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Ionoregulatory physiology of two species of African lungfishes *Protopterus dolloi* and *Protopterus annectens*

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Basic ionoregulatory physiology was characterized in two species of African lungfish, slender African lungfish *Protopterus dolloi* and West African lungfish *Protopterus annectens*, largely under aquatic conditions. There were no substantive differences between the two species. Plasma [Na], [Cl] and [Ca] were only 60–80% of those typical of freshwater teleosts, and plasma Ca activity was particularly low. Unidirectional Na and Cl influx rates from water were also very low, only *c*. 10% of teleost values, whereas unidirectional Ca influx rates were comparable with teleost rates. *Protopterus* spp. were fed a 3% ration of bloodworms every 48 h. The bloodworm diet provided similar amounts of Na and Ca as uptake from water, but almost no Cl. Efflux rates of Na and Cl through the urine were greater than *via* the faeces, whereas the opposite was true for Ca. Net ion flux measurements and ionic balance sheet calculations indicated that (1) both water and dietary uptake routes are important for Na and Ca acquisition; (2) the waterborne route predominates for Cl uptake; (3) unidirectional ion effluxes across the body surface (gills and skin) rather than urine and faeces are the major routes of loss for Na, Cl and Ca. Tissues (muscle, liver, lung, kidney, intestine and heart) and plasma ions were also examined in *P. dolloi* 'terrestrialized' in air for up to 5 months, during which plasma ion concentrations (Na, Cl, Ca and Mg) did not change and there were only a few alterations in tissue ions, that is, increased [Na] in intestine, decreased [Cl] in kidney and increased [Ca] in liver and kidney.

Key words: calcium; chloride; feeding; ion-fluxes; sodium; terrestrialization.

INTRODUCTION

Molecular evidence suggests that lungfishes are the closest relative to the ancestor of tetrapods, although the exact relationships of lungfishes, coelocanths and tetrapods remain unclear (Takezaki *et al*., 2004). Furthermore, they appear to have changed very little over the course of 300 million years (DeLaney *et al*., 1977). Thus, research involving lungfishes allows for a better understanding of adaptive mechanisms involved in the transition from aquatic to terrestrial environments. There

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are six extant species of lungfishes, the Australian lungfish, Queensland lungfish *Neoceratodus forsteri* (Krefft), South American lungfish, American mud fish *Lepidosiren paradoxa* Fitzinger, and four African lungfish: marbled lungfish *Protopterus aethiopicus* Heckel, East African lungfish *Protopterus amphibius* (Peters), West African lungfish *Protopterus annectens* (Owens) and slender lungfish *Protopterus dolloi* Boulenger. The African lungfishes have characteristically underdeveloped internal gills compared with those of freshwater teleosts but like amphibians possess a well-vascularized skin that is capable of ion, nitrogenous waste and water exchange (Smith, 1930; Laurent *et al*., 1978; Sturla *et al*., 2001; Wood *et al*., 2005*a*, *b*; Wilkie *et al*., 2007). The African lungfishes are also obligatory air breathers, possessing a primitive lung, which is a diverticulum of their air bladder (Johansen, 1970). In addition, they exhibit a remarkable ability to survive in air and to aestivate for long periods of time (Smith, 1930; Janssens, 1963; Janssens & Cohen, 1966; DeLaney *et al*., 1974, 1977; Laurent *et al*., 1978; Fishman *et al*., 1986; Greenwood, 1986; Chew *et al*., 2004; Wood *et al*., 2005*a*; Wilkie *et al*., 2007). Thus, they are able to survive in both aquatic and terrestrial environments.

Currently, little is known about ion and water balance under aquatic conditions in the lungfishes and even less is known about this area during periods of air exposure or aestivation (Wright, 2007). The present study examined the basic ionoregulatory physiology of two African lungfishes, *P. dolloi* and *P. annectens*. *Protopterus dolloi* are most commonly found in central Africa in equatorial conditions (usually hot and wet throughout the year, but with unpredictable severe droughts), whereas *P. annectens* are commonly found in regions of north-west, north-east and southern Africa under more temperate conditions (seasonally fluctuating climate with a regular, less extreme dry season every year; Greenwood, 1986). In accord with these habitat differences, there are some behavioral differences between the two species including aestivation frequency and duration, as well as nest size and mating (Greenwood, 1986).

Freshwater fishes exchange Na and Cl across their gills, linking Na uptake to acid and ammonia excretion (H^+ and NH_4^+) and Cl uptake to base excretion (HCO_3^-), to maintain acid and base balance; Ca is also largely taken up by the gills (Perry, 1997; Marshall, 2002; Evans *et al*., 2005). Although the reduced gill area of the African lungfishes plays a minor role in O_2 uptake (*c*. 0–40%), it is theorized to still play an important role in CO_2 excretion (*c*. 60≥ %) and ionic exchange (McMahon, 1970; Laurent & Dunel, 1976; Laurent *et al*., 1978; Fishman *et al*., 1986; Wood *et al*., 2005*a*; Gilmour *et al*., 2007; Iftikar *et al*., 2008). In *P. dolloi*, however, Wilkie *et al*. (2007) reported very low uptake rates of Na and Cl from the aquatic environment, suggesting that nutritional uptake of these ions may normally be very important in this regard. At present, there appear to be no studies on the role of feeding in ion acquisition by the African lungfishes, on the relative importance of ion uptake from the water *v*. the diet or on the loss of ions in the faeces (Wright, 2007). There have been a few previous studies (Sawyer *et al*., 1982) of the ionoregulatory component of renal function in African lungfishes (Patel *et al*., 2009), but only one in *P. annectens* (Babiker & Rankin, 1979) and none in *P. dolloi*.

With this background in mind, the following objectives were established. First, the basic ionoregulatory physiology of *P. annectens* and *P. dolloi* was investigated under aquatic conditions: plasma Na, Cl and Ca levels, unidirectional and net uptake rates of these ions from the external water, potential uptake rates from food and loss rates *via*

the faeces and urine. Renal data are provided from a companion study (Patel *et al.*, 2009) examining water balance and renal function in these same two species. An important conclusion of that study was that although osmotic permeability and urine flow rates were similar to those of teleosts, the diffusive water permeabilities of these two lungfish species were very low relative to those of teleosts. This was interpreted as a key pre-adaptation to life on land, and similarly low diffusive permeability of the epithelium to ions may also be expected. A second goal was to use these various measurements to construct ion balance budgets for the two species; in view of their differences in habitat and behaviour, it was hypothesized that there might well be differences in ion budgets. In view of the scarcity of these animals, the techniques used were largely non-lethal, although an opportunity arose to obtain terminal tissue samples from *P. dolloi* as part of study of its responses to prolonged air exposure ('terrestrialization'; Wilkie *et al.*, 2007; Staples *et al.*, 2008). Therefore, a final goal was to examine tissue ion levels following this extended period of air exposure.

MATERIAL AND METHODS

EXPERIMENTAL ANIMALS

African lungfishes (*P. dolloi* and *P. annectens*) with an average mass of 150 g (range: 68–210 g) were collected in central and western Africa and were then shipped from a commercial dealer in Singapore (where they had been kept in aquaria for 1 month) to McMaster University (Hamilton, Ontario, Canada). All lungfishes were held at 27–30° C under a 12L:12D photoperiod in individual aquaria containing 3 l of dechlorinated tap water supplemented with a small amount of commercial sea salts that helped to prevent fungal infections. This resulted in a water composition of *c*. [Na] = 2·0 mmol l^{-1}, [Cl] = 1·8 mmol l^{-1}, [Ca] = 1·2 mmol 1^{-1}, hardness = 170 mg l^{-1} as $CaCO_3$ equivalents and a pH of 7·8. The fish holding water was changed every second day, and following the water change fishes were fed a diet of recently thawed chironomid *Chironomus* sp. larvae (frozen bloodworms) at a ration of 3% of body mass every second day. All experimental and holding procedures followed Canadian Council on Animal Care guidelines and were approved by the McMaster University Animal Care Committee.

PLASMA IONS

Fishes ($n = 6$ for each species) held under aquatic conditions were lightly anaesthetized using 0·25 g l^{-1} MS-222. Blood samples (100–300 μl) were taken by caudal puncture using lithium-heparinized syringes (Sigma-Aldrich; www.sigmaaldrich.com) fitted with 23 G needles. Blood samples were centrifuged at 13 000 **g** for 5 min and the plasma was transferred to a separate 500 μl centrifuge tube and stored at −80° C until it was analysed for Na, Cl and Ca concentrations. The fishes were returned to their tanks and allowed to recover for a month prior to any other experimentation.

TISSUE IONS IN *P. DOLLOI* AND TERRESTRIALIZATION

The purpose of this study was to better understand the distribution of ions (Na, Cl and Ca) through various tissues (muscle, liver, lung, kidney, intestine and heart) of *P. dolloi*, and how they might change during prolonged air exposure ('terrestrialization'). Samples were collected in conjunction with a 'terrestrialization' study (Wilkie *et al.*, 2007; Staples *et al.*, 2008). To achieve 'terrestrialization' conditions, each tank was drained and then 20 ml of water was added to the tanks so that a thin film of water covered the bottom of the tank. The animals were sprayed with a fine mist of water every 6 days to maintain the humid conditions. The

'terrestrialized' fish were held under complete darkness at a temperature of 24–27° C. The cocoon formation began 1–2 days after initiating terrestrialization and following 1 week the cocoon had completely covered the animal's dorsal body surface, whereas the ventral surface remained in contact with the water film. Following terrestrialization, the fish were briefly re-immersed in water for terminal anaesthesia (0·5 g l^{-1} MS-222), followed by caudal puncture blood sampling as above, then a cephalic blow and decapitation. Aquatic *P. dolloi* were similarly killed to serve as a comparison. Plasma was processed as above, and tissues (white muscle, liver, lung, kidney, intestine and heart) were quickly dissected, frozen immediately in liquid N_2 and then preserved in a −80° C freezer. Three experimental conditions were examined: (1) aquatic fish, (2) fish that were terrestrialized for 1 month and (3) fish that were terrestrialized for 5 months ($n = 2–5$ at each time period for various tissues).

Plasma samples were analysed for total Na, Cl and Ca concentrations. Tissue samples were digested in 1 N HNO_3 (5:1 volume to tissue ratio; Fisher Scientific; trace metal grade; www.fishersci.com) at 65° C for 48 h and then homogenized by vortexing at which time a 2 ml aliquot was removed and centrifuged at 13 000 **g** for 10 min. The supernatant was then appropriately diluted and analysed for Na, Cl and Ca.

NET ION FLUXES FOLLOWING FEEDING

The objective here was to monitor potential changes related to feeding in net ion balance (Na, Cl and Ca) with the water. Aquatic fishes (six *P. dolloi* and six *P. annectens*) adapted to the regular feeding schedule described above were each set up in 2 l of continually aerated water. At $t = 0$ h, each fish was fed 5 g of thawed bloodworms (which approximates their usual 3% ration). All food was consumed within the ensuing 30 min. Starting immediately before feeding at $t = 0$ h, two 5 ml water samples were taken hourly for 8 h and then every 12 to 48 h following the feeding event. The experiment was then repeated with the same protocol, but there was no feeding event in the second series. All samples were stored at −20° C until analysis was carried out for ions (Na, Cl and Ca). The initial sample taken from each tank immediately before feeding was used as the 0 h reference value for each individual fish. Therefore, ion data at each consecutive sample time have been presented as differences from the initial time point (*i.e.* cumulative change) in units of concentration (mmol l^{-1}) in Figs 1 and 2.

Calculations of net flux rates (J_{net}, μmol kg^{-1} h^{-1}) for the ion budgets were based on changes of total ion concentrations (Na, Cl and Ca) in the water when compared with the initial ion concentrations: $J_{net} = ([X_i] - [X_f])V(TW)^{-1}$, where $[X_i]$ = concentration of total ion in the water at the beginning of the flux period (μmol l^{-1}), $[X_f]$ = concentration of total ion in the water at the end of the flux period (μmol l^{-1}), V = volume of water (l), T = time of flux period (h) and W = mass of the fish (kg).

A 'blank' experiment was performed to check on whether the addition of the bloodworms themselves to the water affected water ion levels during the 0·5 h feeding period in the preceding study. Six tanks (identical to the aquaria housing the fishes) were set up with water but no fishes. Three of those tanks were used as a control (test water) and the other three were used for a leaching test (test water plus 5 g of bloodworms). Water ion levels were measured before and after 0·5 h, with and without the addition of bloodworms.

NET IONS AVAILABLE FROM FOOD

In a separate test, 5 g of bloodworms (three replicates) were digested in concentrated nitric acid (100 μL mg^{-1} dry mass) for 72 h. Subsequently, hydrogen peroxide (40 μL mg^{-1} dry mass) was added to complete the digestion and the samples were incubated a further 24 h before being analysed for ions. This more vigorous digestion than used for fish tissue was found to be necessary to liberate all ions from these insect larvae. Net ions available from the diet were estimated based on the measured ionic composition of the bloodworms, a meal size of 5 g of bloodworms for a 150 g fish, and a 48 h feeding interval.

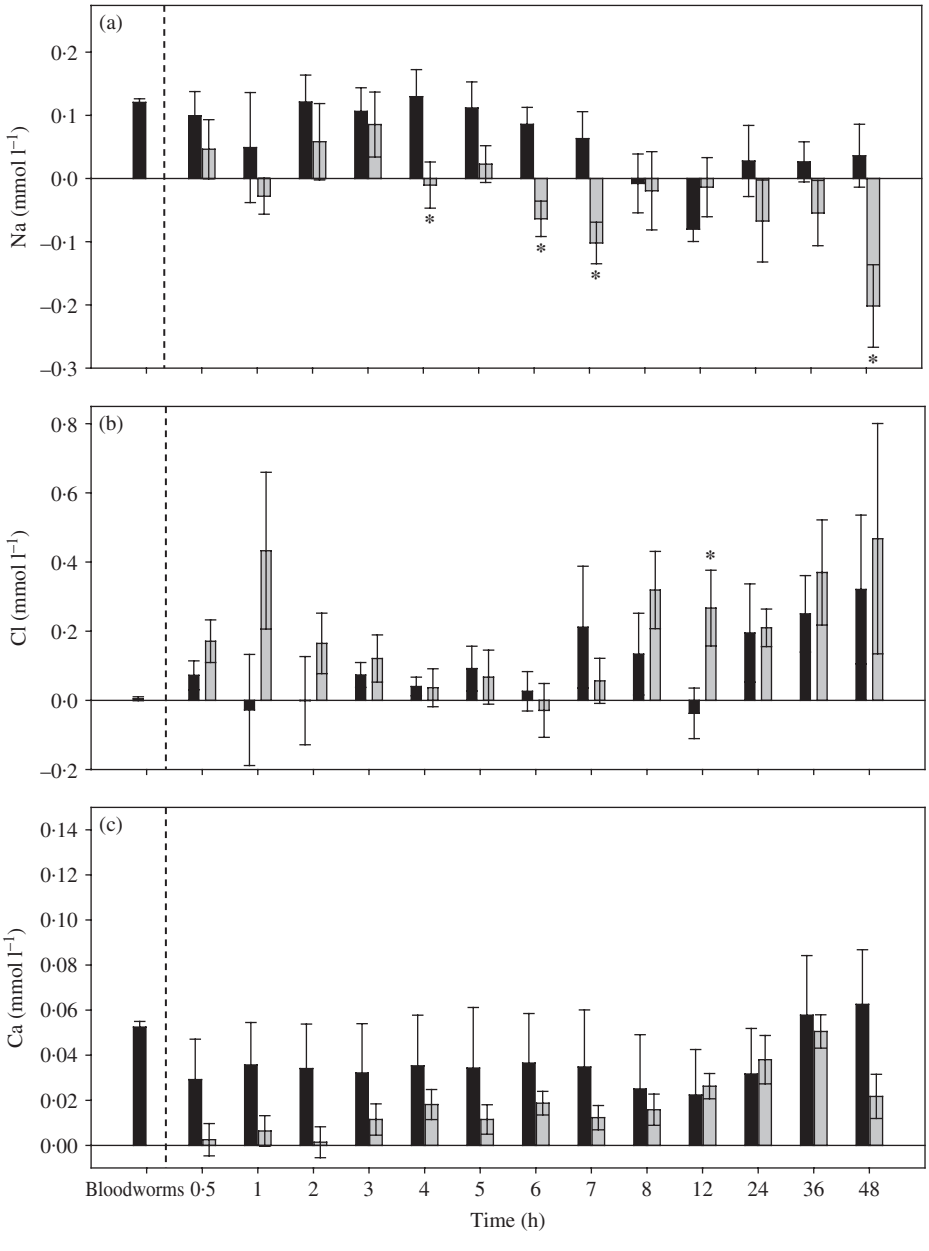

FIG. 1. Cumulative changes in water ion concentrations: (a) Na (b) Cl and (c) Ca relative to initial water composition over 48 h following a feeding ▒ and non-feeding ■ event for *Protopterus dolloi.* Values are means ± S.E. ($n = 6$). Positive values represent gains by the water (*i.e.* losses from the fish); negative values represent losses from the water (*i.e.* gains by the fish). On the far left, before the dashed line, the bar illustrates the maximum change in water ion concentration expected if the total ion content of the bloodworm *Chironomus* sp. meal (5 g = 3% ration) were to be released to the 2 l water volume. *significant difference (unpaired t-test, $P < 0{\cdot}05$) between fasted and fed *P. dolloi*.

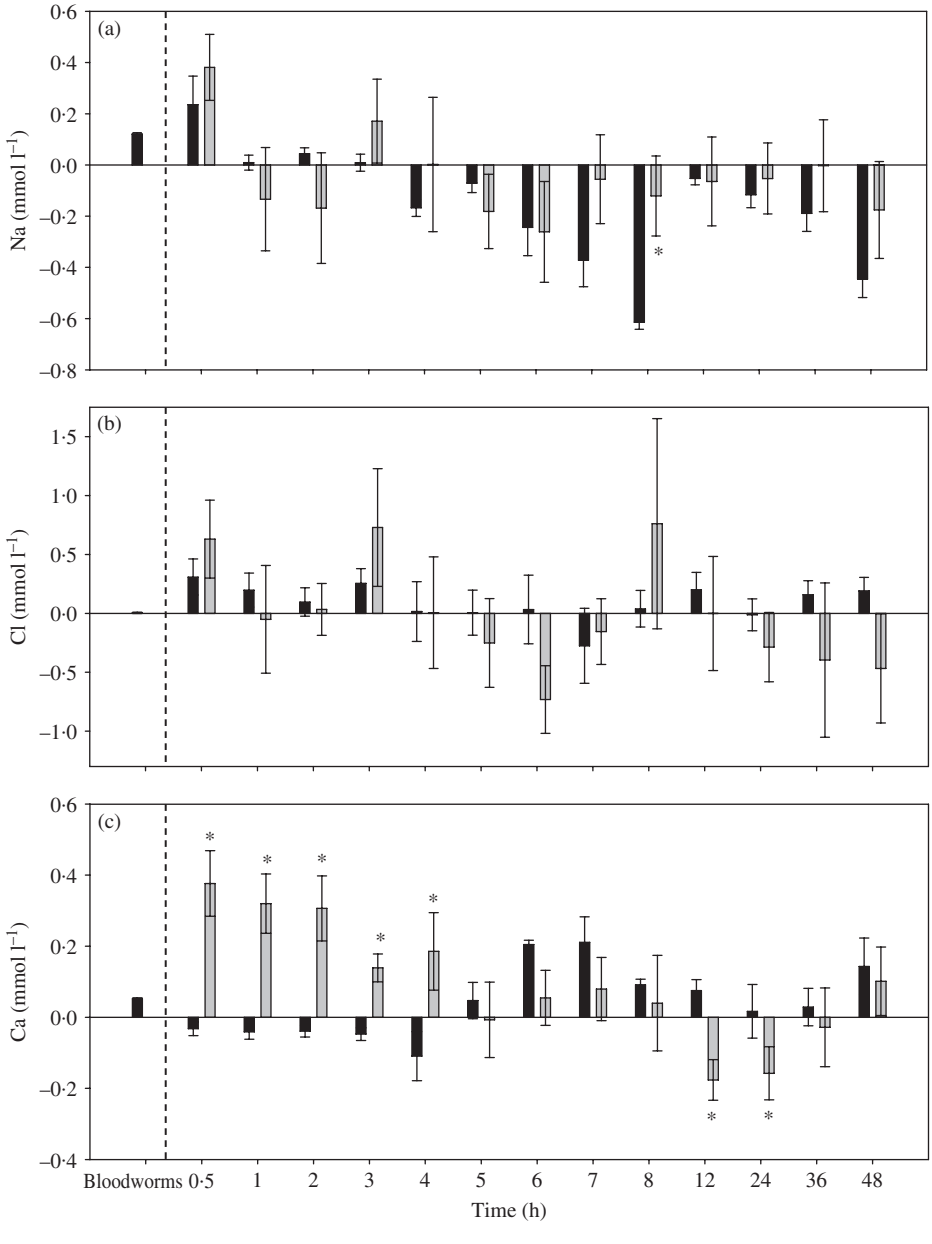

FIG. 2. Cumulative changes in water ion concentrations: (a) Na, (b) Cl and (c) Ca relative to initial water composition over 48 h following a feeding ▭ and non-feeding ■ event for *Protopterus annectens*. Values are means ± S.E. ($n = 6$). Positive values represent gains by the water (*i.e.* losses from the fish); negative values represent losses from the water (*i.e.* gains by the fish). On the far left, before the dashed line, the bar illustrates the maximum change in water ion concentration expected if the total ion content of the bloodworm *Chironomus* sp. meal (5 g = 3% ration) were to be released to the 2 l water volume. * significant difference (unpaired t-test, $P < 0{\cdot}05$) between fasted and fed *P. annectens*.

UNIDIRECTIONAL ION UPTAKE RATES

Three separate radiotracer experiments were carried out to determine unidirectional influx rates of Na, Cl and Ca, respectively, from the external water. In each experiment, six *P. dolloi* and six *P. annectens* were exposed to one radiotracer. In the first experiment, ^{22}Na (1 μCi l^{-1}; PerkinElmer Life and Analytical Sciences; http://las.perkinelmer) was used. In the second, ^{36}Cl (1 μCi l^{-1}; American Radiolabeled Chemicals Inc; www.arc-inc.com), and in the third, ^{45}Ca (3 μCi l^{-1}; PerkinElmer Life and Analytical Sciences), always in 2 l of water. For each animal in each experiment, three 5 ml samples were taken hourly for 8 h; two of the samples were used for radioactive counting and the other sample stored until analysis of the total concentration of the relevant ion. Influx rates (J_{influx}, μmol kg^{-1} h^{-1}) were determined by monitoring the disappearance of radioactivity from the water, and calculated as: $J_{\text{influx}} = (C_{\text{PMi}} - C_{\text{PMf}})V(A_{\text{S}}TW)^{-1}$, where C_{PMi} = radioactive counts of the ion in the water (cpm l^{-1}) at the beginning of the flux period, C_{PMf} = radioactive counts of the ion (cpm l^{-1}) in the water at the end of the flux period and A_{S} = mean specific activity of each isotope in the water (cpm μmol^{-1}).

A ratio of ≤1:10 of estimated A_{S} (cpm μmol^{-1}) in the fish relative to measured A_{S} in the water was set as the threshold for termination in the experiment as this was when recycling of radioactivity would become a significant source of error (Wood, 1988). Because Na, Cl and Ca influx rates were very low, however, this threshold was never reached, and the mean uptake of C_{PM} from the water over the entire 8 h period was used to provide the most accurate estimates of influx rates.

FAECAL ION LOSS RATES

In initial observations, it was found that in fishes on the standard feeding regime never defecated prior to 12 h post-feeding, but most had defecated by 24 h, with little excreta passed thereafter. Therefore, 12 h post-feeding was chosen as the time for faecal sampling. *Protopterus dolloi* and *P. annectens* ($n = 6$ for each) were fed the normal bloodworm meal (3% ration), and then at 12 h post-feeding were taken from their tanks without anaesthesia. Gentle pressure was applied to the body cavity so as to extrude all available faecal materials from the posterior intestine through the cloaca. This material was then weighed and frozen for later ionic analysis. Faecal samples were digested in 1 N HNO_3 and analysed for Na, Cl and Ca by the same methods as used for tissue samples.

Faecal ion loss rates (R_{fi}) for each ion (x) were calculated based on the assumption that by gently massaging the fishes all of the faeces that would accumulate for that feeding session would be extruded. Because the fishes were fed only once every 48 h, this was the time interval used to calculate the time averaged egestion rates: $R_{\text{fi}} = X(WT)^{-1}$.

URINARY ION EXCRETION RATES

In the companion study (Patel *et al.*, 2009), external urinary catheters similar to those developed by Curtis & Wood (1991) were used to determine urine composition and flow rate, and thereby measurements of renal excretion rates. The experimental fishes had been starved for 5 days to avoid the potential for faeces to block the catheters that were sewn onto the cloaca. Excretion rates (μmol kg^{-1} h^{-1}) were calculated as the product of urine flow rate (UFR; ml kg^{-1} h^{-1}) and the relevant ion (Na, Cl and Ca) concentration (μmol ml^{-1}) in the urine. Detailed methods for this experiment have been reported by Patel *et al.* (2009).

ANALYTICAL TECHNIQUES AND CALCULATIONS

Concentrations of Na and Ca in water, urine, plasma, faecal and tissue digests were determined by flame atomic absorption spectroscopy (FAAS) using a 220 FS SpectrAA (Varian; www.varian.com). Water, urine, plasma, faecal and tissue digests were diluted with 0·2% lanthanum for Ca determinations. Water, urine and plasma Cl concentrations were analysed spectrophotometrically by the mercuric thiocyanate method (Zall *et al.*, 1956).

Ca activity was measured in *P. annectens* and *P. dolloi* plasma samples, and for comparison, in five plasma samples from the teleost rainbow trout *Oncorhyncus mykiss* (Walbaum), using Ca^{2+} selective microelectrodes. Borosilicate glass capillaries (TW150-4; WPI; www.wpiinc.com) were pulled to a tip diameter of *c*. 5–8 μm on a P-97 Flaming-Brown pipette puller (Sutter Instruments Co.; www.sutter.com). Micropipettes were silanized with *N*, *N*-dimethyltrimethylsilylamine at 200° C for 60 min. Micropipettes were backfilled and tips were loaded *via* capillary action with a column (*c*. 50–100 μm) of Ca ionophore cocktail (Ca^{2+} ionophore I cocktail A; Fluka; Sigma-Aldrich) and backfilled with 500 mmol l^{-1} $CaCl_2$. Ca^{2+} selective microelectrodes were calibrated in solutions of 5, 0·5 and 0·05 mM $CaCl_2$ and 120 mM NaCl giving slopes of *c*. 25 mV decade^{-1}, close to near-Nernstian (29 mV decade^{-1}). Reference electrodes were constructed from 1·5 mm × 0·86 mm × 10 cm glass capillaries pulled to a tip diameter of 1–3 μm and filled with 500 mmol l^{-1} KCl. All voltages were measured on high impedance ($>10^{13}$ Ω) electrometers and were recorded and analysed using a PC-based data acquisition system (PowerLab 4/25) with Chart version 5 software (ADInstruments Inc.; www.adinstruments.com). A more detailed description of ion selective microelectrode is given by O'Donnell & Rheault (2005). Calcium activity in the calibration solutions was calculated with the use of CHEAQS for calculating chemical equilibria in aquatic systems (http://home.tiscali.nl/cheaqs). The activity values of the calibration solutions predicted by CHEAQS were 1·567, 0·1617 and 0·01623 mmol l^{-1} for 5, 0·5 and 0·05 mmol l^{-1} total Ca, respectively. Using the activity values predicted by CHEAQS, the activity of Ca in the plasma (A_p) was calculated by: $A_p = A_c\, 10^{(\Delta V S^{-1})}$, where A_c is the activity of Ca in the 0·5 mM Ca calibration solution (0·1617 mmol l^{-1}), ΔV is the voltage difference between the plasma sample and the calibration solution (mV) and S is the slope of the Ca^{2+} selective microelectrode.

For radioactivity measurements, ^{22}Na is a γ and β emitter and was counted by a Wallac Wizard 1480 Automatic Gamma Counter (PerkinElmer Precisely; www.perkinelmer.com). ^{36}Cl and ^{45}Ca, which are solely β emitters, were counted by a QuantaSmart (Tricarb Liquid Scintillation Analyzer; PerkinElmer Precisely,) after the addition of 10 ml of scintillation fluid (ACS Amersham Biosciences; www.gelifesciences.com) to the water samples. Tests showed that quench was constant for these samples, so no quench correction was applied.

STATISTICAL ANALYSES

All data are expressed as the mean ± S.E. (n, where n represents the number of experiments, each on a different animal). A two-tailed t-test was used to compare control with corresponding experimental values or to make comparisons between the two species. Time-dependent responses were compared with initial values using a one-way ANOVA followed by a Dunnett's test and pair-wise comparisons were made using a one-way ANOVA followed by Tukey's test. All statistical significance was calculated at $P < 0{\cdot}05$.

RESULTS

PLASMA IONS

Plasma ion composition was very similar between *P. dolloi* and *P. annectens* under aquatic conditions (Table I). Plasma Na and Cl levels ranged from 85 to 134 mmol l^{-1} and 78 to 145 mmol l^{-1}, respectively. The Ca levels ranged from 0·9 to 1·9 mmol l^{-1} for both species. In view of the unusually low plasma Ca levels in both species (*P. dolloi* = 1·03 ± 0·12 mmol l^{-1}, $n = 6$; *P. annectens* = 1·22 ± 0·19 mmol l^{-1}, $n = 6$), Ca activity measurements were made. Ca activities were 3·71 ± 1·57 and 3·55 ± 1·46 of total plasma Ca concentrations in *P. dolloi* and *P. annectens*, respectively. By way of comparison, in *O. mykiss* plasma where total

TABLE I. A comparison of plasma ($n = 6$), faecal ($n = 7$) and urine ($n = 5$) ion concentrations in *Protopterus dolloi* and *Protopterus annectens*. Values are means ± S.E. There were no significant differences between the two species (unpaired t-test, $P < 0{\cdot}05$)

		Protopterus dolloi	*Protopterus annectens*
Plasma ion concentration ($mmol\,l^{-1}$)	Na	$109{\cdot}7 \pm 4{\cdot}1$	$103{\cdot}5 \pm 8{\cdot}9$
	Cl	$105{\cdot}0 \pm 5{\cdot}8$	$105{\cdot}0 \pm 11{\cdot}2$
	Ca	$1{\cdot}03 \pm 0{\cdot}12$	$1{\cdot}22 \pm 0{\cdot}19$
Faecal ion concentration ($mmol\,kg^{-1}$)	Na	$47{\cdot}2 \pm 6{\cdot}2$	$40{\cdot}9 \pm 4{\cdot}7$
	Cl	$20{\cdot}0 \pm 2{\cdot}6$	$14{\cdot}6 \pm 1{\cdot}9$
	Ca	$76{\cdot}6 \pm 14{\cdot}7$	$78{\cdot}9 \pm 11{\cdot}7$
Urine ion concentration ($mmol\,l^{-1}$)	Na	$1{\cdot}71 \pm 0{\cdot}75$	$1{\cdot}24 \pm 0{\cdot}35$
	Cl	$3{\cdot}58 \pm 0{\cdot}79$	$2{\cdot}54 \pm 0{\cdot}58$
	Ca	$0{\cdot}51 \pm 0{\cdot}11$	$0{\cdot}35 \pm 0{\cdot}06$

Ca concentration was more than twice as high ($2{\cdot}65 \pm 0{\cdot}12$ $mmol\,l^{-1}$, $n = 5$), Ca activity was $10{\cdot}43 \pm 0{\cdot}53\%$ of this higher total value. Tests with *O. mykiss* plasma showed that the Ca activity values were not affected by freezing.

TISSUE IONS IN *P. DOLLOI* AND TERRESTRIALIZATION

In a separate series, plasma and tissues (muscle, liver, lung, kidney, intestine and heart) were sampled from *P. dolloi* for ion concentrations under aquatic conditions and after 1 and 5 months of terrestrialization. Plasma Na, Cl and Ca concentrations remained unchanged from aquatic control levels during terrestrialization (Table II). Plasma Mg levels, which were measured only in this series, also remained constant (aquatic $= 0{\cdot}93 \pm 0{\cdot}09$; 1 month terrestrialized $= 0{\cdot}96 \pm 0{\cdot}02$; 5 month terrrestrialized $0{\cdot}90 \pm 0{\cdot}04$ $mmol\,l^{-1}$, $n = 4 - 6$). Tissue ion levels were lower than plasma levels for Na and Cl, but higher than plasma levels for Ca. In general, tissue ion levels were fairly stable during terrestrialization. There was a significant increase, however, in the intestine Na concentration following 1 month of terrestrialization and this trend was maintained following 5 months of terrestrialization (Table II). There were no other significant changes in tissue Na concentration as a result of terrestrialization. Kidney Cl concentration exhibited a significant decrease following 1 month of terrestrialization, but this decrease was no longer evident after 5 months (Table II). Other tissues showed no differences in Cl following terrestrialization. The Ca concentration of the kidney increased more than three-fold by 5 months. Liver Ca concentration significantly increased following 1 month terrestrialization, a trend which was maintained but no longer significant by 5 months (Table II).

NET ION FLUXES FOLLOWING FEEDING

Before beginning the series of feeding experiments, a study was conducted to ensure the bloodworms were not leaching ions into the water. Table III shows that there was no detectable leaching from the bloodworms as the ion levels from the test water and the test water containing the bloodworms were virtually identical. The actual ionic composition of the digested bloodworms exhibited high levels of Na and Ca and very low level of Cl (Table III).

TABLE II. The influence of terrestrialization for 1 and 5 months on ion concentrations in different tissues and blood plasma in *Protopterus dolloi*. Values are mean ± S.E. except where $n < 3$ values are mean (range)

Na	Plasma ($mmol\,l^{-1}$)##	Muscle ($mmol\,kg^{-1}$)#	Liver ($mmol\,kg^{-1}$)	Lung ($mmol\,kg^{-1}$)	Kidney ($mmol\,kg^{-1}$)	Intestine ($mmol\,kg^{-1}$)	Heart ($mmol\,kg^{-1}$)
Aquatic	101·2 ± 4·3	28·7 ± 4·8	41·3 (36·5, 46·0)	84·6 ± 14·7	57·8 ± 9·1	43·7 ± 2·1	58·5 ± 9·7
	$n = 4$	$n = 4$	$n = 2$	$n = 5$	$n = 4$	$n = 4$	$n = 5$
1 month terrestrialized	93·9 ± 0·9	26·4 ± 3·6	40·8 ± 4·2	80·0 ± 2·5	47·7 ± 2·3	76·7 ± 16·4*	79·7 ± 30·5
	$n = 5$	$n = 4$	$n = 4$	$n = 5$	$n = 5$	$n = 5$	$n = 3$
5 month terrestrialized	103·8 ± 2·3	28·5 ± 5·2	51·8 ± 4·3	71·6 ± 16·2	61·0 ± 5·7	66·1 ± 5·0*	75·9 ± 4·9
	$n = 6$	$n = 4$	$n = 4$	$n = 3$	$n = 5$	$n = 5$	$n = 5$
Cl							
Aquatic	90·7 ± 7·8	20·6 ± 3·0	49·7 (48·0, 51·4)	28·6 ± 6·8	79·2 ± 20·0	50·9 ± 2·3	43·4 ±10·5
	$n = 4$	$n = 4$	$n = 2$	$n = 5$	$n = 5$	$n = 4$	$n = 5$
1 month terrestrialized	85·7 ± 5·5	13·6 ± 2·0	33·0 ± 14·9	39·5 ± 9·0	22·2 ± 6·7*	36·4 ± 22·5	46·4 (40·7, 52·1)
	$n = 4$	$n = 4$	$n = 3$	$n = 4$	$n = 4$	$n = 4$	$n = 2$
5 month terrestrialized	86·9 ± 2·8	22·5 ± 4·4	27·9 ±10·4	52·4 (56·0, 48·8)	42·8 ± 8·3	38·1 ± 8·5	48·7 ± 3·8
	$n = 6$	$n = 3$	$n = 3$	$n = 2$	$n = 3$	$n = 3$	$n = 4$
Ca							
Aquatic	2·29 ± 0·10	2·7 (3·04, 2·29)	2·0 (1·72, 2·26)	3·3 ± 0·5	2·4 ± 0·3	2·6 ± 0·3	2·3 ± 1·0
	$n = 4$	$n = 2$	$n = 2$	$n = 5$	$n = 4$	$n = 4$	$n = 5$
1 month terrestrialized	2·08 ± 0·05	2·3 ± 0·2	4·7 ± 0·6*	2·5 ± 0·3	4·9 ± 1·9	2·2 ± 0·2	5·1 ± 3·4
	$n = 5$	$n = 4$	$n = 4$	$n = 5$	$n = 5$	$n = 4$	$n = 4$
5 month terrestrialized	2·24 ± 0·14	9·8 ± 8·4	6·9 ± 2·4	2·6 ± 0·3	8·8 ± 0·7*	11·2 ± 5·1	6·8 ± 4·9
	$n = 6$	$n = 3$	$n = 5$	$n = 4$	$n = 5$	$n = 5$	$n = 5$

*difference from aquatic *P. dolloi* (one-way ANOVA plus Dunnett's test; $n = 2 - 5$; $P < 0{\cdot}05$).

Muscle and liver Na and Cl concentrations have previously been reported in Staples *et al.* (2008) for the aquatic and 5 month terrestrialized conditions.

Plasma Na and Cl data differ from those reported by Wilkie *et al.* (2007) because they are from a subset of fish sacrificed for tissue samples.

TABLE III. The influence of addition of bloodworms *Chironomus* sp. (representative of the 3% ration used during *Protopterus dolloi* and *Protopterus annectens* feeding experiments) on the ionic composition of the experimental water. The ionic concentrations found in the bloodworms are also shown. Values are means ± S.E. ($n = 3$). There were no significant differences between the experimental water and experimental water with bloodworms (paired t-test, $P < 0{\cdot}05$)

	Na	Cl	Ca
Experimental water (mmol l^{-1})	1·77 ± 0·00	1·49 ± 0·02	1·02 ± 0·01
Experimental water + bloodworms (mmol l^{-1})	1·75 ± 0·01	1·50 ± 0·03	1·04 ± 0·01
Bloodworms (mmol kg^{-1})	48·20 ± 2·30	1·86 ± 0·22	20·9 ± 1·00

TABLE IV. Net ion flux rates (μmol kg^{-1} h^{-1}) with the external water averaged over the course of 48 h following a feeding and non-feeding event for *Protopterus dolloi* ($n = 6$) and *Protopterus annectens* ($n = 6$). Values are means ± 1 S.E.

		Net ion flux rates (μmol kg^{-1} h^{-1})		
		Na	Cl	Ca
Protopterus dolloi	Fasted	−9 ± 10	−57 ± 37	−11 ± 4
	Fed	38 ± 14	−75 ± 53	−4 ± 2
Protopterus annectens	Fasted	88 ± 14*	−38 ± 22	−28 ± 15
	Fed	44 ± 40	100 ± 96	−21 ± 20

*significant difference (unpaired t-test, $P < 0{\cdot}05$) between the two species.

In Figs 1 (*P. dolloi*) and 2 (*P. annectens*), the cumulative changes in water ion concentrations (relative to starting values) are depicted over a 48 h period following feeding (or absence of feeding in fasted animals). In Table IV, these cumulative data have been used to calculate net rates of uptake or loss per kilogram per hour, averaged over the 48 h period. On the far left of Figs 1 and 2, the bars illustrate the maximum changes in water ion concentration expected (calculated from the measurements of Table III) if the total ion content of the bloodworm meal (5 g = 3% ration) were to be released into the 2 l water volume.

The fed *P. dolloi* showed significant net removal of Na from the external water compared with fasted fish at 4, 6, 7 and 48 h [Fig. 1(a)]. Clearly, the Na content of the bloodworm meal was not lost to the water. Overall, these data yielded a net Na uptake rate from the water of +38 μmol kg^{-1} h^{-1} in fed fish (Table IV) over the 48 h period. In *P. annectens*, both fed and fasted animals exhibited net removal of Na from the water over 48 h [Fig. 2(a)]. Again the Na content of the meal was not lost to the water. Average net Na uptake rates over 48 h (+44 μmol kg^{-1} h^{-1}) in fed *P. annectens* were comparable with those in fed *P. dolloi*, but less than those in fasted *P. annectens* (Table IV).

The situation was very different for Cl and Ca balance. In *P. dolloi*, both fed and fasted animals lost far more Cl to the water than could be obtained from the small Cl content of the bloodworm meal [Fig. 1(b)]. Over 48 h, the net loss rate was −75 μmol kg^{-1} h^{-1} in fed animals (Table IV). In *P. annectens*, the data were very variable [Fig. 2(b)], and although the net flux rate was positive (100 μmol kg^{-1} h^{-1}), this value was not significantly different from zero (Table IV). With respect to

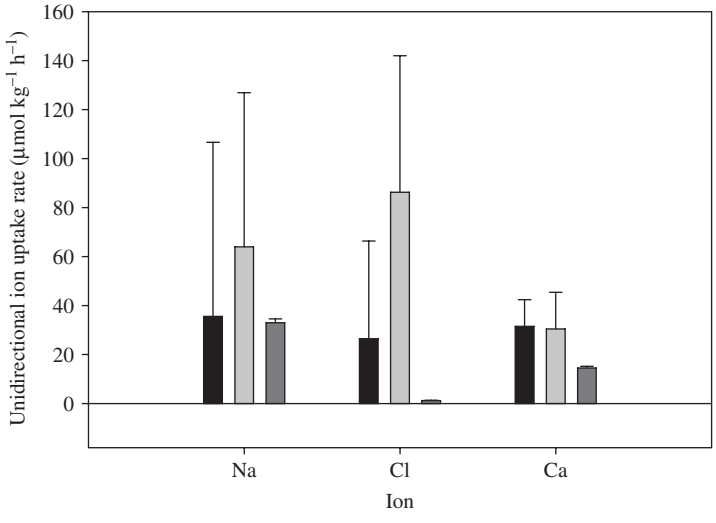

FIG. 3. Unidirectional ion uptake rates from the water for *Protopterus dolloi* (■) and *Protopterus annectens* (□). Values are means ± S.E. ($n = 6$). For comparison, the maximum ion uptake rate that could be achieved from a single 3% ration bloodworm *Chironomus* sp. meal (▩) is also shown, assuming that the total ionic content of the 3% ration meal was absorbed over the 48 h period. There were no significant differences (unpaired t-test, $P < 0{\cdot}05$) between the two species.

Ca, both fed and fasted *P. dolloi* lost Ca to the external water, and the cumulative losses were comparable with the Ca content of the meal [Fig. 1(c)]. Averaged over 48 h, the Ca loss was quite low in fed animals (-4 μmol kg^{-1} h^{-1}). The pattern in *P. annectens* was different; from 0·5 to 4 h post-feeding, fed animals lost Ca to the external water, although this pattern was reversed, with net uptake of Ca from the water at 12 and 24 h [Fig. 2(c)]. By 48 h, the cumulative loss was comparable with the Ca content of the meal. Averaged over this time period, fed *P. annectens* exhibited a negative though variable Ca loss rate (-21 μmol kg^{-1} h^{-1}), which was not significantly different from zero.

UNIDIRECTIONAL ION UPTAKE RATES AND NET IONS AVAILABLE FROM FOOD

Rates of unidirectional ion uptake from the water were low and quite variable; there were no significant differences between *P. dolloi* and *P. annectens* (Fig. 3). For comparison, the maximum ion uptake rate that could be achieved from the bloodworms is also shown in Fig. 3, assuming that the total ionic content of the 3% ration meal was absorbed *via* the gastrointestinal tract over the 48 h period. For both species, this analysis suggests that although unidirectional uptake rates are low, the water and the diet are of comparable importance for Na and Ca acquisition, respectively (Fig. 3), whereas waterborne unidirectional uptake predominates for Cl acquisition, because Cl concentrations in bloodworms are very low (Table III). It should be noted, however, that the ions taken up from the food and the water are not completely retained by the fishes (Figs 1 and 2), and there is some ion loss to the water through the gills and skin, urine and faeces.

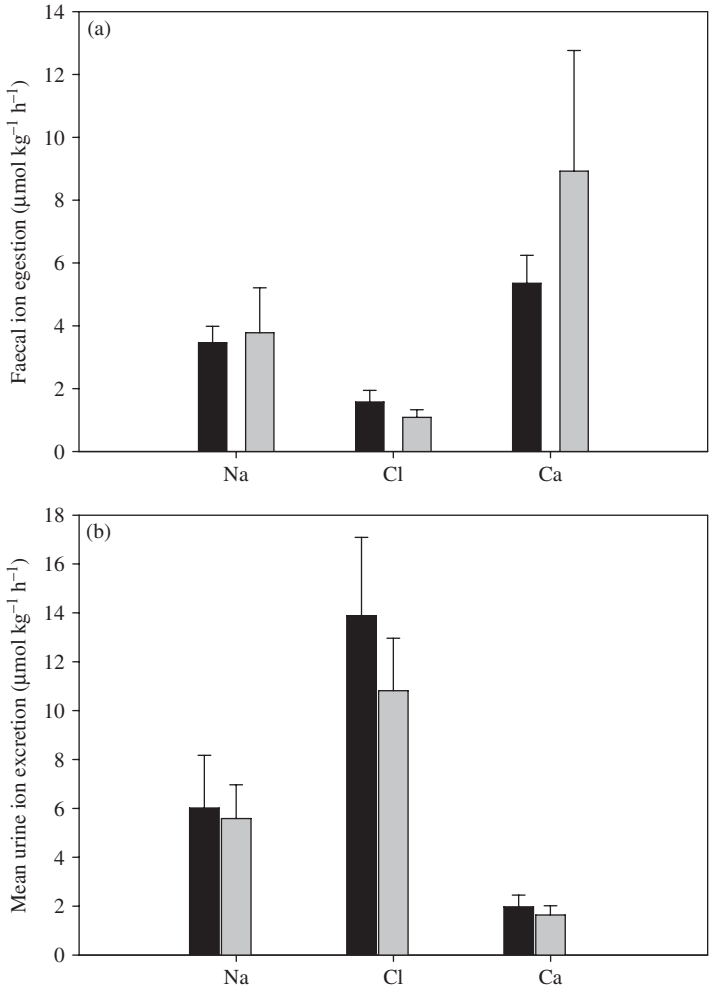

FIG. 4. (a) Estimated faecal ion loss rates ($n = 7$) and measured urinary ion excretion rates ($n = 6$; data from Patel *et al*., 2009) in *Protopterus dolloi* ■ and *Protopterus annectens* □. Values are means ± S.E. There were no significant differences (unpaired t-test, $P < 0{\cdot}05$) between the two species.

FAECAL ION LOSS RATES

Analysis of faecal ion content (Table I) indicated that in both species, Na concentration was very similar to that in the ingested blood worms (see Table III), whereas Cl and Ca concentrations (especially Ca) were considerably higher. The mass of the faeces (*c*. 4 g kg^{-1}), however, was only a small fraction of the mass of the ingested bloodworms (30 g kg^{-1} fish for a 3% ration). There were no differences in faecal ion content between *P. dolloi* and *P. annectens* (Table I). Estimated faecal ion loss rates [Fig. 4(a)] were low relative to measured uptake rates from water and potential uptake rates from food (Fig. 3).

URINARY ION EXCRETION RATES

Using urinary data from Patel *et al*. (2009), there were no significant differences in Na, Cl or Ca concentrations or excretion rates between *P. dolloi* and *P. annectens* [Table I and Fig. 4(b)]. Urinary concentrations of Na and Cl were very low (1–4%) relative to plasma concentrations, whereas urinary Ca levels were *c*. 30–50% of plasma values (Table I). Mean urinary excretion rates were highest for Cl, intermediate for Na and lowest for Ca [Fig. 4(b)]. Loss rates of Na and Cl through the urine [Fig. 4(b)] were greater than *via* the faeces [Fig. 4(a)], although the opposite was true for Ca. Overall, urinary loss rates [Fig. 4(b)] were low relative to measured uptake rates from water and potential uptake rates from food (Fig. 3).

IONIC BALANCE SHEETS

On the basis of the various measurements in this study, Table V presents a balance sheet for ion uptake (+) and loss rates (−) ($\mu mol\,kg^{-1}\,h^{-1}$) over a 48 h period for *P. dolloi* and *P. annectens* fed a standard bloodworm meal (3% ration) at 0 h. Uptake rates from food assumes 100% assimilation of bloodworm ion content as in Fig. 3. Uptake from the water is based on unidirectional ion uptake rates measured with radiotracers in Fig. 3. Ion egestion rates through the faeces and ion excretion rates through the urine were taken from Fig. 4. Estimated rates of net gain from or loss to the water, assuming steady state conditions, are based on the difference: (uptake rate from bloodworms + unidirectional uptake rate from water)−(egestion rate through faeces + excretion rate through urine). Actual rates of net gain or loss to the water are calculated from the data of Figs 1 and 2 in Table IV. Any discrepancy between the two (actual−estimated) should represent the unidirectional efflux rate across the body surface (gills and skin), which was not measured. Note that in Table V, the estimated net uptake exceeded the measured net uptake in both species for all ions (with one exception). This implies that there is an important unidirectional efflux across the skin and gills for these ions. The one exception, for Cl balance in *P. annectens*, may be explained by data variability of unknown origin, as noted earlier. Overall, this analysis indicates the importance of both unidirectional uptake from the water and uptake from the food in Na and Ca acquisition, and from the water only for Cl acquisition, whereas unidirectional loss across the skin and gills appears to be a more important route of loss for all ions than either the urine or faeces. There do not appear to be substantive differences in this regard between *P. dolloi* and *P. annectens*.

DISCUSSION

The present study provides basic information on the ionoregulatory physiology of two species of lungfishes, an area in which there has been little study (Wright, 2007) until recently (Wilkie *et al*., 2007). Contrary to the original hypothesis, there appear to be no important differences in ionoregulatory strategies between the two species, despite their differences in distribution and behaviour (Greenwood, 1986). In general, the overall patterns observed in these fishes during aquatic life appear similar to those in the well-studied freshwater teleosts, although there are some important differences, including very low rates of unidirectional Na and Cl uptake

TABLE V. A balance sheet for ion uptake (+) and loss rates (−) over a 48 h period for *Protopterus dolloi* and *Protopterus annectens* fed a standard *Chironomus* sp. bloodworm meal (3% ration) at 0 h. Uptake from bloodworms assumes 100% assimilation of bloodworm ion content as in Fig. 3. Uptake from the water is based on unidirectional ion uptake rates measured with radiotracers in Fig. 3. Ion egestion rates through faeces were estimated in [Fig. 4(a)] and *via* the urine were measured in [Fig. 4(b)]. Actual rates of net uptake or loss by the fishes with respect to the water are calculated from the data of Figs 1 and 2 (in Table IV). Estimated rates of uptake or loss by the fishes with respect to the water, assuming steady state conditions, are based on the difference: (uptake rate from bloodworms + unidirectional uptake rate from water)−(ion loss through faeces + excretion rate through urine). Any discrepancy between the two (actual−estimated) should represent the unidirectional efflux rate across the body surface (gills and skin) which was not measured

Protopterus dolloi

	Ion uptake Bloodworms	Unidirectional ion uptake Water	Net uptake or loss Water (estimated)	Net uptake or loss Water (actual)	Difference Actual-estimated	Ion excretion Urine	Ion loss Faeces
Na(μmol kg^{-1}h^{-1})	33	36	59	38	−21	−6	−4
Cl(μmol kg^{-1}h^{-1})	1	26	11	−75	−86	−14	−2
Ca(μmol kg^{-1}h^{-1})	15	32	40	−4	−44	−2	−5

Protopterus annectens

	Ion uptake Bloodworms	Ion uptake Water	Net uptake or loss Water (estimated)	Net uptake or loss Water (actual)	Difference Actual-estimated	Ion excretion Urine	Ion loss Faeces
Na(μmol kg^{-1}h^{-1})	34	64	88	44	−44	−6	−4
Cl(μmol kg^{-1}h^{-1})	1	86	75	100	+25	−11	−1
Ca(μmol kg^{-1}h^{-1})	15	30	34	−21	−55	−2	−9

from the water and low plasma ion concentrations. The food appears to be an important source of ions. Low active influx rates of Na and Cl through the external epithelium (*i.e.* gills and skin) correlate with low efflux rates, so the low diffusive permeability to water reported by Patel *et al.* (2009) extends to these monovalent ions as well.

PLASMA IONS

Plasma Na, Cl and Ca concentrations in aquatic *P. dolloi* and *P. annectens* were in broad agreement with earlier measurements in *Protopterus* sp. summarized in Table VI; these values were generally only 50–80% of the comparable concentrations in freshwater teleosts (Table VI). Regulation of such low extracellular ion levels minimizes the ionic gradients that must be maintained against the environment by active transport, and therefore may be a metabolically favourable trait in a taxonomic group that is characterized by extraordinarily low metabolic rates (Iftikar *et al.*, 2008). Urist *et al.* (1972) postulated that this characteristic of low plasma ion concentrations reflected the much longer evolutionary history of the Dipnoi in fresh water in contrast to more modern fishes such as the teleosts.

Ca concentrations in *Protopterus* spp. plasma were particularly low (Table VI), as earlier noted by Urist *et al.* (1972). Therefore, a surprising finding is that Ca activity in *Protopterus* spp. plasma was only *c.* 3·6% of this low total, whereas in the plasma of the teleost *O. mykiss*, Ca activity was 10·4% of a much higher total. No previous measurements of true Ca activity in fish plasma have been reported. There are many measurements, however, of ionized *v.* protein bound Ca. Using the approach of Greenaway (1972) to relate Ca activity to ionized Ca, it can be estimated that ionized Ca was only *c.* 12% of the total in *Proptopterus* spp. plasma, but 33% of the total in *O. mykiss* plasma, the latter in agreement with typical ranges reported in teleosts (25–80%; Hanssen *et al*, 1989; Abbink *et al.*, 2004). This may have implications for cardiac, neural and bone physiology, and may be indicative of unusual plasma proteins and an ability to store Ca in a complexed form for use during periods of terrestrialization.

TABLE VI. A comparison of plasma ion levels in *Protopterus dolloi* and *Protopterus annectens* with plasma ion levels reported for various freshwater teleosts

Name	Na (mM)	Cl (mM)	Ca (mM)	Reference
Protopterus dolloi	110	105	1	Present study
Protopterus annectens	103	105	1·2	Present study
P. annectens	110	123	2·8	Babiker & Rankin (1979)
P. annectens	100	92	1·8	Urist *et al.* (1972)
P. annectens	115	89	—	Odulye (1977)
Protopterus aethiopicus	101	77	2·4	DeLaney *et al.* (1977)
Oncorhynchus mykiss	156	128	2·7	Rogers *et al.* (2003)
Salmo trutta	155	124	5·3	Gordon (1959)
Anguilla anguilla	150	105	2·8	Sharratt *et al.* (1964)
Carassius auratus	130	116	2·5	Houston & Koss (1984)
Cyprinus carpio	130	125	2·1	Houston *et al.* (1970)

TERRESTRIALIZATION AND ITS EFFECTS ON TISSUE IONS IN *P. DOLLOI*

Terrestrialization is probably the last resort by the African lungfishes to water shortage prior to aestivation; the fishes lie virtually motionless in a cocoon in air, without feeding, and with only the ventral skin in contact with a film of water. During this time, fishes, which are normally ammoniotelic, suppress ammonia production, and store urea in their body, a much less toxic N waste product (Chew *et al*., 2003, 2004; Wood *et al.,* 2005*a*; Staples *et al.,* 2008). Remarkably, Wilkie *et al*. (2007) reported that for up to 5 months of this treatment, there were no changes in plasma Na, Cl or osmolality levels, and muscle water content actually increased considerably, due to the continuation of ion and water exchange through the ventral abdominal skin in contact with the water film. Terrestrialization presents a rather different picture from true aestivation, where progressive dehydration and increases in plasma ion concentrations occur (DeLaney *et al*., 1977).

The present data (Table II) augments present understanding of terrestrialization physiology, showing that plasma Ca and Mg concentrations are also maintained, and that Na, Cl and Ca concentrations in most tissues are well regulated despite substantial increases in tissue water content associated with urea retention. With respect to exceptions, the increases in Ca concentrations in liver and kidney suggest that some re-organization of internal Ca distribution occurs during terrestrialization; calcium metabolism in lungfishes is rather different from other fishes as noted earlier (Urist *et al*., 1972). The apparent loss of Cl from the kidney could be the result of diversion of blood flow away from a non-working kidney. The significant increase in Na levels in intestinal tissue during terrestrialization could simply be the result of undigested food remaining in a gut with little blood flow, as there is a considerable amount of Na in the bloodworms (Table III). The milder one-step acid digestion process used for *P. dolloi*, and *P. annectens* tissue may not have liberated all the ions present in the trapped bloodworms, which require a more severe digestion procedure.

UNIDIRECTIONAL ION UPTAKE RATES

The unidirectional Na and Cl uptake rates from the water measured for both *P. dolloi* and *P. annectens* were slightly higher than those reported by Wilkie *et al*. (2007) for aquatic *P. dolloi* but in both studies there was very high variability (Fig. 3). These rates are very low (*c*. 10%) compared with those of a selection of teleosts summarized in Table VII and correlate with the lower plasma Na and Cl concentrations. In the only previous measurement in African lungfishes, Odulye (1977) reported a directly measured unidirectional Na efflux rate of $-500\ \mu mol\ kg^{-1}\ h^{-1}$ in aquatic *P. annectens*, again with high variability. Normally, influx and efflux rates are fairly similar, as indirectly measured in *P. dolloi* (Wilkie *et al*., 2007) and indirectly calculated ('actual−estimated') for both species in Table V. Possibly, the much higher value reported by Odulye (1977) reflected the smaller size of the fish (4−150 g) and experimental stress.

These are the first measurements of unidirectional Ca uptake in any African lungfish (Fig. 3). In contrast to Na and Cl, influx rates of Ca from water were less variable and typical of rates reported for teleosts (Table VII), despite the low plasma Ca concentrations (Table VI). In an immunohistochemical study, the gills and the skin of *P. annectens* showed the presence of two different types of

TABLE VII. A comparison of unidirectional ion uptake rates ($\mu mol\,kg^{-1}h^{-1}$) in *Protopterus dolloi* and *Protopterus annectens* with ion uptake rates reported for various freshwater teleosts

Name	Na	Cl	Ca	Reference
Protopterus dolloi	35	26	31	Present study
P. dolloi	15	20	—	Wilkie *et al.* (2007)
P. annectens	63	86	30	Present study
Fundulus heteroclitus	425	—	—	Scott *et al.* (2004)
Fundulus heteroclitus	375	—	—	Scott *et al.* (2004)
Oncorhynchus mykiss	944	1006	34	Rogers *et al.* (2003)
Danio rerio	525	75	—	Boisen *et al.* (2003)
Tilapia mossambica	—	—	27·9	Flik *et al.* (1986)
F. heteroclitus	—	—	32·5	Pang *et al.* (1981)
Salmo salar smolts	680	760	—	Potts *et al.* (1970)
Anguilla anguilla	480	30–50	—	Motais *et al.* (1966)
Platichthys flesus	220	10–20	—	Motais *et al.* (1966)

mitochondria rich cells (Sturla *et al.*, 2001). These two cell types both stained with a Ca-ATPase antibody appeared to be similar to the α and β 'chloride' cells described in freshwater teleosts (Pisam *et al.*, 1987, 1990, 1993; Pisam & Rambourg, 1991; Perry, 1997; Sturla *et al.*, 2001). More recent work on freshwater *O. mykiss* using binding of peanut lectin agglutinin (PNA) suggested that α cells are PNA positive (PNA+) and β cells are PNA negative (PNA−) (Goss *et al.*, 2001). α PNA+ cells appear to be associated with Ca and Cl uptake and β PNA− cells with Na uptake, respectively (Galvez *et al.*, 2002, 2006). Although the gills of *P. annectens* contain both α and β-type chloride cells (potentially providing uptake of all three ions), only α cells are present on the skin (Sturla *et al.*, 2001). This suggests that the skin may be a major site of trans-epithelial Ca and Cl uptake, by analogy to studies on the skin of other fish species (McCormick *et al.*, 1992; Marshall *et al.*, 2002).

URINARY ION EXCRETION RATES

The kidney appears to function in a very similar manner to those in freshwater teleosts (Hickman & Trump, 1969; Patel *et al.*, 2009), strongly reabsorbing Na and Cl, but reabsorbing Ca to a lesser extent (Table I). Nevertheless, efflux rates of Na and Cl through the urine [Fig. 4(b)] were greater than *via* the faeces [Fig. 4(a)], whereas the opposite was true for Ca. Overall ionic loss rates through the urine were low.

AVAILABILITY OF IONS FROM FOOD AND FAECAL ION LOSSES

The potential uptake of ions from food is an area that has received scant attention in fish ionoregulatory physiology, although its importance is now starting to be recognized in teleosts (Smith *et al.*, 1989; Bucking & Wood, 2006, 2007). In both species, the potential availability of Na and Ca from the food is comparable with the unidirectional uptake of these ions from the water (Fig. 3), and probably plays a valuable supplementary role (Table V). The water is clearly the main source for Cl uptake given the very low concentrations of Cl in the bloodworm diet (Table III).

The actual concentrations of Na in the bloodworms (Table III) are almost identical to those in the faeces (Table I), but because the mass of the ingested material is reduced about seven-fold (from *c*. 30 to *c*. 4 $g\,kg^{-1}$) as it moves through the gut, by simple mass-balance calculations (*i.e.* unchanged concentration factored by reduced mass), *c*. 85% of the Na appears to be absorbed. Concentrations of Ca in the faeces (Table I) are higher than in the food (Table III), but a comparable calculation suggests that *c*. 50% of the ingested Ca is absorbed. For Cl, faecal losses slightly exceed the small intake in the bloodworm diet. In the wild, lungfishes have been reported to eat a variety of small invertebrates and fishes (Smith, 1935), so the relative dietary uptakes of the three ions may differ in natural environments.

NET ION FLUXES FOLLOWING FEEDING

Following feeding there were fluctuations in net ion balance for the fed and fasted fishes of both species (Figs 1 and 2). The fishes, under natural aquatic conditions, are very calm, moving to obtain air from the surface a few times per hour (Iftikar *et al.,* 2008). During a feeding event, however, the fishes become quite vigorous, which could disturb ion balance. The fishes are acclimated to a scheduled feeding every second day which immediately follows a water change, but there may be some handling disturbance which affects ionoregulation, for example, the net losses of Na to the external water during the first 7 h in the fasted *P. dolloi.* When these fishes are kept in water, plasma catecholamines remain unchanged in the face of levels of hypoxia which would cause massive catecholamine mobilization in teleosts (Perry *et al*., 2005), so these animals may be fairly resistant to stress related disturbances.

Despite the high levels of Na in bloodworm meal, the fed *P. dolloi* exhibited a net uptake of Na from the water (Table IV), which started about 4 h after the meal (Fig 1). This could have been in response to the initial net loss of Na that was observed in the first 3 h following feeding (Fig. 1). This trend of net Na uptake was also observed in the fed and fasted *P. annectens* (Table IV), although the temporal pattern was not as clear cut (Fig. 2). Overall, there was a significant difference in net Na balance with the water over 48 h between fasted specimens of the two species (Table IV). When comparing this finding to urine ion excretion [Fig. 4(b)], which showed no differences between species, it is evident that this difference is not due to renal handling and thus requires further study.

All experimental groups showed a net excretion of Cl which was greater than the Cl content of the meal (Figs 1 and 2 and Table IV), except for the fed *P. annectens* which showed a net uptake of Cl over 48 h (perhaps an artifact of data variability; Fig. 2). This net Cl uptake observed for the fed *P. annectens*, however, was not significantly different from the fasted fish which exhibited a net excretion of Cl (Fig. 2). *Protopterus annectens* exhibited a significant Ca loss for the first 4 h following feeding when compared with the fasted fish (Fig. 2); this was far more than could be explained by dispersal of the Ca content of the bloodworms into the water. This transitory disturbance could have been associated with feeding activity. By 48 h, however, the net Ca loss in both species approximately matched the available Ca in the meal. Overall, all of these long-term time-averaged flux rates in Table IV are very low, in accord with the idea that the animals were not unduly stressed during these experiments.

IONIC BALANCE SHEETS

The budget calculations of Table V are instructive in demonstrating several points. First, both unidirectional (presumably active) uptake from the water and dietary uptake routes are important for Na and Ca acquisition, whereas the waterborne route predominates for Cl uptake. Second, the calculations suggest that unidirectional ion effluxes (the 'actual–estimated' column of Table V) across the body surface (gills and skin) rather than in waste products such as urine and faeces are the major route of loss of Na, Cl and Ca. Because efflux rates are so low, indirect measurements such as those performed by Wilkie *et al*. (2007) are beset by variability. In future experiments, it will be instructive to directly measure these effluxes *via* radioisotopically labelling the animals and monitoring appearance in the external water. It will also be of interest to use divided chambers (Wood *et al*., 2005*a*; Patel *et al*., 2009) to determine the relative fractions of unidirectional influx and efflux occurring at the gills *v*. the skin. Lastly, these calculations emphasize that all the flux rates are very low in *Protopterus*, reflective of species with reduced gills, low permeability, low metabolic rates and a sedentary lifestyle. Again, the low overall diffusive permeability of the external epithelium to both water (Patel *et al*., 2009) and ions may be an important pre-adaptation to life on land.

Although respiratory physiology has been relatively well studied in Ceratodontiformes, the paucity of information on ionoregulatory and osmoregulatory physiology in this important transitional group has been emphasized in a recent review (Wright, 2007). Studies such as the present address this issue, but relative to the vast database available on teleosts, it barely scratches the surface. So far, the similarities in basic ionoregulatory strategies between Dipnoi and teleosts appear to be greater than the differences. The principal differences appear to be the much lower ionic exchange rates, and the ability to resist air exposure almost indefinitely in a state of dormancy without marked disturbance of internal ion concentrations.

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