Water balance and renal function in two species of African lungfish Protopterus dolloi and Protopterus annectens

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ABSTRACT

The basic physiology of water balance and kidney function was characterized in two species of African lungfish, Protopterus dolloi and Protopterus annectens. Diffusive water efflux rate constants were low (0.13 h⁻¹–0.38 h⁻¹ in various series) relative to values in freshwater teleost fish. Efflux rate constants increased approximately 3-fold after feeding in both species, and were greatly decreased after 8 months terrestrialization (P. dolloi only tested). Urine flow rates (UFR, 3.9–5.2 mL kg⁻¹ h⁻¹) and glomerular filtration rates (GFR, 6.6–9.3 mL kg⁻¹ h⁻¹) were quite high relative to values in most freshwater teleosts. However urinary ion excretion rates were low, with net re-absorption of >99% Na⁺, >98% Cl⁻, and >78% Ca²⁺ from the primary filtrate, comparable to teleosts. Net water re-absorption was significantly greater in P. dolloi (56%) than in P. annectens (23%). We conclude that renal function in lungfish is similar to that in other primitive freshwater fish, but there is an interesting dichotomy between diffusive and osmotic permeabilities. Aquatic lungfish have low diffusive water permeability, an important pre-adaptation to life on land, and in accord with greatly reduced gill areas and low metabolic rates. However osmotic permeability is high, 4–12 times greater than diffusive permeability. A role for aquaporins in this dichotomy is speculated.

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1. Introduction

The lungfish evolved during the Devonian period, a time when parts of the world were subject to extreme weather conditions including cycles of heavy rainfall followed by periods of drought (Campbell et al., 1999). There are only six extant species of lungfish, and the four within the family Prototereidae are all found in Africa. The traditional view was that these animals are “living fossils” close to the main line of tetrapod evolution and have changed very little over the course of 300 million years (e.g. Parker, 1891; DeLaney et al., 1977). The more modern view is that they may be a sister taxa of the tetrapods (e.g. Takezaki et al., 2004; Friedman et al., 2007). Regardless, their physiology is of evolutionary interest. As adults, all of the African lungfish are obligatory air breathers and thus, possess a primitive lung which is a diverticulum of their air bladder (Johansen, 1970). By understanding the physiology of the lungfish, we can gain a better understanding of the adaptive mechanisms employed in the transition from aquatic to terrestrial environments.

The origin of terrestrial vertebrates has been attributed to selection pressures such as extreme climate changes leading to droughts and depletion of oxygen levels in the water, as well as escape from predators and the greater availability of food on land (Johansen and Lenfant, 1968; Campbell et al., 1999). As early as 1928, Gray postulated that reduction of epithelial permeability to water was probably a necessary precondition for the successful invasion of land (Gray, 1928). It is therefore surprising while there have been many investigations of the air-breathing (e.g. Johansen and Lenfant, 1968) and aestivation physiology (e.g. Fishman et al., 1986) of African lungfish dating back to the classic work of Homer Smith (1930, 1931), there have been only a few studies on their water balance physiology (Godet, 1961; Oduleye, 1977; Wilkie et al., 2007) and kidney function (Sawyer, 1966, 1970; DeLaney et al., 1977; Babiker and Rankin, 1979). Overall, little is known about water balance under aquatic conditions in the lungfish, and even less is known about osmoregulation during air exposure (Wright, 2007).

Therefore the picture which has emerged is far from clear. On the one hand, the few measurements of tritiated water (³H₂O) efflux rate constants available suggest that when the animals are in water, permeability is either low (turnover rate, k=0.44 h⁻¹ in P. annectens; Oduleye, 1977) or extremely low (k=0.18 h⁻¹ in P. dolloi; Wilkie et al., 2007) relative to teleosts, and decreases further during air exposure (Wilkie et al., 2007). These trends seem appropriate for a fish with greatly reduced gills (Laurent et al., 1986) and low metabolic rate, and a dependency on air-breathing for the
bulk of its O₂ uptake (Smith, 1930; Perry et al., 2005; Itifak et al., 2008). A priori one might expect H₂O efflux rate and UFR to co-vary. However, the sparse data available on renal function suggest that both glomerular filtration rates (GFR) and urine flow rates (UFR) are either high (GFR=11 mg kg⁻¹ h⁻¹; UFR=5.9 mg kg⁻¹ h⁻¹ in P. aethiopicus; Sawyer, 1966) or typical of freshwater teleost values (GFR=3.8 mg kg⁻¹ h⁻¹; UFR=2.5 mL kg⁻¹ h⁻¹ in P. annectens; Babiker and Rankin, 1979). There were also discrepancies in renal ion handling between the urinary studies of Sawyer (1966) and Babiker and Rankin (1979). In an early review, Forster and Goldstein (1969) concluded that rates in Proteogerus sp. are very similar to those in freshwater teleosts.

Against this background, the goals of the present study were to characterize water efflux rate constants and renal function, including the handling of major electrolytes, in two species of African lungfish which might differ in their water budgets. P. dolloi inhabit equatorial areas and only aestivate if their body of water dries out, whereas P. annectens are found in temperate areas of Africa and aestivate seasonally (Greenwood, 1986). A divided chamber technique was used to separate gill versus skin H₂O efflux rates. The influence of feeding, which is known to greatly stimulate metabolic rate (“specific dynamic action,” SDA) in lungfish (Itifak et al., 2008), was also examined. By the “osmo-respiratory compromise” (Wood and Randall, 1973; Gonzalez and MacDonald, 1992), an increase in water efflux might well be associated with increased gill or skin O₂ flux during SDA. In view of the scarcity of these animals, the techniques employed were largely non-lethal, though an opportunity arose to also obtain terminal tissue samples for water content from P. dolloi as part of a study of its responses to prolonged air exposure (“terrestrialization”; Wilkie et al., 2007; Staples et al., 2008), as well as to measure H₂O efflux rate constants in this species immediately following 8 months of air exposure.

2. Materials and methods

2.1. Experimental animals

African lungfish (P. dolloi and P. annectens) with an average mass of 150 g (range: 68–210 g), as well as juvenile P. dolloi with an average mass of 9 g (range: 5–11 g) were collected in central and western Africa and were air-freighted first to a commercial dealer in Singapore (where they were held in an aquarium for a month), and then on to McMaster University in Canada. All lungfish were held at 27–30 °C under 12 L:12D photoperiod in individual aquaria containing 3 L of dechlorinated water for terminal anaesthesia (0.5 g L⁻¹ MS-222). A blood sample was taken by caudal puncture and the animal was then killed by a cephalic blow and decapitation. Aquatic lungfish (N=5) were similarly sacrificed to serve as a comparison. Tissues (white muscle, liver, lung, intestine, and kidney) were quickly dissected, frozen immediately in liquid N₂, and then stored at −80 °C. To determine % water content, approximately 150 mg samples of each tissue were weighed on an analytical balance, dried at 65 °C for 96 h, then re-weighed.

2.3. Tritiated water efflux

Diffusive water efflux rate constants were determined in 7 mature P. dolloi, 6 mature P. annectens, and 6 juvenile P. dolloi using intraperitoneal injections of tritiated water (0.25 mCi kg⁻¹ H₂O; PerkinElmer Life and Analytical Sciences, Boston, USA) made up as an isotonic saline. A standardized injection volume equivalent to 1% of the lungfish body weight was used. The fish were then placed in 3 L of water. The 8-h flux period began after a 1-hour equilibration period was given to distribute the injected fluid. This 1-hour equilibration period was selected based on previous studies looking at tritiated water efflux (for example Oduleye, 1977; Wilkie et al., 2007 both of whom specifically worked on lungfish), and our own preliminary experiments. Samples were taken every hour to monitor the appearance of H₂O in the external water. A ratio of 1:10 of estimated specific activity in the fish relative to specific activity in the water was set as the threshold for termination in the experiment (c.f. Wood, 1988). This threshold was reached by 6 h post-injection, so only the 1 to 6 h data sets were used in the calculations. The stock solution was also counted so as to accurately determine the total cpm of H₂O injected.

A study of tritiated water efflux during and after feeding was also carried out using identical techniques on 6 mature P. dolloi and 6 mature P. annectens. However in these experiments, a meal of 5 g of bloodworms was delivered after a 1-h equilibration period following the H₂O injections. In these experiments, the termination criterion was reached by 4 h post-feeding, so calculations could only be performed up to this time point.

In a separate experiment, a comparison of tritiated water efflux was also made between aquatic (N=6) and 8-month terrestrialized P. dolloi (N=5), all of which were mature animals. Approximately 1 h before being reimmersed in water, the terrestrial P. dolloi were injected in their intraperitoneal cavities with 0.25 mCi kg⁻¹ H₂O in isotonic saline (1% of body weight) as above. The fish, which were very weak after being woken up from aestivation, were then placed on a mesh support close to the surface of 3 L of water. Thus, the water was covering the lungfish but there was no great effort needed for the lungfish to come up for air, and therefore no danger of drowning. Samples were taken every hour to monitor the appearance of H₂O in the water for a period of 8 h. The termination criterion was not exceeded throughout this period.

A separate experiment was performed on mature P. dolloi (N=6) and mature Proteogerus annectens (N=6) to distinguish the relative importance of different parts of the body in water efflux. Lungfish were injected in their intraperitoneal cavities with 0.25 mCi kg⁻¹ H₂O in isotonic saline (1% of body weight) as above, and placed individually in 3 L of water. Following a 1-h equilibration period, water in the tank was replaced with only 1 L of water and a polyethylene bottle containing 400 mL of water was sealed around the posterior end of the lungfish using dental dam and an elastic band. The segregation was placed directly behind the external gills so that the entire body posterior to this point lay in the bottle. Thus, the anterior region (1 L) would show excretion by the gills and the posterior region (0.4 L) would show excretion by the bulk of the skin and the kidney of the fish. Water samples were taken at t=0 h, 1 h, 2 h, and 3 h to monitor...
the appearance of $^3$H$_2$O in the water bathing the head end. After 3 h, the bottle was removed and water samples were obtained from the bottle as well to detect the appearance of $^3$H$_2$O in the water bathing the tail end. A preliminary study was conducted for this protocol using water containing 10% blue dye in the polyethylene bottle. The samples taken from this study were run against a control water sample on a spectrophotometer at 650 nm. At this wavelength a 1% leak could be detected. The preliminary study proved that there was no leak present.

2.4. Renal function studies

In a separate experiment using mature $P.$ dolloi ($N=6$) and mature $P.$ annectens ($N=6$), external urinary catheters similar to those developed by Curtis and Wood (1991) for trout were used to determine glomerular filtration rate (GFR), urine flow rate (UFR) and composition, and thereby measurements of renal ion excretion rates. In teleosts, it is well known that UFR and urinary ion excretion rates can be overestimated due to stress-induced "laboratory diuresis" as well as by the use of internal bladder catheters which incapacitate the reabsorptive function of the urinary bladder (Wood and Patrick, 1994). Therefore external catheters (Curtis and Wood, 1991) and a relatively long 3-day measurement period were used to overcome these potential complications. This technique avoids compromising any reabsorptive functions of the urinary bladder as it collects urine vented through the cloaca. The experimental lungfish were fasted for 5 days to avoid the potential for feces to block the catheter that was sewn onto the cloaca. Fish were first anesthetized using MS222 (0.30 g L$^{-1}$). An external catheter (12FR urethral catheter; C.R. Bard Inc., Covington, GA) was sewn onto the cloaca of the lungfish using tightly spaced 2-0 silk sutures (Ethicon; Johnson and Johnson Co., Somerville, USA) and further fastened in place using a commercial animal glue (Vetbond – veterinary tissue adhesive; 3 M Animal Care Products Corp., St. Paul, USA). After being secured in place, each catheter was filled with water and held upright to ensure there was no leak. While still anesthetized, the fish were injected with a 125 $\mu$Ci kg$^{-1}$ of $[3H]$polyethylene glycol-4000 (PEG-4000; PerkinElmer, Life and Analytical Sciences, Boston, USA) into the caudal haemal arch to act as a glomerular filtration rate (GFR) marker (Curtis and Wood, 1991; Wood et al., 2005). The catheters were then allowed to recover from the anaesthetic for 12 h, a period sufficient for the GFR marker to equilibrate throughout the extracellular space (Munger et al., 1991). Urine and water samples were collected subsequently every 12 h for a period of 72 h. At this time, the fish was re-anaesthetized, a blood sample was taken by blind caudal puncture of the haemal arch, and plasma was separated by centrifugation (13000 g for 5 min). The catheter was removed and the lungfish treated with antibiotics for several weeks until the suture scars healed.

Urine samples were frozen at $-80^\circ$C for later ion (Na$^+$, Cl$^-$, Ca$^{2+}$) concentration measurements. Urinary volume was measured gravimetrically for calculation of UFR, and plasma and urine samples were measured for $[3H]$PEG-4000 radioactivity for calculation of GFR.

2.5. Analytical techniques and calculations

Rate constants for the diffusive efflux of water in the $^3$H$_2$O experiments were determined based on the following equation from Evans (1967b):

$$k = (2.3/(t_f-t_0))\log(10)(C_{\text{PM}} \text{ in fish at } t_0)/C_{\text{PM}} \text{ in fish at } t_1)$$

where:

$k =$ the rate constant of the efflux in h$^{-1}$
$t_0 =$ the start of the experiment
$t_1 =$ any point in time after $t_0$ in h

Stock saline solutions used for $^3$H$_2$O injections were counted to accurately determine the CPM injected. CPM in the fish was determined by measuring the appearance of CPM to the surrounding water. In other words, the difference between the amount injected in the lungfish and the amount excreted into the water will give the amount remaining in the lungfish. $^3$H$_2$O radioactivity was counted on the QuantaSmart (Tricarb) Liquid Scintillation Analyzer following the addition of 10 mL of scintillation fluid (ACS Amershams) to 5 mL water samples. Tests demonstrated that quench was constant so no quench correction was applied.

Rate constants of efflux ($k$) were converted to actual diffusive water efflux rates ($J_{\text{efflux}}$) in mL kg$^{-1}$ h$^{-1}$ by assuming the water space was equal to 750 mL kg$^{-1}$, as recommended by Evans (1967a).

$$J_{\text{efflux}} = k \times 750 \text{ mL kg}^{-1}$$

(Eq.2)

The initial 1 h equilibration period was not used when calculating rate constants or water efflux rates, though CPM loss to the water was monitored for inclusion in the above calculation of CPM in the fish.

Urine flow rate (UFR, in mL kg$^{-1}$ h$^{-1}$) was calculated from the cumulative volume of urine collected from the lungfish over discrete time intervals based on the following equation:

$$\text{UFR} = \frac{\text{Volume of urine}}{(\text{Wt (kg)} \times \text{Time})}$$

(Eq.3)

Urine excretion rate ($U_x$, in units kg$^{-1}$ h$^{-1}$) for any substance (x) in the urine was given by the following equation:

$$U_x = \frac{[x]_{\text{urine}} \times \text{UFR}}{}$$

(Eq.4)

where $[x]_{\text{urine}}$ is concentration of x per mL.

Glomerular filtration rate (GFR) was then calculated based on $[3H]$PEG4000 radioactivity in the urine and plasma. Plasma radioactivity ($C_{\text{PM plasma}}$ in cpm mL$^{-1}$) at the midpoint of each urine collection period was calculated based on measurements of the radioactivity of the $[3H]$PEG-4000 injection stock and measured total losses of radioactivity to the water ($\Sigma C_{\text{PM water}}$) and via the urine ($\Sigma C_{\text{PM urine}}$) up until that time, using this equation:

$$C_{\text{PM plasma}} = \frac{(C_{\text{PM injected}} - (\Sigma C_{\text{PM water}} + \Sigma C_{\text{PM urine}}))}{\text{wt (g)} \times 0.25}$$

(Eq.5)

The equation assumes that this extracellular space marker (Munger et al., 1991) was distributed in an extracellular fluid volume of 250 mL kg$^{-1}$, a typical value for fish (Olson, 1992). $[3H]$PEG-4000 losses to the water were small, amounting to 7.38% of the total in $P.$ dolloi and 28.65% in $P.$ annectens. Plasma radioactivity was confirmed by the CPM found in the terminal plasma samples. Radioactivity was counted on the QuantaSmart (Tricarb) Liquid Scintillation Analyzer following the addition of 10 mL of scintillation fluid (ACS Amershams) to 5 mL water samples, 100 $\mu$L urine samples, and 30 $\mu$L plasma samples, which were all made up to the same 5 mL total water volumes. Again, quench was constant.

GFR (in mL kg$^{-1}$ h$^{-1}$) was calculated from the following equation, using the appropriate values for each collection period:

$$\text{GFR} = \frac{\text{UFR} \times C_{\text{PM urine}}}{C_{\text{PM plasma}}}$$

(Eq.6)

where $C_{\text{PM plasma}}$ and $C_{\text{PM urine}}$ are in cpm mL$^{-1}$.

The clearance rate of an ion (in mL kg$^{-1}$ h$^{-1}$) was calculated using mean plasma ion levels for each species ($[x]_{\text{plasma}}$) measured in a separate study (M. Patel, unpublished data) and the measure urinary excretion rate ($U_x$) for that particular ion:

$$\text{Clearance Rate} = U_x \times [x]_{\text{plasma}}$$

(Eq.7)
The corresponding clearance ratio (CRx) was calculated by using the urinary excretion rate (Ux), the concentration of the substance (x) in the plasma ([x]plasma), and the GFR:

$$CR_x = \frac{U_x}{([x]_{plasma} \cdot GFR)}$$  (Eq. 8)

This analysis relates the clearance rate of a substance (x) to the clearance rate of a non-reabsorbed marker, [3H]-PEG 4000 (i.e. GFR). Values of CRx greater than 1.0 indicate that the substance (x) is secreted on a net basis by the renal system, while CRx values less than 1.0 indicate that the substance (x) is reabsorbed on a net basis (Wood and Patrick, 1994; Wood, 1995).

2.6. Statistical analyses

All data are expressed as the mean±SEM (N). Student’s two-tailed t-test (paired or unpaired as appropriate) was used to compare control with corresponding experimental values, or to make comparisons between the two species, or within a species. Time-dependent responses were compared to initial values using a one-way ANOVA followed by a Tukey’s test. All statistical significance was calculated at $P<0.05$.

3. Results

3.1. Tissue water content in P. dolloi and terrestrialization

Tissues were sampled from P. dolloi under aquatic conditions after one month and after five months of terrestrialization. Water contents increased significantly with terrestrialization in all tissues except the lung (Fig. 1). In the liver, the increase in water content was significant after only one month of terrestrialization, while in the other tissues, the elevations became significant at 5 months. It should be noted that these increases in water content were large, but may appear deceptively small because they are expressed on a % water basis. For example, on an absolute basis, the water content per unit dry matter in the liver increased from 3.76 mL/g to 5.79 mL/g after five months of terrestrialization.

3.2. Tritiated water efflux rates

Plots of log body 3H2O content against time were linear for the 5 h experimental period (1 h to 6 h post-injection) in lungfish under standard aquatic conditions, as well as during the 8-h re-immersion period following terrestrialization, thus allowing the calculation of a single rate constant (k) for each animal. Average tritiated water efflux rates for adult P. annectens and P. dolloi were similar, around 0.35 h⁻¹ (Fig. 2A), corresponding to diffusive water efflux rates of about 265 mL kg⁻¹ h⁻¹. Efflux rate constants in juvenile P. dolloi (not shown) were not significantly lower (0.21±0.05 h⁻¹ (N=6)), yielding diffusive water efflux rates of about 170 mL kg⁻¹ h⁻¹.

Notably, following terrestrialization for 8 months, in adult P. dolloi, there was a significant decrease of 70% in the rate constant for resting aquatic lungfish (0.12±0.03 to 0.18±0.02 h⁻¹ (N=6)) was lower than corresponding rates in the preceding series (0.34±0.03 to 0.38±0.04 h⁻¹ (N=6); Fig. 2A and B). Efflux rates increased approximately 3-fold in the first and second hour after feeding, declining somewhat in the third hour (Fig. 3). There were no differences between the two species of fish.
3.3. Renal function studies

Values of GFR (Fig. 5A) and UFR (Fig. 5B) in adult *P. dolloi* and adult *P. annectens* fluctuated somewhat but did not vary significantly over the 72-h experimental period. From 12 h after the start of collection, GFR tended to be higher and UFR lower in *P. dolloi*, but there were no significant differences between the two species at any time. However, the overall data summary of Table 1 demonstrates that the combined effect of these two differences was significant in terms of renal water handling. The mean water clearance ratio values for *P. dolloi* and *P. annectens* were 0.44 ± 0.08 and 0.77 ± 0.07 (*N* = 6) respectively, indicating 56% and 23% reabsorption of the filtered water load respectively. These values were significantly different between the two species.

Urinary Na⁺, Cl⁻, and Ca²⁺ excretion rates also fluctuated considerably, and there was a tendency for rates to be lower from 24 h to 72 h, than from 0 h to 24 h (Fig. 6). There were no significant differences between the two species, except at one time period (24 h–36 h) where both UC₃ and UCa were higher in *P. annectens* than in *P. dolloi*.

### Table 1

A comparison of overall mean renal and urinary measurements in *Protopterus dolloi* and *Protopterus annectens* obtained by use of external urinary catheters

<table>
<thead>
<tr>
<th></th>
<th><em>Protopterus dolloi</em> (N=5)</th>
<th><em>Protopterus annectens</em> (N=6)</th>
<th><em>Oncorhynchus mykiss</em> (N=7–10)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>GFR (mL kg⁻¹ h⁻¹)</strong></td>
<td>9.27 ± 1.25</td>
<td>6.58 ± 0.63</td>
<td>4.05 ± 0.48</td>
</tr>
<tr>
<td><strong>UFR (mL kg⁻¹ h⁻¹)</strong></td>
<td>3.88 ± 0.84</td>
<td>5.19 ± 0.98</td>
<td>2.01 ± 0.17</td>
</tr>
<tr>
<td><strong>Urinary Na⁺ (mmol L⁻¹)</strong></td>
<td>1.71 ± 0.75</td>
<td>1.24 ± 0.35</td>
<td>4.84 ± 0.74</td>
</tr>
<tr>
<td><strong>Urinary Cl⁻ (mmol L⁻¹)</strong></td>
<td>3.58 ± 0.79</td>
<td>2.54 ± 0.58</td>
<td>4.79 ± 0.44</td>
</tr>
<tr>
<td><strong>Urinary Ca²⁺ (mmol L⁻¹)</strong></td>
<td>0.51 ± 0.11</td>
<td>0.35 ± 0.06</td>
<td>1.45 ± 0.11</td>
</tr>
<tr>
<td><strong>Na⁺ excretion rate (mmol kg⁻¹ h⁻¹)</strong></td>
<td>6.01 ± 1.38</td>
<td>5.58 ± 0.51</td>
<td>8.70 ± 1.20</td>
</tr>
<tr>
<td><strong>Cl⁻ excretion rate (mmol kg⁻¹ h⁻¹)</strong></td>
<td>13.89 ± 1.90</td>
<td>10.82 ± 0.49</td>
<td>8.35 ± 0.71</td>
</tr>
<tr>
<td><strong>Ca²⁺ excretion rate (mmol kg⁻¹ h⁻¹)</strong></td>
<td>1.97 ± 0.29</td>
<td>1.64 ± 0.18</td>
<td>2.91 ± 0.25</td>
</tr>
<tr>
<td><strong>CR₄NaO</strong></td>
<td>0.436 ± 0.084</td>
<td>0.770 ± 0.069</td>
<td>0.496</td>
</tr>
<tr>
<td><strong>CR₄Cl⁻</strong></td>
<td>0.006</td>
<td>0.009</td>
<td>0.008</td>
</tr>
<tr>
<td><strong>CR₄Ca²⁺</strong></td>
<td>0.015</td>
<td>0.017</td>
<td>0.009</td>
</tr>
<tr>
<td><strong>CR₄CaO⁻</strong></td>
<td>0.218</td>
<td>0.213</td>
<td>0.267</td>
</tr>
</tbody>
</table>

Comparable data collected by the same technique in rainbow trout (*Oncorhynchus mykiss*) by Curtis and Wood (1991) are included for reference. Means ± 1 SEM.

* Indicates a significant difference (*P* < 0.05) between the lungfish species.

* Calculated using mean plasma data (M. Patel, unpublished).
Fig. 6. Urinary excretion rates of (A) sodium, (B) chloride, and (C) calcium in *Protopterus dolloi* (*N* = 5) and *Protopterus annectens* (*N* = 6) over a 72 h period. Means ± 1 SEM. *⁎* indicates significant difference between the two species (*P* < 0.05; unpaired *t*-test). Means sharing the same letter of the same case are not significantly different from one another (one way ANOVA plus Dunnett’s test).
Overall, average excretion rates for all three ions were low and not significantly different between the two species (Table 1). Slightly higher concentrations of all three ions in the urine of *P. dolloi* were counter-balanced by slightly higher UFR in *Protopterus annectens*. Urinary [Cl\(^-\)] was approximately 2-fold greater than urinary [Na\(^+\)] and 7-fold greater than urinary [Ca\(^{2+}\)] in both species. The clearance ratio analysis demonstrated that all three ions were strongly reabsorbed from the urine on a net basis in both *P. annectens* and *P. dolloi*. The efficiency of Na\(^+\) reabsorption from the primary urine was >99% (i.e. CRNa\(^+\) = 0.01), of Cl\(^-\) reabsorption >98% (i.e. CRCl\(^-\) = 0.02) and of Ca\(^{2+}\) reabsorption >78% (i.e. CRCa\(^{2+}\) = 0.22) (Table 1).

4. Discussion

The present study provides much-needed basic information on water balance and renal physiology in two species of African lungfish, especially under aquatic conditions, a topic on which previous information was very sparse (Wright, 2007). A particular focus on diffusive and osmotic water flux rates has confirmed an interesting dichotomy suspected from diverse studies in the early literature (see Introduction).

4.1. Tissue water content in *P. dolloi* and terrestrialization

In aquatic *P. dolloi*, tissue water contents (76–81%) were very typical of those recorded in a variety of freshwater fish (Olson, 1992), but increased markedly during 1–5 months of terrestrialization, as previously reported by Wilkie et al. (2007) for muscle only. The present results extend this observation to liver, intestine, and kidney. While this general increase in body water during air-exposure and cocoon coverage was initially unexpected, it is important to note that terrestrialization is different from aestivation, and the accompanying physiological responses may also be different, as discussed by Loong et al. (2008). Terrestrialization is thought to be the precursor to aestivation in nature, and is a less stressful experimental treatment with less risk of mortality to these valuable animals. In terrestrialization, the ventral abdominal skin is not covered by cocoon material and remains in contact with a thin water film, allowing water exchange to continue. Indeed there was no increase in plasma osmolality over the 5 months of terrestrialization (Wilkie et al., 2007), in contrast to substantial increases during a comparable period of aestivation in congeric lungfish (Smith, 1931; Janssens, 1964; DeLaney et al., 1977).

Wilkie et al. (2007) related the increase in muscle water to the accumulation of high concentrations of urea in both blood plasma and the muscle intracellular compartment for the purposes of osmotic water retention during terrestrialization. The same explanation most likely applies to the intracellular compartments of liver, intestine, and kidney. Interestingly, body weight measurements indicated that the extra body water of terrestrialized lungfish was lost quickly after re-immersion (Wilkie et al., 2007), in concert with excretion of the retained urea burden (Wood et al., 2005). This contrasts with the rapid water uptake exhibited by aestivated lungfish upon re-immersion (Godet, 1961).

4.2. Tritiated water efflux rates in *P. dolloi* and *P. annectens*

Measurements of diffusive water efflux rates with \(^3\)H\(_2\)O were somewhat variable between different series (Figs. 2–4). Nevertheless, the values clearly demonstrated that there were no consistant differences between *P. dolloi* and *P. annectens*, and confirmed that rates in these lungfish were very low relative to those in teleost fish (Table 2). Under standard aquatic conditions, mean rates varied from 0.38 h\(^{-1}\) in mature *P. dolloi* (Fig. 2A) down to 0.13 h\(^{-1}\) in mature *P. annectens* immediately prior to feeding (Fig. 3). These k rates are in the same range as rates of 0.18 h\(^{-1}\) for *P. dolloi* and 0.44 h\(^{-1}\) for *P. annectens* reported by Wilkie et al. (2007) and Oduleye (1977) respectively. The greatly reduced gill areas, low metabolic rates, and reliance on the air phase for the greater part of O\(_2\) uptake (MO\(_2\)) likely explains these low diffusive water efflux rates in lungfish (see Introduction).

The reasons for the differences in k values among series are not fully understood, but our impression is that the values were positively correlated with the degree of activity of the animals during the measurement period. Normally aquatic lungfish move only a few times per hour to obtain a breath of air from the air phase, but these predatory animals exhibit vigorous activity and increased ventilation when feeding. Thus, k values increased about 3-fold in the first 2 h after feeding (Fig. 3). In post-feeding *P. annectens*, Iftekhar et al. (2008) found that SDA started at this time with a 50% increase in MO\(_2\) from the water phase. Thereafter, activity ceased but water phase MO\(_2\) continued to rise as SDA continued for many more hours (Iftekhar et al. 2008), whereas the \(^3\)H\(_2\)O efflux rate constants (k) started to fall in the present study (Fig. 3). These observations suggest that diffusive water efflux relates more to activity level than to MO\(_2\). However, similar time-dependent adjustments in the “osmo-respiratory compromise” in favour of osmoregulation have been reported previously in teleost fish, and are thought to have a hormonal basis (Wood and Randall, 1973; Gonzalez and MacDonald, 1992).

The divided chamber studies showed that 75–80% of \(^3\)H\(_2\)O flux occurred in the head end (Fig. 4). General belief holds that virtually all diffusive water movements occur at the gills in fish (Motaïs et al., 1969; Haywood et al., 1977; Evans 1979; Isaia, 1984). The lungfish may differ to some degree in this regard. The anterior compartment also encompassed the very small external gills and about 15% of the total skin area of the lungfish, so some of the \(^3\)H\(_2\)O efflux may occur via these pathways, in addition to the modest internal gills. The 20% occurring via the rest of the body must occur mainly through the skin, as the UFR accounts for less than 1% of the diffusive water flux (see below). In a similar experiment, Wood et al. (2005) found that 51% of ammonia efflux and 59% of urea efflux occurred into the posterior compartment in aquatic *P. dolloi*. In the same species, Wilkie et al. (2007) demonstrated that during short term air exposure, the ventral abdominal skin alone, which was still in contact with water, was capable of maintaining \(^3\)H\(_2\)O efflux at 67%, and Na\(^+\) and Cl\(^-\) fluxes at 100% of the submerged whole body rates. All of these functions are normally associated with gills rather than skin in other fish, suggesting unusual skin anatomy and physiology in *Protopterus sp.*

In this regard, an abundance of mitochondria-rich cells has been reported in the skin of *P. annectens* (Sturla et al., 2001). When *P. dolloi* were re-immersed after 8 months of terrestrialization, \(^3\)H\(_2\)O efflux was reduced about 70% relative to control aquatic rates (Fig. 2B). These data confirm comparable observations of Wilkie et al. (2007) on 6-month terrestrialized animals. This decrease is most likely due to the lungfish maintaining to some degree the reorganized physiology that occurs during air exposure. This would include a continuation of blood shunting away from exchange surfaces at the gills (Laurent et al., 1978), a continued reliance on only ventral

<table>
<thead>
<tr>
<th>Name</th>
<th>Rate constant k (h(^{-1}))</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lungfish (<em>Protopterus dolloi</em>)</td>
<td>0.18–0.38</td>
<td>Present study</td>
</tr>
<tr>
<td>Lungfish (<em>Protopterus annectens</em>)</td>
<td>0.13–0.34</td>
<td>Present study</td>
</tr>
<tr>
<td>Lungfish (<em>Protopterus dolloi</em>)</td>
<td>0.18</td>
<td>Wilkie et al. (2007)</td>
</tr>
<tr>
<td>Lungfish (<em>Protopterus annectens</em>)</td>
<td>0.44</td>
<td>Oduleye (1977)</td>
</tr>
<tr>
<td>Tilapia (<em>Tilapia mossambica</em>)</td>
<td>1.86</td>
<td>Potts et al. (1967)</td>
</tr>
<tr>
<td>Minnow (<em>Pseudonxius poissonius</em>)</td>
<td>1.39</td>
<td>Evans (1969)</td>
</tr>
<tr>
<td>Killfish (<em>Fundulus cyanus</em>)</td>
<td>0.38</td>
<td>Potts and Fleming (1970)</td>
</tr>
<tr>
<td>Goldfish (<em>Carassius auratus</em>)</td>
<td>0.92</td>
<td>Evans (1969)</td>
</tr>
<tr>
<td>Atlantic salmon (<em>Salmo salar</em>)</td>
<td>0.48</td>
<td>Potts et al. (1967)</td>
</tr>
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* Now: Oreoichromis mossambicus.
4.3. Renal function in *P. dolloi* and *P. annectens*

Glomerular filtration rate (GFR) and urine flow rate (UFR) were not significantly different between *P. dolloi* and *P. annectens*, though the former exhibited a significantly lower CRH₂O (Table 1, Fig. 5) indicating that net water reabsorption was more than twice as efficient in *P. dolloi* (56% versus 23%). In freshwater fish, we normally consider that the renal system should be fine-tuned to maximize ion reabsorption (i.e., low CR₂H₂O values) while minimizing water reabsorption (i.e., high CRH₂O values) (Hickman and Trump, 1969; Wood, 1995). A higher water reabsorption in *P. dolloi* may be adaptive for water conservation in a species which may face less frequent but more severe aestivation scenarios (i.e., forced aestivation when its body water dries out) in view of its equatorial distribution, in contrast to the temperate distribution of *P. annectens* which aestivates seasonally (Greenwood, 1986).

There have been several studies on renal anatomy in lungfish (Smith, 1930; Guyton, 1935; Wake, 1986; Ojeda et al., 2006), but the functional role of the kidney in *Protopterus* has only been sparsely investigated until now (see Introduction). The present GFR and UFR data for *P. dolloi* and *P. annectens* (Table 1) fit midway within the range of the few previous reports in lungfish of comparable size (GFR = 11 mL kg⁻¹ h⁻¹; UFR = 5.9 mL kg⁻¹ h⁻¹ in *P. aethiopicus*; Sawyer, 1966; GFR = 3.8 mL kg⁻¹ h⁻¹; UFR = 2.5 mL kg⁻¹ h⁻¹ in *P. annectens*; Babiker and Rankin, 1979). Somewhat lower values in 10-fold larger lungfish (GFR = 3.6 mL kg⁻¹ h⁻¹; UFR = 1.3–2.2 mL kg⁻¹ h⁻¹ in *P. aethiopicus*; DeLaney et al., 1977; Sawyer, 1970) likely reflect the impact of allometry. Notably, all of these earlier studies collected urine by implanting catheters inside the urinary bladders, which at least in teleosts, reduces the overall efficiency of ion and water reabsorption (Curtis and Wood, 1991; Wood and Patrick, 1994), whereas we used external catheters to collect naturally voided urine, as well as a long collection period to negate any influence of “laboratory diuresis” (Wood and Patrick, 1994). A recent study of acid–base physiology in *P. annectens* used similar external catheters, and reported a resting UFR (5.90 mL kg⁻¹ h⁻¹) essentially identical to the present measurements (Gilmour et al., 2007; Table 1). Wood et al. (2005) reported a [H]PEG-4000 clearance rate of 5.64 mL kg⁻¹ h⁻¹ into the rear part of a divided chamber by non-catheterized *P. dolloi*.

A recent review concluded that some primitive freshwater fish have a higher GFR and UFR when compared to freshwater teleosts (Wright, 2007). The present results extend this conclusion to African lungfish. Although these results are in accord with the lungfish having an extremely permeable glomerular filtration barrier (Sawyer et al., 1976; Sawyer et al., 1982; Ojeda et al., 2006), it is surprising in light of the reduced gill area (Smith, 1930; Laurent et al., 1978) and relatively low metabolic rates of lungfish (Smith, 1930; Johansen and Lenfant, 1968; Oduleye, 1977; Perry et al., 2005; Ifitiker et al., 2008). Relative to the many freshwater teleosts which have been studied, the present GFR and UFR measurements are quite high for animals of comparable size, even though almost all the teleost data were collected by internal bladder catheters (Hickman and Trump, 1969; Wood, 1995), which would tend to elevate the values. In Table 1, we have included reference data from a study which used the external catheter technique (Curtis and Wood, 1991) on size-matched rainbow trout *Oncorhynchus mykiss*. Again the higher GFR and UFR values in lungfish are evident. However urinary ion concentrations, excretion rates, and clearance ratios for ions are all very low and comparable to those in the rainbow trout, indicative of highly efficient electrolyte conservation by the renal systems of these two lungfish species. This is likely due to the lungfish having very narrow ciliated distal and collecting segments of the proximal tubule (Ojeda et al., 2006). Also our current data on renal ion handling are closer to those of Sawyer (1966) on *P. aethiopicus* than to those of Babiker and Rankin (1979) on *P. annectens*, where Na⁺ reabsorption was less efficient.

4.4. Discrepancy between ¹H₂O fluxes and UFR relative to teleost fish

In accord with the scattered evidence based on different studies with different species of *Protoperus sp.* summarized in the Introduction, the current investigation has confirmed the discrepancy between ¹H₂O fluxes and UFR in lungfish relative to values in teleost fish. Thus, ¹H₂O efflux rate (k), representing unidirectional diffusive water flux, is low relative to teleosts (Table 2), whereas UFR, representing net osmotic water flux, is high relative to teleosts (Table 1). As first explained by Potts et al. (1967), it is possible to directly compare the two measurements. The net diffusive flux is the difference between the two unidirectional diffusive fluxes. Unidirectional diffusive water flux is proportional to the mole fraction of water on the relevant side of the semi-permeable membrane. The mole fraction of water in the external medium (freshwater) where the osmolality is about 5 mOsm kg⁻¹ is 55.560/[55.560+0.005]=0.9999. The mole fraction of water in the blood plasma of the lungfish where the osmolality is about 250 mOsm kg⁻¹ (Wilkie et al., 2007) is 55.560/[55.560+0.250]=0.9955. The difference between the two mole fractions is 0.0044, and therefore the net diffusive flux of water should be 0.44% of the total unidirectional diffusive water flux. Using the range of k values (0.13–0.38 h⁻¹) recorded for aquatic lungfish in the present study (Table 2) and a water space of 750 mL kg⁻¹, then the predicted net diffusive water flux would be 0.43–1.25 mL kg⁻¹ h⁻¹. This is far below the recorded mean net osmotic water flux, as estimated by the UFR values of 3.88–5.19 mL kg⁻¹ h⁻¹. Thus osmotic water permeability appears to be 4–12 times greater than diffusive water permeability in lungfish. A summary of comparative calculations for four freshwater teleosts (trout, eel, goldfish, and flounder) yielded a mean ratio of 2.75 (range 2.1–3.3), whereas the ratio was close to 1.0 in seven seawater teleosts (Isaia, 1984).

While the modest discrepancy in freshwater teleosts (but not in seawater teleosts) has been recognized for many years, its explanation has been controversial (Potts et al. 1967; Evans, 1969; Motais et al. 1969; Loretz, 1979; Isaia, 1984). Long before the discovery of aquaporins, one of the early explanations was that there were “water-filled pores” in freshwater but not seawater gill epithelia (Motais et al., 1969). This reflects the idea that diffusive exchange mainly occurs through the cell membrane, whereas osmotic water flux may additionally involve bulk flow through channels. Recently, Evans et al. (2005) have suggested that this hypothesis should be revisited with modern molecular techniques. In future work, it will be of interest to see if aquaporins are particularly abundant in the gills or skin of *Protopterus sp.*, thereby explaining the larger discrepancy. An alternate explanation could be that UFR provides an overestimate of osmotic water flux because lungfish drink significant amounts of freshwater. Drinking rates have never been measured in lungfish to our knowledge. There seems to be no reason why they should do this, but certainly this possibility could also be evaluated in future tests.

Overall, our results re-inforce the dichotomy between rates of ¹H₂O exchange and UFR in African lungfish, and therefore emphasize that these two indices measure different parameters, the former representing diffusive water permeability and the latter osmotic permeability (Isaia, 1984). They further illustrate that the kidneys of *P. dolloi* and *P. annectens* are well-designed for a freshwater existence requiring efficient ion conservation and water excretion. Finally, in
accord with the prediction of Gray (1928) about the preconditions needed for successful invasion of land, we conclude that African lungfish have reduced the diffusive permeability of their epithelium to water.

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References


