PHYSIOLOGICAL AND MOLECULAR CHARACTERIZATION OF UREA TRANSPORT BY THE GILLS OF THE LAKE MAGADI TILAPIA (*ALCOLAPIA GRAHAMI*)

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Summary

The Lake Magadi tilapia (*Alcolapia grahami*) is an unusual fish, excreting all its nitrogenous waste as urea because of its highly alkaline and buffered aquatic habitat. Here, using both physiological and molecular studies, we describe the mechanism of branchial urea excretion in this species. *In vivo*, repeated short-interval sampling revealed that urea excretion is continuous. The computed urea permeability of *A. grahami* gill is $4.74 \times 10^{-5} \pm 0.38 \times 10^{-5}$ cm s^{-1} (mean ± S.E.M., $N=11$), some 10 times higher than passive permeability through a lipid bilayer and some five times higher than that of even the most urea-permeable teleosts studied to date (e.g. the gulf toadfish). Transport of urea was bidirectional, as demonstrated by experiments in which external [urea] was elevated. Furthermore, urea transport was inhibited by classic inhibitors of mammalian and piscine urea transporters in the order thiourea>*N*-methylurea>acetamide. A 1700 base pair cDNA for a putative Magadi tilapia urea transporter (mtUT) was cloned, sequenced and found to display high homology with urea transporters from mammals, amphibians and other fishes. When cRNA transcribed from mtUT cDNA was injected into *Xenopus laevis* oocytes, phloretin-inhibitable urea uptake was enhanced 3.4-fold relative to water-injected controls. Northern analysis of gill, red blood cells, liver, muscle and brain using a portion of mtUT as a probe revealed that gill is the only tissue in which mtUT RNA is expressed. Magadi tilapia gill pavement cells exhibited a trafficking of dense-cored vesicles between the well-developed Golgi cisternae and the apical membrane. The absence of this trafficking and the poor development of the Golgi system in a non-ureotelic relative (*Oreochromis niloticus*) suggest that vesicle trafficking could be related to urea excretion in *Alcolapia grahami*. Taken together, the above findings suggest that the gills of this alkaline-lake-adapted species excrete urea constitutively *via* the specific facilitated urea transporter mtUT.

Key words: *Alcolapia grahami*, urea transporter gene, UT-A2, gills, nitrogen excretion, Lake Magadi tilapia.

Introduction

The Lake Magadi tilapia (*Oreochromis alcalicus grahami*, now *Alcolapia grahami*; Seegers and Tichy, 1999) is a truly remarkable fish. It is the only fish species that survives (and thrives) in the highly alkaline (pH 10) and highly buffered (CO_2 content 180 mmol l^{-1}) waters of Lake Magadi in the Rift Valley of Kenya (Coe, 1966). These waters would rapidly kill standard teleostean species, largely because most fish are unable to excrete ammonia under these conditions, resulting in

neurotoxicity. Several publications have now documented that a central part of the survival/adaptational strategy of this species is to be completely ureotelic, i.e. to excrete all their nitrogen waste as urea and none as ammonia, an unusual condition for a fully aquatic teleostean fish (Randall et al., 1989; Wood et al., 1989; Wood et al., 1994; Wright et al., 1990). Furthermore, the rates of urea synthesis and excretion approach mammalian excretion rates, perhaps because of the

high temperature of the environment (Narahara et al., 1996) and nitrogen loading by the predominant diet, cyanobacteria (Wood et al., 1994).

The biochemical mechanisms of urea production in this species are beginning to be relatively well understood. The fish possess a fully functional hepatic ornithine–urea cycle (O-UC) (Randall et al., 1989; Walsh et al., 1993), but two unusual aspects of urea synthesis have recently been discovered. First, the key O-UC enzyme carbamoyl phosphate synthetase (CPSase) and other O-UC enzymes are also expressed in muscle, and the total muscular CPSase activity far outweighs the total hepatic activity (Lindley et al., 1999). This observation led these authors to suggest that most urea is indeed synthesized in the muscle. They further postulated that this architecture was due to the inefficiency of vertebrate CPSase; if CPSase were packaged in the liver, the mass of liver required to meet the huge rates of urea production observed would be incompatible with a laterally compressed perciform body plan (Lindley et al., 1999). The second unusual aspect of urea synthesis in this species is that the enzyme CPSase, while appearing to be of the piscine CPSase III isoenzyme type, uncharacteristically has a high capacity to use ammonia as a substrate, in addition to the more usual substrate glutamine (Lindley et al., 1999).

While information is accumulating on the mechanisms of urea production in this unusual species, very little is known about the mechanism of urea excretion. To date, only one study has addressed urea excretion. Using a divided chamber design separating the anterior and posterior of the fish, Wood et al. (Wood et al., 1994) demonstrated that approximately 80 % of urea was excreted from the anterior half of the fish, with 20 % being excreted from the posterior half, suggesting a minimal role for urinary urea excretion. Given the rapid progress being made on the physiological and molecular mechanisms of urea excretion in both mammalian and piscine systems (for reviews, see Sands et al., 1997; Walsh and Smith, 2000), the aims of the present study were to begin to analyze mechanisms of urea excretion in the Lake Magadi tilapia. Derived from a combination of physiological and molecular approaches, our results suggest that urea excretion is by the continuous operation of a specific facilitated transport mechanism in the gills. Furthermore, since the specific cellular organization of the gill may be related to this transport, we undertook ultrastructural studies of the cells lining the gills in a comparison between the urea-excreting fish *Alcolapia grahami* and its ammoniotelic relative *Oreochromis niloticus.*

Materials and methods

Animals and holding conditions

Lake Magadi tilapia (*Alcolapia grahami*, Seegers and Tichy, 1999; formerly *Oreochromis alcalicus grahami*) were captured from the Fish Springs Lagoon site (Narahara et al., 1996) at Lake Magadi, Kenya, by seine net in January and February 1997. The fish were immediately transported to an outdoor laboratory site several kilometers away (at the Magadi Soda Co., PLC) in large plastic containers filled with aerated water from Fish Spring Lagoon (typical water chemistry is reported in Table 1 of Wood et al., 1989). Water freshly collected from this site was also used for all experiments. Typically, fish were used in physiological experiments within 24 h of capture. In addition, selected tissues were frozen on site in liquid N_2 and returned to North America at liquid N_2 temperatures in a dry shipper. A simple saline, 200 mmol l^{-1} NaCl (similar to Magadi tilapia plasma; Wright et al., 1990), was used for all physiological procedures, and metomidate-HCl (5 mg l^{-1} in lagoon water) was used for anesthesia. All blood samples were taken by caudal puncture using a 100 µl gas-tight Hamilton syringe with a customized needle. The syringe was wetted with sodium heparin (1000 i.u. ml^{-1} in saline).

In vivo *physiological studies*

Series 1: *pattern of urea excretion*

To establish normal temporal patterns and rates of urea-N excretion, 11 tilapia (1.3–4.4 g) were placed in individual amber Tusker chambers (Wood et al., 1994) filled with 500 ml of continuously aerated lagoon water and allowed to settle for 1 h. Water samples (5 ml) for urea-N analysis were drawn every 15 min for the next 6.5 h. The temperature averaged 37.5 °C (36–39 °C). At the end of the experiment, the fish were quickly anesthetised and weighed, and the blood was sampled (10–20 µl) for plasma urea-N analysis. Plasma was separated by allowing the blood to settle at 4 °C in vertical hematocrit tubes (as the power failed that day), and then frozen for later analysis.

Series 2: *thiourea* versus *urea permeability*

To compare the relative permeability of the gills to urea and the urea analog thiourea, nine larger tilapia (4.9–15.7 g) were anesthetized briefly, weighed and injected with [^{14}C]thiourea (NEN; specific activity 58.0 mCi mmol^{-1}; 1 mCi=3.7×10^7 Bq). The [^{14}C]thiourea stock was made up at a concentration of 20 µCi ml^{-1} in saline; each fish received 10 µl g^{-1} injected into the hemal arch (i.e. 0.2 µCi g^{-1}) using the customized Hamilton syringe. The fish were transferred first to fresh lagoon water for 1 h for recovery, to allow the radiolabel to distribute in the bloodstream and to wash off any leaking radiolabel, and then to individual shielded beakers, each containing 300 ml of continually aerated lagoon water. Two water samples (each 5 ml) were drawn at 0, 1.5 and 3 h for simultaneous measurements of urea-N and [^{14}C]thiourea fluxes, and then the fish were anesthetized again for terminal blood sampling (50–150 µl) by caudal puncture. Plasma was separated by centrifugation at 10 000 *g* for 2 min and then frozen for later analysis of urea-N and [^{14}C]thiourea radioactivity. The temperature was 34 °C during the flux measurements in the outdoor laboratory.

Series 3: *urea influx and the effects of urea analogs*

This series employed an elevated external urea concentration (20 mmol l^{-1}) radiolabeled with [^{14}C]urea (NEN; specific activity 8.60 mCi mmol^{-1}), to test whether

gill urea flux was bidirectional, and evaluated whether urea influx could be inhibited by the simultaneous presence of urea analogs in the external water at three times the concentration of water urea. Influx rates were determined by measuring the appearance of [^{14}C]urea in the fish. There were five different treatment groups, each containing 7–10 fish (0.61–2.54 g), placed in shielded beakers containing 1.01 of continuously aerated lagoon water spiked with 20 mmol l^{-1} urea (40 mmol l^{-1} urea-N) plus 20 μCi l^{-1} [^{14}C]urea. This water came from a single stock to give identical concentrations of urea and [^{14}C]urea. In addition, the water contained 60 mmol l^{-1} acetamide, 60 mmol l^{-1} *N*-methylurea, 60 mmol l^{-1} thiourea, 30 mmol l^{-1} NaCl (as an osmotic pressure control) or no addition (as an absolute control). Three water samples (each 5 ml) were taken at 0, 1.5 and 3 h for measurement of water urea and [^{14}C]urea radioactivity; values did not change appreciably. Immediately after the 3 h sample, the fish were rapidly rinsed with fresh lagoon water (to remove surface-bound radioactivity), anesthetized, weighed and preserved in liquid N$_2$ for later measurement of [^{14}C]urea radioactivity. Because of a procedural error, most of the samples from the thiourea test were lost, so this treatment, together with a simultaneous control, was repeated. The results of the tests were identical, and the data were therefore combined. The temperature during these tests averaged 32 °C (31–33 °C). For the sake of comparison, normal urea flux rates to the water over a 3 h period (in the absence of added external urea or analogs) and plasma urea-N levels were measured in 10 fish of the same size, and at the same temperature, by the methods outlined in series 1.

Calculations

Urea flux rates and concentrations were expressed in units of nitrogen for consistency with previous studies (e.g. Wood et al., 1989; Wood et al., 1994). Urea-N flux rates to the water (in μmol-N kg h^{-1}=nmol-N g^{-1} h^{-1}) in series 1, 2 and 3 were calculated from changes in concentration in the water (μmol-N l^{-1}) multiplied by volume (l) and factored by time (h) and fish mass (kg). Urea permeabilities (in cm s^{-1}) of the gill in series 1 and series 2 were obtained by assuming that 80 % of the measured flux rate (μmol-N kg h^{-1}) occurred across the gills (Wood et al., 1994). This value was divided by the measured plasma urea-N concentration (μmol-N l^{-1}) and the gill area (cm^2 kg^{-1}), while converting hours (×3600) to seconds and liters (×1000) to cm^3. The thiourea permeability of the gill in series 2 was calculated using an identical approach, substituting counts of [^{14}C]thiourea for μmol urea-N. The gill area of each fish was estimated from its body mass using the allometric relationship of total secondary lamellar surface area (SLSA) to body mass established by Maina et al. (Maina et al., 1996) for this species at Fish Springs Lagoon. Urea-N influx rates in series 3 were calculated by dividing the terminal concentration of [^{14}C]urea radioactivity in the whole bodies (counts g^{-1}) by the mean specific activity of the water (counts mmol^{-1}) and time (h).

Analytical techniques

Urea-N in lagoon water (1 ml samples) and plasma (10 μl added to 1 ml of lagoon water) was measured using the diacetyl monoxime method (Rahmatullah and Boyde, 1980; Price and Harrison, 1987) with standards made up in lagoon water. The final volume (1 ml of sample plus 1 ml of reagents) was adjusted to 3 ml by the addition of 1 ml of lagoon water so that the assay could be read on a Spectronic 20 spectrophotometer (Bausch & Lomb).

[^{14}C]urea and [^{14}C]thiourea radioactivity were measured by scintillation counting (LKB Rackbeta 1217) in samples of lagoon water (1 ml) and plasma (20–100 μl plus 1 ml of lagoon water) made up to 11 ml with Ready-safe aqueous fluor (Beckman). [^{14}C]Urea in whole bodies was measured by first solubilizing the whole carcass in 6 ml g^{-1} NCS tissue digest media (Amersham) in a sealed vial overnight at 42 °C; 2 ml samples of clear digest were then neutralized with 30 μl of glacial acetic acid and made up to 20 ml with OCS organic fluor (Amersham). Quench correction was performed by the external standard method, and equalization of counting efficiencies in the two fluor systems was performed by internal standardization.

Molecular studies

Polymerase chain reaction cloning

Initial attempts to screen a Magadi tilapia (whole-body) cDNA library using the toadfish urea transporter (tUT; Walsh et al., 2000) as a probe were unsuccessful, so a reverse transcription/polymerase chain reaction (RT–PCR) approach was taken. Total RNA was isolated from the gill by homogenization of 0.2 g of tissue in 1.2 ml of phenol-guanidinium thiocyanate (Trizol Reagent, Gibco BRL) followed by standard extraction with chloroform and precipitation with isopropanol (Sambrook et al., 1989). mRNA was enriched from total RNA using an mRNA purification kit based on oligo(dT)–cellulose column chromatography (Pharmacia Biotech). cDNA was synthesized using MMLV reverse transcriptase and oligo(dT) primers (Stratagene). PCR was performed on this cDNA using degenerate primers designed using sequences for mammalian, piscine and amphibian urea transporters. These were sense (UTPS) (corresponding to the third base pair coding for amino acid residue 175 to the second base pair coding for amino acid residue 183 of tUT), 5′GGAYYTICCNGTNTTYACNYTI-CC3′; antisense (UTDA2) (corresponding to amino acid residues 316–310 of tUT), 5′GCIARITRRTGIRYYTGCC-AIGT3′. PCR conditions were 94 °C for 4 min, followed by 50 °C for 5 min, at which point *Taq* polymerase was added followed by the mineral oil layer in a hot-start protocol, and then 30 cycles of 94 °C for 30 s, 50 °C for 1 min, 72 °C for 1 min, followed by 72 °C for 7 min in a Perkin-Elmer 480 PCR machine using 0.5 ml GeneAmp tubes (Perkin-Elmer).

PCR products were separated by gel electrophoresis (1 % agarose gel in TAE buffer (40 mmol l^{-1} Tris-acetate, 1 mmol l^{-1} EDTA, pH 8.0), and the major band of 420 base pairs (bp) was gel-purified and subcloned into the plasmid vector pCR 2.1

(Invitrogen, Carlsbad, CA, USA). The resultant plasmid (m17-1) was transfected into Epicurean Ultracompetent *Escherichia coli* (Stratagene, La Jolla, CA, USA). Standard blue/white screening on Luriabroth (LB) containing 25 mg ml^{-1} kanamycin plates identified colonies with the potential insert, which were then cultured in LB. The plasmid DNA was isolated by the alkaline lysis method (Qiagen Kit, Chatsworth, CA, USA), and restriction digests were performed to ensure the presence of an appropriately sized insert. Both strands of insert DNA of several clones were sequenced using an automated dideoxy chain-termination sequencing method (Sanger et al., 1977) and found to be homologous to the toadfish urea transporter (tUT) gene (see Results).

Rapid amplification of cDNA ends PCR

5′ and 3′ rapid amplification of cDNA ends (RACE) PCR was performed to amplify 5′ and 3′ ends using the Marathon cDNA amplification kit (Clontech, Palo Alto, CA, USA) and adaptor-ligated gill cDNA (adaptor from the kit). Gene-specific primers were synthesized on the basis of the known sequences of m17-1 and were mtUTGSP1 (the antisense-gene-specific primer for 5′RACE), 5′CCAATGGCGATGCAGGCTA-ACACACAGT3′, corresponding to base pairs 380–352 of m17-1, and mtUTGSP2 (the sense-gene-specific primer for 3′RACE), 5′GGAGGCATCTTCATCATCTCCCTCTTTA3′, corresponding to base pairs 211–238 of m17-1. The sense primer for 5′RACE and the antisense primer for 3′RACE were complementary to an adaptor supplied with the kit (which was ligated to the 5′ and 3′ cDNA ends). PCR conditions were: 94 °C for 1 min, followed by five cycles of 94 °C for 30 s, 72 °C for 4 min, followed by five cycles of 94 °C for 30 s, 70 °C for 4 min, followed by 25 cycles of 94 °C for 30 s, 68 °C for 4 min with machine and tubes as above. Generally, PCR reactions were performed in duplicate to exclude PCR errors. The 5′RACE reactions produced a band of approximately 1.2 kilobase pairs (kb) and the 3′RACE reactions produced a band of approximately 1 kb, which were purified, subcloned and sequenced as above. Once sequences were confirmed to overlap, gene-specific primers near to the 5′ and 3′ ends were designed to obtain a 'full-length' clone, and a PCR product of 1.6 kb (mtUT 2Ex-2), which included the putative open reading frame (ORF), was generated and similarly cloned into pCR 2.1.

Northern analysis

Total RNA was extracted from several Magadi tilapia tissues (gill, liver, skeletal muscle, blood cells and brain) and poly(A$^+$)-enriched as above. Equal amounts (3 µg) of poly(A$^+$) RNA were loaded onto a formaldehyde–agarose gel, electrophoresed and transferred to a nylon membrane (Hybond-N, Amersham, Arlington Heights, IL, USA) using standard methods (Sambrook et al., 1989). Following prehybridization, the membrane was hybridized at 42 °C in hybridization buffer containing 30 % formamide with random-primed ^{32}P-labeled m17-1. [^{32}P]m17-1 was produced by extracting the excised bands from *Eco*RI-digested minipreps

electrophoresed as above, labeling with the RadPrime kit (Gibco, Frederick, MD, USA) and [α-^{32}P]dCTP (ICN, Irvine, CA, USA) and purification on G-50 Sephadex. Final washes were in 0.1× SSC/0.1 % SDS at 65 °C (1× SSC is 0.15 mol l^{-1} NaCl, 0.015 mol l^{-1} sodium citrate).

Functional expression in Xenopus laevis *oocytes*

After linearization of mtUT plasmid with *Kpn*I, cRNA was synthesized *in vitro* using T7 polymerase (T7-mMessage mMachine, Ambion) and microinjected (30 ng) into collagenase-treated defolliculated *Xenopus laevis* oocytes, as described previously (Smith et al., 1995). Parallel groups of oocytes were injected with water (50 nl) as a negative control. Oocytes were incubated in isotonic frog saline (in mmol l^{-1}: Na$^+$ 104, Cl$^-$ 104, Ca^{2+} 1.8, Mg^{2+} 1, pyruvate 2.5, Hepes 5, pH 7.4, containing 50 µg ml^{-1} gentamycin) for 2–3 days at 18 °C prior to experimentation. For urea flux studies, oocytes were preincubated in buffer (in mmol l^{-1}: 200 mannitol, 2 KCl, 1 MgCl$_2$, 1 CaCl$_2$, 10 Hepes, 5 Tris, pH 7.4) for 15 min and then transferred to uptake solution consisting of the preincubation medium with added urea (1 mmol l^{-1}) and [^{14}C]urea (2.7 µCi ml^{-1}). After 1 min, oocytes were rapidly washed three times with ice-cold uptake solution containing 10 mmol l^{-1} unlabeled urea and dissolved in 0.5 ml of 10 % SDS, and the radioactivity was measured by scintillation counting. Inhibition of [^{14}C]urea uptake by 1.0 mmol l^{-1} phloretin dissolved in ethanol was measured by addition of phloretin to the preincubation solution, as described previously (Smith et al., 1995). As a vehicle control, ethanol was included in the control experiments. In some experiments, oocytes were preincubated for 15 min with the phorbol ester 12-*O*-tetradecanoylphorbol 13-acetate (PMA) (a potent activator of protein kinase C), or with forskolin (an activator of adenyl cyclase used to investigate the effect of cyclic-AMP-dependent protein kinase A, PKA), prior to uptake measurements in the continued presence of these drugs. Both drugs were added as a solution in dimethylsulfoxide (DMSO).

Ultrastructure of the gill lining

Fish weighing 1–5 g were captured early in the morning, killed with an overdose of MS222 (0.5 g l^{-1} in Fish Spring Lagoon water) and processed immediately for transmission electron microscopy of the gill filaments according to methods described previously (Laurent et al., 1995).

Chemicals, analyses of sequence information and statistical analyses

Unless stated otherwise, all chemicals were purchased from Sigma Chemical Co. (St Louis, MO, USA). Routine analyses of DNA and protein sequences was accomplished by MacVector and DNASTAR programs. The sequences of urea transporters for other animals were obtained by a basic BLAST 2.0 search (Altschul et al., 1997) of GenBank. Statistical significance was tested by Student's *t*-test or analysis of variance (ANOVA), and *post-hoc* comparisons were made using the Student–Newman–Keuls or Bonferroni methods as
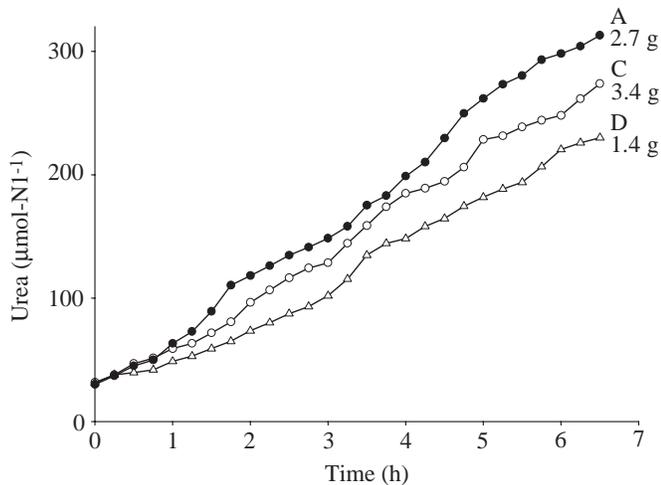
Fig. 1. Typical records of the appearance of urea-N in the external water at 15 min intervals over 6.5 h for three individual Lake Magadi tilapia (A, C and D), each placed in 500 ml of lagoon water in series 1. Note that urea excretion is continuous, occurring at a more-or-less steady rate over time.

appropriate. Values are quoted as means ± S.E.M., and statistical significance is assumed at the $P<0.05$ level.

Results

In vivo physiology

Series 1: pattern of urea excretion

Serial sampling at closely timed intervals (15 min) demonstrated that urea-N accumulates in the external water at a more-or-less constant rate (Fig. 1), rather than as discrete pulses. Overall net urea-N excretion rate in this series at 37.5 °C was $6603\pm526\,\mu$mol-N kg^{-1} h^{-1} (N=11), and plasma urea-N was $7900\pm841\,\mu$mol-N l^{-1} (N=11), yielding an estimated branchial permeability of $4.74\times10^{-5}\pm0.38\times10^{-5}$ cm s^{-1} (N=11) (means ±1 S.E.M.). Compared with the gills of most teleosts and the even lower permeability of elasmobranch gills, the value in the Magadi tilapia is extremely high (Fig. 2). Indeed, the value is over 10 times that for standard lipid bilayers and approximately five times higher than that of the actively pulsing gulf toadfish (Fig. 2).

Series 2: thiourea versus urea permeability

In individual tilapia, the permeability of the gills to urea was closely correlated with that of its analog thiourea (Fig. 3). However, in this series at 34 °C, absolute thiourea permeability ($6.92\times10^{-6}\pm0.89\times10^{-6}$ cm s^{-1}, N=9) was less than 20 % of urea permeability ($3.69\times10^{-5}\pm0.37\times10^{-5}$ cm s^{-1}, N=9). Notably, this low ratio of thiourea-to-urea permeability in the continuously excreting Magadi tilapia is nearly identical to that of the gulf toadfish during an active pulse event (Fig. 4). At the same time, it is very different from the situation in the non-pulsing toadfish, in which the ratio is close to 1.2, the expected value for these very similar molecules when simple diffusion alone is involved in their movement.
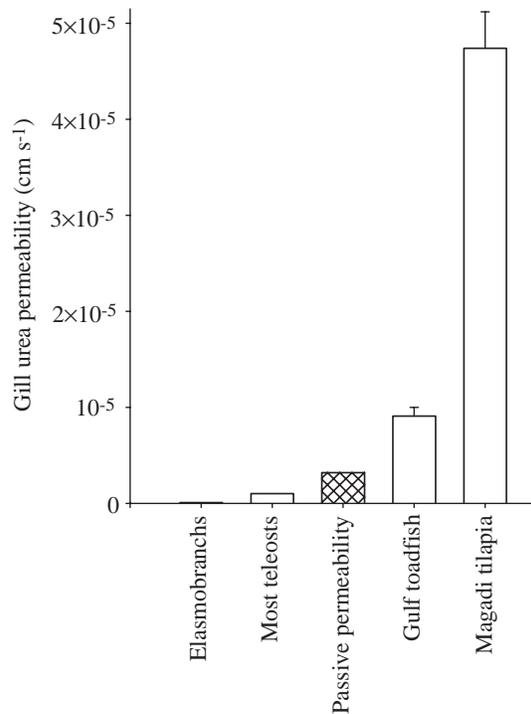
Fig. 2. A comparison of the urea permeability of the gills of the Lake Magadi tilapia (means +1 S.E.M., N=11; series 1 data) with literature values for the gills of elasmobranchs and most teleosts (Isaia, 1984; Wood et al., 1995; Pärt et al., 1998), passive permeability in lipid bilayers (Galluci et al., 1971; Finkelstein, 1976) and the gills of the gulf toadfish when its facilitated diffusion carrier is fully activated (Wood et al., 1998; McDonald et al., 2000).
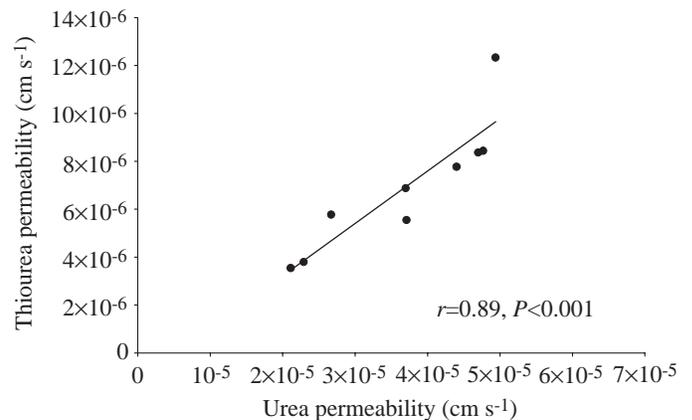
Fig. 3. The relationship between thiourea permeability and urea permeability in the gills of individual Lake Magadi tilapia in series 2.

Series 3: urea influx and the effects of urea analogs

Unidirectional influx rates measured in the presence of a high external urea concentration (20 mmol l^{-1}=40 000 μmol-N l^{-1}) in the control group were $6718\pm125\,\mu$mol-N kg^{-1} h^{-1} (N=19). In the reference group run at the same temperature (32 °C), net efflux rates were $4346\pm538\,\mu$mol-N kg^{-1} h^{-1} (N=10) and plasma urea-N concentration was
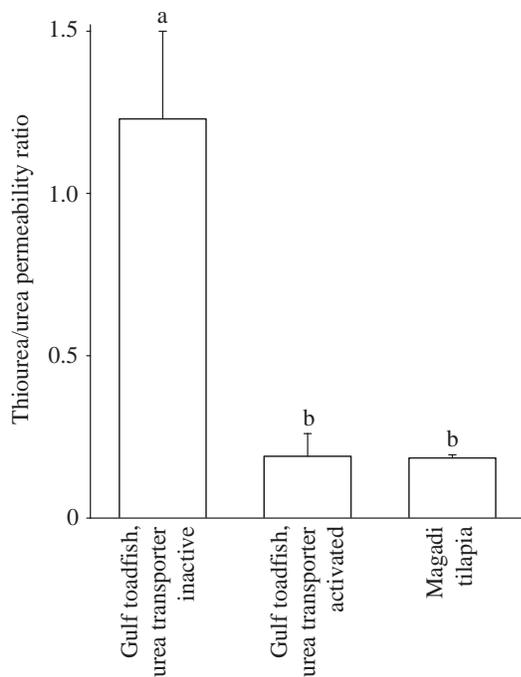
Fig. 4. The ratio of thiourea-to-urea permeability in the gills of the Lake Magadi tilapia (series 2 data) in comparison with the ratio in the gills of the gulf toadfish (Wood et al., 1998) when the latter's facilitated diffusion transporter is either inactive or activated (means +1 S.E.M., $N=6$). Values sharing the same letter are not significantly different.

Fig. 5. The effects of three urea analogs (acetamide, N-methylurea and thiourea, present in the external water at a concentration of $60\,mmol\,l^{-1}$) on unidirectional urea-N influx (urea was present in the external water at a concentration of $20\,mmol\,l^{-1}$) in the Magadi tilapia of series 3. Values are given as μmol-$N\,kg^{-1}\,h^{-1}$, equivalent to $nmol$-$N\,g^{-1}\,h^{-1}$. The $30\,mmol\,l^{-1}$ NaCl treatment served as an osmotic pressure control. Values are means +1 S.E.M. (control, $N=19$; NaCl, $N=9$; acetamide, $N=8$; N-methylurea, $N=7$; thiourea, $N=13$). Values sharing the same letter are not significantly different.

$20\,584\pm735\,\mu mol$-$N\,l^{-1}$ ($N=10$). Thus, in the presence of an inward concentration gradient (from water to blood plasma), influx was larger than efflux and in approximate proportion to the relative concentrations on the two sides of the gill, consistent with a bidirectional transport system. When the effects of urea analogs (at $60\,mmol\,l^{-1}$, three times the urea concentration in the water) on this influx were examined, influx was significantly reduced by all three, with the potency order thiourea>N-methylurea>acetamide (Fig. 5). Addition of $30\,mmol\,l^{-1}$ NaCl had no effect relative to control rates (Fig. 5), indicating that the effects were unrelated to the small elevation of osmotic pressure.

Molecular cloning

PCR and RACE PCR yielded a sequence of 1699 bp (GenBank accession no. AF278537) with high homology to published sequences for urea transporters, specifically the UT-A2 of mammals (rabbit and rat), the shark kidney urea transporter (ShUT) and the toadfish gill urea transporter (Smith et al., 1995; Smith and Wright, 1999; Walsh et al., 2000; You et al., 1993). The Magadi tilapia urea transporter has modified polyadenylation sequences at base pairs 1552–1557 (ATTAAT) and at base pairs 1654–1659 (ATTTAA). The longest open reading frame (ORF) begins at nucleotide 103 and has a modified Kozak site upstream of the ATG (GCCCCTG) and a G at position +4 (whereas the consensus site is GCCA/GCC and a G at position +4). This ORF ends at base
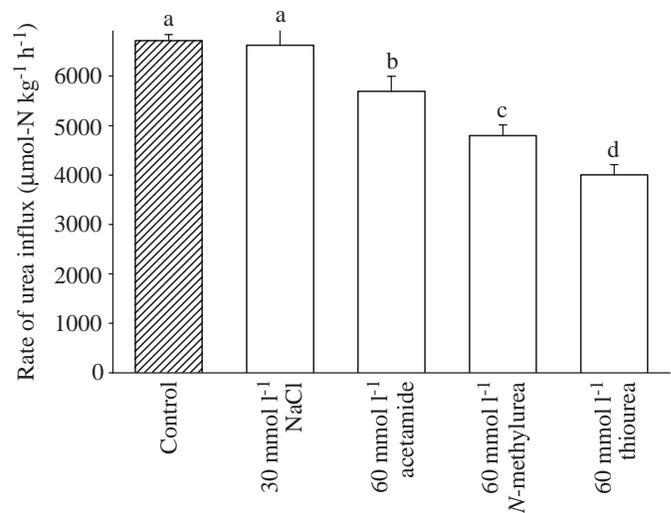
pair 1530, encoding a 475-amino-acid-residue protein that we have named 'mtUT' (Magadi tilapia urea transporter) (Fig. 6). A second possible initiation codon in the same frame occurs at nucleotide 244 and is preceded by the sequence GGAGAC and a G at position +4. Although the first 47 amino acid residues of the 475-residue protein do not have high homology with other vertebrate urea transporter proteins, a region of higher homology with other vertebrate urea transporter proteins begins at the second and third methionines (amino acid residues 46 and 53, respectively) of the deduced polypeptide sequence (Fig. 6). Overall, mtUT has 75 % amino acid identity with tUT (toadfish UT) and 56–61 % amino acid identity with human UT-A2, $Rana$ UT, rat UT-A2, rat UT-B and ShUT. This homology approximates the level of identity between ShUT or tUT and mammalian urea transporters (Smith and Wright, 1999; Walsh et al., 2000).

Oocyte studies

Injection of mtUT cRNA into $Xenopus\ laevis$ oocytes resulted in a 3.4-fold increase in urea uptake rates relative to water-injected controls, and this increase was inhibitable by phloretin (Fig. 7A). Futhermore, when the effectors PMA and forskolin were applied to oocytes injected with mtUT cRNA, PMA had the effect of depressing urea uptake to approximately 60 %, whereas the effect of forskolin was not signficant (Fig. 7B).

Northern analysis

Of all the tissues studied (gill, liver, muscle, red blood cells and brain), only the gill showed a signal in northern analyses

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mtUT        1  MAHPDLPSQNGNSQEDGQEDRVQPGGATALQKARASFLKGVSYFSGDMEVFGKWMEKQFF
tUT         1  MAHPDEHEN-QQADGKENV-PVQPAGPTRLQKARACFLKGVSYFSGDMKAFGKWMEKRCI
ShUT        1  ------MEQ-KKAAIVE---EISTLNPDNLKET---FFKGIGYLSGDMSEFGYWLKQQNI
RanUT       1  ------MESHIPINMAEKELKKPVESPNHCSSGLGFFCTAIHYISGEMKEFGNWLKDKPI
ratUT-A2    1  ------MEESSEIKVETNTTRTTWIQSSMIAGGK-RVSKALSYITGEMKECGEGLKDKSP
humanUT-A2  1  ------MEESSEIKVETNISKTSWIRSSMAASGK-RVSKALSYITGEMKECGEGLKDKSP
ratUT-B     1  ----MAMED-IPTMVKVDRGESQILSCRGRRCGL----KVLGYVTGDMKEFANWLKDKPV

mtUT       61  LLRLLDWVLRGAAQVMFVNNPLSGLVIFAGLILQNYWWALNGFVGTLFATISALLLQQNR
tUT        59  LLQLLDWVLRGASQVMFVNNPLSGLIIFAGLILQNYWWALNGFVGTLAATVSALIIRQNR
ShUT       48  IFQFIDWILRGAAQVMFVNNPLSGLIILVGLIVQNPWWALNGFVGTVFSTLAALLLSQNR
RanUT      55  IFQFIDWVLRGASQVMFVSNPLSGLIIIAALFLQNPWWAIAGCLGTAVSTLTALLLSQDR
ratUT-A2   54  VFQFLDWVLRGTSQVMFVNNPLSGILIVLGLFVQNPWWAISGCLGTIMSTLTALILSQDK
humanUT-A2 54  VFQFFDWVLRGTSQVMFVNNPLSGILIILGLFIQNPWWAISGCLGTIMSTLTALILSQDK
ratUT-B    52  VLQFMDWILRGISQVVFVSNPISGILILAGLLVQNPWWALCGCVGTVSTLTALLLSQDR

mtUT      121  GAIAAGLYGYNGILVGLLMAVFSNAGDWYWWLLLPNIFMSMMCPIVSSALASINSRWDLP
tUT       119  GAIAAGLYGYNGILVGLLMAVFSNAGNWYWWLLLPNTFMSMMCPIVSSALASINSRWDLP
ShUT      108  SAIAAGLYGYNGILVGLLMAVFCDKGDWYWWLLLAVIVMSMACPIISSALAAVMGKWDLP
RanUT     115  SAIAAGLHGYSAMLVGLLMAVFSTKGDWYGWLLFPVAVMSMTCPLLSSALGNIFSKWDLP
ratUT-A2  114  SAIAAGLHGYNGVLVGLLMAVFSDKGNYYWWLLLPVIVMSMTCPILSSALSTVFSKWDLP
humanUT-A2 114 SAIAAGFHGYNGVLVGLLMAVFSDKGDYYWWLLLPVIIMSMSCPILSSALGTIFSKWDLP
ratUT-B   112  SAITAGLQGYNATLVGILMAVFSDKGDYFWWLIFPVSAMSMTCPVFSSALSSLFSKWDLP

mtUT      181  VFTLPFNILVCLHMVATGHYNNYFPQVLIQPRSELPNITWAELDVAKLFMSVPVGIGQVY
tUT       179  VFTLPFNILVCLHMVATGHYNNHFPQVLIQPRSELPNITWSEIDLPKLFMSIPVGIGQVY
ShUT      168  VFTLPFNISVGLFMAATGHYNQHFPQVLIEPVTSSQNITWPDLSIPMLLRAIPVGVGQVY
RanUT     175  VFTLPFNIAVCLHLAATGPNNEFFPTVDFRPTGVVPNISWSDVEISQLLKAVPVGVGQVY
ratUT-A2  174  VFTLPFNIAVTLYLAANGHYNLFFPTKLLQPAVTTPNITWSDVQVPLLLRAIPVGIGQVY
humanUT-A2 174 VFTLPFNITVTLYLAATGHYNLFFPTTLLQPASAMPNITWSEVQVPLLLRAIPVGIGQVY
ratUT-B   172  VFTLPFNMALSLYLPATGHYNTFFPSKLFMPVSSVPNITWSELSALELLKSLPVGVGQIY

mtUT      241  GCDNPWTGGIFIISLFISSPITCAHAVLGSAAGMVSGLALAAPFGDIYFGLWGYNCVLAC
tUT       239  GCDNPWTGGIFIISLFISSPITCIHAVLGSAAGMVSGLALAAPFGDIYFGLWGYNCVLAC
ShUT      228  GCDNPWTGGIFLVALTISSPIICLHAAIGSCVGILAGLSLASPFQKIYDGLWSYNCVLAC
RanUT     235  GCDNPWSGGIMLVGLFISSPIICMHAAMGASMGMLAGLSIAAPFESIYFGLWGYNSVLGC
ratUT-A2  234  GCDNPWTGGIFLVALFVSSPLICLHAAIGSTIGMLAALSIATPFDSIYFGLCGFNSTLAC
humanUT-A2 234 GCDNPWTGGIFLIALFISSPLICLHAAIGSTMGMLAALTIATPFDSIYFGLCGFNSTLAC
ratUT-B   232  GCDNPWTGAIFLCAILLSSPLMCLHAAIGSLLGVIAGLSLAAPFKDIFSGLWGFNSSLAC

mtUT      301  IAIGGMFYALTWQVHLLAITCAFFCAYLGSAIANIMSRFGLPACTWPFCLSALTFLLLTT
tUT       299  IAIGGMFYALTWQVHLLALTCAFFCAYLGSAIANVMSTFGLPACTWPFCLSALTFLLITT
ShUT      288  IAVGGMFYALTWQTHLLAIICSIFCAYLFEALMNIMSVVGLPACTWPFCLSTIVFLLLTT
RanUT     295  IAVGGMFYALTWQTHLLAIACALMCAYLGAALANVMAVIGLPSCTWPFCLTTLIFVLLTT
ratUT-A2  294  IAIGGMFYVITWQTHLLAIACALFAAYLGAALANMLSVFGLPPCTWPFCLSALTFLLLTT
humanUT-A2 294 IAIGGMFYVITWQTHLLAIACALFAAYLGAALANMLSVFGLPPCTWPFCLSALTFLLLTT
ratUT-B   292  IAIGGMFMALTWQTHLLALACALFTAYFGACMTHLMAAVHLPACTWSFCLATLLFLLLTT

mtUT      361  GTKRIFKLPLAKVTYPEKNLRFFWKLKKQEKTEKAEKERKEREEQQRAVEEEVILNEKEQ
tUT       359  ETQKIFKLPLAKVTYPEKNLCYVWKLKKQKKQEKADKLKKECEEAQRALEEEEILNAKEQ
ShUT      348  NNKAIYKLVLCEVTYPEKNRRIYQEMKKME-QS---K----------------------
RanUT     355  NNPAIYKLPLSKVTYPEDNRIYLNLKKERKKQ--------------------------
ratUT-A2  354  NNPGIYKLPLSKVTYPEANRIYFLSQEKNRRASMITKYQAYDVS----------------
humanUT-A2 354 NNPAIYKLPLSKVTYPEANRIYYLSQERNRRASIITKYQAYDVS----------------
ratUT-B   352  ENPNIYRMPLSKVTYSEENRIFYLQNKKRVVDSVCVHVCVCGCMCILPVCVLLHVYLCVS

mtUT      421  LKVELERMEQGKTENEAQEVKSD--STEVEVPQTAEGPREERNTENDLIEVTLTDYV
tUT       419  LRLALHRMEEGKKMNGQADVKNEEKETEHNVPDRVQVATEDETRQDNPTEVGDNHV
ShUT           --------------------------------------------------------
RanUT          --------------------------------------------------------
ratUT-A2       --------------------------------------------------------
humanUT-A2     --------------------------------------------------------
ratUT-B   412  VSS-----------------------------------------------------
```
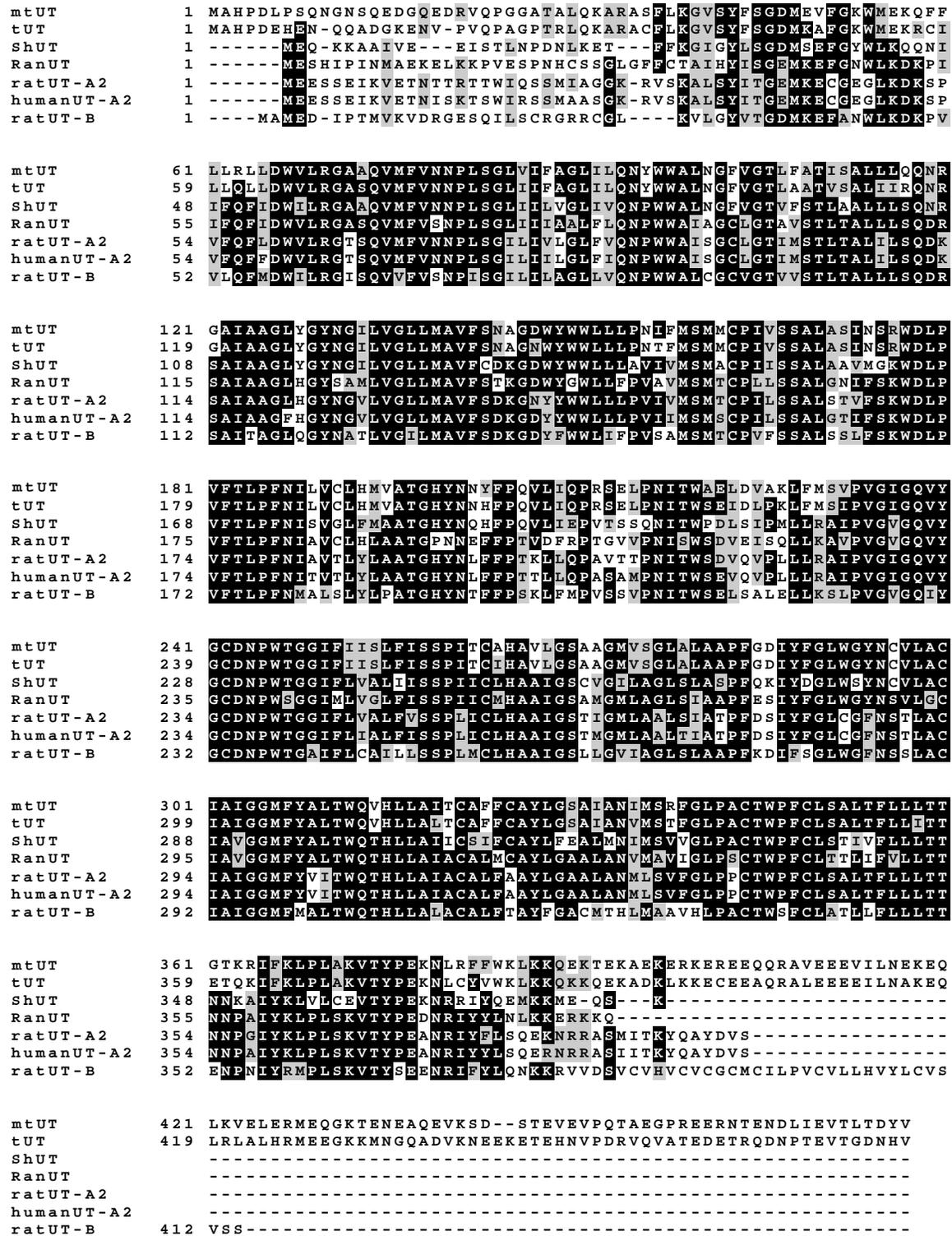
Fig. 6. Alignment of the Lake Magadi tilapia urea transport protein (mtUT) with other urea transporter (UT) proteins (references and accession numbers are as follows: tUT, toadfish, Walsh et al., 2000, AF165893; ShUT, shark, Smith and Wright, 1999, AF257331; RanUT, fUT *Rana*, Verbavatz et al., 1996, Y12784.1; ratUT-A2, rUT, Smith et al., 1995, Q62668; humanUT-A2, HUT11, Olives et al., 1994, Q15849; ratUT-B, UT11, Couriaud et al., 1996, X98399.1). Black boxes enclose identical amino acid residues, grey boxes enclose conservative substitutions, dashes represent deletions and white areas represent non-identity.
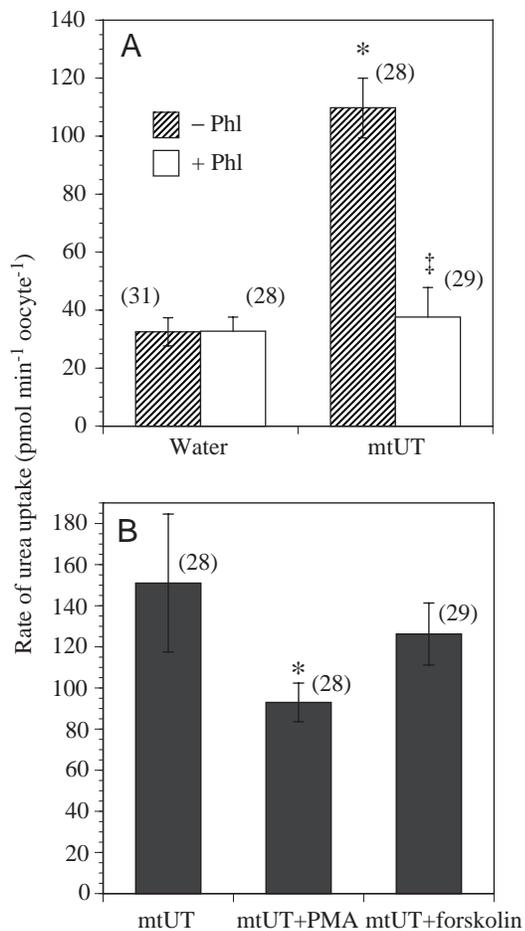
Fig. 7. Effects of Lake Magadi tilapia urea transport protein (mtUT) expression on urea uptake by *Xenopus laevis* oocytes. (A) Summary of the urea accumulated in a 1 min period by water-injected control oocytes or oocytes expressing mtUT. Hatched columns show uptakes under control conditions. Open columns show uptakes in the presence of $1.0 \, \text{mmol} \, l^{-1}$ phloretin for 15 min. An asterisk indicates a significant increase ($P<0.01$) in urea uptake compared with the water-injected control group and a double dagger denotes that phloretin caused a significant reduction in uptake in comparison with the paired control group. (B) Effects of the phorbol ester 12-*O*-tetradecanoylphorbol 13-acetate (PMA) ($10^{-6} \, \text{mol} \, l^{-1}$) and forskolin ($2 \times 10^{-5} \, \text{mol} \, l^{-1}$) in a 15 min preincubation on 1 min urea uptake rates. An asterisk indicates a significant decrease ($P<0.01$) in urea uptake compared with the control group. Values are presented as means ± S.E.M. with the number of oocytes tested in parentheses.

under high stringency (Fig. 8). A single band was seen at approximately 1.9 kb, which we believe corresponds to mtUT cDNA. Notably, a signal near to 4.0 kb often seen in other species was absent from *A. grahami*. When lower-stringency washes (e.g. $1 \times$ SSC, $50\,°C$) were used, the pattern was the same (results not shown).

Gill ultrastucture

Two cell types are examined when the function of gills is
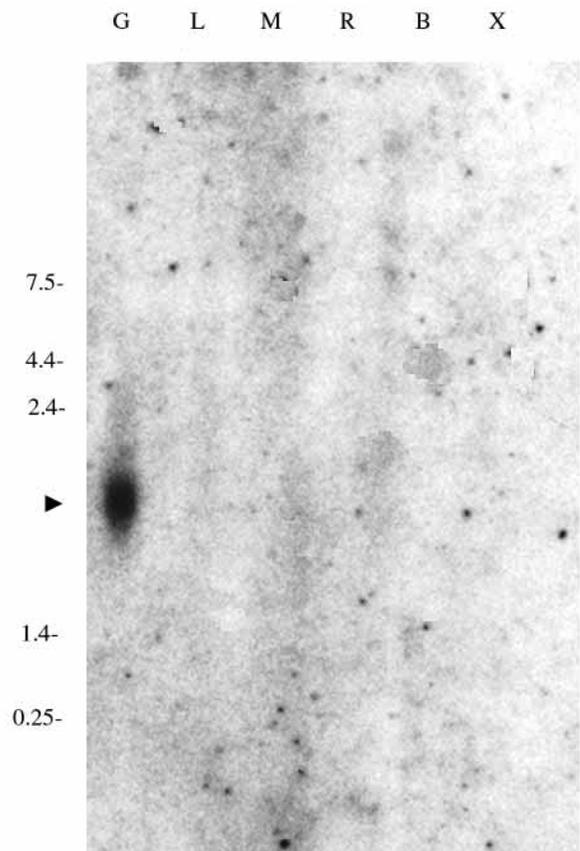
Fig. 8. Tissue distribution of Lake Magadi tilapia urea transport mRNA (mtUT) using high-stringency northern analysis. Membranes were probed using ^{32}P-labeled full-length random-primed m17-1 cDNA probe and hybridized at $42\,°C$ with 30 % formamide. Final washes were in $0.1 \times$ SSC, 0.1 % SDS at $65\,°C$. Each lane contains $3 \, \mu g$ of poly(A^+) RNA. G, gill; L, liver; M, muscle; R, red blood cells; B, brain; X, a negative control with no tissue. Numbers on the left are the positions of known molecular mass RNA standards (in kb), and the arrowhead marks the band in gill at approximately 1.9 kb. Ethidium-bromide-stained gels were examined prior to blotting for RNA degradation, particularly for 18S and 28S rRNA, and degradation was not evident.

studied as a barrier between the fish and the milieu (Laurent and Perry, 1995). The chloride cell has previously been studied in some detail (Laurent et al., 1995) and will not be considered further in the present study because it did not appear to display any particular specific features that might be related to urea secretion. However, pavement cells (PVCs) showed more interesting details. Fig. 9A displays an ultrathin section of a pavement cell of the Lake Magadi tilapia in which three Golgi apparatus profiles are visible side by side in the same cell. This density was representative of the great density of Golgi stacks in the PVCs of this species. Numerous vesicles (50–200 nm in diameter) are scattered around the Golgi apparatus and between these organelles and the apical membrane. They are variably filled with electron-dense material. Fig. 9B demonstrates the relationships between the Golgi saccules and vesicles pinching off from them. Some vesicles, usually the smallest, display a

Fig. 9. (A) A gill pavement cell (PVC) of *Alcolapia grahami* showing three Golgi apparatus profiles (G) giving rise to vesicles of variable size engaged in the process of filling (arrowhead). Note the poor conservation of the plasma membrane, which contrasts with the better fixation of intracellular membranes. W, water; m, mitochondria. (B) A well-developed Golgi apparatus (G, long arrow) close to a profile of rough endoplamic reticulum and producing many vesicles of variable size and varied density (short arrow) some of them close to the apical membrane. The arrowheads point to dense vesicles apposed to the membrane, an aspect reminiscent of what is observed in the urea-secreting toadfish when they are pulsing urea (Laurent et al., 2000). m, mitochondria. (C) A gill pavement cell of the ammoniotelic related species *Oreochromis niloticus*. In this case, the PVC is contiguous with a chloride cell (CC). Note the non-active Golgi apparatus and its associated reticulum. Vesicles are scanty and clear. Note the presence of glycocalyx on the plasma membrane indicating a better preservation of ultrastructure due to appropriate working conditions. Scale bars, 1 μm (A,B), 500 nm (C).
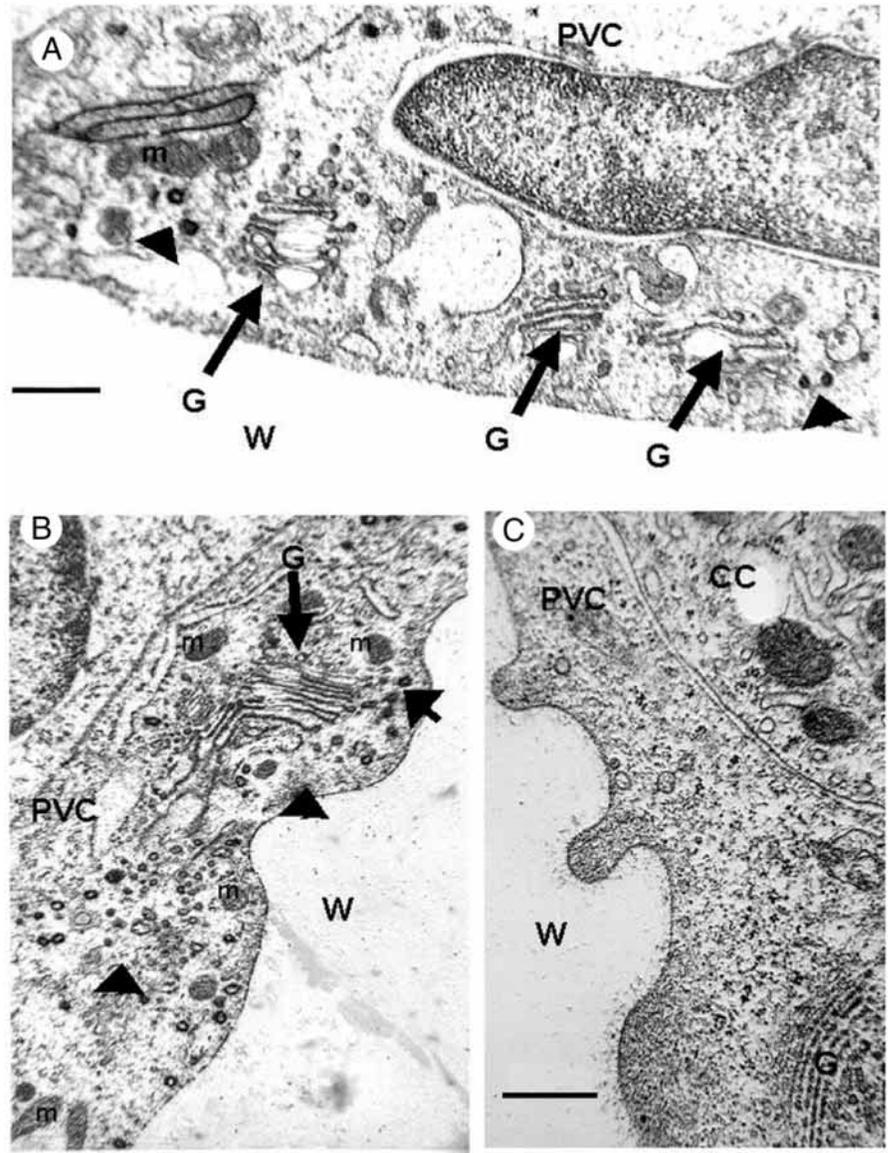
well-contrasted electron-dense periphery but an electron-lucent center. Some others, usually the largest, are almost completely filled with an electron-dense material. In some cases, a dense core floats inside an electron-lucent vesicle. Lysosomes are absent from, or at least very rare in, the PVCs.

For comparison, Fig. 9C shows a representative ultrathin section of a pavement cell of *Oreochromis niloticus*, a fully ammoniotelic fish closely related to the urea-secreting species. Golgi stacks are present but do not display great activity. A few vesicles are scattered within the cell, but they never exhibit an electron-dense filling.

Discussion

In vivo *physiology*

The present physiological studies on the Lake Magadi tilapia reveal several new facets of urea excretion mechanisms. First, urea is excreted continuously in this species (Fig. 1), rather than in discrete pulses as has been observed for the gulf toadfish (Wood et al., 1995). It is likely that the extremely high rates of nitrogen metabolism and urea-N production in the Magadi tilapia necessitate continuous nitrogen excretion, and this necessity is reflected in the exceptionally high urea permeability values in the gills compared with those of other fishes, including ureoteles (Fig. 2). The observation of a urea permeability more than 10 times greater than traditional 'passive' values (Fig. 2) by itself suggests that a transport system for urea must be present in the gills, a conclusion supported by the other data presented here.

When external urea concentrations were experimentally elevated above plasma levels, unidirectional influx measurements revealed a bidirectional transport system, with rates proportional to the relative concentrations on the two sides of the gill epithelium. This result is identical to that of a similar experiment performed on the 'pulsing' gulf toadfish (Wood et al., 1998), in which a facilitated diffusion

mechanism is present in the gills. Both the inhibitory effects of urea analogs on urea transport (Fig. 5) and the correlation between the permeability for thiourea and urea (Fig. 3) further suggest a specific urea-transport mechanism. Indeed, the greater inhibitory potency of thiourea than acetamide is identical to the response of toadfish gill urea transport (McDonald et al., 2000). In the rat kidney inner medullary collecting duct, from which a facilitated diffusion urea transporter has been cloned and fully characterized (Smith et al., 1995), all three of the analogs tested here (Fig. 5) inhibited urea transport with the same potency order (thiourea>N-methylurea>acetamide; Chou and Knepper, 1989). The low thiourea-to-urea permeability (0.18; Fig. 4) continuously present in the gills of the Magadi tilapia was identical to the ratio seen in the gulf toadfish when its facilitated diffusion urea transporter is activated during pulsatile excretion events (Wood et al., 1998; McDonald et al., 2000) and very different from the value of close to 1.0 seen when the transporter is inactive. A value of 1.0 (or higher) is expected when only passive diffusion is occurring in phospholipid and cholesterol membranes (Lippe, 1969). In summary, all these pieces of physiological evidence point to the presence of a continuously active facilitated diffusion transport mechanism for urea in the gills of the Magadi tilapia similar to that present in the rat kidney and the toadfish gill.

Molecular and oocyte expression studies

The molecular data presented here provide further confirmation of this conclusion. A transporter was cloned that, when expressed in *Xenopus laevis* oocytes, facilitated urea transport and showed the characteristic phloretin inhibition of urea transporters (Fig. 7A). Several signature sequences of characteristic domains of urea transporters (Karakashian et al., 1999) are highly conserved in the Lake Magadi urea transporter (mtUT; Fig. 6), e.g. the LP box (amino acid residues 179–181), the TYPE domain (amino acid residues 374–377), which is a potential casein kinase II phosphorylation site, the P/YWW domain (amino acid residues 96–98), which is part of a leucine zipper motif (amino acid residues 93–114), and the NIT potential *N*-glycosylation site (amino acid residues 217–220). Notably, the ALE domain, which is diagnostic of UT-B proteins (amino acid residues 215–217 in rat UT-B), is absent from mtUT and all the other urea transporters compared, strongly suggesting that mtUT is a UT-A. The mtUT protein also exhibits a primary structure consistent with several hydrophobic regions in a pattern similar to other urea transporters. mtUT is very similar to tUT, with both putative proteins consisting of 475 amino acid residues; however, the above signature regions are displaced by two amino acids in mtUT, since it has two fewer amino acid residues in the N terminus and two extra amino acid residues in the C terminus compared with tUT. A final notable difference between the teleostean mtUT/tUT and other urea transporters is a much longer C-terminal amino acid sequence. However, there are differences between mtUT and tUT in this C-terminal region. In particular, mtUT lacks the potential amidation site seen in tUT (EGKK), but has four potential casein kinase II phosphorylation sites (TENE, TEVE, TEND and TLTD) compared with two such potential phosphorylation sites in tUT (TEDE and TRQD) (Fig. 6).

There is another difference in the C-terminal amino acid sequence that is potentially important; mtUT has potential protein kinase C (PKC) phosphorylation sites at amino acid residues 362–364 (TKR) and 392–394 (TEK), whereas only one site is noted in tUT (360–362; TQK) (Fig. 6). Notably, these sites are absent from other non-teleost vertebrate forms of urea transporter reported to date. We have speculated previously that this additional C-terminal sequence may be related to the regulation of urea transport in the toadfish gill (Walsh et al., 2000). Given the differences between the apparent modes of excretion in the Lake Magadi tilapia (continuous) and the toadfish (pulsatile), it will be interesting to determine whether these differences are related to sequence differences in the gill urea transporters or whether, alternatively, regulation of transport is at some other level of control (e.g. transcription, translation or insertion of the transporters into the gill cell membranes). In this regard, the oocyte expression studies offer some preliminary information. First, the effect of PMA was to downregulate urea transport by 60 % (Fig. 7B). Whether this deactivation occurred by the action of PKC on a PKC phosphorylation site on mtUT is the subject of further studies because an alternative mechanism in our studies may be *via* PKC effects which increase membrane recycling and possibly reduce the number of mtUT molecules in the membrane. There was no significant effect of forskolin on urea transport (Fig. 7B), and this result is not surprising given the lack of typical PKA sites (RRXS) in the mtUT sequence.

The lack of detectable UT-A2 mRNA in liver and muscle (Fig. 8) demands attention, given that these are the proposed sites of urea production (Lindley et al., 1999). However, Walsh et al. (Walsh et al., 2000) noted a similar expression pattern in the toadfish and speculated that other pathways, e.g. a piscine analog of Aquaporin 9 (Ishibashi et al., 1998; Tsukaguchi, 1998), may allow urea exit from tissues that are the sites of urea production.

Gill ultrastucture

Comparative gill morphological evidence points to the involvement of the Golgi apparatus and vesicular trafficking in the urea excretion process. Fig. 9A,B demonstrates that PVCs are the site of vesicular trafficking between the Golgi cisternae and the apical membrane in *Alcolapia grahami*. As for the ureotelic gulf toadfish (Walsh et al., 2000; Laurent et al., 2000), we speculate that this rich Golgi apparatus and the intense vesicular trafficking are the structural manifestations of the facilitated diffusion urea-transport process, representing either insertion of the transporter into the apical membrane and/or the packaging of urea into vesicles for transport. Studies to examine urea transporter localization by immunocytochemistry with urea transporter antibodies would test these hypotheses and clarify the precise role of vesicular trafficking in urea transport.

Concluding remarks

In summary, we have presented evidence supporting the hypothesis that urea excretion in the Lake Magadi tilapia is a specific facilitated transport mechanism at the gill. We have cloned and functionally expressed mtUT, a candidate mediator of this urea-transport mechanism. Several characteristics of this transport system (e.g. high capacity, continuous operation) appear to differ from other systems (e.g. the toadfish and elasmobranchs), suggesting that additional study will yield clues into the evolution of urea transport mechanisms in the vertebrates.

References

Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D. J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res*. **25**, 3389–3402.

Chou, C.-L. and Knepper, M. A. (1989). Inhibition of urea transport in inner medullary collecting duct by phloretin and urea analogues. *Am. J. Physiol.* **257**, F359–F365.

Coe, M. J. (1966). The biology of *Tilapia grahami* Boulenger in Lake Magadi, Kenya. *Acta Trop.* **23**, 146–177.

Couriaud, C., Ripoche, P. and Rousselet, G. (1996). Cloning and functional characterization of a rat urea transporter: expression in the brain. *Biochim. Biophys. Acta* **1309**, 197–199.

Finkelstein, A. (1976). Water and nonelectrolyte permeability of lipid bilayer membranes. *J. Gen. Physiol.* **68**, 127–135.

Galluci, E., Micelli, S. and Lippe, C. (1971). Non-electrolyte permeation across thin lipid membranes. *Arch. Int. Physiol. Biochim.* **79**, 881–887.

Isaia, J. (1984). Water and non-electrolyte permeation. In *Fish Physiology*, vol. 10B (ed. W. S. Hoar and D. J. Randall), pp. 1–38. New York: Academic Press.

Ishibashi, K. M., Kuwahara, Y., Gu, Y., Tanaka, F. and Sasaki, S. (1998). Cloning and functional expression of a new aquaporin (AQP9) abundantly expressed in the peripheral leukocytes permeable to water and urea, but not to glycerol. *Biochem. Biophys. Res. Commun.* **244**, 268–274.

Karakashian, A., Timmer, R. T., Klein, J. D., Gunn, R. B., Sands, J. M. and Bagnasco, S. M. (1999). Cloning and characterization of two new isoforms of the rat kidney urea transporter: UT-A3 and UT-A4. *J. Am. Soc. Nephrol*. **10**, 230–237.

Laurent, P., Maina, J. N., Bergman, H. L., Narahara, N., Walsh, P. J. and Wood, C. M. (1995). Gill structure of a fish from an alkaline lake: effect of short-term exposure to neutral conditions. *Can. J. Zool.* **73**, 1170–1181.

Laurent, P. and Perry, S. F. (1995). Morphological basis of acid–base and ionic regulation in fish. In *Advances in Comparative and Environmental Physiology. Mechanisms of Systemic Regulation in Lower Vertebrates II*, vol. 22 (ed. N. Heisler), pp. 91–118. Berlin, Heidelberg: Springer-Verlag.

Laurent, P., Wood, C. M., Wang, Y., Perry, S. F., Gilmour, K. M., Pärt, P. and Walsh, P. J. (2000). A new type of intracellular vesicular trafficking in the gill of urea-excreting fish (*Opsanus* sp.) in the family Batrachoididae. *Cell Tissue Res*. (in press).

Lindley, T. E., Scheiderer, C. L., Walsh, P. J., Wood, C. M., Bergman, H. G., Bergman, A. N., Laurent, P., Wilson, P. and Anderson, P. M. (1999). Muscle as a primary site of urea cycle enzyme activity in an alkaline lake-adapted tilapia, *Oreochromis alcalicus grahami. J. Biol. Chem.* **274**, 29858–29861.

Lippe, C. (1969). Urea and thiourea permeabilities of phospholipid and cholesterol bilayer membranes. *J. Mol. Biol.* **39**, 669–672.

Maina, J. N., Kisia, S. M., Wood, C. M., Narahara, A., Bergman, H. L., Laurent, P. and Walsh, P. J. (1996). A comparative allometric study of the morphometrics of the gills of a hyperosmotic adapted cichlid fish, *Oreochromis alcalicus grahami* of Lake Magadi, Kenya. *Int. J. Salt Lake Res.* **5**, 131–156.

McDonald, M. D., Wood, C. M., Wang, Y. X. and Walsh, P. J. (2000). Differential branchial and renal handling of urea, acetamide and thiourea in the gulf toadfish *Opsanus beta*: evidence for two transporters. *J. Exp. Biol.* **203**, 1027–1037.

Narahara, A. B., Bergman, H. L., Laurent, P., Maina, J. N., Walsh, P. J. and Wood, C. M. (1996). Respiratory physiology of the Lake Magadi Tilapia, (*Oreochromis alcalicus grahami*), a fish adapted to a hot, alkaline and frequently hypoxic environment. *Physiol. Zool.* **69**, 1114–1136.

Olives, B., Neau, P., Bailly, P., Hediger, M. A., Rousselet, G., Cartron, J. P. and Ripoche, P. (1994). Cloning and functional expression of a urea transporter from human bone marrow cells. *J. Biol. Chem.* **269**, 31649–31652.

Pärt, P., Wright, P.A. and Wood, C. M. (1998). Urea and water permeability of dogfish (*Squalus acanthias*) gills. *Comp. Biochem. Physiol.* **119**, 117–123.

Price, N. M. and Harrison, P. J. (1987). Comparison of methods for the analysis of urea in seawater. *Mar. Biol.* **94**, 307–313.

Rahmatullah, M. and Boyde, T. R. (1980). Improvements in the determination of urea using diacetyl monoxime; methods with and without deproteinization. *Clin. Chim. Acta* **107**, 3–9.

Randall, D. J., Wood, C. M., Perry, S. F., Bergman, H., Maloiy, G. M. O., Mommsen, T. P. and Wright, P. A. (1989). Urea excretion as a strategy for survival in a fish living in a very alkaline environment. *Nature* **337**, 165–166.

Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual*, 2nd edition. New York: Cold Spring Harbor Laboratory Press.

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

Sanger, F., Nicklen, S. and Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74**, 5463–5467.

Seegers, L. and Tichy, H. (1999). The *Oreochromis alcalicus* flock (Teleostei: Cichlidae) from lakes Natron and Magadi, Tanzania and Kenya, with descriptions of two new species. *Ichthyol. Expl. Freshwaters* **10**, 97–146.

Smith, C. P., Lee, W.-S., Martial, S., Knepper, M. A., You, G., Sands, J. M. and Hediger, M. (1995). Cloning and regulation of expression of the rat kidney urea transporter (rUT2). *J. Clin. Invest.* **96**, 1556–1563.

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

Tsukaguchi, H. C., Shayakul, U. V., Berger, B., Mackenzie, S., Devidas, W. B., Guggino, A. N. and Hediger, M. A. (1998). Molecular characterization of a broad selectivity neutral solute channel. *J. Biol. Chem.* **273**, 24737–24743.

Verbavatz, J. M., Couriaud, C., Berthonaud, V., Gobin, P., Bailly, C., Ibarra, C., Silberstein, M., Simon, M., Hediger, M., Ripoche, P., Rousselet, G. and de Rouffignac, C. (1996). Cloning, functional characterization and localization of an Amphibian urea transporter (Abstract). *J. Am. Soc. Nephrol.* **7**, 1274.

Walsh, P. J., Bergman, H. L., Narahara, A., Wood, C. M., Wright, P. A., Randall, D. J., Maina, J. N. and Laurent, P. (1993). Effects of ammonia on survival, swimming and activities of enzymes of nitrogen metabolism in the Lake Magadi tilapia *Oreochromis alcalicus grahami*. *J. Exp. Biol.* **180**, 323–327.

Walsh, P. J., Heitz, M., Campbell, C. E., Cooper, G. J., Medina, M., Wang, Y. S., Goss, G. G., Vincek, V., Wood, C. M. and Smith, C. P. (2000). Molecular identification of a urea transporter in gill of the ureotelic gulf toadfish (*Opsanus beta*). *J. Exp. Biol.* **203**, 2357–2364.

Walsh, P. J. and Smith, C. P. (2000). Urea transport. In *Fish Physiology*, vol. 18, chapter 8, *Nitrogen Excretion* (ed. P. M. Anderson and P. A. Wright). New York: Academic Press (in press).

Wood, C. M., Bergman, H. L., Laurent, P., Maina, J. N., Narahara, A. and Walsh, P. J. (1994). Urea production, acid–base regulation and their interactions in the Lake Magadi Tilapia, a unique teleost adapted to a highly alkaline environment. *J. Exp. Biol.* **189**, 13–36.

Wood, C. M., Gilmour, K. M., Perry, S. F., Pärt, P., Laurent, P. and Walsh, P. J. (1998). Pulsatile urea excretion in gulf toadfish (*Opsanus beta*): evidence for activation of a specific facilitated diffusion transport system. *J. Exp. Biol.* **201**, 805–817.

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

Wood, C. M., Perry, S. F., Wright, P. A., Bergman, H. L. and Randall, D. J. (1989). Ammonia and urea dynamics in the Lake Magadi tilapia, a ureotelic teleost fish adapted to an extremely alkaline environment. *Respir. Physiol.* **77**, 1–20.

Wright, P. A., Perry, S. F., Randall, D. J., Wood, C. M. and Bergman, H. (1990). The effects of reducing water pH and total CO_2 on a teleost fish adapted to an extremely alkaline environment. *J. Exp. Biol.* **151**, 361–369.

You, G., Smith, C. P., Kanai, Y., Lee, W.-S., Steizner, M. and Hediger, M. A. (1993). Cloning and characterization of the vasopressin-regulated urea transporter. *Nature* **365**, 844–847.