Ionoregulatory strategies and the role of urea in the Magadi tilapia (*Alcolapia grahami*)

Chris M. Wood, Paul Wilson, Harold L. Bergman, Annie N. Bergman, Pierre Laurent, George Otiang'a-Owiti, and Patrick J. Walsh

Abstract: The unique ureotelic tilapia *Alcolapia grahami* lives in the highly alkaline and saline waters of Lake Magadi, Kenya (pH ~10.0, alkalinity ~380 mmol·L^{-1}, Na^+ ~350 mmol·L^{-1}, Cl^- ~110 mmol·L^{-1}, osmolality ~580 mosmol·kg^{-1}). In 100% lake water, the Magadi tilapia maintained plasma Na^+, Cl^-, and osmolality at levels typical of marine teleosts and drank the medium at 8.01 ± 1.29 mL·kg^{-1}·h^{-1}. Gill chloride cells were predominantly of the seawater type (recessed, with apical pits) but a few freshwater-type chloride cells (surficial, with flat apical exposure) were also present. Whole-body Na^+ and Cl^- concentrations were relatively high and exhibited larger relative changes in response to salinity transfers than did plasma ions. All fish succumbed upon acute transfer to 1% lake water, but tolerated acute transfer to 10% lake water well, and gradual long-term acclimation to both 10 and 1% lake water without change in plasma cortisol. Plasma osmolytes were here maintained at levels typical of freshwater teleosts. Curiously, drinking continued at the same rate in fish adapted to 1% lake water, but chloride cells were now exclusively of the freshwater type. Significant mortality and elevated cortisol occurred after acute transfer to 200% lake water. However, the fish survived well during gradual adaptation to 200% lake water, although plasma cortisol remained chronically elevated. Urea levels accounted for only 2–3% of internal osmolality in 100% lake water but responded to a greater extent than plasma ions during exposure to 10 and 200% lake water, decreasing by 28–42% in the former and increasing by over 500% in the latter relative to simultaneous-control values. Urea thereby played a small but significant role (up to 8% of internal osmolality) in osmoregulation.

Résumé : Le remarquable tilapia uréotélique *Alcolapia grahami* vit dans les eaux salées très alcalines du lac Magadi au Kenya (pH ~10,0, alcalinité ~380 mmol·L^{-1}, Na^+ ~350 mmol·L^{-1}, Cl^- ~110 mmol·L^{-1}, osmolalité ~580 mosmol·kg^{-1}). Dans de l'eau du lac à 100 %, les tilapias de Magadi gardaient stables leurs concentrations de Na^+, de Cl^- et leur osmolalité à des valeurs typiques des téléostéens marins et buvaient l'eau du milieu à raison de 8,01 ± 1,29 mL·kg^{-1}·h^{-1}. Les cellules à chlorure des branchies étaient en majorité de type marin (encastrées, avec des fosses apicales), mais quelques-unes étaient de type d'eau douce (superficielles, à surface apicale plate). Les concentrations totales de Na^+ et de Cl^- étaient relativement élevées et elles ont subi des modifications relatives plus importantes en réaction à des changements de salinité que les concentrations plasmatiques d'ions. Tous les poissons sont morts à la suite d'un transfert brutal à de l'eau du lac à 1 %, mais ont bien supporté un transfert subit à de l'eau à 10 % et une acclimatation graduelle aussi bien à l'eau à 10 % qu'à l'eau à 1 % sans modifier leur cortisol plasmatique. Les osmolytes du plasma se sont maintenus à des concentrations typiques de téléostéens d'eau douce. Assez curieusement, les poissons adaptés à de l'eau à 1 % ont continué de boire au même rythme, mais leurs cellules à chlorure étaient alors toutes de type d'eau douce. Une mortalité importante et une augmentation du cortisol ont été observées après un transfert brusque à de l'eau du lac à 200 %. Cependant, les poissons ont survécu à une adaptation graduelle à l'eau du lac à 200 %, mais les concentrations de cortisol plasmatique sont restées élevées en permanence. Les taux d'urée n'étaient responsables que de 2–3 % de l'osmolalité interne dans de l'eau du lac à 100 %, mais ils ont réagi plus fortement que les ions plasmatiques au cours d'expositions à de l'eau du lac à 10 % et à 200 %, diminuant de 28–42 % dans le premier cas, et augmentant de plus de 500 % dans l'autre cas, par comparaison à des valeurs témoins enregistrées en même temps. L'urée

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a donc un rôle réduit, quoique important, à jouer dans l'osmorégulation (expliquant jusqu'à 8 % de l'osmolalité interne).

[Traduit par la Rédaction]

Introduction

Lake Magadi, which is home to a single teleost species, *Alcolapia grahami* (Seegers and Tichy 1999; formerly *Oreochromis alcalicus grahami* and *Tilapia grahami*), presents one of the most hostile aquatic environments on earth. Environmental chemistry is both unusual and severe (pH ~10.0, alkalinity ~380 mmol·L^{-1}, Na^+ ~350 mmol·L^{-1}, Cl^- ~110 mmol·L^{-1}, osmolality ~580 mosmol·kg^{-1}) and additional stressors include daytime hyperoxia and nighttime hypoxia, intense predation by birds, and temperatures that may reach the upper lethal limit of 42.5°C for this species (Coe 1966; Reite et al. 1974; Johansen et al. 1975; Wood et al. 1989; Narahara et al. 1996). The diet is composed almost exclusively of nitrogen-rich cyanobacteria (Coe 1966). Routine rates of O_2 consumption and nitrogenous waste excretion are the highest ever recorded for teleost fish of comparable size, even when allowance is made for the high environmental temperature (Wood et al. 1989, 1994; Franklin et al. 1995; Narahara et al. 1996).

Over the past three decades, numerous papers have illuminated a host of remarkable physiological and behavioural adaptations that allow the Magadi tilapia to thrive in the face of extremity. Physiological adaptations include regulation of extremely high blood plasma and tissue pH levels (Johansen et al. 1975; Wood et al. 1994) and the absence of a Bohr effect in the blood (Narahara et al. 1996), an extremely thin blood-to-water diffusion barrier and high surface area in the gills (Maina 1990; Maina et al. 1996), a capacity for air breathing via accessory respiratory epithelia (Maina et al. 1995; Narahara et al. 1996), a marked depression of metabolic demand when temperature falls during nighttime hypoxia (Narahara et al. 1996), and most importantly, 100% ureotelism (Randall et al. 1989; Wood et al. 1989). The latter is achieved by expression of the ornithine urea cycle throughout the white-muscle mass (Lindley et al. 1999) as well as the liver (Randall et al. 1989; Walsh et al. 1993) and by the expression of a facilitated diffusion transporter for urea in the gill epithelium (Walsh et al. 2001). This is a critical adaptation, because it allows urea-N production and excretion to occur continuously at a very high rate, thereby overcoming the difficulties of excreting ammonia-N into a highly alkaline, highly buffered environment (Wilkie and Wood 1996).

Ionoregulatory and osmoregulatory strategies are less clear-cut. For example, it is not known whether *A. grahami* exploits urea as a regulated osmolyte. Despite the unusual ionic composition of the lake water (high Na^+, high HCO_3^- and CO_3^{2-}, low Cl^-), some studies have reported plasma ions and osmolality at levels fairly typical of those in marine teleosts (Leatherland et al. 1974; Eddy and Maloiy 1984; Wright et al. 1990). However, others have reported that plasma Na^+ levels are twice plasma Cl^- levels (Maloiy et al. 1978) or only 75% of plasma Cl^- levels (Skadhauge et al. 1980), both of which would be unusual for fish. Furthermore, the reported whole body concentration ratio of Na^+ to Cl^- (approximately 2:1) also appears to be unusual (Eddy et al. 1981; Eddy and Maloiy 1984). Gill chloride cell structure appears to be typical of marine teleosts (Maina 1990; Laurent et al. 1995), and the occurrence of drinking at a rate much higher than in marine teleosts has been reported (Maloiy et al. 1978). However, gill ion flux rates appear to be much lower than in marine teleosts (Maetz and DeRenzis 1978; Eddy et al. 1981; Eddy and Maloiy 1984; Wright et al. 1990).

Curiously, despite this tolerance of extreme alkalinity and unusual environmental ion levels, the Magadi tilapia dies within 12–36 h after transfer to neutralized lake water (Wood et al. 1989, 1994; Wright et al. 1990). This may be an unnatural situation, for here HCO_3^- plus CO_3^{2-} in the environment are replaced with a strong anion (e.g., Cl^-). A more natural situation may be one in which salinity and alkalinity change in proportion, as will occur with simple dilution or concentration of the lake water. For example, torrential rainstorms may occasionally dilute the lagoons where the fish normally live (Coe 1966). In this regard, there are several reports that Magadi tilapia can be adapted to diluted lake water, although there is conflicting information on whether they can adapt to fresh water (Coe 1966; Leatherland et al. 1974; Maloiy et al. 1978; Maina 1990). However, rainstorms also cause flooding over the floating "trona" precipitate that covers more than 90% of Lake Magadi's surface, and the resulting dissolution of sodium carbonate and bicarbonate salts may result in very high salinities and alkalinities, which the fish will encounter if they venture into these surface waters. Coe (1966) reported finding many dead fish after such flooding events, and Reite et al. (1974) reported that fish died about 10 h after experimental transfer to a NaCl solution with twice the Na^+ concentration of standard lake water (but no alkalinity).

With this background in mind, in the present study we assessed the iono- and osmo-regulatory performance of *A. grahami* in "standard" (100%) lake water and during acute exposure and after gradual long-term adaptation to both dilute lake water (nominally 10 and 1%) and concentrated lake water (200%). Particular goals were (*i*) to describe the fish's normal plasma and whole-body ionic composition in the face of conflicting earlier reports (see above); (*ii*) to check the very high drinking rates reported by Maloiy et al. (1978) and to see whether drinking continued in dilute media; (*iii*) to evaluate the time course of hyper- and hypo-regulation during exposures to altered salinity–alkalinity; (*iv*) to assess the possible role of urea in osmoregulation in these situations; (*v*) to assess the degree of stress involved by measuring plasma cortisol levels; and (*vi*) to test whether the typical "seawater" morphology of the chloride cells in the branchial epithelium, as described by Maina (1990) and Laurent et al. (1995), changed after adaptation to these altered media. Because *A. grahami* does not tolerate transportation and indoor laboratory holding well (cf. Leatherland et al. 1974; Johansen et al. 1975), we performed these experiments at an outdoor field laboratory close to the lakeside. Experimental conditions were less than ideal, but the fish survived well and the

Table 1. Measured water-chemistry data for Magadi tap water, 200% lake water, and 100% lake water (from Fish Springs Lagoon), and for the various dilutions made from these media.

	Na^+ ($mmol \cdot L^{-1}$)	Cl^- ($mmol \cdot L^{-1}$)	Total CO_2 ($mmol \cdot L^{-1}$)	pH	Osmolality ($mosmol \cdot kg^{-1}$)	Alkalinity[a] ($mequiv \cdot L^{-1}$)
Tap water	3.5	0.3	0.9	6.96	11	0.9
Lake water						
100%	355	113	216	9.86	581	378
10%	49.8 (38.6)	15.9 (11.6)	19.3 (22.4)	9.74	76.1 (68.0)	21.5 (38.6)
1%	8.1 (7.0)	1.2 (1.4)	4.7 (3.1)	9.15	18.9 (16.7)	5.0 (4.7)
200%	696 (710)	216 (226)	369 (432)	10.14	933 (1162)	692 (756)

Note: Nominal values (calculated from mixing ratios) are shown in parentheses.
[a]See the text (Experimental media) for the method of calculation.

results reveal remarkable iono- and osmo-regulatory flexibility in this remarkable species.

Methods and materials

Experimental animals

Adult *A. grahami* were netted by beach seine from Fish Springs Lagoon at the edge of Lake Magadi, Kenya, in January–February 1997. This lagoon harbours one of the largest populations in the lake and was the standard collecting site used in almost all previous physiological studies on this species (for a detailed description of the site see Coe 1966 and Narahara et al. 1996). It provided the standard reference water termed 100% lake water in Table 1. Most fish were in the 1- to 4-g range; larger fish (5–20 g) were rare and were reserved specifically for drinking-rate measurements. The fish were transferred immediately to a nearby outdoor laboratory with natural photoperiod (set up on a covered balcony kindly supplied by Magadi Soda PLC), where they were subsequently held during all experiments. Ambient temperature typically varied from about 30 to 36°C over the day, similar to the diurnal fluctuation at the collection site (cf. Narahara et al. 1996). Procedures were in accord with the guidelines of the Canadian Council on Animal Care.

Experimental media

In all experiments, samples were taken to check water chemistry at the outdoor laboratory, using equipment and methods identical with those described by Wood et al. (1994). Water chloride was measured with a Radiometer CMT10 coulometric titrator and temperature with a Fisher probe. Water pH was measured with a Radiometer GK2401C electrode and water total CO_2 with a Radiometer CO_2 electrode, using the acid reaction chamber system of Cameron (1971); both electrodes were displayed on Radiometer pHM 71 or pHM 84 meters. Alkalinity, expressed as HCO_3^- equivalents (i.e., $[HCO_3^-] + 2[CO_3^{2-}]$), was calculated from pH and total CO_2 measurements, using values for αCO_2 and pK^I and pK^{II} at the appropriate temperature and chlorinity (from Skirrow 1975). Samples were frozen and transported back to McMaster University for later measurement of sodium (by atomic absorption spectroscopy (AAS); Varian 1275-AA) and osmolality (by vapour-pressure osmometry; Wescor 5100A). Samples for sodium analysis were diluted to the linear range of the instrument (0–200 $\mu mol \cdot L^{-1}$); osmolality samples were read without dilution.

Diluted lake water was made by mixing the appropriate volumes of 100% lake water with local fresh water, Magadi tap water (composition as in Table 1). Concentrated lake water was made by adding the appropriate amounts of NaCl and "trona powder" (kindly supplied by Magadi Soda PLC) to 100% lake water. Trona is the floating precipitate that covers 90% of the surface of Lake Magadi; trona powder is this precipitate but with the NaCl removed, and therefore consists of $NaHCO_3$ and $(Na)_2CO_3$ plus other minor natural salts in the lake water. Our nominal target concentrations for experiments were 1, 10, 100, and 200% lake water. Water-chemistry measurements in these various salinities–alkalinities (Table 1; data from the long-term adaptation experiments) were reasonably close to nominal values calculated from the mixing ratios used in these field experiments, and deviations likely reflected procedural variations in making the mixtures. However, minor discrepancies in osmolality and alkalinity also likely resulted from concentration-dependent alterations in activity coefficients and pK^I and pK^{II} values. It should be noted that since Magadi tap water contained appreciable concentrations of some moieties (e.g., 3.5 mmol $Na^+ \cdot L^{-1}$, 0.9 mmol total $CO_2 \cdot L^{-1}$, 11 $mosmol \cdot kg^{-1}$ osmolality), the concentrations of these substances in the 1% mixture were actually about 2% of full lake-water concentrations (Table 1).

Acute exposure to altered salinity–alkalinity

A large batch of fish caught on a single day were allowed to settle for about 12 h in 100 L of 100% lake water from Fish Springs Lagoon. Vigorous aeration was provided. Ten fish were sacrificed for whole body and plasma composition measurements, while the remainder (approximately 30 fish per treatment) were transferred either to another 50-L bucket of 100% lake water or to similar volumes of either 1, 10, or 200% lake water. Again, each chamber was vigorously aerated. Fish (N = 10–15 at each time) were sacrificed from all tanks at approximately 5 h (range 4–6 h) and 15 h (range 14–16 h) after transfer. These fish were not fed.

Long-term adaptation to altered salinity–alkalinity

Another large batch of fish was divided into four groups (25 fish in each group) intended for four different treatments (control, dilution to 1%, dilution to 10%, and concentration to 200% lake water) and each was initially placed in a polyethylene bucket filled with 100 L of 100% lake water from Fish Springs Lagoon. Vigorous aeration was provided. Fish in each tank were fed 1 g of a commercial "Cichlid Diet" twice each day (8:00 a.m. and 8:00 p.m.), which corresponds

to a ration of approximately 3.2% body mass per day (dry food/wet mass). As all food was eaten, control and experimental groups consumed the same amount. The water was changed daily by removing and replacing 90 L approximately 2 h after the 8:00 a.m. feeding. One group, designated control, was kept in 100% lake water throughout. The two dilution groups were gradually acclimated to dilute media by reductions of 10% every 24 h, achieved by the addition of the appropriate proportion of Magadi tap water (composition as in Table 1). Once 10% (i.e., 90% dilution) was reached, subsequent daily steps for one of the groups were 5, 2.5, and 1%. The fourth group was gradually acclimated to more concentrated media by increases of 10% every 24 h until 200% was reached. Once the nominal target dilution or concentration was reached, fish were held for a further 10 days (with daily water changes and feeding) to ensure that acclimation was complete. As outlined below, these fish were then either sacrificed for whole body and plasma composition measurements (all groups), sacrificed for determinations of branchial morphology (all groups), used for drinking-rate measurements (100 and 1% groups only), or used for enzymatic measurements and swimming performance trials in experiments reported by Wood et al. (2002).

Measurements of plasma and whole-body composition

Fish were rapidly anaesthetized in a solution of metomidate-HCl (5 $mg \cdot L^{-1}$) made up in the appropriate salinity–alkalinity, then blotted and weighed. A 100-μL gas-tight Hamilton syringe fitted with a customized needle and wetted with lithium heparin (1000 IU/mL in 200 mM NaCl) was used to draw a blood sample from the caudal arch. Plasma was separated by centrifugation and either analyzed immediately for urea-N (by the diacetyl monoxime method; Rahmatullah and Boyde 1980; Price and Harrison 1987) or frozen in liquid nitrogen for shipment back to Canada using a dry-shipper (Minnesota Valley Engineering). At McMaster University, plasma was thawed, mixed, and immediately aliquotted for measurement of osmolality (by vapour-pressure osmometry, as above) and Na^+ (by AAS, using the same dilution range as for water samples above), Cl^- (by the colorimetric assay of Zall et al. 1956), and cortisol (by ICN Immunocorp radioimmunoassay with the modifications outlined by Wood et al. 1997) concentrations. As sample volumes ranged from 20 to 220 μL, it was not always possible to measure all parameters on all fish, so the following priority order was assigned for analyses: urea > Cl^- > cortisol > osmolality > Na^+.

Immediately following blood sampling, the whole fish was freeze-clamped in liquid nitrogen for later determination of whole-body urea-N, Na^+, and Cl^- concentrations. At the field laboratory, each frozen fish was weighed and ground to a fine powder under liquid nitrogen; a weighed aliquot of the frozen powder was then extracted in 9 volumes of 10% trichloracetic acid (TCA) at 4°C for 30–60 min. The extract was centrifuged for clarification and then assayed on site for urea-N by the diacetyl monoxime method (as above). The extracts were returned to McMaster University, where they were appropriately diluted and assayed for Na^+ by AAS (as above) and Cl^- (by the colorimetric assay of Zall et al. 1956). In all cases, standards were made up in the appropriate dilution of TCA.

Branchial morphology

Branchial morphology was examined in fish (1–4 g; $N = 3$ per treatment) taken from the long-term acclimations to 200, 100, 10, and 1% lake water and also from a freshly collected 100% lake water group. Unfortunately, samples from the 10% lake water treatment were lost in transit. Fish were killed rapidly with an overdose of MS 222 (0.5 $g \cdot L^{-1}$); the gills were removed and processed immediately for light microscopy and transmission and scanning electron microscopy (TEM amd SEM, respectively), according to methods described previously (Laurent et al. 1985, 1995). In brief, the tissue was kept on ice throughout processing. Gill arches (right side) were excised and quickly rinsed in ice-cold sodium cacodylate (0.15 $mol \cdot L^{-1}$). The individual gill filaments were then carefully dissected away from each gill arch in the buffer. Only the anterior and posterior rows of filaments remained attached to the septum of the arch. Each piece was then fixed in 5% iced glutaraldehyde in cacodylate buffer for 1 h and processed for TEM (Siemens Elmiskop 101 and Jeol 200TM) or SEM (Cambridge Stereoscan 100), as outlined by Laurent and Hebibi (1990) and Laurent et al. (1994).

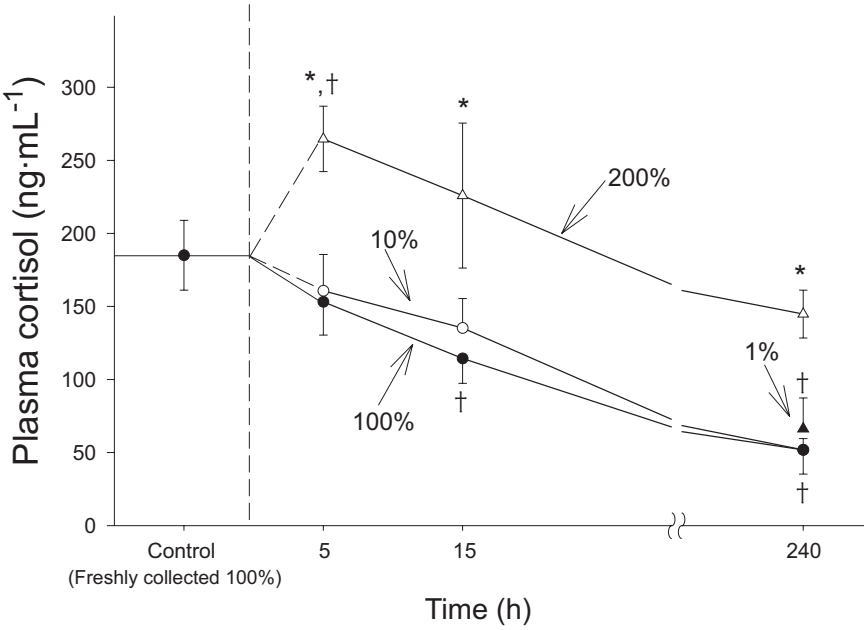
Measurements of drinking rate

Measurements were performed on three groups: freshly collected fish in 100% lake water ($N = 5$), fish that had undergone long-term acclimation to 100% lake water ($N = 11$), and fish that had undergone long-term acclimation to 1% lake water ($N = 6$). The largest fish available (5–20 g) were used and the method of Wilson et al. (1996) was followed. The fish were placed in 1–2 L of the appropriate water labelled with 30 $\mu Ci \cdot L^{-1}$ (1 Ci = 37 GBq) of [^{3}H]-labelled polyethylene glycol 4000 (PEG-4000, NEN Dupont) for 4–7 h. Water samples for radioactivity measurements (3 × 1.5 mL each) were collected at the start, middle, and end of the exposure periods. Each fish was netted out individually (exact time noted), rinsed in isotope-free water, and weighed. The gut was exposed, ligated with suture at both ends to contain the contents, weighed, and then frozen in liquid nitrogen for shipment back to McMaster University. The frozen gut was later homogenized in 10 mL of 10% $HClO_3$ for 5 min, allowed to settle for 24 h, and then centrifuged at 500 × g for 5 min to yield a clear protein-free supernatant. Triplicate 1.0-mL aliquots of the supernatant were added to 10 mL of ACS fluor (Amersham), together with 4 mL of distilled water, and analysed for radioactivity ([^{3}H]-labelled PEG-4000) on an LKB Rackbeta scintillation counter. Water samples were counted in the same scintillant mixture; quench correction was performed by the external-standard method and checked by internal standardization. Drinking rate was calculated from total disintegrations per minute (1 dpm = 0.0167 Bq) of the gut, factored by time, fish mass, and mean disintegrations per minute of the external water. In practice, the radioactivity of the external water changed by less than 10% during the exposure periods and no disintegrations per minute were detected in the faecal samples removed from the rectum, indicating that there was no loss of radioactivity by this route and, hence, no underestimation of drinking rate.

Statistical analyses

Data are expressed as mean ± 1 SEM (N), where N is the number of fish. Comparisons with simultaneous-control val-

Fig. 1. The influence of acute transfer (from 100% lake water) and chronic acclimation to 200% (△), 10% (○), 1% (▲, chronic only), or 100% (control, ●) lake water on plasma cortisol in freshly collected Magadi tilapia. Only chronic data were obtained for the 1% treatment, as the fish died after acute transfer to 1% lake water. Chronic data are plotted at 240 h, as fish were held at the appropriate dilution for 10 days after gradual adaptation. Values are mean ± 1 SEM (N = 7–15 at all points, except at 15 h in 200% lake water, where N = 3.) Asterisks indicate significant differences ($P < 0.05$) from the simultaneous 100% lake water control value; daggers indicate significant differences ($P < 0.05$) from the initial value for freshly collected fish in 100% lake water.



ues were made by Student's unpaired t test (two-tailed); multiple comparisons were performed by one-way analysis of variance, followed by the Bonferroni post hoc test to identify specific differences. Cortisol data were normalized by logarithmic transformation prior to statistical testing. A significance level of $P \leq 0.05$ was used throughout.

Results

Tolerance of acute exposure versus gradual acclimation to altered salinity–alkalinity

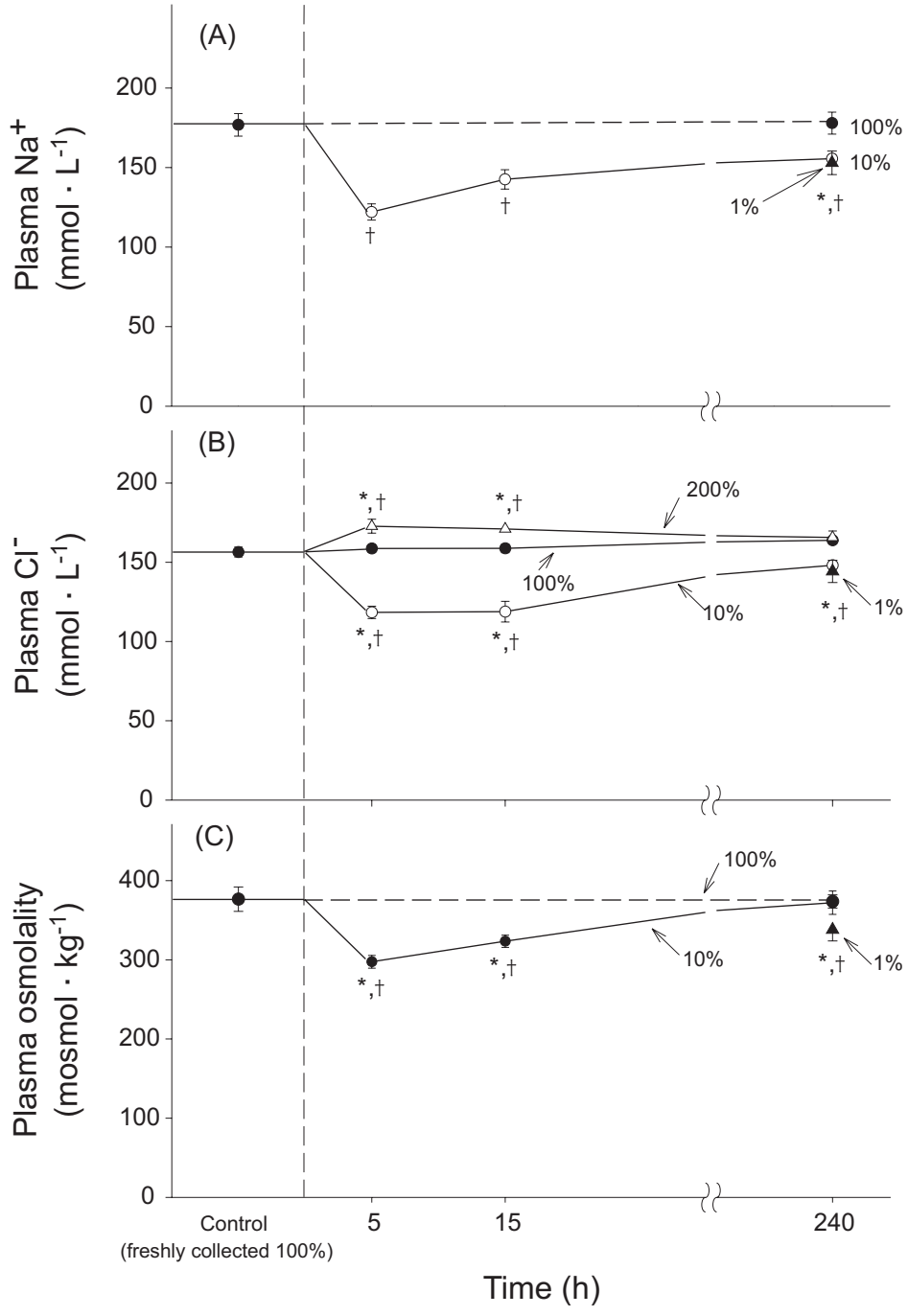
In the acute-exposure experiment, all fish were dead within a few hours after direct transfer to 1% lake water, and thus for this experiment, blood and whole-body samples were not taken. However, the fish tolerated gradual acclimation to 1% lake water well, with a cumulative mortality of only 18%, which is not significantly different from the 12% mortality in the long-term control group in the same period (3 weeks, the last 10 days of which were at the target dilution). In contrast, acute transfer to 10% lake water was well tolerated: by 15 h, mortality was only 5% and all surviving fish looked healthy. There was no mortality during long-term acclimation to 10% lake water. Acute transfer to 200% lake water was clearly a lethal challenge for many of the fish, with mortality reaching 58% in the first 15 h. Furthermore, the sluggish surface-seeking behaviour of the surviving fish at this time indicated that this was a highly stressful treatment. However, gradual acclimation to 200% lake water was again well tolerated, with a cumulative mortality of only 12% over 3 weeks, identical with the control treatment. In experiments not reported here, subsequent handling of these long-term 200% lake water fish resulted in high mortality, suggesting the persistence of chronic stress; this was not seen in the other long-term treatment groups.

Plasma cortisol data were in accord with this interpretation (Fig. 1). In surviving fish, cortisol levels increased by 65% (relative to simultaneous-control measurements) within 5 h after acute transfer to 200% lake water, and remained significantly elevated relative to simultaneous controls even after long-term acclimation. In the 10% lake water treatment, cortisol levels remained very similar to those in the 100% lake water control group throughout, and simply tracked the decline over time seen in the latter. Even in fish of the 1% lake water treatment, which survived after gradual adaptation only, cortisol levels were not chronically elevated relative to simultaneous-control values.

Iono- and osmo-regulatory status during exposure to altered salinity–alkalinity

There were no significant changes in plasma Na^+ (initially 177 ± 7 mmol·L^{-1}; Fig. 2A), Cl^- (initially 157 ± 3 mmol·L^{-1}; Fig. 2B), or osmolality (initially 376 ± 6 mosmol·kg^{-1}; Fig. 2C) levels in the control treatment (100% lake water) during the exposures. At 5 h after acute transfer to 10% lake water, plasma Na^+ and Cl^- levels fell significantly by 24% relative to either initial or simultaneous-control values, with only slight recovery at 15 h (Figs. 2A and 2B). After long-term acclimation to 10% lake water, there was further recovery, but the levels of both ions remained significantly depressed. Plasma osmolality underwent very similar changes but recovered completely by 24 h (Fig. 2C). Long-term acclimation to 1% lake water resulted in levels of plasma Na^+ and Cl^- that were very similar to those measured at the same time in the 10% lake water treatment (Figs. 2A and 2B), but

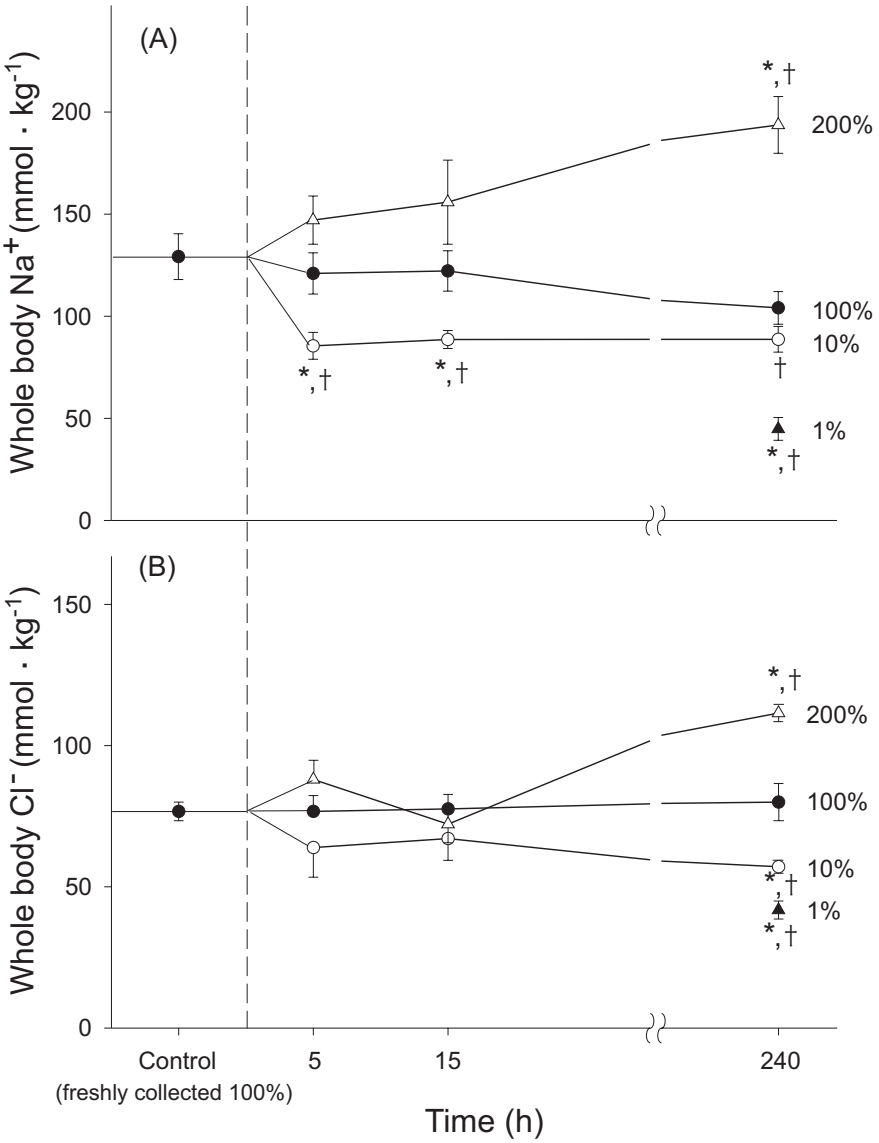
Fig. 2. The influence of acute transfer (from 100% lake water) and chronic acclimation to 200% (△), 10% (○), 1% (▲, chronic only), or 100% (control, ●) lake water on plasma Na^+ concentration (A), plasma Cl^- concentration (B), and plasma osmolality (C) in freshly collected Magadi tilapia. Only chronic data were obtained for the 1% treatment, as the fish died after acute transfer to 1% lake water. Chronic data are plotted at 240 h, as fish were held at the appropriate dilution for 10 days after gradual adaptation. Values are mean ± 1 SEM (N = 4–7 for Na^+ and osmolality and N = 7–15 for Cl^- at all points.) Asterisks indicate significant differences ($P < 0.05$) from the simultaneous 100% lake water control value; daggers indicate significant differences ($P < 0.05$) from the initial value for freshly collected fish in 100% lake water.



here osmolality was chronically depressed by 10% relative to simultaneous-control values (Fig. 2C). After acute transfer to 200% lake water, levels of plasma Cl^- increased by 8–10% at 5 and 15 h relative to both initial and simultaneous-control values, but recovery was complete after long-term acclimation (Fig. 2B). Plasma Na^+ and osmolality were not measured in the 200% lake water treatment, owing to limited sample volumes.

In comparing whole-body and plasma concentrations, we assumed that concentrations on a per kilogram basis were directly comparable with those on a per litre basis, recognizing that the small errors due to differences in density were within

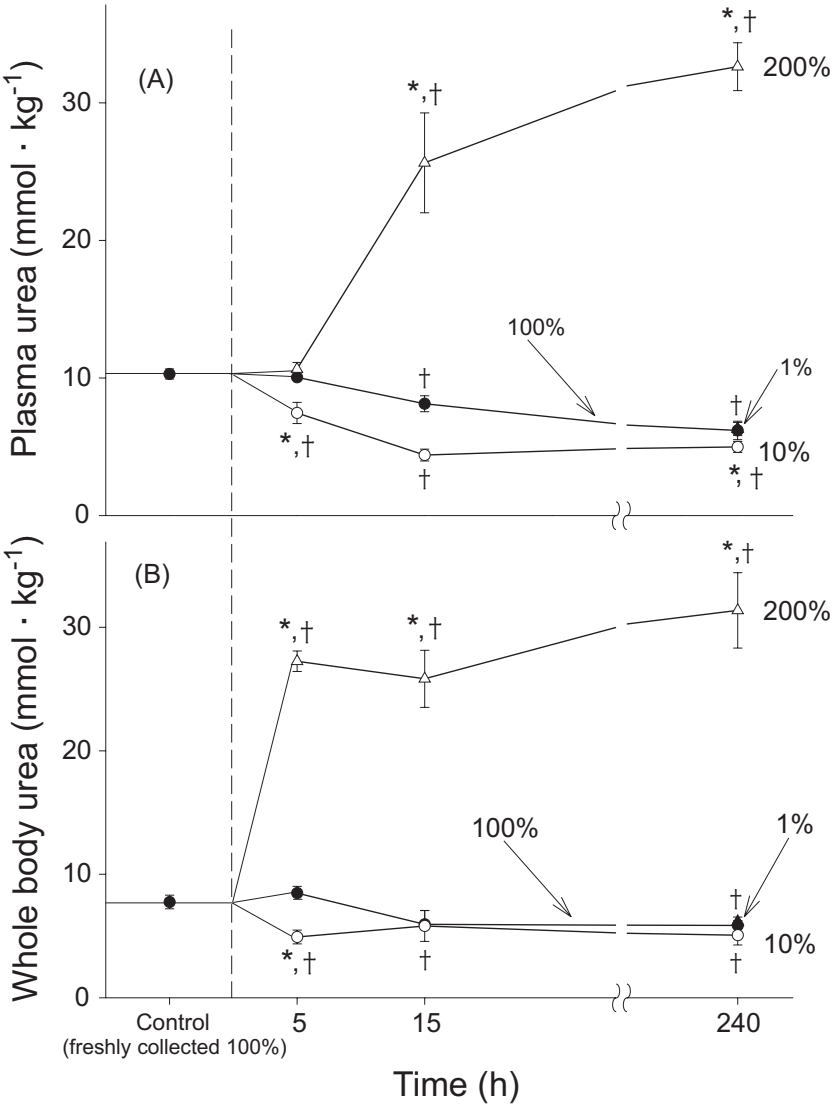
Fig. 3. The influence of acute transfer (from 100% lake water) and chronic acclimation to 200% (△), 10% (○), 1% (▲, chronic only), or 100% (control, ●) lake water on whole-body Na^+ concentration (A) and whole-body Cl^- concentration (B) in freshly collected Magadi tilapia. Only chronic data were obtained for the 1% treatment, as the fish died after acute transfer to 1% lake water. Chronic data are plotted at 240 h, as fish were held at the appropriate dilution for 10 days after gradual adaptation. Values are mean ± 1 SEM (N = 8–15 at all points.) Asterisks indicate significant differences ($P < 0.05$) from the simultaneous 100% lake water control value; daggers indicate significant differences ($P < 0.05$) from the initial value for freshly collected fish in 100% lake water.



the error of the analyses. Whole-body Na^+ (initially 129 ± 11 mmol·kg^{-1}; Fig. 3A) and Cl^- (initially 77 ± 3 mmol·kg^{-1}; Fig. 3B) concentrations were lower than plasma concentrations in 100% lake water (cf. Fig. 2) but were very high relative to values in "standard" teleosts (see Discussion) and did not change significantly throughout the exposures. The Na^+ to Cl^- ratio (averaging 1.5 in the 100% lake water control group and 1.1–1.5 in the other groups) did not appear to be unusual. Nevertheless, upon adaptation to both more concentrated and more dilute environments, ion levels in the whole body were more variable and showed larger relative changes than those in the plasma. At 5 and 15 h after acute exposure to 10% lake water, only the 29% decrease in whole-body Na^+ concentration relative to the simultaneous-control value was significant (Fig. 3A), whereas after long-term acclimation, only the 29% drop in whole-body Cl^- concentration remained significant relative to the simultaneous-control group (Fig. 3B). However, after chronic acclimation to 1% lake water, both levels had fallen by 48–57% relative to simultaneous controls. After acute transfer to 200% lake water, whole-body Na^+ and Cl^- concentrations tended to rise, but only the increases after long-term acclimation (86% for Na^+ and 39% for Cl^- relative to simultaneous controls) were significant (Figs. 3A and 3B).

In freshly collected control fish, urea concentrations in plasma (10.3 ± 0.4 mmol·L^{-1}; Fig. 4A) and the whole body (7.8 ± 0.6 mmol·kg^{-1}; Fig. 4B) were similar and fell slightly during laboratory holding, with the declines becoming significant by 15 h. These urea levels therefore accounted for only 2–3% of internal osmolality. However, internal urea

Fig. 4. The influence of acute transfer (from 100% lake water) to 200% (△), 10% (○), 1% (▲, chronic only), or 100% (control, ●) lake water on plasma urea concentration (A) and whole-body urea concentration (B) in freshly collected Magadi tilapia. Only chronic data were obtained for the 1% treatment, as the fish died after acute transfer to 1% lake water. Chronic data are plotted at 240 h, as fish were held at the appropriate dilution for 10 days after gradual adaptation. Values are mean ± 1 SEM (N = 8–15 at all points, except 240 h in 200% lake water, where N = 5.) Asterisks indicate significant differences ($P < 0.05$) from the simultaneous 100% lake water control value; daggers indicate significant differences ($P < 0.05$) from the initial value for freshly collected fish in 100% lake water.



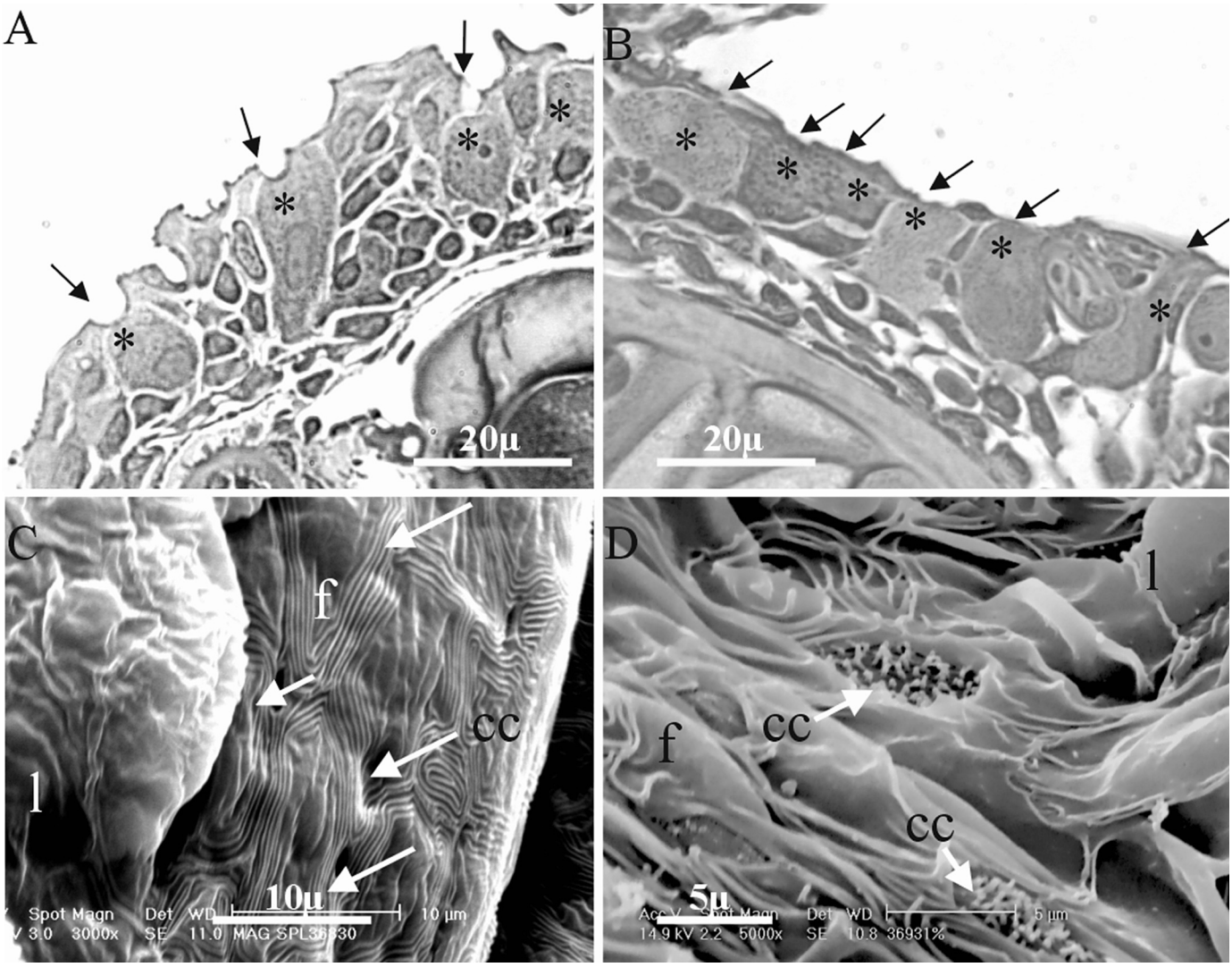
levels exhibited greater relative changes in response to environmental challenge than did internal ion levels. Plasma and whole-body urea fell significantly by 28 and 42%, respectively, at 5 h after acute transfer to 10% lake water (Figs. 4A and 4B). After long-term acclimation, only the depression of urea in the plasma of the 10% lake water group (Fig. 4A) remained significant relative to the simultaneous value in the 100% lake water control group; there were no significant differences in the 1% lake water group relative to simultaneous controls. However, the long-term control group also exhibited plasma and whole-body urea concentrations lower than those in freshly collected fish.

Urea changes were much more dramatic in fish acutely transferred to 200% lake water; by 5 h, the whole-body urea concentration had increased 3.3-fold. In animals having undergone long-term acclimation, a 5.3-fold elevation was seen. Both comparisons are relative to the simultaneous controls (Fig. 4B). In contrast, the plasma urea concentration did not change at 5 h, rose by 3.2-fold at 15 h, and reached the same 5.3-fold increase relative to simultaneous controls after long-term acclimation, thereby accounting for about 8% of internal osmolality (Fig. 4A).

Drinking rates

Drinking rates were measured only in control fish and in fish chronically adapted to 1% lake water. In Magadi tilapia adapted to 100% lake water, drinking rates were not significantly different between freshly collected fish and those having undergone long-term acclimation in the laboratory, averaging 8.01 ± 1.29 $mL \cdot kg^{-1} \cdot h^{-1}$ (N = 16) overall. These rates were comparable with or higher than those of marine teleosts living in full-strength seawater (see Discussion). After

Fig. 5. Representative semi-thin sections (A and B) and scanning electron micrographs (C and D) of the gill filament epithelium of Magadi tilapia in 100% lake water (A and C) or after long-term acclimation to 1% lake water (B and D). In 100% lake water (A and C), note that the apical membrane surfaces of chloride cells are burrowed in pits (arrows), whereas in 1% lake water (B and D), the apical surfaces of the chloride cells are flat and level with the filament epithelium (arrows). Note also villi ornamenting the apical membrane. CC and *, chloride cell; *l*, lamella; *f*, filament.



acclimation to 1% lake water, drinking still occurred at a similar rate, viz. 9.64 ± 2.97 $mL \cdot kg^{-1} \cdot h^{-1}$ (N = 6).

Branchial morphology

There were no substantive differences in branchial morphology between control fish freshly collected from 100% lake water, control fish held long-term in 100% lake water in the laboratory, and fish that had undergone long-term acclimation to 200% lake water. In all three groups, the basic morphology was similar to that described earlier (see Discussion), but with the important new finding of a second type of chloride cell. Magadi tilapia that had been adapted to 100 or 200% lake water displayed two distinct populations of chloride cells: either the apical surface of the chloride cell is recessed at the base of a pit edged by pavement cells (Figs. 5A and 5C) or the apical membrane lies level with the filament epithelium and is ornamented with microvilli, as is typically seen in fish acclimated to 1% lake water (Figs. 5B and 5D). In fish acclimated to 100 and 200% lake water, chloride cells of the first type were numerous and generally associated with neighbouring accessory cells, while cells of the second type were very rare. However, in fish having undergone long-term acclimation to 1% lake water, only chloride cells belonging to the second type were seen.

Discussion

Overview

Alcolapia grahami exhibits impressive tolerance, iono- and osmo-regulating effectively when gradually adapted to a range of salinity and alkalinity from 1 through 200% of its already extreme environment. In 100 and 200% lake water, it clearly hyporegulates, and in 10 and 1% lake water, it clearly hyperregulates. Plasma ions and osmolality are therefore subject to strong homeostatic regulation, being held at levels typical of standard seawater teleosts in 100 and 200% lake water

and levels typical of standard freshwater teleosts in 10 and 1% lake water, respectively. As outlined below, chloride-cell morphology varies in parallel with this hypo- and hyperregulation, with seawater-type cells predominating in fish acclimated to 100 and 200% lake water and freshwater-type cells being the only ones present in fish acclimated to 1% lake water (note that the 10% lake water samples were lost). In 100% lake water, the fish drinks at high rates and, curiously, this drinking continues unchanged after adaptation to 1% lake water. During acute exposures to altered salinity–alkalinity, there is a typical pattern of disturbance of plasma composition followed by recovery, and urea is exploited as an osmolyte to a small but significant extent. While whole-body Na^+ to Cl^- ratios are not unusual, absolute concentrations are unusually high and not as well regulated as plasma ions. Although the fish does not survive direct transfer to 1% lake water, it tolerates direct transfer to 10% lake water very well, and can be gradually adapted to both these media, as well as to 200% lake water. Clearly, however, the Magadi tilapia finds the downward transition easier than the upward one, based on mortalities occurring after acute transfer and rapid sustained elevations in plasma cortisol in fish exposed to 200% lake water.

Two differences worthy of note between our experimental treatments and the natural situation in Lake Magadi lagoons were the use of an artificial food rather than natural cyanobacteria and the constant O_2 regime achieved by continuous vigorous aeration of the holding tanks. In nature, the O_2 regime cycles from daytime hyperoxia to nighttime hypoxia (Narahara et al. 1996). It is difficult to predict how these factors may have affected the present ionoregulatory data, although it is noteworthy that there were no significant ionic changes in the long-term control group kept in 100% lake water. These fish did, however, exhibit modest declines in plasma and whole-body urea concentrations, which were probably in accord with the decreased metabolic rates and urea-production rates measured in these same fish and attributed to dietary limitation by Wood et al. (2002). Plasma cortisol levels also tended to fall with time, although it is not clear whether this was the result of an initial elevation due to the stress of capture and confinement or a reduction of stress due to the constancy of the laboratory holding conditions.

Iono- and osmo-regulation in 100% lake water

The present results confirm those of earlier studies (Leatherland et al. 1974; Eddy and Maloiy 1984; Wright et al. 1990) showing that Magadi tilapia living in 100% lake water, an environment with an osmolality equivalent to about half-strength seawater, hyporegulate overall (Fig. 2 versus Table 1) and maintain plasma Na^+ and Cl^- concentrations and osmolality at levels similar to those in marine teleosts in full-strength seawater (Holmes and Donaldson 1969). However, they do not confirm reports of very high (Maloiy et al. 1978) or very low (Skadhauge et al. 1980) plasma Na^+ to Cl^- ratios or reports that the whole-body Na^+ concentration is twice that of Cl^- (Eddy et al. 1981; Eddy and Maloiy 1984). Some of these discrepancies may relate to analytical techniques (e.g., the use of an electron microprobe by Skadhauge et al. 1980; incomplete extraction of Na^+ and (or) Cl^- by water or HNO_3 homogenization, discussed by Eddy and Maloiy 1984). However, sample contamination may also be an issue; in our experience, particular care must be taken at this field site, where not only the water but also the air is rich in Na^+ and Cl^- because of the prevalence of trona dust.

One unusual ionoregulatory feature appears to be the very high whole-body Na^+ and Cl^- concentrations maintained in 100% lake water (Fig. 3), values that are 1.5- to 2.0-fold higher than in standard marine teleosts (Holmes and Donaldson 1969). Furthermore, whole-body ion levels were not well regulated during long-term exposure to altered salinities. Eddy et al. (1981) reported a similarly high whole-body Na^+ level: 141 mmol·kg^{-1} (versus 129 mmol·kg^{-1}in the present study). Since these fish drink, the presence of imbibed lake water (100% lake water = 355 mmol·L^{-1} Na^+, 113 mmol·L^{-1} Cl^-; Table 1) in the gastrointestinal tract may be a contributing factor. However, to explain the "elevation" in these whole-body values for Na^+ alone, about 20% of the body mass would have to be ingested lake water, which seems unlikely. Interestingly, there is a single report of muscle Na^+ concentration in this species and it is also very high: 117 mmol·kg^{-1} (Maloiy et al. 1978; versus <30 mmol·kg^{-1} in standard marine teleosts, Holmes and Donaldson 1969). A study of ionoregulation at the tissue and cell levels in this species may be profitable.

At the whole-animal level, the present data help clarify the ionoregulatory mechanisms of the Magadi tilapia. We here confirm that these fish do drink in 100% lake water, at a mean rate of 8 mL·kg^{-1}·h^{-1}. While this rate is below the exceptional values (mean 24 mL·kg^{-1}·h^{-1}, range 7–50 mL·kg^{-1}·h^{-1}) reported by Maloiy et al. (1978) for this species, it is nevertheless at the high end of typical values for marine teleosts in full-strength seawater (2–10 mL·kg^{-1}·h^{-1}; Wilson et al. 1996; Fuentes and Eddy 1997). Recently, Bergman (2000) has shown that in the Magadi tilapia, imbibed alkaline lake water can bypass the acidic stomach by virtue of a unique gastrointestinal morphology and that about 75% of the imbibed water, Na^+, Cl^-, and HCO_3^-/CO_3^{2-} are absorbed from the gastrointestinal tract. Thus, about 2200 μmol·kg^{-1}·h^{-1} of Na^+ are absorbed through the gut. According to Eddy et al. (1981) and Eddy and Maloiy (1984), branchial Na^+ efflux rates are about 4000 μmol·kg^{-1}·h^{-1}, and according to Wright et al. (1990), branchial Na^+ influx rates are about 2100 μmol·kg^{-1}·h^{-1}, so there appears to be a good balance for Na^+. For Cl^-, approximately 700 μmol·kg^{-1}·h^{-1} is absorbed through the gut, and according to Eddy et al. (1981), branchial Cl^- efflux rates are about 1650 μmol·kg^{-1}·h^{-1}, so gill Cl^- influx must be about 950 μmol·kg^{-1}·h^{-1}. This synthesis of data from various sources strongly supports the view that a very low gill permeability, and accompanying low branchial Na^+ and Cl^- turnover rates, are important adaptations to this extreme environment (Eddy et al. 1981; Eddy and Maloiy 1984). Gill turnover rates for typical marine teleosts at comparable salinity are 5- to 10-fold higher (Evans 1984).

Iono- and osmo-regulation in concentrated and dilute environments

In accord with Maina (1990) but in contrast with Maloiy et al. (1978), we found that the fish do not survive direct transfer to very dilute water (1% lake water). Maloiy et al. (1978) worked at a much lower temperature (22°C), which may explain this difference. Nevertheless, it is clear that the

Magadi tilapia is quite tolerant of more dilute environments, osmoregulating well after direct transfer to 10% lake water and, after gradual adaptation, transfer to 1% lake water (Fig. 2). The latter observation agrees with Leatherland et al. (1974), who used a similar gradual-dilution scheme for adaptation to fresh water. It is curious that the relatively high drinking rate observed in 100% lake water continued unchanged in fish that had undergone long-term acclimation to dilute lake water (1%). Osmotically, this appears distinctly disadvantageous, but is perhaps related in some way to the normal functioning of the digestive tract, given the unique gastrointestinal anatomy (stomach bypass system) of this species described by Bergman (2000).

In parallel studies, Wood et al. (2002) found that plasma total CO_2 (and thus HCO_3^-) was regulated at unchanged levels after long-term acclimation to 1, 10, or 100% lake water. Balancing Cl^- versus HCO_3^-/CO_3^{2-} regulation may be a particular problem for these fish, because Cl^- must normally be taken up against an electrochemical gradient (plasma-to-water) yet HCO_3^-/CO_3^{2-} must be excreted against an electrochemical gradient (Wood et al. 1994). For the latter, Laurent et al. (1995) suggested that the Magadi tilapia uses seawater-type chloride cells to operate a branchial Na^+ plus HCO_3^-/CO_3^{2-} active excretion scheme analogous to the standard Na^+ plus Cl^- active excretion mechanism seen in the gills of marine teleosts (Marshall 1995). Laurent et al. (1995) also hypothesized a freshwater type Cl^-/HCO_3^- exchanger on the apical membrane of the chloride cells for Cl^- uptake. Certainly, the ability of the fish to hyper-regulate plasma Cl^- (and Na^+) after acute transfer to lower dilutions (Fig. 2B) demonstrates that a branchial capacity for active Cl^- uptake (and Na^+ uptake) can occur.

In this regard, the morphological observations on the gills in the present study (Fig. 5) are relevant. The basic ultrastructure of the gills of *A. grahami* is similar to that described earlier by Maina (1990) and Laurent et al. (1995) but with the important new finding of a second type of chloride cell; cells of this new type were present in low numbers in fish acclimated to concentrated media but became more abundant (indeed the only type present) in fish acclimated to dilute media (1% lake water). This second type of cell, with a relatively flat apical exposure ornamented by microvilli, appears to be typical of chloride cells identified by other authors as the freshwater type, while the recessed form with an apical pit appears to be typical of the seawater type; similar chloride-cell pleomorphism has been well documented in other euryhaline cichlids (Laurent 1984; Perry et al. 1992; Kültz et al. 1995; Van Der Heijden et al. 1997). The few chloride cells of the freshwater type present in fish acclimated to 100% lake water may provide the fish with a Cl^-/HCO_3^- exchange mechanism for active Cl^- uptake in lake water, while the dominant seawater type performs active Na^+ plus HCO_3^-/CO_3^{2-} excretion, as hypothesized by Laurent et al. (1995). After acute exposure to dilute media (Fig. 2), these freshwater-type chloride cells would allow active uptake of Na^+ (by direct or indirect Na^+/H^+ exchange) and Cl^- exchange (by Cl^-/HCO_3^-) in the normal freshwater manner (Marshall 1995), thereby limiting the immediate decline in these plasma ions. Essentially perfect hyperosmotic regulation occurs in the longer term (Fig. 2), after the freshwater type has become the sole type present.

In the present study, the fish regulated plasma Cl^- effectively (Fig. 2B) when exposed to 200% lake water, probably by shutting down the uptake mechanism and activating excretion mechanisms, since in this medium, external Cl^- exceeded internal levels by about 50 $mmol \cdot L^{-1}$ (cf. Table 1). The failure of Magadi tilapia to survive in lake water neutralized with HCl (i.e., replacement of HCO_3^-/CO_3^{2-} with an additional 300–400 $mmol \cdot L^{-1}$ Cl^-; Wood et al. 1989, 1994) is probably due to Cl^- invading the body fluids (Wright et al. 1990), that is, the animal's physiology is designed mainly to acquire rather than to excrete Cl^- and a limited excretory capacity is overwhelmed. Unfortunately, plasma Na^+ and total CO_2 (i.e., HCO_3^-/CO_3^{2-}) were not measured in the 200% lake water series. It is possible that a failure to control either or both of these during acute exposure to more concentrated and more alkaline lake water led to stress and mortality. In this regard, the sustained surge in plasma cortisol (Fig. 1), which is well known to cause seawater-type chloride cell proliferation and increased Na^+,K^+-ATPase activity in euryhaline teleosts (McCormick 1995), may be adaptive in increasing the capacity for Na^+ and HCO_3^-/CO_3^{2-} excretion by the scheme of Laurent et al. (1995). This proliferation takes a few days to occur (McCormick 1995), which may explain why gradual acclimation to 200% lake water was much better tolerated than acute transfer.

The osmoregulatory role of urea

Wood et al. (2002) demonstrated that the Magadi tilapia remains 100% ureotelic, continuing to produce and excrete only urea (albeit at a reduced rate) even when acclimated to environments as dilute as 1% lake water. The present observations (Fig. 4) demonstrate that urea plays a small but significant role in osmoregulation. Although urea accounts for only 2–3% of plasma osmolality in fish acclimated to 100% lake water, urea concentrations respond to a greater extent than plasma ions to both hypo- and hyper-osmotic challenges. The increases in both plasma and whole-body urea upon exposure to 200% lake water were particularly marked (over 500%; Fig. 4) and much larger than the corresponding increases in plasma (8–10%) and whole-body (39–86%; Figs. 2 and 3) Na^+ and Cl^- levels. Obviously, these urea changes can account for only a small fraction of plasma and tissue osmolality (maximum 8%) and thus are much less important than the 30–40% contribution commonly seen in marine elasmobranchs (Wood 1993). Nevertheless, the rapidity of the response may be critical to survival. Recently, Wilson et al. (submitted for publication)[2] found a direct positive correlation between whole-body urea levels and survival time of Magadi tilapia during acute challenge with 500% lake water. To our knowledge, this is the first definitive evidence that urea plays an osmoregulatory role in salinity adaptation in any teleost (Wood 1993; Korsgaard et al. 1995), although some studies on euryhaline marine teleosts (e.g., Wright et al. 1995) have suggested that urea may be involved.

[2] P.A. Wilson, C.M. Wood, P.J. Walsh, H.L. Bergman, A.N. Bergman, P. Laurent, S. Kisia, and B.N. White. Discordance between genetic structure and morphological and physiological adaptation in Lake Magadi tilapia. Submitted for publication.

Since measurements of urea fluxes were not made during the acute-exposure experiments, it is not clear whether urea production rate in the tissues, excretion rate via the gills, or both, were adjusted. However, plasma and whole-body levels of urea appear to be normally about equal (Fig. 4), in accord with earlier measurements of equilibrium between concentrations in plasma and white muscle (Wood et al. 1989). Therefore, the fact that whole-body urea concentration increased more rapidly than plasma urea concentration during acute challenge with 200% lake water, and fell to a greater relative extent during acute challenge with 10% lake water, suggests that both processes may be modulated. Regulation of the UT-A type facilitated diffusion transport system for urea in the gills of the Magadi tilapia (Walsh et al. 2001) may play a role in this regard.

Ecological relevance

Recently, we found an isolated population of Magadi tilapia that lives in the surface water flow from a small dilute spring (26% lake water) that overlies a toxic underlying environment (700–1000% lake water; Wilson et al., submitted for publication[2]). These fish likely exploit a "freshwater" osmoregulatory strategy most of the time but can tolerate brief excursions into the underlying toxic environment to feed on algae. We have also discovered separate populations of Magadi tilapia living in isolated lagoon systems with osmolalities ranging from 278 to 1689 mosmol·kg^{-1}, that is, approximately 50–290% lake water. Thus, given a long enough adaptation time, Magadi tilapia can exploit the "seawater" osmoregulatory strategy to adapt to concentrations even higher than the 200% lake water tested here. However, we also found that following a torrential rainstorm that flooded the surface of the trona, the overlying floodwater had an osmotic pressure greater than 3000 mosmol·kg^{-1} (due to trona dissolution). Lake water of this concentration (approximately 500%) was acutely toxic (<30 min) to all populations, in accord with the original anecdotal observations of Coe (1966). The limits to physiological tolerance may therefore serve as a barrier to gene flow between different lagoon populations (Wilson et al., submitted for publication[2]).

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