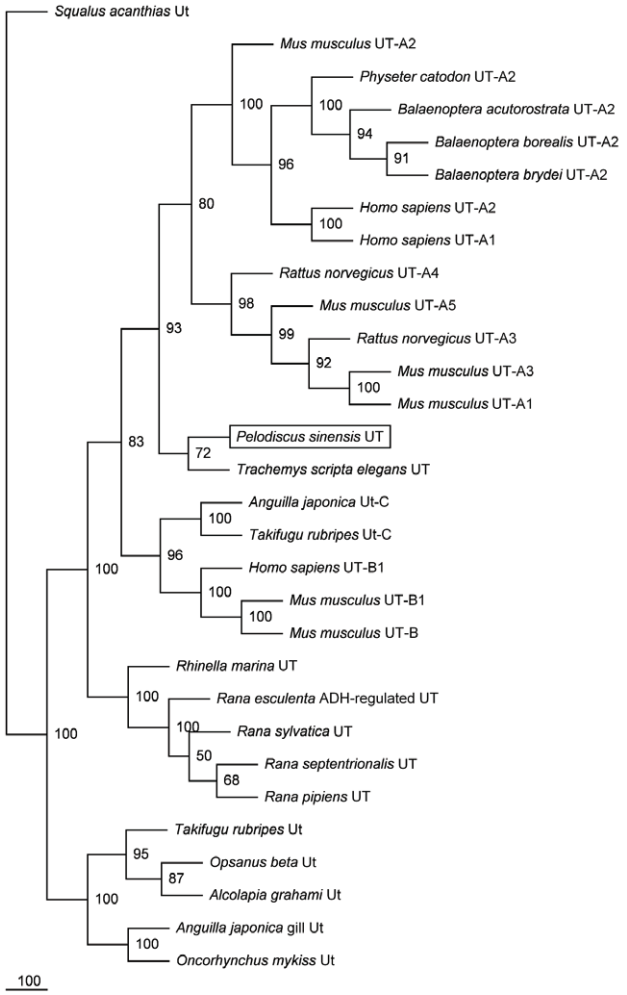

Fig. 4. A phylogenetic tree that illustrates the relationship between the amino acid sequence of the putative urea transporter (UT) obtained from the buccopharyngeal epithelium of *Pelodiscus sinensis* and those of UTs of other animals, with that of *Squalus acanthias* (elasmobranch) as the outgroup.

excretion in *P. sinensis*. However, two components of active urea transport located in the inner medullar collecting duct of the mammalian kidney, one through a basolateral Na^+/urea counter transporter (Kato and Sands, 1998a; Kato and Sands, 1998b) and the other through an apical Na^+/urea counter transporter (Kato and Sands, 1998a), are also known to be sensitive to phloretin inhibition. Therefore, it was essential to test whether the buccopharyngeal epithelium of *P. sinensis* had the ability to excrete urea against a concentration gradient of urea.

The buccopharyngeal epithelium is capable of active urea excretion

Because the concentration (up to 36 mmol $N l^{-1}$) of urea in the saliva collected from the buccopharyngeal epithelium of turtle injected intraperitoneally with saline was significantly (~14.5-fold) higher than that in the plasma (2.48 mmol $N l^{-1}$), and the urea concentration of the saliva increased to a phenomenal level of 614 mmol $N l^{-1}$ with a plasma urea concentration of only 45.5 μmol $N ml^{-1}$ 6 h after intraperitoneal injection with urea, it can be concluded that the buccopharyngeal urea epithelium of *P. sinensis* was capable of active urea transport. Active urea transport processes have been described in a variety of tissues, which include: (1) Na^+-linked active urea transport in the renal tubule (Schmidt-Nielsen et al., 1972; Morgan et al., 2003a; Morgan et al., 2003b) and gills (Fines et al., 2001) of the spiny dogfish, *Squalus acanthias*; (2) phloretin-inhibitable active urea secretion in rabbit proximal straight tubules (Kawamura and Kokko, 1976); (3) H^+-dependent and/or phloretin-inhibitable active urea transport in the skins of *Bufo bufo*, *Bufo marinus*, *Bufo viridis* and *Rana esculenta* (Ussing and Johansen, 1969; Garcia-Romeu et al., 1981; Rapoport et al., 1988; Lacoste et al., 1991; Dytko et al., 1993); (4) active urea transport in kidney tubules from dogs (Goldberg et al., 1967; Beyer and Gelarden, 1988) and frogs (Forster, 1954; Schmidt-Nielsen and Shrauger, 1963); (5) active urea transport in the yeast *Saccharomyces cerevisiae* (Pateman et al., 1982; ElBerry et al., 1993); and (6) active urea transport in bacteria (Jahns et al., 1988). However, to date, molecular characterizations of these active UTs have not been performed and therefore a molecular understanding of these transporters in comparison of UT-A, UT-B and UT-C is not possible at present.

A putative UT is expressed in the buccopharyngeal epithelium of *P. sinensis*

In mammals, UT-A, which includes six isoforms, and UT-B, which includes two isoforms, are derived from two distinct genes, *SLC14A2* and *SLC14A1*, respectively (Sands, 1999; Bagnasco, 2003; Smith, 2009; Stewart, 2011). Both UT-A and UT-B family members are expressed in the mammalian kidney. UT-A is differentially expressed along the nephron (Shayakul et al., 1996), whereas UT-B is expressed in blood vessel of the descending vasa recta (Xu et al., 1997). In non-mammalian vertebrates, several UT cDNAs, including a novel UT-C, have been isolated from kidneys, urinary bladder and gills (Couriaud et al., 1999; Smith and Wright, 1999; Mistry et al., 2001; Mistry et al., 2005; Hyodo et al., 2004; Konno et al., 2006).

Here, we report for the first time the full-length cDNA sequence of a putative *UT*, whose deduced amino acid sequence shared ~70% similarity with UT-A2 from the kidneys of human and mouse, from the buccopharyngeal epithelium of *P. sinensis*. Similar to the UT obtained from the kidney of the red-eared slider turtle (Uchiyama et al., 2009), it has only 10 transmembrane domains as compared with 11 found in mammalian UT-A2. Due to the presence of various potential phosphorylation sites in the translated amino acid sequence of this putative UT-A2 from *P. sinensis*, it is probable that its activity could be regulated through phosphorylation and dephosphorylation, although the details of the regulatory mechanisms await future study. Indeed, in mammalian kidneys, vasopressin, acting through cyclic AMP, stimulates urea transport across rat terminal inner medullary

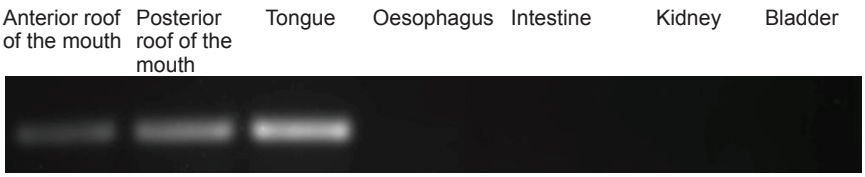

Fig. 5. mRNA expression of the urea transporter gene (*UT*) in the anterior roof of the mouth, posterior roof of the mouth, tongue, oesophagus, intestine, kidney and bladder of *Pelodiscus sinensis*.

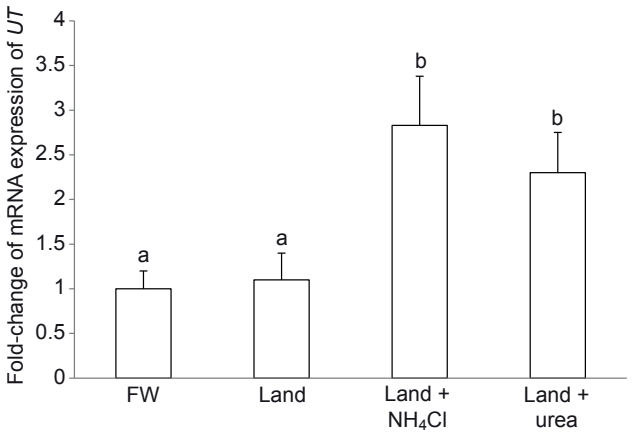

Fig. 6. Relative quantification (fold-change) of the mRNA expression of the putative *UT* in the anterior roof of the mouth of *Pelodiscus sinensis* kept in freshwater (FW; control), or in terrestrial conditions for 24 h followed by an injection of physiological saline (0.09% NaCl; land), NH_4Cl (7.5 μmol N g^{-1} turtle; land + NH_4Cl) or urea (40 μmol N g^{-1} turtle; land + urea), and kept in terrestrial conditions for another 24 h. Data are means + s.e.m. (*N*=4). Means not sharing the same letter are significantly different (P<0.05).

collecting ducts by increasing the phosphorylation, mediated by the MEK-ERK pathway, and accumulation at the apical plasma membrane of UT-A1 (Blount et al., 2008; Klein et al., 2010). Because this putative *UT-A2* was not expressed in the eosophagus and gut, it corroborates the proposition that buccopharyngeal urea excretion involved the rinsing of the mouth of *P. sinensis* with, and not the drinking of, the external medium. In addition, the absence of its expression in the kidney of *P. sinensis* is in support of the conclusion that the kidney has a limited role in urea excretion.

The mRNA expression of this putative *UT-A2* in the buccopharyngeal epithelium was upregulated (~2.5–3-fold) in *P. sinensis* challenged with an exogenous load of NH_4Cl or urea and simultaneously exposed to terrestrial conditions for an extended period (a total of 48 h). Being ureogenic, *P. sinensis* could detoxify exogenous ammonia to urea (Ip et al., 2008), which, like exogenous urea, needed to be excreted through the buccopharyngeal route during emersion. These results are of physiological significance because they imply that buccopharyngeal urea excretion in *P. sinensis* can be enhanced in response to increased endogenous urea production after feeding (Lee et al., 2007) or increased detoxification of ammonia during environmental ammonia exposure (Ip et al., 2008).

An active urea transporter yet to be cloned could be present in the buccopharyngeal epithelium

UT-A, UT-B and UT-C are facilitative urea transporters that allow the rapid movement of urea molecules across cell membranes, and a net equilibrative urea movement can only occur down a concentration gradient (for reviews, see Smith, 2009; Stewart, 2011). These transporters are distinct from the undefined class of proteins responsible for the active transport of urea against a urea concentration gradient (Stewart, 2011). Because transepithelial transport involves the movement of a substance across the basolateral (blood-facing) and apical (lumen-facing) membranes of epithelial cells, we speculate that the putative UT-A2 was expressed in the basolateral membrane of the buccopharyngeal epithelium of *P. sinensis*. We also hypothesize that an active urea transporter yet to be cloned and sequenced was present in the apical membrane of the buccopharyngeal epithelium; only then could urea be actively transported from the cytosol to the saliva to achieve high concentrations of urea in the latter. Consequently, the urea concentration in the cytosol would remain low, and the transport of urea from the plasma into the cytosol could be facilitated by the putative UT-A2 expressed in the basolateral membrane.

Indeed, there is evidence that supports the presence of both facilitated urea transport [through a homolog of UT-A2 (Smith and Wright, 1999)] and Na^+-coupled secondary active urea transport in the elasmobranch kidney (Morgan et al., 2003a; Morgan et al., 2003b). Elasmobranchs retain ~300–600 mmol l^{-1} urea in the plasma for the purpose of isoosmotic and hypoionic osmoregulation. Because the urea concentration in seawater is extremely low (<0.01 mmol l^{-1}), elasmobranchs need an effective mechanism to hold these elevated concentrations of urea in the plasma against a huge urea concentration gradient (McDonald et al., 2006). Classic studies by Schmidt-Nielsen et al. (Schmidt-Nielsen et al., 1972) and Hays et al. (Hays et al., 1977) revealed that urea retention/reabsorption in elasmobranchs involved an active Na^+-coupled urea transport process in the kidney. However, Smith and Wright (Smith and Wright, 1999) uncovered a gene that showed high homology to mammalian UT-A2 in the kidney of spiny dogfish, *S. acanthias*, and similar UT has been reported subsequently for other elasmobranchs (Morgan et al., 2003a; Hyodo et al., 2004), supporting the proposition that urea reabsorption occurs through facilitated diffusion. Using an *in vitro* isolated brush-border (apical) membrane vesicle preparation, Morgan et al. (Morgan et al., 2003b) found phloretin-sensitive, non-saturable urea transport in the bundle zone supporting the expression of UT-A in this zone of the kidney (Morgan et al., 2003a). In the sinus zone, they demonstrated phloretin-sensitive, Na^+-linked urea transport that behaved according to Michaelis–Menten saturation kinetics with a low K_m (0.7 mmol l^{-1}) or high affinity for urea. Morgan et al. (Morgan et al., 2003a) also found UT-A expression in the sinus zone. Taken together, these results suggest that both facilitated diffusion and Na^+-coupled secondary active transport may be responsible for renal urea transport in the elasmobranch kidney. In the current model for active urea absorption in the kidney of elasmobranchs, the Na^+/urea counter-transporter is localized along the basolateral membrane while a UT-A facilitative transporter is positioned in the apical membrane (McDonald et al., 2006), which is apparently opposite to the case of active urea excretion in the buccopharyngeal epithelium of *P. sinensis*.

Because phoretin sensitivity is a characteristic of both facilitative (i.e. UT-A) and secondary active Na^+/urea counter-transport systems (Chou and Knepper, 1989; Isozaki et al., 1993; Isozaki et al., 1994a; Isozaki et al., 1994b; Sands et al., 1996; Kato and Sands, 1998a; Kato and Sands, 1998b), but not Na^+/urea cotransporters, and because water with a salinity of 1 contained ~15 mmol l^{-1} Na^+, which was higher than the normal intracellular Na^+ concentration (10 mmol l^{-1}), the possibility of a Na^+/urea counter-transporter being expressed in the apical membrane of the buccopharyngeal epithelial cells of *P. sinensis* cannot be eliminated at present. However, active buccopharyngeal urea excretion resulted in extraordinarily high urea concentrations (~614 mmol l^{-1}) in the saliva of turtles injected intraperitoneally with urea; therefore, the possibility of the presence of a novel active urea transporter independent of Na^+ movement in the apical surface of the buccopharyngeal epithelium cannot be ignored.

Why would *P. sinensis* evolve to have the ability to excrete urea through the mouth?

It is generally accepted that the kidney is responsible for the excretion of urea in vertebrates (except fish). However, contrary to this common notion, our results suggest that the mouth can be a major route of urea excretion in soft-shelled turtles. What makes soft-shell turtles different from other vertebrates? Turtles of the family Trionychidae (soft-shelled turtles) are often found in brackish water or even the sea (Minnich, 1979), and *P. sinensis* can indeed survive in water of salinity 15 (Lee et al., 2006). Although invading the brackish and/or marine environment would provide extra resources for growth and reproduction, it also produces new challenges. If urea, as a major nitrogenous waste, was to be excreted through the kidney, *P. sinensis* would need to continuously imbibe water, which is hyperosmotic and hyperionic to its body fluid, for urine production. This would lead to an accelerated increase in plasma Na^+ and Cl^- concentrations. Because reptilian kidneys are incapable of excreting monovalent ions and producing hyperosmotic urine (Dantzler, 1995; Lee et al., 2006), it would be difficult for *P. sinensis* to maintain internal Na^+ and Cl^- homeostasis after drinking brackish water, leading to deleterious consequences. Because the buccopharyngeal urea excretion route involves only rinsing the mouth with the ambient water, problems associated with drinking brackish water and the consequential disruption of ionic homeostasis can be avoided. Furthermore, if indeed a Na^+/urea counter transport system was involved, it would mean that buccopharyngeal urea excretion could be more effective when *P. sinensis* is in brackish water (e.g. salinity 15) because of the presence of a larger Na^+ gradient between the saliva and the cytosol, which drives the counter transport of urea across the apical membrane of the buccopharyngeal epithelium.

Evolutionary significance of buccopharyngeal urea excretion in *P. sinensis*

In ureotelic fishes, urea excretion occurs mainly through gills (McDonald et al., 2006). In adult amphibians, urea excretion takes place mainly in the kidney, and some taxa can excrete urea through the cutaneous surface (Garcia-Romeu et al., 1981; Rapoport et al., 1988; Lacoste et al., 1991; Dytko et al., 1993). In this study, we demonstrated that urea excretion occurred mainly through the buccopharyngeal cavity of *P. sinensis*. Buccopharyngeal urea excretion in soft-shelled turtles could have an evolutionary link with the ability of some mammals, e.g. ruminants (Kennedy and Milligan, 1980; Marini and Van Amburgh, 2003) and certain species of bat (Beal, 1991), to excrete urea into the saliva for the purpose of urea recycling. In ruminants, urea secreted into the saliva is subsequently swallowed to support protein synthesis by the fermentative microbes present in the rumen (Marini and Van Amburgh, 2003). However, in *P. sinensis*, the behavioral adaptation of submerging the head into water with rhythmic pharyngeal movements during emersion facilitates the excretion of urea through the mouth. Thus, *P. sinensis* can be an appropriate model for studies on the expression of various types of facilitative and active urea transporters in the buccopharyngeal cavity. Such studies would provide information on how urea excretion could occur through the mouth, which may have applications in the treatment of conditions under which urea accumulates, such as renal failure.

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