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UREA PRODUCTION, ACID–BASE REGULATION AND THEIR INTERACTIONS IN THE LAKE MAGADI TILAPIA, A UNIQUE TELEOST ADAPTED TO A HIGHLY ALKALINE ENVIRONMENT

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Summary

The Lake Magadi tilapia, *Oreochromis alcalicus grahami*, thrives in highly alkaline geothermal springs and pools surrounding Lake Magadi, Kenya (control pH=9.9, C_{CO_2}=173 mmol l^{-1}), has a functional hepatic ornithine–urea cycle (OUC) and excretes all nitrogenous waste as urea-N at variable rates (J_{Urea}) related to O_2 consumption ($\dot{M}_{O_2}$). The mean value of $J_{Urea}/\dot{M}_{O_2}$ (N/O_2=0.183) was high for fish but below the theoretical maximum (approximately 0.27) for 100 % aerobic respiration of protein, so an exogenous source of substrates is not required to explain the observed J_{Urea}. J_{Urea} was insensitive to thiourea. Urea excretion occurred largely (80 %) through the gills, but urea-N was also present in bile and urine. Control blood pHe, pHi and [HCO_3^-] (approximately 8.1, 7.6 and 15 mmol l^{-1}, respectively, at approximately 32 °C) were extremely high. When fish were exposed to lake water titrated with HCl and aerated to remove CO_2, N/O_2 progressively declined. At a lake water pH of 7.05 and C_{CO_2} of 0 mmol l^{-1}, N/O_2 was reduced by 80 % and an intense metabolic acidosis occurred (pHe=7.04, [HCO_3^-]=1.5 mmol l^{-1}). Restoration of control water pH 9.9 at a C_{CO_2} of 0 mmol l^{-1} resulted in intermediate levels of N/O_2 and internal acid–base status. Additional experiments confirmed that urea production was inhibited by low pHe, was dependent on blood [HCO_3^-] with a K_m of 3.06 mmol l^{-1} and was insensitive to acetazolamide. While metabolic acidosis clearly inhibited OUC ureagenesis, the system appeared to be saturated with HCO_3^- under control conditions so that additional basic equivalent loading would not stimulate ureagenesis. Urea production in the Lake Magadi tilapia does not appear to remove exogenous HCO_3^- or to play a role in normal acid–base regulation.

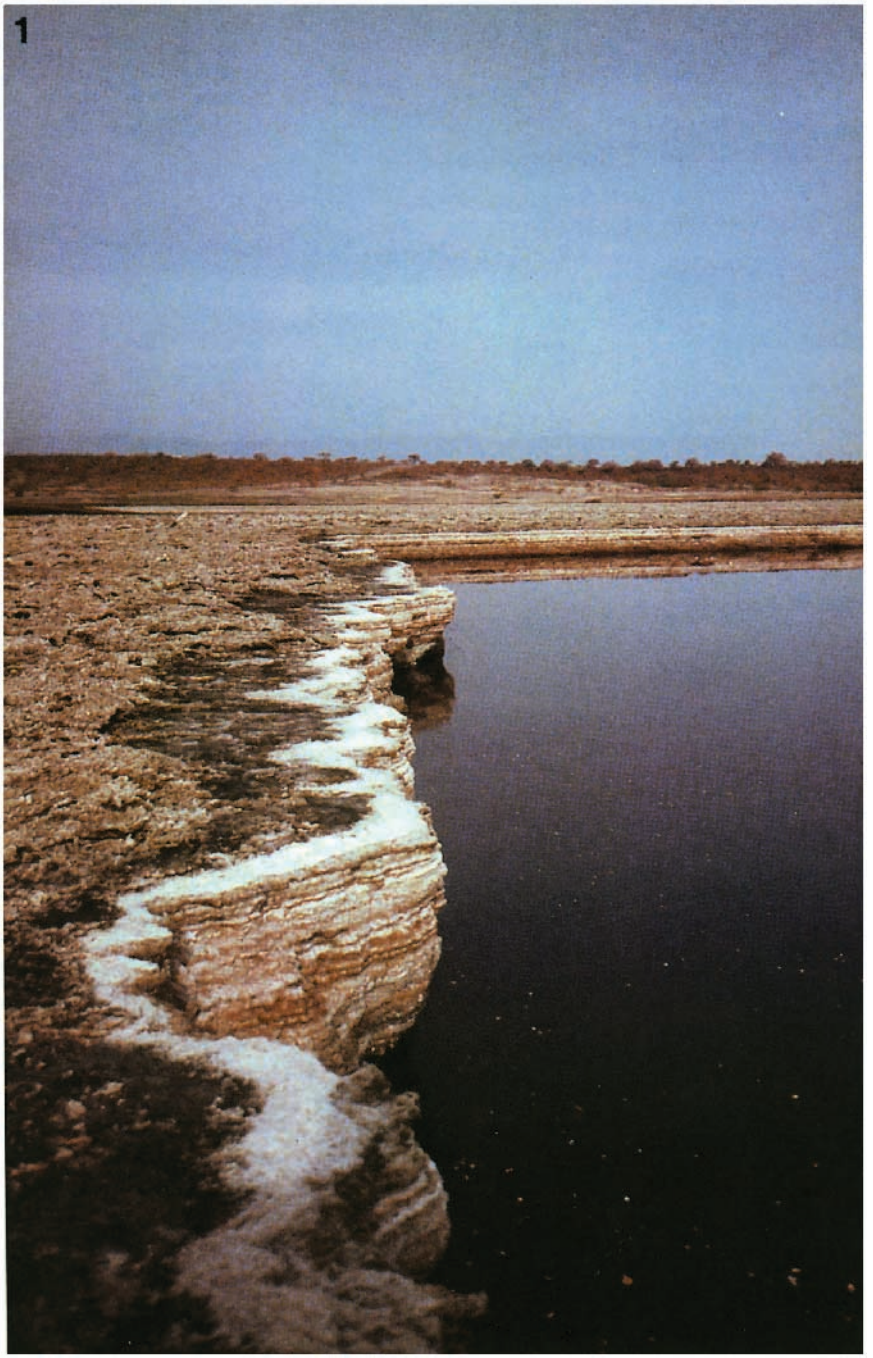
Key words: Lake Magadi, urea, acid–base balance, high pH, liver, ornithine–urea cycle, ammonia, glutamine, *Oreochromis alcalicus grahami.*

Introduction

The tilapia *Oreochromis alcalicus grahami* lives in the highly alkaline Lake Magadi in the southern Rift Valley of Kenya (Coe, 1966). Most of the lake's surface is covered with a solid crust of crystalline 'trona' which is up to 5 m in depth (Fig. 1). The trona is composed largely of Na_2CO_3 and $NaHCO_3$; these salts enter the lake in an alkaline liquor flowing from volcanic hot springs around the margin of the lake and form a floating precipitate as the water evaporates and cools. The only natural areas of open water are small lagoons close to these geothermal inlets, which are kept open by the constant flow from the springs. It is here that the Lake Magadi tilapia thrives under some of the most hostile conditions ever described for fish life (pH=10, C_{CO_2}=180 mmol l^{-1}, osmolality=525 mosmol kg^{-1}, temperatures up to 42 °C; Coe, 1966). Such conditions would undoubtedly be fatal to most other teleost fishes. One important adaptation to the extreme alkalinity is that the species excretes all nitrogenous waste as urea-N rather than as ammonia-N; this excretion occurs at an exceptionally high and variable rate (Randall *et al.* 1989; Wood *et al.* 1989). The Lake Magadi tilapia possesses a complete complement of ornithine–urea cycle (OUC) enzymes in the liver and appears to produce urea mainly by the OUC (Randall *et al.* 1989; Walsh *et al.* 1993). However, the sites, mechanisms and all of the possible functions of urea excretion remain unknown. Complete reliance on ureotelism in the normal environment makes this fish species unique; most teleosts are ammoniotelic and have negligible hepatic OUC activity. Indeed, only a handful are capable of OUC-based ureagenesis, and these are generally ammoniotelic under normal conditions (e.g. the toadfish, Read, 1971; Mommsen and Walsh, 1989, and certain air-breathing fish of India, Saha and Ratha, 1987, 1989). We have argued that *O. a. grahami* excretes urea-N because it would be difficult or impossible to excrete ammonia across the gills into highly buffered water where the pH (10) is so much greater than the pK (9.1) of the NH_3/NH_4^+ reaction (Wood *et al.* 1989; Wood, 1993).

Besides nitrogen derived from amino acid metabolism, the other substrate for urea synthesis is HCO_3^-, which raises the possibility that urea production also plays a role in acid–base regulation. Blood pH and HCO_3^- levels are unusually high in *O. a. grahami* compared with those of other teleosts, presumably as a result of the highly alkaline environment (Johansen *et al.* 1975; Wood *et al.* 1989). In mammalian physiology, the possible contribution of OUC-based ureagenesis to systemic acid–base regulation and the influence of acid–base status on ureagenesis have been subjects of controversy over the past decade (e.g. Atkinson and Camien, 1982; Halperin *et al.* 1986; Walser, 1986; Knepper *et al.* 1987; Atkinson and Bourke, 1987; Marsh and Knepper, 1992). In brief, the controversy revolves around the assertion of Atkinson and colleagues that urea synthesis is central to acid–base balance because it removes endogenously produced HCO_3^- that

Fig. 1. A photograph of the 'trona', a solid crust up to 5 m thick which covers about 90 % of the surface area of Lake Magadi, Kenya. The trona consists of a floating precipitate of Na_2CO_3 and $NaHCO_3$. In this particular location, the crust has been cut open and removed by a mining operation, but the only natural areas of open water are lagoons at the lake margins kept open by flow from geothermal springs. The Magadi tilapia *Oreochromis alcalicus grahami* lives in the lagoons.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would otherwise swamp the body fluids with an overwhelming alkalosis. Very recently, the unusual reliance of the Lake Magadi tilapia on OUC-based ureagenesis has been cited as support for the theory, inasmuch as the animal is presumably threatened by being swamped with exogenous HCO_3^- from the highly alkaline environment as well as by endogenously produced HCO_3^- (Meijer *et al.* 1990; Atkinson, 1992). Mammalian studies indicating that hepatic ureagenesis is inhibited by acidosis and stimulated by alkalosis, especially of the 'metabolic' varieties, are cited as additional evidence that ureagenesis is dynamically manipulated so as to achieve acid–base homeostasis (reviewed by Haussinger, 1990). In this regard, it is interesting that urea excretion in *O. a. grahami* was severely reduced by exhaustive exercise and completely blocked by exposure to water of pH 7.0 (Wood *et al.* 1989). These observations raise the possibility that the occurrence of OUC-based ureagenesis in the Lake Magadi tilapia relates as much to acid–base regulation as it does to waste nitrogen excretion.

The objectives of the present study were therefore several-fold. The first was to establish whether the gills were the major site of urea excretion (J_{Urea}), using a simple divided chamber system for these very small fish. In the light of emerging evidence in higher vertebrates that urea movement across many epithelia is carrier-mediated, we also tested whether J_{Urea} was altered by thiourea, a common competitive blocker of urea transporters (Marsh and Knepper, 1992). Putative urea-excreting cells have recently been described in the gills of *O. a. grahami* (Maina, 1991). The second objective was to confirm the very high rates of urea-N production that we reported earlier (Randall *et al.* 1989; Wood *et al.* 1989) and to understand the great variability in urea excretion amongst individuals and groups. In particular, we reasoned that metabolic rate might be an important variable, given the high temperatures and voracious feeding habits of the species. We further reasoned that if urea production were driven by exogenous HCO_3^- entering from the external environment as well as by endogenous HCO_3^- production from protein metabolism, then the ratio $J_{Urea}/\dot{M}_{O_2}$ (N/O_2) ratio might be unusually high and perhaps even exceed the theoretical aerobic maximum (approximately 0.27), where 100 % of the $\dot{M}_{O_2}$ is devoted to the oxidation of protein (van Waarde, 1983). The final, and most extensive, objective was to explore possible relationships amongst J_{Urea}, the acid–base status of the environment and the systemic acid–base status of the fish. To do this, we titrated Lake Magadi water with HCl to alter the total carbonate alkalinity ($HCO_3^-+CO_3^{2-}$) and the pH levels of the environment. The acid–base status of the fish was also manipulated independently of the environment by injection with $NaHCO_3$ and acetazolamide. The activities of several key enzymes of nitrogen metabolism in liver and brain were measured in two treatments so as to clarify the relationship between acid–base balance and ureagenesis. All experiments were performed outdoors, on fish freshly caught from the wild, with only locally available chemicals, so experimental methods were at times less than ideal.

Materials and methods

Experimental animals

Adult *Oreochromis alcalicus grahami* were collected by seine between 19 January and

16 February 1992 either from Fish Springs Lagoon at the edge of Lake Magadi, Kenya (see Coe, 1966), or from a large outdoor tank continually filled with water from the Lagoon. This tank serves as a reservoir for 'process water' for the operations of Magadi Soda PLC and contains a population of *O. a. grahami* derived and continually replenished from the Fish Springs population. Screening protects the tank from avian predators, so the fish grow unusually large (typically 10–30 g), in contrast to the small adult fish (1–10 g) of the Lagoon. These tilapia deteriorate rapidly in captivity (see Wood *et al.* 1989), so fish were collected each morning and experiments were started within 3 h of capture, except for tests where there was a need for pre-exposure. Tilapia were held in groups of 10–20 in aerated 20 l plastic buckets filled with Lagoon water prior to testing. The fish were maintained, and all experiments were performed, in an outdoor laboratory set up on the balcony of the chemistry building of Magadi Soda PLC, where ambient temperature varied from about 30 to 36 °C over the day, similar to the diurnal variation at the collection sites.

Water chemistry

The acid–base titration curve of Lake Magadi Lagoon water under the conditions of our experiments was investigated by titrating a 25 ml sample with approximately 2.27 mol l^{-1} HCl, using a Gilmont digital microburette and a Radiometer GK2401C combination electrode and PHM 84 meter. Total dilution of the sample with titrant was about 10 %. As standardized HCl was not available, the exact amount of acid added was monitored by measurement of $[Cl^-]$. Subsamples (200 μl) were removed for assay of C_{CO_2} and $[Cl^-]$ after each of the 15 addition steps in the titration. The sample was vigorously aerated throughout titration to drive off free CO_2 and, on the basis of preliminary trials, a minimum of 100 min was allowed between additions to achieve stable pH and C_{CO_2} levels. The entire titration took approximately 36 h.

The resulting titration curves (Fig. 2) were used as a guideline for manipulating water acid–base chemistry in physiological experiments. The desired pH and C_{CO_2} levels were attained by adding the appropriate amount of concentrated HCl to about 20 l of fresh Lake Magadi Lagoon water and then aerating it vigorously for 24 h. To achieve essentially CO_2-free water at pH 7.0 or 9.9, the water was titrated to slightly below 7.0 and then raised back to the desired pH by titration with 1 mol l^{-1} NaOH. This elevated water $[Na^+]$ by no more than 20 mmol l^{-1}. Water pH and C_{CO_2} levels were monitored routinely at the start and end of experiments.

Standard flux protocol

The standard flux protocol involved the simultaneous measurement of J_{Urea} and $\dot{M}_{O_2}$ on 9–18 small tilapia (1–3 g) subjected to a variety of different treatments. At least six fish were exposed to each treatment. Flux experiments were conducted in 530 ml amber chambers (Tusker Beer bottles) filled with the appropriate water and each sealed with a rubber bung fitted with an aeration line and sampling port. A single fish was added to each Tusker chamber 1 h prior to the start of flux measurements. Drugs and saline vehicle were administered by intraperitoneal injections (10 μl g^{-1}) immediately prior to placing the fish in the chambers. Water samples were drawn for measurement of urea at 1 h and 4 h.

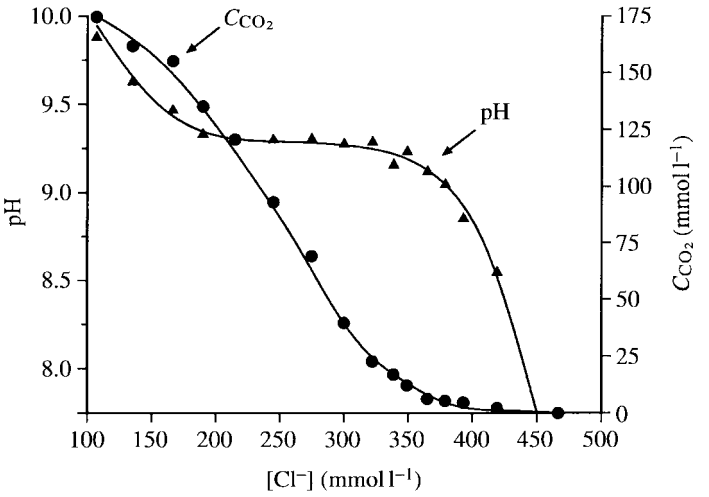

Fig. 2. Titration curves of Lake Magadi Lagoon water with HCl showing the change in water pH and water C_{CO_2} (i.e. CO_3^{2-}+HCO_3^-) with the increase in water [Cl^-]. Note the flat region in the pH curve at pH 9.1–9.4 despite the steady decline in C_{CO_2} over this same range.

Between 2 h and 3 h, the chambers were sealed and aeration was stopped for 30–60 min for measurement of $\dot{M}_{O_2}$. A chamber containing water but no fish was included in each series to serve as a blank. Water pH was monitored and adjusted as necessary with small amounts of 1 mol l^{-1} HCl and 1 mol l^{-1} NaOH. At the end of the experiment, fish were weighed and returned to the wild, and a pooled water sample from each treatment was analysed for C_{CO_2}.

Standard blood sampling protocol

Larger tilapia (10–30 g) were exposed to a subset of the treatments from the above flux protocol and then sampled for blood. Exposures were conducted in small groups (minimum of five fish per treatment) in aerated 20 l plastic buckets, and the sampling time was designed approximately to coincide with the midpoint of the exposure period in the flux protocol (i.e. 2–3 h). At sampling, the fish were gently transferred to a 300 ml beaker of treatment water containing 200 μl of Transmore, a locally purchased aquarist's anaesthetic containing α-methyl quinoline (concentration unknown) as the active ingredient. Loss of equilibrium occurred within 2 min, and then a blood sample (20–70 μl) for acid–base analysis was drawn anaerobically into a gas-tight syringe (Hamilton) from the haemal arch. The syringe was pre-rinsed with 1000 i.u. ml^{-1} sodium heparin (Richter) in 180 mmol l^{-1} NaCl. Most fish subsequently recovered from the anaesthetic and were returned to the wild. However, in a few cases, larger samples (200 μl) were drawn and pooled for more extensive analyses (whole-blood and plasma C_{CO_2}, red blood cell intracellular pH, plasma urea, ammonia, glutamine, glutamate), and the gall bladder and urinary bladder from the same fish were punctured to yield bile and urine samples. Plasma, bile and urine samples were frozen at −20 °C, shipped to Hamilton, Canada, in a block of ice and stored at −80 °C for 10 months prior to assay.

Tissue sampling for enzymes

At the end of two treatments, brains and liver were dissected from small fish, placed in cryotubes and quick-frozen in liquid N_2. These samples were shipped to Miami, USA, in a dry shipper (Minnesota Valley Engineering) and stored at −80 °C for less than 2 months prior to assay.

Experimental treatments

The standard flux protocol experiments with small fish were performed in each of the following treatments. Tissue samples were taken after treatments vii and viii. The standard blood sampling protocols with larger fish were performed in treatments i, v, vi, ix, x, xi and xii. Mean measured water pH and C_{CO_2} are given. (i) Control (pH 9.94, C_{CO_2} 172.6 mmol l^{-1}); (ii) pH 9.12, C_{CO_2} 140.7 mmol l^{-1}; (iii) pH 9.16, C_{CO_2} 50.8 mmol l^{-1}; (iv) pH 8.76, C_{CO_2} 12.1 mmol l^{-1}; (v) pH 7.05, C_{CO_2} 0.4 mmol l^{-1}; (vi) pH 9.91, C_{CO_2} 0.6 mmol l^{-1}. Treatments ii, iii and iv were designed to test the effects of large changes in C_{CO_2} with only small changes in pH. In addition, the following longer-term experiments were performed: (vii) 24 h control; fish were tested in control water after 24 h of holding in the laboratory in control water; (viii) 24 h pH 7.0 exposure; fish were tested in pH 7.0 water (see v) after 24 h of exposure to this water; (ix) long-term holding; fish were held in the laboratory in control water for at least 4 days and then tested in control water. The data were obtained from the approximately 20 % of the initial group which survived this extended holding period. Our original intention was to compare a starved group and a group fed with Tetramin tropical fish food flakes. However, since both groups were observed to feed on blue-green algae (cyanobacteria), which grew in the holding tanks, and on dying fish (in preference to Tetramin) and yielded almost identical data, they were combined.

The following injection experiments (10 μl g^{-1}, intraperitoneal) were performed in an attempt to manipulate internal acid–base status without altering external water chemistry. All tests were run in control water: (x) saline control: injected with 180 mmol l^{-1} NaCl; (xi) 10 mmol kg^{-1} $NaHCO_3$: injected with 1000 mmol l^{-1} $NaHCO_3$; (xii) 1 mmol kg^{-1} acetazolamide: injected with 100 mmol l^{-1} acetazolamide in 180 mmol l^{-1} NaCl (the acetazolamide was obtained as generic tablets of known content from a local pharmacy; tablets were homogenized in saline and filtered prior to injection); (xiii) 3 mmol kg^{-1} thiourea (BDH): injected with 300 mmol l^{-1} in 180 mmol l^{-1} NaCl.

Divided chamber experiments

A simple divided chamber was constructed to determine the site(s) of urea excretion in *O. a. grahami*. Very small adult tilapia, typically about 1 g, were selected. Each fish was first tranquilized in a solution of 10 μl of Transmore in 100 ml of control water for 5 min; this prevented struggling but allowed ventilation to continue. The head was then quickly inserted through a pinhole in a rubber membrane so as to separate the gills and pectoral fins from the remainder of the body. The membrane was sealed into the chamber in such a way that the gills were exposed to 25–50 ml of water containing anaesthetic in the anterior compartment (an Erlenmeyer flask) and the body (including vent and urogenital papilla) was exposed to 10–15 ml of water containing anaesthetic in the posterior

compartment (a centrifuge tube). Fluxes lasted 10–30 min; data were used only from fish that continued to ventilate throughout the flux period and where the membrane seal remained patent. Comparable control experiments were conducted with free-swimming fish in small beakers containing control water alone, or control water with the same concentration of anaesthetic, to evaluate possible disturbing effects of the experimental procedures.

Analytical techniques

The levels of urea, O_2, C_{CO_2}, pH and Cl^- present in the water, and blood and bile acid–base status, were determined on site. The urea in the water was first converted to ammonia using jack-bean urease (BDH); ammonia was then measured by the salicylate–hypochlorite method of Verdouw *et al.* (1978). As different water qualities altered both the colour reaction of the ammonia assay and the enzymatic conversion efficiency for urea, standard curves were constructed for each medium tested. All urea data have been expressed as urea-N. J_{Urea} was calculated from increases in urea-N in the environment factored by mass and time. Water P_{O_2} was determined using a Radiometer O_2 electrode at the experimental temperature. P_{O_2} values were converted to O_2 concentrations using αO_2 values appropriate to the temperature and salinity from Boutilier *et al.* (1984). $\dot{M}_{O_2}$ was calculated from the decrease (blank-corrected) in O_2 during the period of chamber closure, factored by mass and time. Water, blood, plasma and bile C_{CO_2} were measured by the method of Cameron (1971) using a Radiometer CO_2 electrode in the reaction chamber. The outputs from the O_2 and CO_2 electrodes, together with the blood pH assembly (below), were displayed on a single Radiometer pHM 71 meter. Water [Cl^-] was measured with a Radiometer CMT10 coulometric chloridometer, and water pH with a Radiometer GK2401C electrode and pHM 84 meter.

Blood and bile pH values were determined using a Radiometer E5021 capillary electrode assembly at the experimental temperature (31–34 °C for all acid–base measurements). Samples were recovered from the pH capillary and then assayed for C_{CO_2} in the Cameron chamber. P_{CO_2} and HCO_3^- levels were calculated by the Henderson–Hasselbalch equation, using values of pK′ and αCO_2 appropriate to the temperature and ionic strength of the samples (Boutilier *et al.* 1984). Red blood cell intracellular pH (pHi) was measured by pooling blood samples from 3–4 fish in a 500 μl centrifuge tube, centrifuging at 13 000 ***g*** for 2 min, decanting the plasma by aspiration (for metabolite and C_{CO_2} analyses), freezing the plasma and pellet in liquid N_2, and then measuring pHi by the freeze–thaw method of Zeidler and Kim (1977). Haematocrit was determined gravimetrically in these tests. Nernst equilibrium potentials between the blood and the external environment for OH^-, HCO_3^-, CO_3^{2-} and Cl^- were calculated as outlined by Wilkie *et al.* (1993).

Urea-N, total ammonia (T_{Amm}) and glutamine/glutamate levels in plasma, bile and urine samples were determined in Hamilton using the diacetyl monoxime method of Crocker (1967), the glutamate dehydrogenase method of Mondzac *et al.* (1965) and the glutaminase/glutamate dehydrogenase method of Lund (1986), respectively, all using Sigma kits. Activities (at 30±0.2 °C) of the following enzymes in brain and liver samples were determined in Miami using methods identical to those of Walsh *et al.* (1993): malate

Table 1. *The partitioning of urea-N excretion (J_{Urea}) between anterior and posterior compartments in divided chamber experiments with lightly anaesthetized* Oreochromis alcalicus grahami

Treatment	J_{Urea} (μmol N kg^{-1} h^{-1})	Percentage of total
Control	3470±490 (6)	100
Anaesthetic	3254±626 (6)	100
Divided chamber and anaesthetic total	3178±446 (11)	100
Anterior compartment	2479±347 (11)	78.6±2.7
Posterior compartment	678±133 (11)	21.4±2.7

Total J_{Urea} in free-swimming fish in control water, and control water plus anaesthetic, are also given.
Values are mean ± 1 S.E.M. (*N*).
There were no significant differences (P>0.05) amongst the total J_{Urea} values for the three treatments.

dehydrogenase (MDH), citrate synthase (CS), alanine aminotransferase (AlaAT), aspartate aminotransferase (AspAT), glutamate dehydrogenase (GDH), glutamine synthetase (GNS), arginase (ARG) and ornithine–citrulline transcarbamoylase (OCT).

Values are reported as means ±1 S.E.M. (*N*). The significance of differences (P<0.05) was evaluated using Student's two-tailed *t*-test, paired or unpaired design, as appropriate.

Results

Sites of urea excretion

The gills were clearly the major site of urea excretion, as almost 80 % of J_{Urea} appeared in the anterior compartment in the divided chamber experiments (Table 1). Total J_{Urea} of tilapia in the divided chamber was not significantly different from that of free-swimming fish in anaesthetic or control water, indicating that the experimental procedures did not alter urea excretion.

Plasma, bile and urine analyses

Urea concentrations in bile and urine were similar to those in blood plasma (Table 2). Total ammonia (T_{Amm}) was present at about 10 % of urea-N concentration in plasma and bile; there was insufficient sample for urine T_{Amm} analyses. Levels of glutamine were very low in blood plasma, not detectable in urine, but significantly higher in bile. Bile is produced by the liver and glutamine is a proximate nitrogen source for ureagenesis in the liver, specifically for the HCO_3^- fixation reaction catalyzed by carbamoyl phosphate synthetase III (CPS III) in the first step of the OUC (Mommsen and Walsh, 1989).

Table 2. *Nitrogenous metabolites and acid–base status in plasma, bile and urine samples from* Oreochromis alcalicus grahami *in control water*

	Plasma	Bile	Urine
Urea	8.11±0.78	10.61±2.36	19.03
($mmol\,N\,l^{-1}$)	(6)	(6)	11.81
Glutamine	0.109±0.022	2.658±0.831*	ND
($mmol\,l^{-1}$)	(6)	(4)	(2)
Glutamate	0.760±0.074	0.717±0.160	ND
($mmol\,l^{-1}$)	(6)	(4)	(2)
Ammonia	0.86±0.21	1.16±0.22	–
($mmol\,l^{-1}$)	(6)	(6)	
pH	7.995±0.043	7.439±0.050*	–
	(4)	(10)	
[HCO_3^-]	13.63±1.49	5.33±0.74*	–
($mmol\,l^{-1}$)	(4)	(10)	
P_{CO_2}	5.07±0.76	7.72±1.27	–
(mmHg)	(4)	(10)	

Values are mean ± 1 S.E.M. (*N*) where *N* represents the number of different fish for bile and urine samples and the number of different pools for plasma samples. Where *N*=2, both values are given. Each pool represents blood samples from 3–4 fish.

*Significantly different ($P \leqslant 0.05$) from corresponding plasma value. ND, not detectable.

Only urea, glutamine and glutamate were measured in urine.

Glutamate, a direct precursor of glutamine, was present at much higher levels than glutamine in plasma; however, in bile, glutamate levels were lower than glutamine levels, but similar to those of glutamate in plasma. Glutamate was not detectable in urine.

Bile pH was about 0.5 units lower than plasma pH, reflecting a comparable P_{CO_2}, but a 60 % lower HCO_3^- concentration (Table 2). Bile samples from two fish which had been starved for 4 days yielded pH and [HCO_3^-] values similar to those in fed fish. Urine acid–base status was not measured.

Variability of J_{Urea} *and its relationship to* $\dot{M}_{O_2}$

Under control conditions, mean values of J_{Urea} determined on different batches of fish on different days varied from 1909±577 (6) to 3820±618 (8) $\mu mol\text{-}N\,kg^{-1}\,h^{-1}$, with individual values as high as 12000 $\mu mol\text{-}N\,kg^{-1}\,h^{-1}$. These numbers generally agree with the very high, and variable, rates reported earlier by Randall *et al.* (1989) and Wood *et al.* (1989), but the extreme values of 20 000–50 000 $\mu mol\text{-}N\,kg^{-1}\,h^{-1}$ noted for several groups in the earlier studies could not be confirmed. Much of the present variation in J_{Urea} was clearly associated with variations in $\dot{M}_{O_2}$ both within and among groups, at least some of which, in turn, were related to differences in experimental temperature, which could not be controlled in the outdoor laboratory. In separate experiments, we demonstrated that $\dot{M}_{O_2}$ is extremely sensitive to temperature in this species (C. M. Wood,

unpublished results). Differences in activity, feeding status and entrained diel effects may have been additional factors. When J_{Urea} was normalized to $\dot{M}_{O_2}$, variability amongst control groups decreased greatly: N/O_2 ratios ranged from 0.168±0.028 (6) to 0.226±0.060 (6), with an overall mean of 0.183±0.024 for 40 animals in seven different trials. On the basis of these results, J_{Urea} values were normalized to $\dot{M}_{O_2}$ (i.e. N/O_2) for all experimental treatments.

Water chemistry

The chemistry of the Lagoon water varied slightly from day to day (pH=9.85–9.97, C_{CO_2}=169–190 mmol l^{-1}, [Cl^-]=84–109 mmol l^{-1}). The starting conditions for the titration curve of Fig. 2 were pH=9.89, C_{CO_2}=175 mmol l^{-1}, [Cl^-]=107 mmol l^{-1}. This curve served as a practical guide for water acid–base manipulations in physiological experiments, and the pH and C_{CO_2} levels obtained were stable relative to the time scale of the experiments (hours or days). If an infinite equilibration time had been employed, it is possible that a different relationship would have been obtained. It was necessary to raise the Cl^- concentration to about 450 mmol l^{-1} (equivalent to the addition of about 340 mmol l^{-1} H^+) to remove all CO_2 and reach neutrality. Between Cl^- concentrations of 175 and 350 mmol l^{-1} (H^+ addition 65–240 mmol l^{-1}), the water was extremely well buffered, with negligible change in pH despite the almost linear 10-fold drop in C_{CO_2} over this range (150–15 mmol l^{-1}). This well-buffered region occurred in the appropriate pH range (9.1–9.4) for the pK″ of the HCO_3^-/CO_3^{2-} reaction in water of this ionic strength and temperature range (Skirrow, 1975). However, it must be noted that two key determinants of the pK″ could not be held constant during the titration: temperature itself, because of ambient fluctuations, and chlorinity, because of titration with HCl. Nevertheless, this region offered an opportunity to manipulate external C_{CO_2} (i.e. HCO_3^- and CO_3^{2-} levels) over a wide range with minimal effect on external pH in the experimental treatments.

Influence of experimental treatments on urea production

Urea excretion was sensitive to the acid–base status of the environment. Titration of Lake Magadi Lagoon water from pH 9.9 to pH 7.0, and therefore C_{CO_2} from 173 mmol l^{-1} to almost 0 mmol l^{-1}, resulted in a progressive decline in relative J_{Urea}, expressed as N/O_2 (Fig. 3). The first significant decline (about 40 %) was seen at pH 9.1, C_{CO_2}=51 mmol l^{-1}, but a similarly depressed N/O_2 was seen at pH 9.1, C_{CO_2}=141 mmol l^{-1}. There was a further significant decline at pH 8.8, C_{CO_2}=12 mmol l^{-1} and, at pH 7.0, the inhibition reached 80 %. When the water pH was raised back to 9.9 by addition of OH^- (i.e. C_{CO_2} remaining close to 0 mmol l^{-1}), there was a significant stimulation of N/O_2, but only back to the level seen at pH 9.1, C_{CO_2}=141 mmol l^{-1}. This represented 67 % of the control value, but was not significantly different from it. There were no significant differences in $\dot{M}_{O_2}$ values (average about 21 000 μmol kg^{-1} h^{-1}) amongst the treatment groups of Fig. 3, so all changes in N/O_2 reflected real effects on J_{Urea}.

Fig. 4 displays the relationships between water pH and N/O_2, and between water C_{CO_2} and N/O_2, from these experiments. In the absence of knowledge of the exact pK″ in Lake Magadi water during these experiments (which will have a critical effect on the

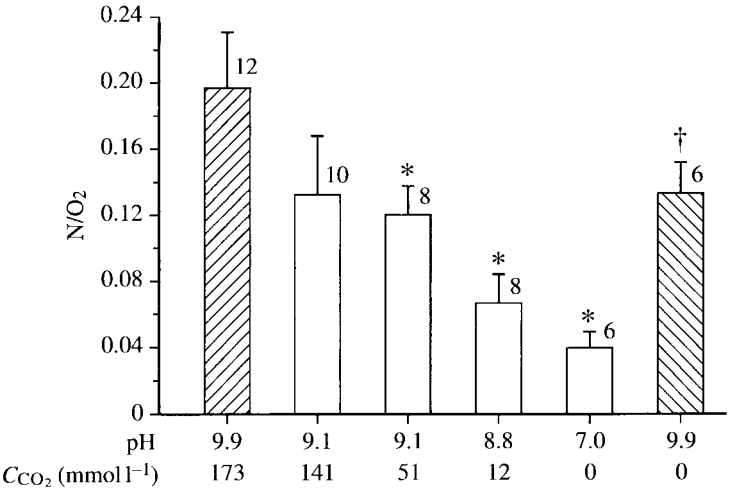

Fig. 3. The influence of the acid–base chemistry of Lake Magadi water on N/O_2, the ratio of urea-N excretion (J_{Urea}) to O_2 consumption ($\dot{M}_{O_2}$) in *Oreochromis alcalicus grahami*. Values are mean +1 S.E.M.; values of *N* are given beside the columns. Asterisks indicate significant differences ($P<0.05$) from the control, normal Lake Magadi Lagoon water (left-hand column, hatched). The right-hand column (reverse hatched) had essentially the same pH as the control, but virtually no C_{CO_2}. The dagger indicates a significant difference from the pH 7.0, $C_{CO_2}=0\,mmol\,l^{-1}$ value.

CO_3^{2-}/HCO_3^- ratio in the mid-range of C_{CO_2} values), we have not separated the CO_3^{2-} and HCO_3^- components of C_{CO_2}. Nevertheless, it is clear that N/O_2 was not simply related to just one *external water* acid–base variable and, furthermore, that the response at pH 9.9, $C_{CO_2}=0\,mmol\,l^{-1}$, did not coincide with either of the relationships. This conclusion should be contrasted with a later analysis of relationships between N/O_2 and *internal* acid–base variables (Fig. 6, see below).

After 24 h of exposure to pH 7.0, $C_{CO_2}=0\,mmol\,l^{-1}$, many of the fish were near death and $\dot{M}_{O_2}$ had dropped to 50 % of the original control value (Table 3). J_{Urea} did not recover and N/O_2 remained significantly depressed. J_{Urea}, $\dot{M}_{O_2}$ and N/O_2 values in 24 h control fish were not significantly different from the original control values. In the 20 % of fish that survived long-term holding (4+ days) in control water, N/O_2 was significantly depressed by about 45 %, though changes in J_{Urea} and $\dot{M}_{O_2}$ were not significant (Table 3).

Intraperitoneal injection with isotonic NaCl tended to lower N/O_2, but the effect was not significant relative to non-injected controls (Fig. 5). $NaHCO_3$ injection, intended to cause metabolic alkalosis, acetazolamide injection, intended to block carbonic anhydrase, and thiourea injection, tested as a putative inhibitor of urea transport, were all without significant effect on N/O_2 relative to either saline-injected fish or non-injected controls (Fig. 5). Indeed, if anything, thiourea treatment tended to raise N/O_2. In none of these treatments was there any significant effect on either J_{Urea} or $\dot{M}_{O_2}$.

Influence of experimental treatments on internal acid–base status

Measured values of pHe were undoubtedly underestimates of true *in vivo* values owing

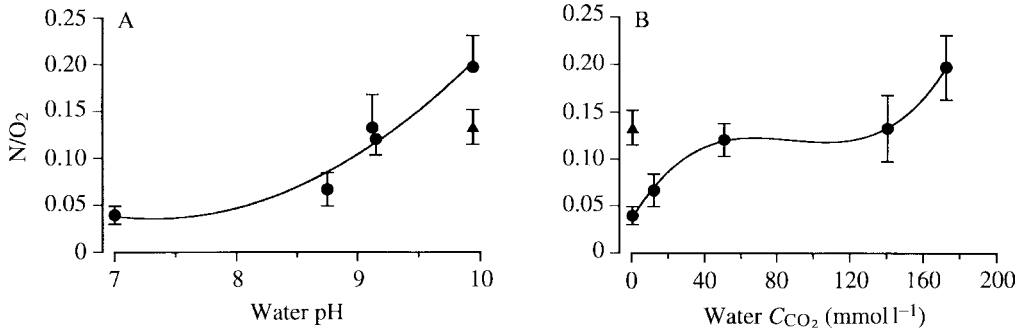

Fig. 4. Relationships between (A) the pH of Lake Magadi water and N/O_2 ratio and (B) the C_{CO_2} of Lake Magadi water and N/O_2 ratio in *Oreochromis alcalicus grahami*. N/O_2 is the ratio of urea-N excretion (J_{Urea}) to O_2 consumption ($\dot{M}_{O_2}$). Note the non-agreement of the points (triangles) obtained by titration of decarbonated water back to pH 9.9 by NaOH with the remainder of the data set. Values are mean ±1 S.E.M. See Fig. 3 for *N* values.

Table 3. *The influence of acute and 24 h of exposure to neutral decarbonated water (pH=7.0, C_{CO_2}=0 mmol l^{-1}), of 24 h of holding in control water, and of long-term holding (4+ days) in control water on urea-N excretion (J_{Urea}), O_2 consumption ($\dot{M}_{O_2}$) and their ratio (N/O_2) in* Oreochromis alcalicus grahami

	J_{Urea} (μmol N kg^{-1} h^{-1})	$\dot{M}_{O_2}$ (μmol kg^{-1} h^{-1})	N/O_2
Control (acute)	2641±314 (12)	16321±2440 (12)	0.197±0.034 (12)
Neutral (acute)	530±141* (6)	13491±1231 (6)	0.039±0.009* (6)
Control (24 h)	4675±1321 (10)	21530±2089 (10)	0.248±0.076 (10)
Neutral (24 h)	1012±340* (9)	8415±2139* (9)	0.124±0.034* (9)
Control (4+ days)	2088±689 (14)	20007±1594 (14)	0.110±0.021* (14)

Values are mean ± 1 S.E.M. (*N*).
*Significantly different ($P \leq 0.05$) from acute control value.

to the elevation in P_{CO_2} accompanying caudal sampling (cf. Wood *et al.* 1989). Nevertheless, measured pHe values (almost 8.1) in control fish were very high for the experimental temperature (approximately 32 °C), reflecting high HCO_3^- levels (Table 4). Acute exposure (2–3 h) to pH 7.0, C_{CO_2}=0 mmol l^{-1}, caused a dramatic metabolic acidosis with a 1.0 unit drop in pHe and almost complete loss of blood HCO_3^-. Acute exposure to decarbonated water at the same pH as the control (pH 9.9, C_{CO_2}=0 mmol l^{-1}) also caused a significant metabolic acidosis, though of lesser extent. Long-term holding

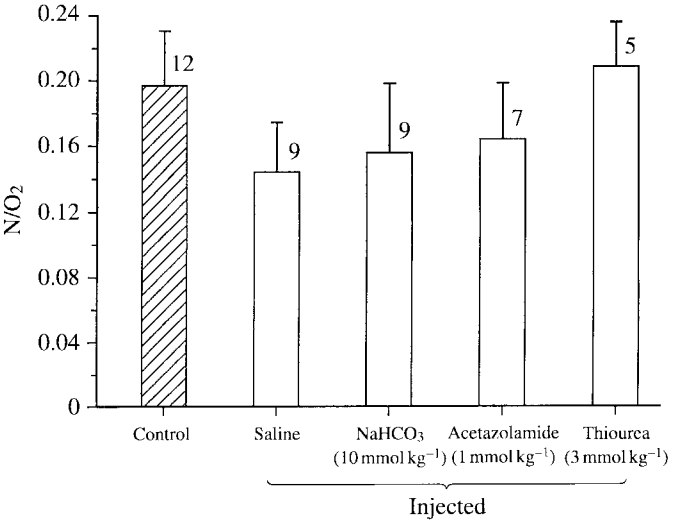

Fig. 5. The influence of intraperitoneal injections (10 μl g^{-1}) of saline and the specified loads of $NaHCO_3$, acetazolamide and thiourea on N/O_2, the ratio of urea-N excretion (J_{Urea}) to O_2 consumption ($\dot{M}_{O_2}$), in *Oreochromis alcalicus grahami*. Values are mean +1 S.E.M.; values of *N* are given beside the columns. There were no significant differences amongst the treatments.

under control conditions (4+ days) resulted in a metabolic acidosis intermediate in magnitude between those in the preceding treatments, but complicated by a significant decline in P_{CO_2} of unknown origin.

Intraperitoneal injection of saline caused a substantial metabolic acidosis, pHe falling by 0.3 units, and blood [HCO_3^-] by almost 50 % (Table 4). Relative to these saline-injected controls, acetazolamide injection caused a significant rise in P_{CO_2}, confirming that the drug was effective in blocking carbonic anhydrase and causing CO_2 retention. As both pHe and HCO_3^- levels were significantly elevated in this treatment, the respiratory acidosis was completely compensated by 'metabolic' mechanisms (i.e. HCO_3^- accumulation). Injection of $NaHCO_3$ also raised pHe and HCO_3^- levels significantly, though the response was complicated by a significant fall in P_{CO_2} of unknown origin.

For the most part, these internal acid–base disturbances associated with different experimental treatments were of 'metabolic' origin; i.e. due to large changes in blood [HCO_3^-] with only small changes in P_{CO_2}. They therefore provided an opportunity to determine whether there was any relationship between the rate of ureagenesis and blood acid–base status over a range of HCO_3^- concentrations (Fig. 6). Ureagenesis was indexed as the N/O_2 values from separate, but identically exposed, groups of the flux experiments, and all data sets were used. N/O_2 was positively related to pHe in a linear fashion (Fig. 6A) and to blood [HCO_3^-] in a hyperbolic fashion (Fig. 6B). The latter was reminiscent of a Michaelis–Menten relationship, so Eadie–Hofstee analysis (Michal, 1983) was applied, yielding K_m=3.06 mmol l^{-1} (as HCO_3^-) and V_{max}=0.204 (as N/O_2). This suggests that the ureagenesis pathway is normally saturated with HCO_3^- under

Table 4. *The influence of experimental treatments on blood acid–base status in* Oreochromis alcalicus grahami

	pHe	$[HCO_3^-]$ (mmol l^{-1})	P_{CO_2} (mmHg)
Not injected			
Control	8.084±0.085 (10)	15.34±2.22 (10)	4.55±0.67 (10)
pH=7.0 C_{CO_2}=0 mmol l^{-1}	7.039±0.036* (7)	1.55±0.07* (7)	5.85±0.51 (7)
pH=9.9 C_{CO_2}=0 mmol l^{-1}	7.718±0.102* (7)	4.61±0.92* (7)	3.29±0.69 (7)
Long-term control	7.540±0.029* (5)	2.79±0.63* (5)	2.95±0.53* (5)
Injected			
Saline control	7.789±0.040* (7)	8.12±0.95* (7)	4.32±0.39 (7)
Acetazolamide (1 mmol kg^{-1})	7.986±0.068† (13)	16.63±2.46† (13)	5.65±0.37† (13)
$NaHCO_3$ (10 mmol kg^{-1})	8.085±0.048† (8)	11.09±1.37† (8)	3.17±0.29*† (8)

Values are mean ± 1 S.E.M. (*N*).
*Significantly different ($P \leqslant 0.05$) from corresponding control value.
†Significantly different ($P \leqslant 0.05$) from corresponding saline-injected control value.

control conditions, so that increases in $[HCO_3^-]$ should have no effect, but that decreases into the K_m range will have a marked inhibitory effect.

In addition to discrete measurements on individual fish, blood acid–base status under control conditions and after acute exposure to pH 7.0, C_{CO_2}=0 mmol l^{-1}, was also determined on pooled samples, each from several fish (Table 5). This allowed analysis of red blood cell (RBC) pHi and of CO_2 distribution between plasma and erythrocytes. Haematocrit ranged from 20 to 30 %. Extracellular pH and whole-blood HCO_3^- levels of these pooled samples were not significantly lower than discrete values from individual fish under comparable conditions (cf. Table 4). In pools from control fish, red blood cell pHi was approximately 7.6, about 0.4 units below pHe (Table 5). Plasma C_{CO_2} was significantly greater than whole-blood C_{CO_2}, suggesting that CO_2 levels (mainly HCO_3^-) were quite low inside the erythrocytes. After acute exposure to pH 7.0, C_{CO_2}=0 mmol l^{-1}, RBC pHi was greatly depressed to about 7.1, now only about 0.27 units below pHe, and there was only a negligible difference between plasma and whole-blood C_{CO_2}.

Preliminary estimates of pHi in epaxial white muscle were made using the homogenate technique of Pörtner *et al.* (1990) on freeze-clamped samples, albeit without benefit of metabolic inhibitors. The mean value under control conditions was 7.646±0.130 (5), similar to that of the red blood cells (Table 5).

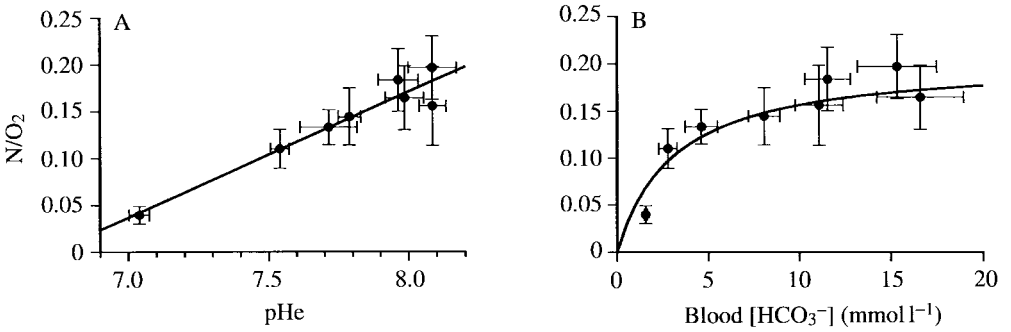

Fig. 6. Relationships between (A) blood pHe and N/O_2 ratio and (B) blood HCO_3^- concentration and N/O_2 ratio in *Oreochromis alcalicus grahami*. Acid–base data were taken from Table 4 and N/O_2 data from the corresponding treatments in the flux experiments of Figs 3 and 5 and Table 2. Values are mean ±1 S.E.M.; *N* values as in the listed sources. N/O_2 is the ratio of urea-N excretion (J_{Urea}) to O_2 consumption ($\dot{M}_{O_2}$). The line in A is a linear regression (r=0.96); the line fitted in B is a Michaelis–Menten equation with K_m=3.06 mmol l^{-1} (as HCO_3^-) and V_{max}=0.204 (as N/O_2).

Table 5. *Whole-blood, plasma and red blood cell acid–base status in blood pools sampled from* Oreochromis alcalicus grahami *in control water and after acute exposure to neutral decarbonated water*

	Control (*N*=4), water pH=9.9, C_{CO_2}=173 mmol l^{-1}		Neutral (*N*=2), water pH=7.0, C_{CO_2}=0 mmol l^{-1}	
	Whole blood	Plasma	Whole blood	Plasma
pHe	7.995±0.043	–	7.382±0.017†	–
Red blood cell pHi	7.586±0.013	–	7.107±0.025†	–
C_{CO_2} (mmol l^{-1})	11.98±0.88	13.80±1.51*	2.26±0.34†	2.12±0.32
$[HCO_3^-]$ (mmol l^{-1})	11.83±0.87	13.63±1.49*	2.14±0.32†	2.01±0.30
Haematocrit (%)	23.7±2.4	–	25.1±2.1	–

Values are mean ± 1 S.E.M. (*N*), where *N* represents the number of different pools. Each pool represented blood samples from 3–4 fish.

*Significantly different ($P \leqslant 0.05$) from corresponding whole-blood value by paired Student's *t*-test.

†Significantly different ($P \leqslant 0.05$) from corresponding control value by unpaired Student's *t*-test.

Enzyme activities

We hypothesized that, if changes in ureagenesis in response to internal acidosis (Fig. 6) were related to alterations in enzyme activities, then 24 h of exposure to neutral decarbonated water (pH 7.0, C_{CO_2}=0 mmol l^{-1}) might cause the greatest alterations. Brain glutamine synthetase (GNS) activity fell significantly by about 20 % relative to that of 24 h control fish (Table 6). Liver ornithine–citrulline transcarbamoylase (OCT) and arginase (ARG) fell by about 50 %, but the differences were not significant because of variability and low *N* values. There were no other changes in enzyme activities in either brain or liver.

Table 6. *Enzyme activities in the brain and liver of* Oreochromis alcalicus grahami *in control water and after 24 h of exposure to neutral decarbonated water (pH=7.0, C_{CO_2}=0 mmol l^{-1})*

	Brain		Liver	
	24 h control	24 h neutral	24 h control	24 h neutral
GDH	17.87±1.54	16.86±0.59	23.06±4.86	24.28±7.09
AspAT	73.17±2.61	74.14±3.93	78.14±16.05	88.48±33.00
AlaAT	5.04±0.14	4.63±0.46	27.23±7.38	19.08±8.39
GNS	51.23±3.98	40.94±1.78*	1.73±0.36	1.82±0.53
MDH	50.16±1.02	51.30±1.73	388.09±72.69	367.96±87.17
CS	11.17±0.56	10.56±0.56	6.49±1.37	5.86±1.97
OCT	–	–	13.36±3.96	6.29±2.47
ARG	–	–	33.18±10.06	17.58±6.05

Values are mean ± 1 S.E.M., N=5; units are μmol of substrate converted to product per minute per gram wet mass.

*Significantly different ($P \leqslant 0.05$) from 24 h control value.

–, no measurements were made.

GDH, glutamate dehydrogenase; AspAT, aspartate aminotransferase; AlaAT, alanine aminotransferase; GNS, glutamine synthetase; MDH, malate dehydrogenase; OCT, ornithine–citrulline transcarbamoylase; ARG, arginase.

Discussion

Sites and mechanisms of urea-N production and excretion

Activities of OUC-related enzymes in the liver of 24 h control fish (Table 6) were similar to those reported earlier for this species, as were concentrations of T_{Amm} and urea in blood plasma (Table 2; Randall *et al.* 1989; Wood *et al.* 1989; Walsh *et al.* 1993). These hepatic OUC activities were much higher, and plasma urea levels moderately higher, than those found in standard ammoniotelic teleosts (Chiu *et al.* 1986; Wood, 1993; Wilkie *et al.* 1993). In contrast, the activity of the uricolytic enzyme allantoicase in liver, measured earlier by Randall *et al.* (1989), was not exceptional. The liver is probably the major site of urea production by the OUC in *O. a. grahami*, though not necessarily the only site, inasmuch as the kidney also exhibits substantial OUC activity in certain ureagenic air-breathing teleosts (Saha and Ratha, 1987, 1989).

T_{Amm} levels in plasma (Table 2) were probably overestimated because of rapid adenylate deamination accompanying the stress of blood sampling, as discussed by Wood *et al.* (1989). In that study, true plasma T_{Amm} was estimated at about 0.39 mmol l^{-1} based on a passive (Nernstian) distribution between white muscle and plasma. The present bile measurements provide an alternative means of calculating true plasma T_{Amm}. As there is no electrical gradient across the teleost gall bladder (Diamond, 1962), a passive ammonia distribution between bile and plasma should be dictated by the pH gradient. Calculations based on this assumption and the measurements in Table 2 yield a true plasma T_{Amm} of 0.34 mmol l^{-1}, similar to the earlier estimate. This plasma T_{Amm} value is still well above

the very low level of plasma glutamine (Table 2). However, as in other ureogenic teleosts and elasmobranchs (Mommsen and Walsh, 1989; Barber and Walsh, 1993), glutamine, rather than ammonia, is the preferred nitrogen substrate of CPS III in the Lake Magadi tilapia (Randall *et al.* 1989), whereas ammonia and glutamate are the substrates for hepatic glutamine synthetase. Very low circulating glutamine levels appear to be characteristic of fish in general, whether they be ureotelic (Leech *et al.* 1979; Walsh *et al.* 1989; Barber and Walsh, 1993) or ammoniotelic (Ash *et al.* 1989). In this regard, it is interesting that glutamine levels were so markedly elevated relative to glutamate in bile (Table 2); we suggest that high biliary glutamine levels may reflect high intracellular glutamine levels in liver, the organ that produces this secretion.

Identification of the gills as the major route (80 %) of J_{Urea} (Table 1) was in general accord with findings in other fish, both those producing small amounts of urea by uricolysis and those, such as elasmobranchs, producing the major portion of their nitrogen wastes in the form of urea-N (see Wood 1993, for a review). Maina (1991) has recently described unusual ovoid cells with electron-dense granules in the gills of the Lake Magadi tilapia and has hypothesised that they may represent the sites of urea-N excretion. As urea-N was found in bile and urine at levels similar to those of blood plasma (Table 2), it is likely that the 20 % of J_{Urea} appearing in the posterior compartment during the divided chamber experiments originated from both intestinal and urinary excretion.

In many higher vertebrate systems, thiourea has been identified as a competitive blocker of urea transport with a similar K_m, and a K_i equal to the K_m (Marsh and Knepper, 1992). The dose of thiourea injected ($3\,mmol\,kg^{-1}$=$6\,mmol\,N\,kg^{-1}$) was intended to produce a circulating thiourea level approximately equal to that of urea (Table 2) and therefore should have reduced J_{Urea} by 50 %. The absence of any inhibitory effect (Fig. 5) demonstrates that urea excretion in the Lake Magadi tilapia is not thiourea-sensitive, but does not completely eliminate the possibility of a transport mechanism. For example, the active urea transporter in the elasmobranch kidney accepts acetamide and methylurea, but not thiourea (Schmidt-Nielsen and Rabinowitz, 1964). Further studies with a range of antagonists are required to determine whether the very high rates of urea excretion across the gills of the Lake Magadi tilapia are carrier-mediated or occur by simple diffusion.

The relationship of J_{Urea} *to* $\dot{M}_{O_2}$

The mean N/O_2 ratio under control conditions, 0.183±0.024 (40), did not exceed the theoretical aerobic maximum of about 0.27 where 100 % of $\dot{M}_{O_2}$ would be devoted to protein oxidation (van Waarde, 1983). Indeed, the present values are quite close to the 'routine' aerobic value of 0.234±0.022 (25) reported by Kutty (1972) for the related but ammoniotelic *Tilapia mossambica* at 30 °C in fresh water. Nevertheless, these N/O_2 ratios were high relative to ratios measured in most other species, where values in the range 0.04–0.12 are typical, even in carnivorous salmonids (Brett and Zala, 1975; van Waarde, 1983; Wiggs *et al.* 1988). Taken at face value, a N/O_2 ratio of 0.183 would indicate that about 68 % of aerobic metabolism was based on protein oxidation, a surprisingly high proportion relative to the 15–45 % determined for most fish. Therefore, although it is not absolutely necessary to invoke external sources of nitrogen and HCO_3^-

to explain the observed rates of J_{Urea}, it certainly remains possible that external substrates made a substantial contribution. While the potential source of exogenous HCO_3^- is obvious, the source of exogenous nitrogen remains unclear. As outlined below, it is difficult to see how the ureagenic system could contribute to acid–base balance without the latter.

Internal acid–base status under control conditions

The present measurements of blood pHe and HCO_3^- in *O. a. grahami* (Table 4) were higher than in our earlier study (Wood *et al.* 1989) and at the upper end of the wide range of values reported by Johansen *et al.* (1975). We attribute this difference to the availability of larger fish and to an excellent anaesthetic in the present study, both of which greatly facilitated rapid blood sampling without struggling and with a minimum of air exposure. The great sensitivity of this species to handling stress is underscored by the persistent metabolic acidosis seen in saline-injected control fish (Table 4) 2–3 h after the initial injection. This phenomenon made it particularly important to compare the experimentally injected fish with the saline-injected controls rather than with the non-injected controls. Nevertheless, non-specific stress effects associated with injection and handling may have complicated or obscured the responses of experimentally injected fish.

True *in vivo* blood pHe values in *O. a. grahami* were probably even higher than measured here. In all treatment groups, blood P_{CO_2} values (Table 4) were undoubtedly elevated above true *in vivo* levels, and therefore pHe depressed, because of caudal puncture sampling, as documented by Wood *et al.* (1989). Water of high pH is essentially a 'P_{CO_2} vacuum' (Johansen *et al.* 1975). In contrast to the present blood P_{CO_2} values in the range 3–6 mmHg (Table 4), recent Pa_{CO_2} measurements in salmonids exposed to water of pH 9.5–10.0 indicate values less than 1 mmHg when sampled by indwelling catheter (Wilkie and Wood, 1991; Wilkie *et al.* 1993). If this were the case in *O. a. grahami*, arterial pHe could be as high as 8.7–9.0, based on recalculation of control data to this P_{CO_2} range. The highest values measured in individual fish, both by Johansen *et al.* (1975) and in the present study, were in the region of 8.4. By way of contrast, the 'standard' fish values normally measured at 32 °C or predictable from alphastat and relative alkalinity relationships would be about 7.5–7.6 (Cameron, 1984).

The present determinations of RBC pHi (Table 5) and preliminary estimates of white muscle pHi, both approximately 7.6, are the first intracellular measurements in a fish living at such high pH. They clearly show that the alkalotic condition in the extracellular fluid was reflected in the intracellular fluid. For example, white muscle pHi in the channel catfish at the same temperature (32 °C) was only about 7.1 (Cameron and Kormanik, 1982), and in rainbow trout at 15 °C, both RBC pHi and white muscle pHi were about 7.3 (Wood and LeMoigne, 1991). Interestingly, Lykkeboe *et al.* (1975) reported that the O_2 affinity of stripped haemoglobin from the Lake Magadi tilapia was greatest at a solution pH of about 7.6 and was virtually insensitive to pH from 7.6 to 8.5. The pH-sensitivity of various enzymes in comparison with those of 'standard' teleosts at comparable temperatures (see, for example, Yancey and Somero, 1978) is obviously an important topic for future investigation (see Somero, 1986).

Table 7. *Nernst equilibrium potentials (–*E*, in mV) across the gills of* Oreochromis alcalicus grahami *for those anions whose distributions were altered during exposure of the fish to decarbonated water at pH 7.0 or pH 9.9*

	Water pH=9.9 C_{CO_2}=173 mmol l^{-1}	Water pH=7.0 C_{CO_2}=0 mmol l^{-1}		Water pH=9.9 C_{CO_2}=0 mmol l^{-1}	
	Control	Initial[a]	2–3 h[b]	Initial[a]	2–3 h[b]
$-E_{OH}$ (mV)	+112	−63	+1	+111	+133
$-E_{HCO_3}$ (mV)	+26	−97	−38	−138	−108
$-E_{CO_3}$ (mV)	+66	−71	−11	−7	+19
$-E_{Cl}$ (mV)	−11	+27	+21	+27	+23

Equilibrium potentials have been changed in sign to indicate the direction of the net driving force across the gills: i.e. TEP–*E*. The force is –*E* when TEP=0 mV. +, inwards; –, outwards.

[a]Initial values were calculated using the control blood composition, i.e. assuming that no changes in blood composition had yet occurred.

[b]The 2–3 h values were calculated using the measured blood composition at that time.

The influence of water acid–base status on internal acid–base status

Exposure of *O. a. grahami* to decarbonated water titrated back to control pH 9.9 or neutral pH 7.0 resulted in internal metabolic acidosis (Table 4). The acidosis was more severe in the latter circumstances and was in quantitative agreement with the observations of Wright *et al.* (1990), who earlier subjected this species to a similar treatment. The present water and blood data, in combination with the plasma Cl^- measurements of Wright *et al.* (1990), allow calculation of Nernst equilibrium potentials (*E*) for those ions (all anions) whose concentrations were altered in the experimental treatments. The difference between the transepithelial potential (TEP) and the Nernst potential (i.e. TEP$-E$) is the driving force on the ion. According to Eddy *et al.* (1981), the TEP across the gills of the Lake Magadi tilapia is negligible under control conditions (1.8 mV) and does not change greatly even when the external medium is greatly altered (e.g. −12 mV in neutral fresh water). Therefore, $-E$ alone provides an estimate of the driving force, as tabulated in Table 7.

Under control conditions, there were strong electrochemical gradients in the order OH^->CO_3^{2-}>HCO_3^- tending to drive these anions into the fish, while Cl^- would tend to move out. Upon initial exposure to pH 7.0, C_{CO_2}=0 mmol l^{-1} water, all gradients were reversed, so that OH^-, HCO_3^- and CO_3^{2-} would tend to move out passively, while Cl^- would enter. By the time blood samples were taken, 2–3 h after the start of exposure, internal pHe and C_{CO_2} had fallen to such an extent that OH^- (and of course H^+) approached a passive distribution and the gradients driving HCO_3^- and CO_3^{2-} out of the fish had declined greatly. In contrast, when the fish were exposed to pH 9.9, C_{CO_2}=0 mmol l^{-1} water, there was little appreciable change in the driving force on OH^-, but the other three gradients again reversed. The largest reversal occurred with the HCO_3^- gradient; the CO_3^{2-} gradient changes were much smaller and the Cl^- gradient changes were very similar to those occurring in the pH 7.0, C_{CO2}=0 mmol l^{-1} treatment. In all treatments, a small driving force for Na^+ entry (−12 mV) remained more or less constant.

Knowledge of permeabilities to the various ions and actual changes in TEP would be required to extend this analysis to a more mechanistic level. Nevertheless, it is clear that the induction of metabolic acidosis cannot be attributed to just one type of acidic or basic equivalent and that invasion of Cl^- may have played an important role. The acid–base regulatory system of *O. a. grahami* appears to be set up to counter continually the entry of basic equivalents and Na^+ from the normally alkaline environment and to counter the loss of Cl^- to the environment. This entry of Na^+ and basic equivalents may occur either by passive diffusion across the gills or as a result of drinking. Indeed the drinking rates measured by Maloiy *et al.* (1978) and Skadhauge *et al.* (1980) in this species in control water would bring approximately $5000\,\mu mol\,kg^{-1}\,h^{-1}$ of basic equivalents into the intestine, some or all of which could be absorbed. When the entry of basic equivalents is reduced, the acid–base regulatory system cannot rapidly adapt, and severe acidosis results. A parallel study has followed alterations in gill morphology during exposure to pH 7.0, C_{CO_2}=0 mmol l^{-1} water, and suggests an important role for gill chloride cells in the observed acid–base changes (P. Laurent, H. L. Bergman, A. Narahara, P. J. Walsh, J. N. Maina and C. M. Wood, in preparation).

The influence of water acid–base status and internal acid–base balance on urea production and excretion

Internal acid–base status appears to link urea production rate to external acid–base status in *O. a. grahami* (Figs 3, 4). This conclusion is based on the observed sensitivity of blood acid–base status to water acid–base status (Tables 4, 5) and on the clear relationships between blood acid–base status and N/O_2 (Fig. 6). The possibility that reductions in N/O_2 were due to inhibition of urea excretion rather than production cannot be completely eliminated, but this seems most unlikely in view of the finding of Wright *et al.* (1990) that there was no internal accumulation of urea-N after 12 h of exposure to pH 7.0, C_{CO_2}=0 mmol l^{-1} water. Calculations based on Tables 2 and 3 indicate that plasma urea-N would have increased more than fourfold if excretion rather than production had been inhibited.

The observed relationships between ureagenesis (indexed as N/O_2) and pHe (Fig. 6A) and between ureagenesis and blood [HCO_3^-] (Fig. 6B) during 'metabolic' acid–base disturbances were remarkably similar to those seen in many mammalian studies (reviewed by Haussinger, 1990). The results indicate that metabolic acidosis inhibits J_{Urea} in the Lake Magadi tilapia and strongly suggest that the rate of ureagenesis depends on the HCO_3^- supply. Indeed the estimated K_m for extracellular HCO_3^- in intact *O. a. grahami* (3.06 mmol l^{-1}) was similar to that (1.30 mmol l^{-1}) for isolated hepatocytes of the toadfish *Opsanus beta*, one of the very few other teleosts capable of OUC-based ureagenesis (Walsh *et al.* 1989). The value also fits well in the range (2–5 mmol l^{-1}) of many mammalian studies for isolated liver mitochondria or free CPS I (Meijer *et al.* 1990).

A major difference from both the toadfish and mammals was the complete lack of an inhibitory effect of acetazolamide in *O. a. grahami* (Fig. 5). A critical dependence of ureagenesis on mitochondrial carbonic anhydrase activity has been seen in a variety of mammalian studies (Haussinger, 1990). The concentration of acetazolamide used here

(1 mmol kg^{-1}) also caused a 60 % inhibition of urea synthesis by toadfish hepatocytes (Walsh *et al.* 1989). In the present study, the drug was clearly effective in blocking systemic carbonic anhydrase, as shown by the blood acid–base data (CO_2 retention; Table 4), but it is possible that the intramitochondrial enzyme escaped inhibition. Alternatively, given the high temperature, abundant HCO_3^- supply and high extra- and intracellular pH in this species, it is possible that HCO_3^- enters the mitochondria directly or is produced inside the mitochondria by the uncatalysed hydration reaction at a rate sufficient to sustain ureagenesis at V_{max}. In this regard, it is interesting that, in perfused rat liver, acetazolamide had little effect on urea synthesis from glutamine, in contrast to its marked inhibitory effect on urea synthesis from NH_4^+, and that, even in the latter pathway, high extracellular [HCO_3^-] completely prevented the inhibitory effect (Haussinger, 1986).

The relationships in Fig. 6 are typical of mammalian data cited by Atkinson (1992) and colleagues to support the view that OUC-based ureagenesis plays a key role in systemic acid–base regulation. We believe that there are several reasons why this interpretation is not appropriate in the case of *O. a. grahami*.

First, if for any reason additional basic equivalents start to enter from the alkaline environment, the rate of the OUC cannot increase automatically to remove them. This is because the ureagenic system is saturated at normal extracellular levels of HCO_3^- (Fig. 6B; Table 4), producing a value of N/O_2 at V_{max} (0.204) equal to the normal control value. However, if fewer basic equivalents than normal enter (for example, as a result of a temporary cessation of drinking or because of acidification and decarbonation of the water, as in our experiments), ureagenesis may be reduced as the substrate (i.e. HCO_3^-) level in the body fluids falls below the saturation point of the curve. This would help conserve HCO_3^-. Part of the mechanism for this response may be a reduction in the activities of GNS and the OUC enzymes (Table 6). Therefore, the system does not appear to be designed to protect the animal from being 'flooded by HCO_3^- from the ambient water' (see Atkinson, 1992). This situation contrasts with the immediate stimulation of ureagenesis seen during ammonia-loading (Wood *et al.* 1989), which suggests that the OUC is poised to remove additional amino-N rather than additional HCO_3^- and that nitrogen supply is normally a limiting factor. As noted by Atkinson himself (1992), while there may be multiple sources of HCO_3^- in animal systems, there are few other sources of NH_4^+ besides protein metabolism. Very recently, Barber and Walsh (1993) have similarly concluded that the primary driving force for urea synthesis in the toadfish is the need for nitrogenous waste excretion rather than HCO_3^- removal.

Second, the normal aerobic metabolism of proteins and amino acids produces approximately equimolar amounts of NH_4^+ and HCO_3^-, while ureagenesis consumes equal amounts of NH_4^+ and HCO_3^- on a net basis. Therefore, ureagenesis will effect *net* removal of exogenous HCO_3^- *only* if the accompanying NH_4^+ is also of exogenous origin, rather than a product of the animal's own protein metabolism. The potential source of exogenous NH_4^+ is unclear, though the possibility that exogenous nitrogen is supplied in excess of HCO_3^- by the cyanobacterial diet (Coe, 1966) deserves attention in future studies. The ability of cyanobacteria to fix atmospheric N_2 as ammonia and to store large amounts of nitrogen in a unique polypeptide (multi-L-arginyl poly-L-aspartic acid) has

been well documented (Fay, 1983). The long-term holding experiment was designed to test this idea by depriving the fish of access to this food item but, unfortunately, small amounts of algae continued to grow on the walls of the tanks despite daily cleaning and water changes. Nevertheless, ureagenesis was reduced as intended (Table 3). Had urea synthesis served as a mechanism to remove HCO_3^- entering from the water, then internal metabolic alkalosis, rather than the observed acidosis (Table 4), would have been predicted as a result of exogenous nitrogen limitation.

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