Ionoregulatory Physiology of the African Lungfish, *Protopterus dolloi* and *Protopterus annectens*

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Abstract:

The origin of terrestrial vertebrates from water-dependent fish involved numerous morphological and physiological modifications (Benton, 1990). Interest in the adaptive mechanisms involved in the transition from aquatic to terrestrial environments has led to research involving lungfish. African lungfish are obligatory air breathers and have a primitive lung and characteristically underdeveloped gills compared to freshwater teleosts. The gills are thought to play an important role in CO_2 excretion and possibly in water and ionic exchange while in aquatic conditions. At present, little is known about the basic ionoregulatory physiology of lungfishes; the aim of this thesis was to describe the basic principles of ion and water balance in two species of African lungfish, *Protopterus dolloi* and *Protopterus annectens*. Patterns and rates are very similar in the two species, apart from differences in water handling at the kidney. In aquatic conditions, plasma ion (Na^+, Cl^-, Ca^{2+}) levels are lower than in teleost fish. The major site of diffusive water exchange appears to be the gills. The skin is well vascularized and also serves as site of water exchange, and likely Cl^- and Ca^{2+} uptake as well. However, water and ion exchange rates are lower than in freshwater teleosts, probably due to the reduced gill area, though glomerular filtration, urine flow rates (an index of osmotic permeability), and urinary ion excretion rates are comparable to those of teleosts. Water exchange rates increase immediately after feeding, likely associated with specific dynamic action, and decrease with prolonged terrestrialization, likely due to disturbances in gill function. A budget analysis of ion balance indicates that both unidirectional uptake from the water and net uptake from the food (especially for Cl^-) are important, whereas unidirectional efflux across the gills and/or skin is a larger route of ion loss than

are feces or urine. Despite many physiological differences between freshwater teleosts and the African lungfish, water and ion balance are maintained in a broadly similar fashion and are achieved by compensating for the reduced gill area by ion acquisition via the skin and by greater ion reabsorption by the kidneys.

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Chapter 1: Introduction

BACKGROUND:

During the Devonian period, parts of the world were subject to extreme weather conditions including cycles of heavy rainfall followed by periods of drought (Campbell *et al.*, 1999). Thus, natural selection favored fish with lungs since they were capable of surviving in both aquatic and terrestrial environments. The origin of terrestrial vertebrates could have been attributed to selection pressures such as escape from predators, the availability of food on land, extreme climate changes leading to droughts and depletion of oxygen levels in the water (Johansen, 1968; Campbell *et al.*, 1999). Anatomically it would seem that the lobe-finned fish, with muscular fins and skeletal extensions, would be very closely related to the ancestor of tetrapods. However, molecular evidence suggests that lungfish are the closest relative (Campbell *et al.*, 1999). Furthermore, 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). Since the discovery of the *Protopterus annectens* by Owen (1839), taxonomists have struggled to phylogenetically identify them as a fish, an amphibian or a close relative to the ancestors of both (Duvernoys, 1846). However, it is indisputable that they are now highly specialized largely due to a change in their habitat (Parker, 1891). Thus, research involving lungfish allows for a better understanding of adaptive mechanisms involved in the transition from aquatic to terrestrial environments.

The distribution of *Protopterus dolloi* and *Protopterus annectens* is spread throughout Africa. The *Protopterus dolloi* are most commonly found in central Africa in equatorial conditions, whereas the *Protopterus annectens* are commonly found in the

North West, North East and in South Africa, which puts them in more temperate regions (Greenwood, 1986). In addition to these habitat differences, there are some visual distinctions between the two species such as coloration, scale size, and morphometric features (Poll, 1961; Greenwood, 1986). However, in terms of breeding, the two species exhibit similar behaviors. The African lungfish spawn when there is substantial accumulation of water due to heavy rainfall or flooding from other bodies of water (Greenwood, 1958, 1986). Their nests, made in the substrate, are "U" shaped and the adult lungfish exhibit parental care by guarding the two openings of the nest (Greenwood, 1986). Water is present in the trough of the U-shaped nest where the eggs are deposited, fertilized, and kept for 8-10 days before they hatch. Once the young lungfish have hatched, they remain in the nest as they are quite small and inactive. During this time the young fish have functional internal and external gills which prove to be sufficient in acquiring oxygen and ion uptake. It has been noted that these young fish are not obligatory air breathers and can survive for more than one month without coming to the surface of the water (Budgett, 1901; Johnels and Svensson, 1954; Greenwood, 1958). The lungfish leave the nest when they reach about 35mm in length, which also corresponds with the time they become obligatory air breathers (Greenwood, 1986).

During the dry season, the African lungfish commonly endures harsh environmental conditions as the body of water they occupy evaporates and becomes hypoxic, which in turn also leads to a food shortage (Smith, 1931; Janssens, 1963; Janssens and Cohen, 1967; DeLaney *et al.*, 1974; Fishman *et al.*, 1986; Greenwood, 1986). To cope, the lungfish often choose to aestivate (enter a state of dormancy or torpor). Similar to the nests made for breeding purposes (and in some cases the same

one), the lungfish will dig a burrow in the substrate, or use one that has been made by other animals, for the purpose of aestivation (Smith, 1931; Janssens, 1963; Janssens and Cohen, 1967; DeLaney *et al.*, 1974; Fishman *et al.*, 1986; Greenwood, 1986). However, in the *Protopterus dolloi* species, it has been observed that only the adult females and immature lungfish aestivate in the burrow, whereas the male *Protopterus dolloi* will stay in the dried out habitat that they occupied during the wet season (Brien *et al.*, 1959; Greenwood, 1986). Furthermore, since aestivation is highly dependent on environmental conditions the two species of lungfish differ. *Protopterus dolloi*, which inhabit equatorial areas, only aestivate if their body of water dries out (Greenwood, 1986). However, the *Protopterus annectens*, which are found in temperate areas of Africa, are known to aestivate seasonally (Greenwood, 1986).

Once inside the burrow, the lungfish will secrete a mucus cocoon to surround itself and to avoid dessication (Smith, 1931; Janssens, 1963; Janssens and Cohen, 1967; DeLaney *et al.*, 1974, 1977; Fishman *et al.*, 1986; Greenwood, 1986). The lungfish is positioned in such a way that its mouth remains open to the entrance of the burrow, allowing ventilation to occur (Smith, 1930,1931; Janssens, 1963; DeLaney *et al.*, 1974, 1977). During the aestivation period the lungfish does not eat or drink and endures many physiological changes including a decrease in metabolic rate, heart rate, blood pressure, ion exchange, water exchange, and oxygen requirements (Smith, 1931; Fishman *et al.*, 1986; Wilkie *et al.*, 2007). The gills also fill with mucus limiting or completely abolishing the ability to uptake oxygen or ions and thus blood flow is shunted away from the internal gill arches (Laurent *et al.*, 1978; Fishman *et al.*, 1986; Sturla *et al.*, 2001). Fecal and urine excretion ceases and waste accumulates in the lungfish body (Smith,

1930; Janssens, 1963; Janssens and Cohen, 1967; DeLaney *et al.*, 1977; Chew *et al.*, 2004; Wood *et al.*, 2005; Wilkie *et al.*, 2007). Furthermore, during the months of aestivation, ammonia is converted to urea via the ornithine urea cycle (Smith, 1930; Janssens and Cohen, 1966). Thus, urea (a much less toxic nitrogen waste product) accumulates in the body and is usually excreted within the first two days of the lungfish being reimmersed in water (Smith, 1930; DeLaney *et al.*, 1977; Wood *et al.*, 2005). Therefore, the lungfish becomes ureotelic upon re-immersion, as opposed to ammoniotelic while aquatic (Smith, 1930; Janssens, 1963; Janssens and Cohen, 1967; DeLaney *et al.*, 1977; Chew *et al.*, 2004; Wood *et al.*, 2005; Wilkie *et al.*, 2007).

The remarkable ability of lungfish to aestivate for long periods of time has been looked at by many investigators (e.g. Smith, 1930; Janssens, 1963; Janssens and Cohen, 1967; DeLaney *et al.*, 1974,1977; Laurent *et al.*, 1978; Fishman *et al.*, 1986; Greenwood, 1986; Chew *et al.*, 2004; Wood *et al.*, 2005; Wilkie *et al.*, 2007). While little is known about ion and water balance under aquatic conditions in the lungfish, even less is known about this area during aestivated periods (Wright, 2007). The literature that is available shows vast differences in ion regulation depending on the techniques used to induce aestivation (Smith, 1930; DeLaney *et al.*, 1977; Wilkie *et al.*, 2007). Thus, terrestrialization is the term now used for different grades of air exposure and the associated physiological changes that occur when the fish is air-exposed but not burrowed in the mud; it is thought that this condition is representative of the changes that take place before aestivation occurs (Chew *et al.*, 2003, 2004; Wilkie *et al.*, 2007). When the lungfish truly aestivates in a mud cocoon there is a significant increase in plasma Na^+, Cl^- and osmolality and a substantial loss of weight (Smith, 1930; DeLaney *et al.*, 1977).

However, during terrestrialization, Wilkie et al. (2007) reported no change in plasma osmolality, Na^+ or weight of the lungfish, but a fall in plasma Cl^-. They also demonstrated that the primary site for water exchange is the ventral body surface, since this is the only region that remains exposed to the water, because the rest of the animal becomes covered with cocoon material (Wilkie *et al.*, 2007). Remarkably, under these conditions, the *Protopterus dolloi* reduces water exchange rates by 95%; this reduction is likely due to a decrease in water permeability across the entire body surface (Wilkie *et al.*, 2007).

Diffusive water flux refers to the unidirectional flux of water transported across membranes by simple or facilitated diffusion. Osmotic water flux, which is generally much smaller, refers to net water flux being transported by the presence of transport-related osmotic and/or hydrostatic gradients. In freshwater fish, the majority of water exchange (90%) takes place across the gills (Motais *et al.*, 1969; Haywood *et al.*, 1977; Evans 1979; Isaia, 1984). However, the African lungfish has characteristically underdeveloped internal gills compared to those of freshwater teleosts, but like amphibians possesses a well-vascularized skin which is capable of ion, nitrogenous waste, and water exchange (Smith, 1930; Laurent *et al.*, 1978; Sturla *et al.*, 2001; Wood *et al.*, 2005; Wilkie *et al.*, 2007). Therefore, in aquatic conditions, water flux can take place via the gills, skin and urine of the lungfish. A rate constant refers to the water fraction from the inside of the fish that is excreted out by diffusive exchange in one hour. For example, a rate constant of 0.34 h^{-1} means 34% of the water inside is excreted out by diffusive exchange in one hour, even though the net rate of osmotic water excretion by urine flow may only be 1% per hour. Recent studies by Wilkie et al. (2007) on

Protopterus dolloi showed diffusive water flux rates to be 125 ml/kg/h and rate constants to be 0.16 h^{-1}. These rates are comparable to those of some freshwater and seawater teleosts including the euryhaline flounder and eel (Evans, 1979). However, most teleost fish (e.g. Salmonids, Cichlids, Cyprinids) have much higher water exchange and rate constants (Evans, 1979). Thus, even though the lungfish is able to use its skin for water exchange, the reduced gill area results in lower water exchange rates compared to freshwater teleosts (Evans, 1979; Wilkie *et al.*, 2007). To date, intraspecific differences between *Protopterus dolloi* and *Protopterus annectens* have not been examined. Since there are differences in habitat and frequency of aestivation, there may be differences in physiology that may contribute to variation in water exchange between the two species.

In fish, the ability to regulate extracellular Na^+ and Cl^- levels is essential to maintain osmoregulation. Freshwater fish exchange these ions across their gills, linking Na^+ uptake to acid excretion (NH_4^+ or H^+) and Cl^- uptake to base excretion (HCO_3^-), to maintain acid/base balance (Payan *et al.*, 1984; Perry, 1997; Marshall, 2002). These exchanges appear to take place mainly in mitochondria rich cells which are found on the filamental epithelium of the gills (Payan *et al.*, 1984). Ca^{2+} is also an important nutrient ion, which is transported across the branchial epithelium via the branchial epithelial calcium channel found in both the mitochondria rich cells and pavement cells of the gill (Payan *et al.*, 1984; Shahsavarani *et al.*, 2006). Although the reduced gill area of the African lungfish plays a minor role in O_2 uptake (~20%), it is theorized to have an important role in CO_2 (~60 %) excretion and ionic exchange (McMahon, 1970; Laurent and Dunel, 1976; Laurent *et al.*, 1978; Fishman *et al.*, 1986; Perry *et al.*, 2005). In an immunohistochemical study conducted on the *Protopterus annectens* by Sturla et al.

(2001), the gills and the skin show the presence of two different types of mitochondria rich cells. These two cell types appear to be similar to mitochondria rich cells designated as α and β "chloride" cells that are found in freshwater fish (Pisam *et al.*, 1987; Pisam and Rambourg, 1991; Pisam *et al.*, 1990; Pisam *et al.*, 1993; Perry, 1997; Sturla *et al.*, 2001). The gills of the *Protopterus annectens* contains both α and β type "chloride cells", however, the skin contain only α type "chloride" cells (Sturla *et al.*, 2001). These α type "chloride" cells seem to play a large role in ion regulation as they are the site for Cl^- absorption as well as Ca^{2+} uptake (Perry, 1997). Despite the presence of "chloride" cells in both the gill and the skin, Wilkie et al. (2007) showed very low uptake rates of Na^+ and Cl^- from their aquatic environment for the *Protopterus dolloi*. However, since both of these species of African lungfish are commonly found in swampy backwaters, in which they can be subject to hypoxic and ion poor surroundings, it is likely that they would exhibit decreased ion permeability in comparison to freshwater teleost (Smith, 1931; Janssens, 1963; Janssens and Cohen, 1967; DeLaney *et al.*, 1974; Fishman *et al.*, 1986; Greenwood, 1986, 1987; Wilkie *et al.*, 2007). Further evidence of this low ion uptake is observed from plasma composition data reported for the *Protopterus aethiopicus* in aquatic conditions (DeLaney *et al.*, 1976). Mean plasma calcium levels were 4.8 meq/L, which is typical of most freshwater fish; However, mean sodium and chloride levels were 101 meq/L and 77 meq/L respectively (DeLaney *et al.*, 1976), which are rather low relative to those of most freshwater teleost fish (see tabular summary in Evans, 1979, for example).

Observational studies have reported that African lungfish locate their prey by olfaction which is supplemented with taste buds on their pectoral fins (Johnels and

Svensson, 1954; Greenwood, 1986). It has also been observed that prey type is quite influential in their feeding behavior (O'Reilly *et al.* 2002). African lungfish are sluggish when capturing earthworms but increase their movement when capturing a more active prey, such as goldfish (O'Reilly *et al.* 2002). Therefore, in natural environments the African lungfish must vary their movements since they have been reported to be carnivorous and prey on small invertebrates including crustaceans and mollusks, and also on small fish (Smith, 1935; Greenwood, 1986). They capture their prey by suction and by the use of hydraulic transport they position the food for processing between the tooth plates (Bemis, 1986; Greenwood, 1986). An autostylic jaw suspension, tooth plates, and mobile branchial apparatus are the three major morphological features that allow for feeding (Bemis, 1986).

Presently there appear to be no studies on the role or impact of feeding on energy expenditure, water exchange or ion acquisition in the African lungfish (Wright, 2007). Also, the importance of ion uptake from the water *versus* the diet has not been compared. Possibly, one may be a more important source of ions compared to the other. Furthermore, it is unclear how starvation (either under aquatic or aestivated conditions) affects water and ion balance.

A study looking at nitrogen metabolism in the *Protopterus dolloi* following feeding has shown that there is an initial increase in ammonia excretion; however, after 3 hours the *Protopterus dolloi* became ureotelic and remain so for 21 hours (Lim *et al.*, 2004). The presence of high levels of ammonia in the plasma is observed in some freshwater fish following feeding (Wicks and Randall, 2002). However, because there is an increase in urea synthesis and excretion before an observable increase in plasma

ammonia levels, it is likely that the lungfish uses a different mechanism to perceive and respond to feeding and thus the *Protopterus dolloi* has the capacity to prevent a surge in plasma ammonia concentration (Lim *et al.*, 2004).

Studies addressing kidney function in the lungfish are interesting as they may show adaptive changes involved in the transition from aquatic to terrestrial environments as well as intermediate characteristics of both fish and amphibians (Smith, 1930; Guyton, 1935; Wake, 1986; Ojeda *et al.*, 2006). In freshwater fish the gills play a large role in nitrogen waste (ammonia) excretion and ion exchange whereas the kidney functions in water excretion and ion conservation by reabsorption (Hickman and Trump, 1969; Wood, 1995). The aquatic lungfish excretes nitrogen waste and water from the gills, kidney, and the skin (Smith, 1930; Janssens and Cohen, 1968; Delaney *et al.*, 1976; Wood *et al.*, 2005; Ojeda *et al.*, 2006; Wilkie *et al.*, 2007). However, during the dry season when the lungfish is subject to aestivation, nitrogen excretion ceases and the gill and skin are covered by a mucous cocoon rendering them inoperable. Renal excretory functions appear to be minimal or cease (Smith, 1930; Janssens and Cohen, 1968; Delaney *et al.*, 1976; Wood *et al.*, 2005, Ojeda *et al.*, 2006, Wilkie *et al.*, 2007) Thus, kidney function of the lungfish must be flexible in that it is able to cycle between aquatic and aestivated conditions.

Urine flow rate (UFR) is highly dependent on environmental conditions and the overall water permeability of the fish (Wood, 1995). In other words, UFR gives an index of osmotic water flux. An increase in the osmotic gradient for water influx, and/or an increase in water permeability will result in an increase in overall urine flow rate (Wood, 1995). In the case of the lungfish, water permeability appears to be low which also

decreases urine flow rate (Wood, 1995; Ojeda *et al.*, 2006; Wilkie *et al.*, 2007). Glomerular filtration rate (GFR) refers to the rate at which water is filtered from the plasma into the primary urine by the glomeruli (Wood, 1995). GFR is highly affected by arterial blood pressure and thus is highly dependent on water permeability since water loading would cause an increase in blood pressure; Therefore, GFR has a linear relationship to UFR (Wood, 1995).

Kidney function in freshwater primitive fish is comparable to that in teleosts (Wright, 2007). Overall, it has been found that some primitive fish have a higher GFR and UFR when compared to freshwater teleosts (Wright, 2007). However, studies looking at kidney function in *Protopterus aethiopicus* (Sawyer, 1966; Forster and Goldstein, 1969) have reported UFR and estimated GFR to be comparable to those in representative freshwater teleosts (e.g. Hickman and Trump, 1969; Patel *et al.*, 2006). Similar studies have not been carried out in the *Protopterus dolloi* and *Protopterus annectens*. A fish that maintains ion balance should exhibit ion excretion rates that are similar to ion influx rates (Wood, 1995). However, this may not occur in the kidney as it has been shown that in some primitive fish (i.e. lampreys, bowfin) there is minimal loss of Na^+ and Cl^- as most of it is reabsorbed by the kidney (Logan *et al.*, 1980; Butler and Youson, 1988; Wright, 2007). There is virtually no information on ion handling in the lungfish kidneys but it can be assumed that their response would be typical of freshwater primitive fish in that there would be minimal loss of ions through the urine (Wright, 2007).

Overall, a better understanding is needed of lungfish metabolism as it relates to the regulation of essential ions required for the maintenance of tissue, plasma and overall

function. Thus, studies addressing ion acquisition from the diet and from surrounding waters are needed. Similarly, information is required on the loss rates of these ions to the water via excretion through the skin, gills and kidney, as well as in the feces in order to understand ion balance. Comparable studies on water balance in aquatic lungfish are essential to understanding the unique physiology of the lungfish and how it maintains osmotic balance when metabolic rates are increased (i.e. feeding), or decreased (i.e. aestivation). Overall, this would provide information on the ability of the lungfish to maintain internal osmotic and ion homeostasis.

OBJECTIVES:

Tissue Ion Comparison:

Based on previous findings, during aestivation of the African lungfish, there are many physiological changes that occur (i.e. slowing of metabolic rate, cessation of urine and feces, a build up of urea, loss of gill function) (Smith, 1930; Janssens, 1963; Janssens and Cohen, 1967; DeLaney *et al.*, 1977; Chew *et al.*, 2004; Wood *et al.*, 2005). Given these extreme changes, it is remarkable that plasma osmolality and ion levels are maintained during terrestrialization (Wilkie *et al.* 2007). To confirm that there were no significant changes in ion distribution in the *Protopterus dolloi*, various tissues (muscle, liver, lung, kidney, intestine and heart) were analyzed for ion composition (Na^+, Cl^-, and Ca^{2+}) for aquatic, 1 month and 5 month terrestrialized conditions. Furthermore, tissue ion content was compared to values for some freshwater teleosts to observe changes that may be of evolutionary significance.

During terrestrialisation water exchange is limited to the ventral skin surface (since this is the only area in direct contact with the water) (Wilkie et al., 2007). Tissue water content was examined to learn whether terrestrialization affects water balance. If tissue water content were to be maintained (like plasma osmolality), this would suggest that the observed differences in diffusive water exchange rates during this treatment, as reported by Wilkie et al. (2007), do not markedly alter the internal osmotic status of the animal.

Feeding Studies:

To date, feeding studies looking at ion balance in the African lungfish are non-existent. The purpose of this study was to determine the role of the diet in ion acquisition and to compare uptake to the loss of these ions via the urine (see below) and feces. Since uptake rates of major ions from the external water proved to be extremely low (see next section), it seemed likely that the food would be an important source of ion acquisition. By determining ion levels in the diet and then monitoring the loss of ions to the water we determined the importance of diet in the African lungfish.

In addition, feces were collected (prior to discharge) from both *Protopterus dolloi* and *Protopterus annectens* and analyzed for major ions, so as to provide a measure of ion loss rate in the feces.

Studies on Nitrogenous Waste Excretion:

Nitrogen metabolism during aestivation in the African lungfish has been studied by many researchers (Smith, 1930; Janssens, 1963; Janssens and Cohen, 1967; DeLaney *et al.*, 1977; Chew *et al.*, 2004; Wood *et al.*, 2005; Wilkie *et al.*, 2007). In aquatic conditions the African lungfish is ammoniotelic, however, during aestivation it produces

and stores urea in the body, which is than excreted upon reimmersion to the water (Smith, 1930; Janssens, 1963; Janssens and Cohen, 1967; DeLaney *et al.*, 1977; Chew *et al.*, 2004; Wood *et al.*, 2005; Wilkie *et al.*, 2007). A recent study by Lim et al (2004) also showed that following feeding the *Protopterus dolloi* becomes ureotelic. Thus, to confirm these results a separate experiment was carried out in which levels of ammonia and urea were monitored in fed and starved fish. The experiment was also carried out in the *Protopterus annectens* to see if nitrogen production and excretion was similar.

Plasma Ion Levels:

Plasma ion levels in the African lungfish *Protopterus dolloi* and *Protopterus aethiopicus* were found to be quite low compared to freshwater teleosts (DeLaney *et al.*, 1977; Evans, 1979; Rogers *et al.*, 2003; Wilkie *et al.*, 2007). The plasma was analyzed to confirm these plasma ion levels in the *Protopterus dolloi* and to determine whether this is also true for the *Protopterus annectens*. Furthermore, since ion levels in the plasma of the *Protopterus dolloi* and the *Protopterus annectens* proved to be similar, a study was conducted to see if this was also true of ion acquisition from the food and water.

Waterborne Ion Uptake:

The only available data on unidirectional ion uptake rates from the water by lungfish are those reported by Wilkie et al (2007) in *Protopterus dolloi*, where both Na^+ and Cl^- flux rates were very low relative to those seen in most teleost fish (Evans, 1979; Rogers *et al.*, 2003). Therefore one goal of the present study was to confirm these apparently low rates in *Protopterus dolloi*, and also to see if the same is true in *Protopterus annectens* using standard radiotracer flux techniques. An additional goal was

to measure unidirectional Ca^{2+} flux rates, which have never been determined previously in any species of lungfish.

Diffusive Water Exchange:

Diffusive water exchange rates in the *Protopterus dolloi* have previously been reported to be quite low by Wilkie et al. (2007). In the present comparative study, our goal was to use a similar technique of intraperitoneal injection of tritiated water (^3H_2O) to confirm these previous measurements in *Protopterus dolloi* and to determine whether the *Protopterus annectens* and juvenile *Protopterus dolloi* exhibited similarly low rates. A further goal was to compare diffusive water exchange values with osmotic water flux rates, the latter determined by measurements of the urine flow rate.

In a separate experiment, a divided chamber study was employed to separate the gills from the skin and urinary opening and thus to localize the sites of water exchange. Since water exchange rates have been found to be quite low it is likely that the reduced gill area is efficient in providing the majority of water exchange. However, it is also possible that the skin may play a minor role in water exchange.

Based on recent a recent study conducted by Wilkie et al. (2007), diffusive water exchange was significantly reduced during terrestrialization, but plasma osmolality was maintained. Since water permeability was altered, we predicted that there would be a significant decrease in water exchange rates following return to aquatic conditions.

Also, recent work on *Protopterus annectens* by Iftikar *et al.* (submitted) has shown that following feeding there is an increase in O_2 uptake from both the air and water phases reflecting the extra metabolic energy required to process food for storage and use, a trend that is commonly know as Specific Dynamic Action (SDA). By the

"osmo-respiratory compromise" (Wood and Randall, 1973; Gonzalez and McDonald, 1991), an increase in gill or skin O_2 flux might well be associated with an increase in water exchange rates. Therefore, tritiated water exchange rates were measured in both *Protopterus annectens* and *Protopterus dolloi* before and after a standard meal.

Ion and Waste Excretion Study:

To date, the paper by Sawyer (1966), in which an internal urinary catheter was used, provides the only data on urine flow rate (UFR) and glomerular filtration rate (GFR) for the African lungfish, *Protopterus aethiopicus*. The values obtained in that study were very similar to the values obtained for many freshwater fish (Hickman and Trump, 1969; Patel *et al.*, 2006) which seems surprising in light of the reduced gill area (Smith, 1930; Laurent *et al.*, 1978; Wood *et al.*, 2005; Wilkie *et al.*, 2007) and relatively low metabolic rates of lungfish (Smith, 1930; Johansen and Lenfant, 1968; Oduleye, 1977; Perry et al., 2005). A study was therefore conducted to see if similar results would be obtained for the *Protopterus dolloi* and the *Protopterus annectens*, employing an external urinary catheterization method similar to that Curtis and Wood (1991), which allows the urinary bladder to store and then discharge the urine naturally. The urine collected from this study was analyzed for ions (Na^+, Cl^-, and Ca^{2+}), so as to allow direct measurement of renal ion excretion rates. Glomerular filtration rate was also measured, and in turn this facilitated clearance ratio analyses, which provide a more in depth look at kidney function. Clearance ratio analysis quantifies changes in tubular secretion and reabsorption of ions and water (Wood, 1995). These experiments also provided comparisons between osmotic water flux values, for which UFR provides a measure in

freshwater fish (Wood, 1995), and the unidirectional diffusive exchange rates measured with tritiated water (see above).

Chapter 2: Material and Methods

Experimental Animals:

African lungfish (*Protopterus dolloi* and *Protopterus annectens*) with an average weight of 150 g (range: 68-210 g) were collected in central and western Africa and were then shipped from a commercial dealer in Singapore to McMaster University (ON., Canada). All lungfish were held at 27-30°C under 12L:12D photoperiod in individual aquaria containing 3 L of dechlorinated tap water supplemented with a small amount of commercial sea salts which helped to prevent fungal infections. This resulted in a water composition of approximately Na^+ = 2.0 mmol/L, Cl^- = 1.8 mmol/L, Ca^{2+} = 1.2 mmol/L, hardness = 170 mg/L as $CaCO_3$ equivalents, and a pH of 7.8. The lungfish holding water was changed every second day and following the water change they were fed a diet of frozen chronimid larvae (bloodworms) at a ration of 3% of body weight every second day.

Plasma Ion Comparison:

A study was conducted to compare the plasma composition of the *Protopterus dolloi* and *Protopterus annectans*. The lungfish were held under aquatic conditions and a sample size of N=6 of both species were used. Fish were lightly anaesthetized using MS222 (TMS Veterinary anaesthetic; Aqua-Life, Syndel Laboratories Ltd., Vancouver, Canada) which was added to the water (0.25 g/L). Blood samples (100-300 µl) were taken by caudal puncture using heparinized syringes fitted with 23 G needles. Blood samples were centrifuged at 13 000 x g for 5 minutes and the plasma was transferred to a

separate 500 µl centrifuge tube and stored at –80°C until it was analyzed for ion (Na^+, Cl^-, Ca^{2+}) concentrations.

Tissue Ion Comparison:

The purpose of this study was to better understand the distribution of ions (Na^+, Cl^-, and Ca^{2+}) through various tissues (muscle, liver, lung, kidney, intestine and heart) of the *Protopterus dolloi*. Another goal was to look at possible physiological changes affecting ion balance that might be taking place during prolonged air exposure ("terrestrialization"). Samples were collected in conjunction with a terrestrialization study led by Dr. Mike Wilkie (Wilkie et al., 2007). To achieve terrestrialization conditions, each lungfish tank was drained and then 20 ml of water was added to the tanks so that a thin film of water was at 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 body (Wilkie et al., 2007). Following terrestrialization the lungfish were re-immersed in water and sacrificed to collect tissue samples. Aquatic lungfish were also sacrificed to serve as a comparison of tissue physiological changes. The samples were immediately frozen in liquid N_2 and then preserved in the –80°C freezer. Three experimental conditions were examined: aquatic lungfish, 1 month and 5 month terrestrialized fish. A sample size of N= 2-5 was used to determine ion content of various tissues. Tissue samples were digested in 1N HNO_3 (5:1 volume to tissue ratio; Fisher Scientific; trace metal grade) 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 x g for 10 min. The supernatant was then appropriately diluted and analyzed for ions (Na^+, Cl^-, and Ca^{2+}).

To determine tissue water content a sample size of N = 5 was used. Again three experimental conditions were examined: aquatic lungfish, 1 month and 5 month terrestrialized fish. Approximately 150 mg samples of muscle, liver, lung, intestine and kidney samples were weighed and dried at 65°C for a period of 96 hours. The difference in wet and dry weights was used to determine the % water content.

Feeding Study:

In general, studies looking at the role of the diet in ion balance are limited in fish, and non-existent in lungfish. Determining Na^+, Cl^-, and Ca^{2+} compositions of the food and then monitoring the water ion levels and waste products such as urine and faeces will provide information on how important diet is to maintaining ionic homeostasis. In the laboratory, the lungfish were fed a diet of frozen bloodworms (please see above). In most cases the food was completely eaten within 0.5 hours.

To determine any changes in ion balance with the water related to feeding, 6 *Protopterus dolloi* and 6 *Protopterus annectens* were set up in 2 L of dechlorinated tap water and at t = 0 h the lungfish were fed 5 g of thawed bloodworms (which approximates their usual 3% ration). Two 5 ml water samples were taken hourly for 8 hours and then every 12 hours 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^{2+}), and for nitrogenous waste products (ammonia- N, urea- N). An initial sample was taken from the tank of each fish and the relevant concentration determined was used as the 0 h

reference value. Therefore, each consecutive sample was presented as the difference from the initial time point (i.e. cumulative change) in units of concentration (μmol/L). Ammonia and urea results were presented as excretion rates (μmol/kg/h).

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 lungfish) were set up with 3 L of water. 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.

Finally, in a separate experiment 5 g of bloodworms (3 replicates) were digested in concentrated nitric acid (100 μL/mg dry weight) for 72 h. Subsequently, hydrogen peroxide (40 μL/mg dry weight) was added to complete the digestion and the samples were incubated a further 24 h before being analyzed for ions. 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 lungfish, and a 48 h feeding interval.

Unidirectional Waterborne Ion Uptake Studies:

Several experiments were carried out to determine unidirectional influx rates of Na^+, Cl^-, and Ca^{2+} from the external water. Six *Protopterus dolloi* and 6 *Protopterus annectens* were exposed to one radiotracer - either $^{22}Na^+$ ((1 μCi/L); PerkinElmer Life and Analytical Sciences, Boston,USA) $^{36}Cl^-$ ((1 μCi/L); American Radiolabeled Chemicals Inc., Saint Louis, USA), or $^{45}Ca^{2+}$ ((3 μCi/L)); PerkinElmer Life and Analytical Sciences, Boston,USA) in 2 L of water. Three 5 ml samples were taken

hourly for 8 hours; two of the samples were used for radioactive counting and the other sample stored until analysis of the total concentrations of the relevant ions. Influx rates (in μmol/kg/h) were determined by monitoring the disappearance of radioactivity from the water, and calculated as outlined below A ratio of $\leq$ 1:10 of estimated specific activity (cpm /μmol) in the fish relative to specific activity 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 (c.f. Wood, 1988).

Diffusive Water Exchange:

The objectives of this study were to determine water balance and how it is maintained in the two species of lungfish, *Protopterus dolloi* and *Protopterus annectens*. Firstly, diffusive water exchange rates were determined in the juvenile and mature *Protopterus dolloi* as well as in the *Protopterus annectens* using intraperitoneal injections of tritiated water (^3H_2O) in isotonic saline. Six juvenile *Protopterus dolloi*, 7 mature *Protopterus dolloi*, and 6 mature *Protopterus annectens* were injected into their intraperitoneal cavities with 0.25 mCi/kg titriated water (^3H_2O) (PerkinElmer Life and Analytical Sciences, Boston,USA) made up as an isotonic saline. A standardized dose of 1% of the lungfish body weight was injected. The fish were than placed in 3 L of water. The 8 hour flux period began after a 1 hour equilibration period was given to distribute the injected fluid. Samples were taken every hour to monitor the appearance of ^3H_2O in the water for a period of 8 hours. Again, a ratio of $\leq$ 1:10 of estimated specific activity (cpm /μmol) in the fish relative to specific activity in the water was set as the threshold for termination in the experiment. The stock solution was also counted so as to accurately determine the amount injected.

A study of diffusive water exchange during feeding was also carried out using identical techniques on 6 *Protopterus dolloi* and 6 *Protopterus annectens*. However in these experiments, a meal of 5g of bloodworms was delivered after a 1 hour equilibration period following titriated water (^3H_2O) injections.

In a separate experiment, comparisons of water efflux rates and rate constants were also made between aquatic (N=6) and 8 month terrestrialized *Protopterus dolloi* (N=5). Before being reimmersed in water, five terrestrial *Protopterus dolloi* were injected in their intraperitoneal cavities with 0.25 mCi/kg titriated water (^3H_2O) made up as an isotonic saline (1% of the lungfish body weight). The fish (which were very weak after being woken up from aestivation) were than 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 for the lungfish to come up for air and therefore no danger of drowning. The 8 hour flux period began after a 1 hour equilibration period was given to distribute the injected fluid. Samples were taken every hour to monitor the appearance of ^3H_2O in the water for a period of 8 hours.

A separate experiment was performed on 6 *Protopterus dolloi* and 6 *Protopterus annectens* to distinguish the relative importance of different parts of the body in water exchange. Lungfish were injected in their intraperitoneal cavity with 0.25 mCi/kg titriated water (^3H_2O) as isotonic saline. Again the standard 1% of the lungfish body weight was injected. The fish were than placed in 3 L of water. Following a 1 hour equilibration period, water in the tank was renewed with only 1 L of water and a polyethylene bottle containing 400 ml of water was attached to the posterior end of the lungfish using dental dam and further fastened with an elastic. The segregation was

placed directly behind the external gills. Thus, the anterior region (1 L) would show excretion by the gills and the posterior region (400 ml) would show excretion by the bulk of the skin and the kidney of the fish. The 3 hour flux period began and water samples were taken at the beginning (t = 0 h) and than again after 1,2 and 3 hours to monitor the appearance of ^3H_2O in the water bathing the head end. At 3 hours the bottle was removed and water samples were obtained from the bottle as well to detect the appearance of ^3H_2O 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 the spectrophotometer at 650 nm. At this wavelength a 1% leak could be detected. The preliminary study proved that there was no leak present.

Ion and Waste Excretion Study:

Twelve hours after feeding of the standard bloodworm meal, 6 *Protopterus dolloi* and 6 *Protopterus annectens* were taken from out of their tanks and then gentle pressure was applied to the body cavity so as to collect samples of fecal material from the posterior intestine through the cloaca. This material was then weighed and frozen for later ionic analysis. Feces samples were digested in 1N HNO_3 (5:1 volume to tissue ratio; Fisher Scientific; trace metal grade) 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 x g for 10 min. The supernatant was then appropriately diluted analyzed for ions (Na^+, Cl^-, and Ca^{2+}).

In a separate experiment, externally urinary catheters similar to those developed by Curtis and Wood (1991) were used to determine urine composition and flow rate, and thereby measurements of renal excretion rates. The experimental lungfish were starved

for 5 days to avoid the potential for feces to block the catheters that were sewn on to the cloaca. Fish were first anesthetized using MS222 (0.30 g/L). An external catheter (12FR urethral catheter; C.R. Bard Inc., Covington, USA) was sewn onto the lungfish using 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; 3M Animal Care Products Corp., St. Paul, USA). The fish were than injected with a 125 μCi/kg of [^{3}H]polyethelene 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 fish were then allowed to recover from the anaesthetic for 12 hours, 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 hours for a period of 72 hours. A terminal plasma sample was also taken by blind caudal puncture of the haemal arch. Urinary volume was measured gravimetrically (for calculation of urine flow rate), and samples were then frozen for later analysis of Na^+, Cl^-, Ca^{2+} (for calculation of urinary excretion rates), and [^{3}H]PEG-4000 radioactivity (for calculation of glomerular filtration rates). Excretion rates (in μmol/kg/h) were calculated as the product of urine flow rate (UFR in ml/kg/h) and the relevant concentration (μmol/ml) in the urine.

Analytical Techniques and Calculations:

Concentrations of Na^+, Ca^{2+} and Cl^- in water, urine, plasma, and tissue digests were determined by flame atomic absorption spectroscopy (FAAS) using a 220FS SpectrAA (Varian, Mulgrave, Australia). Water, urine, plasma, and tissue digests were diluted with 0.2% lanthanum for Ca^{2+} determinations. Water, urine and plasma Cl^-

concentrations were analyzed by the mercuric thiocynate spectrophotometric method (Zall et al., 1956). Water and urine ammonia concentrations were determined by the indophenol blue method of Ivancic and Degobbis (1984). The diacetyl monoxime method of Rahmatullah and Boyde (1980) was used to determine water and urine urea levels.

Net flux calculations were based on changes of total ion concentrations (Na^+, Cl^-, and Ca^{2+}) in the water when compared with the initial ion concentrations.

Net fluxes (in μmol/kg/h) were calculated by the following equation:

$$J_{net} = \frac{([X_i] - [X_f]) \times V}{(T)(W)} \qquad \text{Equation 1}$$

Where:
$[X_i]$ =concentration of total ion in the water at the beginning of the flux period (in μmol/L)
$[X_f]$ =concentration of total ion in the water at the end of the flux period (in μmol/L)
V = volume of water (in L)
T = time of flux period (in h)
W = weight of the lungfish (in kg)

Rates of unidirectional ion influx (in μmol/kg/h) were based on the disappearance of radioactivity from the water (into the lungfish) during each flux period.

Influx rates were calculated by the following equation:

$$J_{influx} = \frac{([CPM_i] - [CPM_f]) \times V}{(SA)(T)(W)} \qquad \text{Equation 2}$$

where:
CPM_i = radioactive counts of the ion in the water (in cpm/L) at the beginning of the flux period
CPM_f = radioactive counts of the ion (in cpm/L) in the water at the end of the flux period
V = volume of water (in L)
SA = specific activity of each isotope (in cpm/μmol)
T = time of flux period (in h)
W = weight of the lungfish (in kg)

$^{22}Na^{+}$ is a γ and β emitter and was counted by a Wallac Wizard 1480 Automatic Gamma Counter (PerkinElmer Precisely, Turku, Finland). $^{36}Cl^{-}$ and $^{45}Ca^{2+}$, which are solely β emitters, were counted by a QuantaSmart (Tricarb) Liquid Scintillation Analyzer; PerkinElmer Precisely, Downers Grove, USA) after the addition of 10 ml of scintillation fluid (ACS Amersham Biosciences, Little Chalfont, Buckinghamshire, U.K.) to the water samples. Tests demonstrated that quench was constant for these samples, so no quench correction was applied. Because Na^{+}, Cl^{-}, and Ca^{2+} influx rates were very low, the estimated specific activity (cpm /μmol) in the fish relative to specific activity in the water remained far below the termination threshold $\leq$ 1:10, and mean uptake of CPM from the water over the entire 8h period was used to provide the most accurate estimates of influx rates.

Rate constants for the diffusive efflux of water in the $^{3}H_2O$ experiments were determined based on the following equation from Evans (1967).

$$k = (2.3/t_1-t_0) \log 10 \text{ (CPM in fish at } t_0/\text{CPM in fish at } t_1) \qquad \text{Equation 3}$$

where:
k = the rate constant of the efflux
t_0 = the start of the experiment
t_1 = any point in time after t_0

Stock saline solutions used for $^{3}H_2O$ injections were also 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 in the water will give the amount remaining in the lungfish. Radioactivity was counted on the QuantaSmart (Tricarb) Liquid Scintillation Analyzer following the addition of 10 ml of scintillation fluid (ACS Amersham) to 5 ml water

samples. Again, tests demonstrated that quench was constant so no quench correction was applied.

Rate constants of efflux (k) were converted to actual water efflux rates (J_{efflux}) in ml/kg/h by assuming the water space was equal to 750 ml/kg.

$$J_{efflux} = k * 750 \text{ ml/kg} \qquad \text{Equation 4}$$

where:
k = the rate constant of the efflux in h^{-1}

The initial 1 hour equilibration period was not used when calculating rate constants or water efflux, though CPM loss to the water was monitored for inclusion in the above calculation of CPM in the fish. Additionally, only the subsequent 5 hours were taken into account in calculations of k and J_{efflux} due to the likely occurrence of recycling (specific activity ratio of water to fish generally reached the threshold of 1:10 after this time). However, during the feeding study, where exchange rates were higher, only the subsequent 4 hours of the experiment were used because the specific activity threshold was reached after this time point.

Urine flow rate (UFR) was calculated from the cumulative volume of urine collected from the lungfish over discrete time intervals based on the following equation:

$$UFR = \frac{\text{Volume of urine}}{(\text{Mass x Time})} \qquad \text{Equation 5}$$

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

$$CPM_{Plasma} = \frac{(CPM_{injected} - (\Sigma CPM_{water} + \Sigma CPM_{urine}))}{wt(g)*0.25} \qquad \text{Equation 6}$$

The equation assumes that this extracellular space marker (Munger et al., 1991) was distributed in an extracellular fluid volume of 250 ml/kg, a typical value for fish (Holmes and Donaldson, 1969). 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 Amersham) to 5 ml water samples, 100 µl urine samples, and 30 µl plasma samples, which were all made up to the same 5 ml total water volumes. Again, quench was constant.

GFR was calculated from the following equation, using the appropriate values for each collection period:

$$GFR = \frac{UFR \times CPM_{urine}}{CPM_{plasma}} \qquad \text{Equation 7}$$

Urine excretion rate (U_x) for any substance (x) in the urine was given by the following equation:

$$U_x = [x]_{urine} \times UFR \qquad \text{Equation 8}$$

Fecal ion excretion rates for any substance (x) were calculated based on the assumption that by gently massaging the lungfish we would extrude all of the feces that would accumulate for that feeding session. Since collection of feces had to take place while fish were outside of the tank, we monitored the fish for several days in advance to determine the time when fecal excretion would normally take place. It was determined that about 12 hours after feeding, feces could usually be seen in the surrounding water,

and collections were therefore made at this time. However, since the fish were fed only once every 48 h, we used 48 hours as the appropriate time interval to calculate the time-averaged excretion rates:

$$\text{Fecal ion excretion} = [x]_{feces} / \text{Weight} * \text{Time} \qquad \text{Equation 9}$$

The clearance rate of an ion was calculated from the urinary excretion rate (U_x)of the same ion using mean plasma ion levels from the study described earlier in which 6 *Protopterus dolloi* and 6 *Protopterus annectens* were anaesthetized and blood samples were taken by blind caudal puncture (please see above):

$$\text{Clearance Rate} = U_x * [x]_{plasma} \qquad \text{Equation 10}$$

The corrresponding clearance ratio (CR_x) was calculated by using the urine excretion rate (U_x), the concentration of a substance (x) in the plasma, and the GFR with the following equation:

$$CR_x = U_x / ([x]_{plasma} * GFR) \qquad \text{Equation 11}$$

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

Statistical Analyses:

All data are expressed as the mean ± SEM (N). Student's 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 to initial values

using a one-way ANOVA followed by a Dunnett's test and pairwise comparisons were made using a one-way ANOVA followed by a Tukey's test. All statistical significance was calculated at $P<0.05$.

Chapter 3: Results

Plasma Ion Comparison:

Plasma ion composition was similar between the *Protopterus dolloi* and the *Protopterus annectens* under aquatic conditions (Fig. 1). Plasma Na^+ and Cl^- levels ranged from 85-134 mM and 78-145 mM respectively. The Ca^{2+} levels ranged from 0.9-1.9 mM for both species of lungfish.

Tissue Ion and Water Composition:

Tissues (muscle, liver, lung, kidney, intestine, and heart) were sampled from *Protopterus dolloi* for ion concentrations and water content under aquatic conditions and after one and five months of terrestrialization. In general, ion levels were fairly stable. However, there was a significant increase in the intestine Na^+ concentration following one month of terrestrialization and this trend was maintained following five months of terrestrialization (Fig. 2A). There were no other significant changes in tissue Na^+ concentration as a result of terrestrialization. Kidney Cl^- concentration exhibited a significant decrease following one month of terrestrialization, but this decrease was no longer evident after five months (Fig. 2B). Other tissues showed no differences in Cl^- following terrestrialization. Following one month of terrestrialization, the Ca^{2+} concentration of the kidney doubled from aquatic values and there was a further increase, which became significant at five months (Fig. 2C). Liver Ca^{2+} concentration significantly increased following 1 month terrestrialization but this increase was not present following 5 months of terrestrialization.

Water content significantly increased in the muscle, intestine and kidney following five months of terrestrialization (Fig. 3). In the liver the increase in water

Figure 1. Plasma ion levels for the *Protopterus dolloi* (black bars) (N=6) and *Protopterus annectens* (N=6) (grey bars). There were no significant differences between the two species.

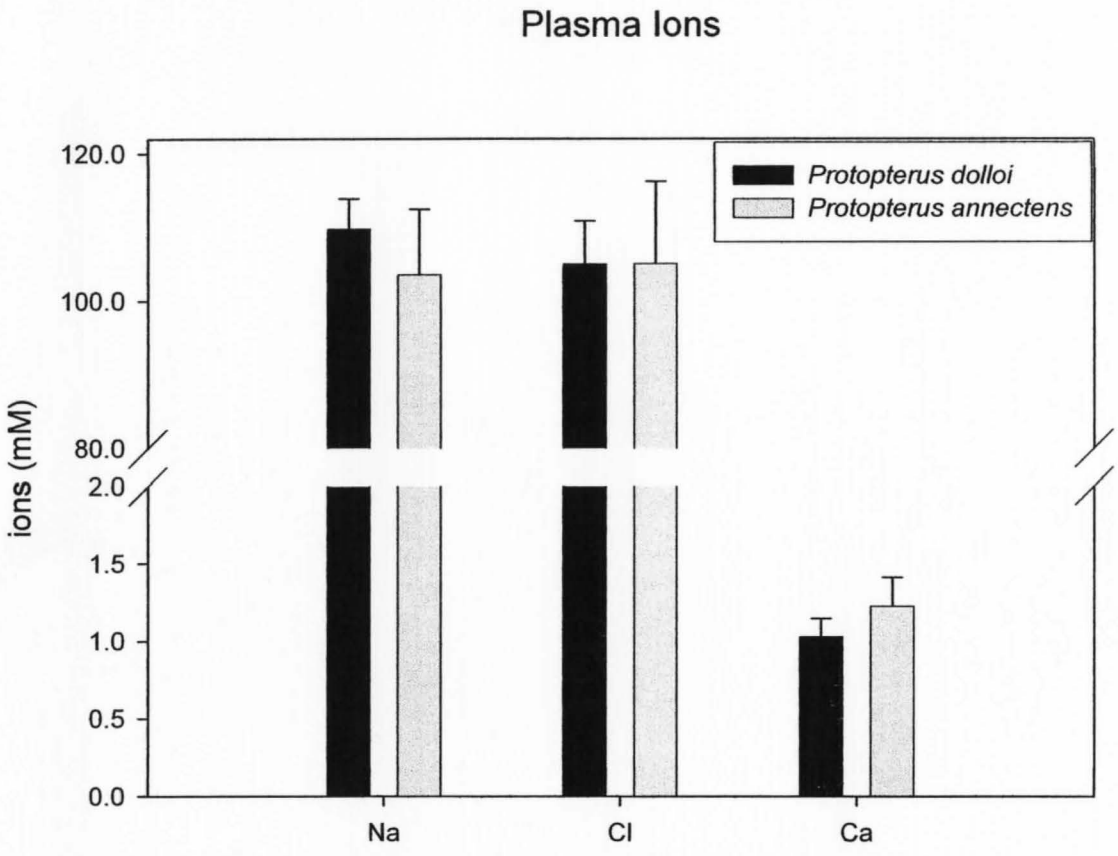

Plasma Ions
Protopterus dolloi
Protopterus annectens
ions (mM)
120.0
100.0
80.0
2.0
1.5
1.0
0.5
0.0
Na
Cl
Ca

Figure 2. Ion distribution of different tissues under aquatic (black bars), 1 month terrestrialized (light gray bars), and 5 month terrestrialized (gray bars) conditions for the *Protopterus dolloi*. N=2-5 ($P<0.05$; one way ANOVA plus Dunnett's test)

* indicates difference from aquatic lungfish

Figure 2

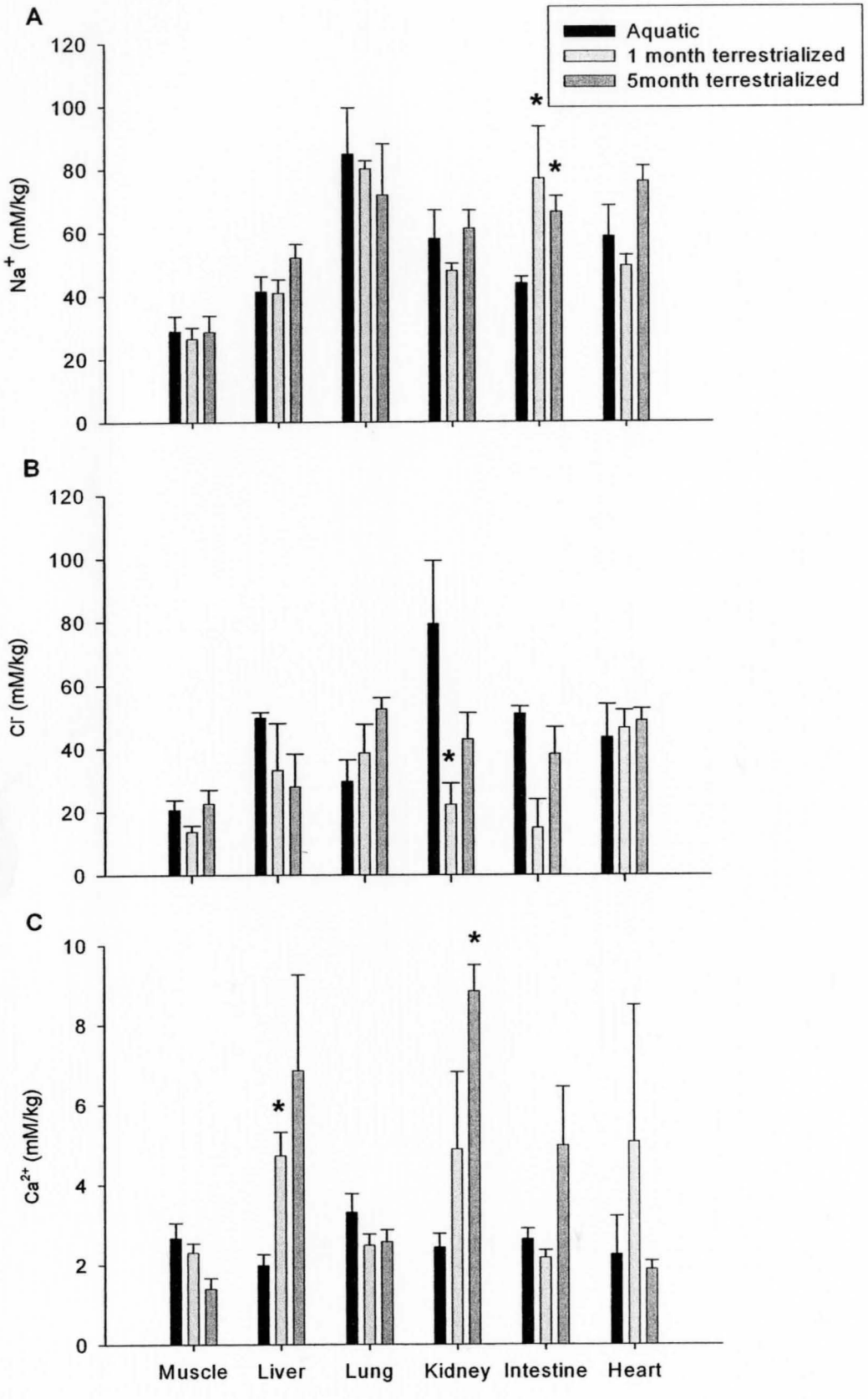
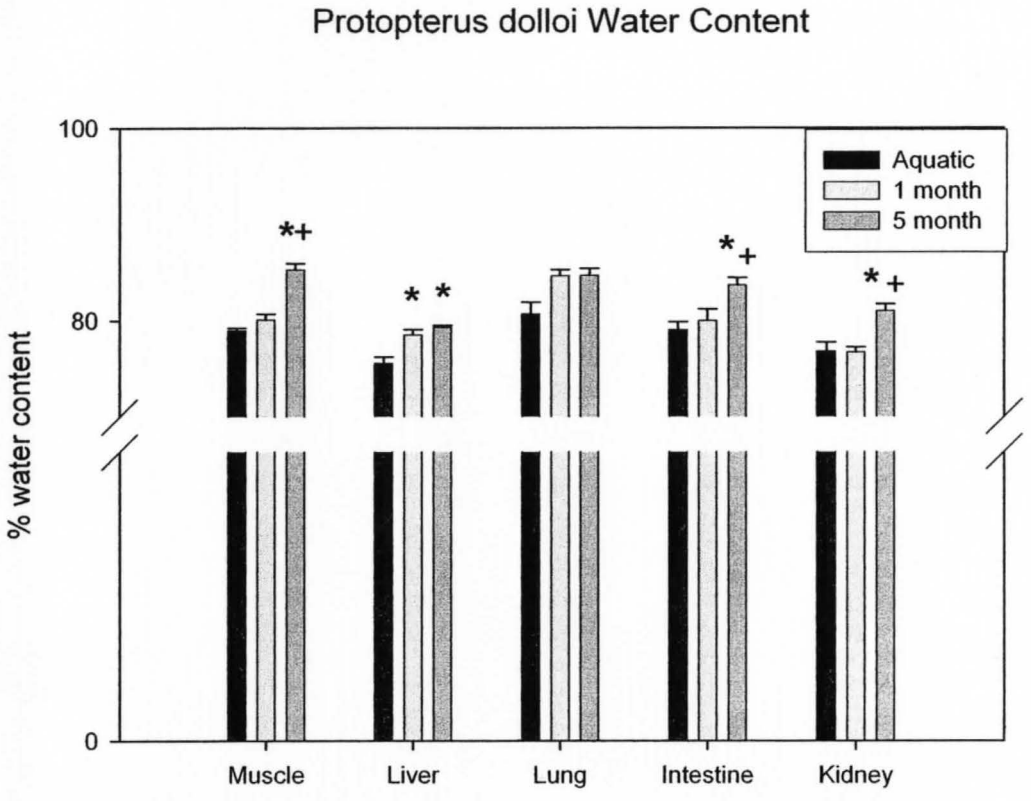

A
Aquatic
1 month terrestrialized
5month terrestrialized
Na^+ (mM/kg)
120
100
80
60
40
20
0
*
*
B
Cl^- (mM/kg)
120
100
80
60
40
20
0
*
C
Ca^{2+} (mM/kg)
10
8
6
4
2
0
*
*
Muscle
Liver
Lung
Kidney
Intestine
Heart

Figure 3. Water tissue content under aquatic (black bars), 1 month terrestrialized (light gray bars), and 5 month terrestrialized (gray bars) conditions for the *Protopterus dolloi*. N=2-5 ($P<0.05$; one way ANOVA plus Dunnett's test).

* indicates difference from aquatic lungfish

+ indicates difference between 1 month terrestrialized and 5 month terrestrialized lungfish.


Protopterus dolloi Water Content
100
80
0
% water content
Aquatic
1 month
5 month
*+
* *
*+
*+
Muscle
Liver
Lung
Intestine
Kidney

content was evident after only one month of terrestrialization and this elevation continued to be significant following five months of terrestrialization. It should be noted that these increases in water content were large, but may appear deceptively small when 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.

Feeding Study:

Before beginning the series of feeding experiments, a pilot study was conducted to ensure the bloodworms were not leaching ions into the water. Table 1 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^{2+} and very little Cl^- (Table 2).

In Figs. 4 and 5, 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 starved animals). In Table 3, these cumulative data have been used to calculated net rates of uptake or loss per kg per h, averaged over the 48h period. On the far left of Figs. 4 and 5, the bars illustrate the maximum changes in water ion concentration expected if the total ion content of the bloodworm meal (5 g = 3% ration) were to be released to the 2 L water volume.

The fed *Protopterus dolloi* showed significant net removal of Na^+ from the external water compared to starved fish at 6, 7 and 48 hours (Fig. 4A). 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 umol/kg/h in fed fish (Table 3) over the 48h period. In *Protopterus annectens* there was no substantive difference (except at 7h) in Na^+ balance between fed and starved animals, and both exhibited net removal of Na^+ from the water over 48h. Again the Na^+ content of the meal was not lost to the water. Average net Na^+ uptake rates over 48 h (44 umol/kg/h) in fed *P. annectens* were comparable to those in fed *P. dolloi* (Table 3).

The situation was very different for Cl^- and Ca^{2+} balance. In *P. dolloi*, both fed and starved animals lost far more Cl^- to the water than could be obtained from the small Cl^- content of the bloodworm meal (Fig. 4B). Over 48 h, the net loss rate was -75 uM/kg/h in fed animals (Table 3). In *P. annectens*, the data were very variable (Fig. 5B), and while the net flux rate was positive (100 umol/kg/h), this value was not significantly different from zero (Table 3). With respect to Ca^{2+}, both fed and starved *P. dolloi* lost Ca^{2+} to the external water, and the cumulative losses were comparable to the Ca^{2+} content of the meal (Fig. 4C). Averaged over 48h, the Ca^{2+} loss was quite low in fed animals (-4 umol/kg/h). The pattern in *P. annectens* was different; from 0.5 through 4h post-feeding, fed animals lost Ca^{2+} to the external water, though this pattern was reversed, with removal of Ca^{2+} from the water at 12h and 24h (Fig. 5C). By 48 h, the cumulative loss was comparable to the Ca^{2+} content of the meal. Averaged over this time period, fed *P. annectens* exhibited a negative though variable Ca^{2+} loss rate (-21 umol/kg/h) which was not significantly different from zero.

Table 1. Bloodworm leaching experiment: Ion levels found in the experimental water and the experimental water with bloodworms (mM/L) (N=3). There were no significant differences between the experimental water and experimental water with bloodworms.

	Na^+	Cl^-	Ca^{2+}
Experimental Water	1.77 ± 0.00	1.49 ± 0.02	1.02 ± 0.01
Experimental Water + Bloodworms	1.75 ± 0.01	1.50 ± 0.03	1.04 ± 0.01

Table 2. Ion levels found in the bloodworm (mM/kg). (N=3)

	Na	Ca	Cl
Bloodworms	48.21 ± 2.27	20.91 ± 1.00	1.86 ± 0.22

Table 3. Net ion flux rates (umol/kg/h) over the course of 48 hours following a feeding and non-feeding event for the *Protopterus dolloi* (N=6) and the *Protopterus annectens* (N=6).

* indicates difference between the two species.

		Na^+	Cl^-	Ca^{2+}
Protopterus dolloi	**Starved**	-9 ± 10	-57 ± 40	-11 ± 0.00
	Fed	38 ± 10	-75 ± 50	-4 ± 0.00
Protopterus annectens	**Starved**	88 ± 10*	-38 ± 20	-28 ± 10
	Fed	44 ± 40	100 ± 100	-21 ± 20

Figure 4. Cumulative changes in ion concentrations relative to initial water composition over 48 hours following a feeding and non-feeding event for the *Protopterus dolloi* (N=6). 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 meal (5 g = 3% ration) were to be released to the 2 L water volume. ($P<0.05$; Paired t-test and one way ANOVA).

* indicates difference between starved and fed lungfish

Figure 4

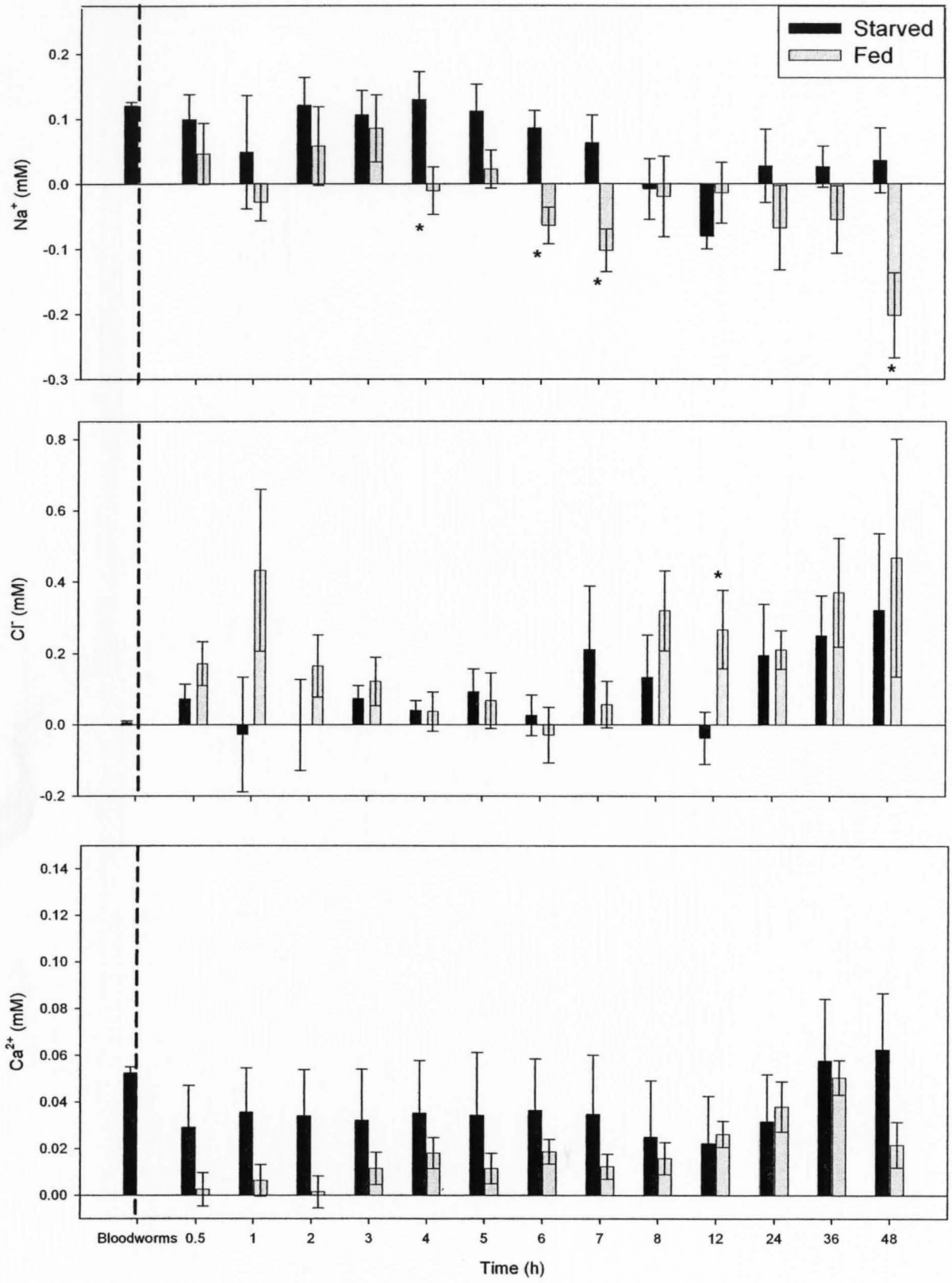
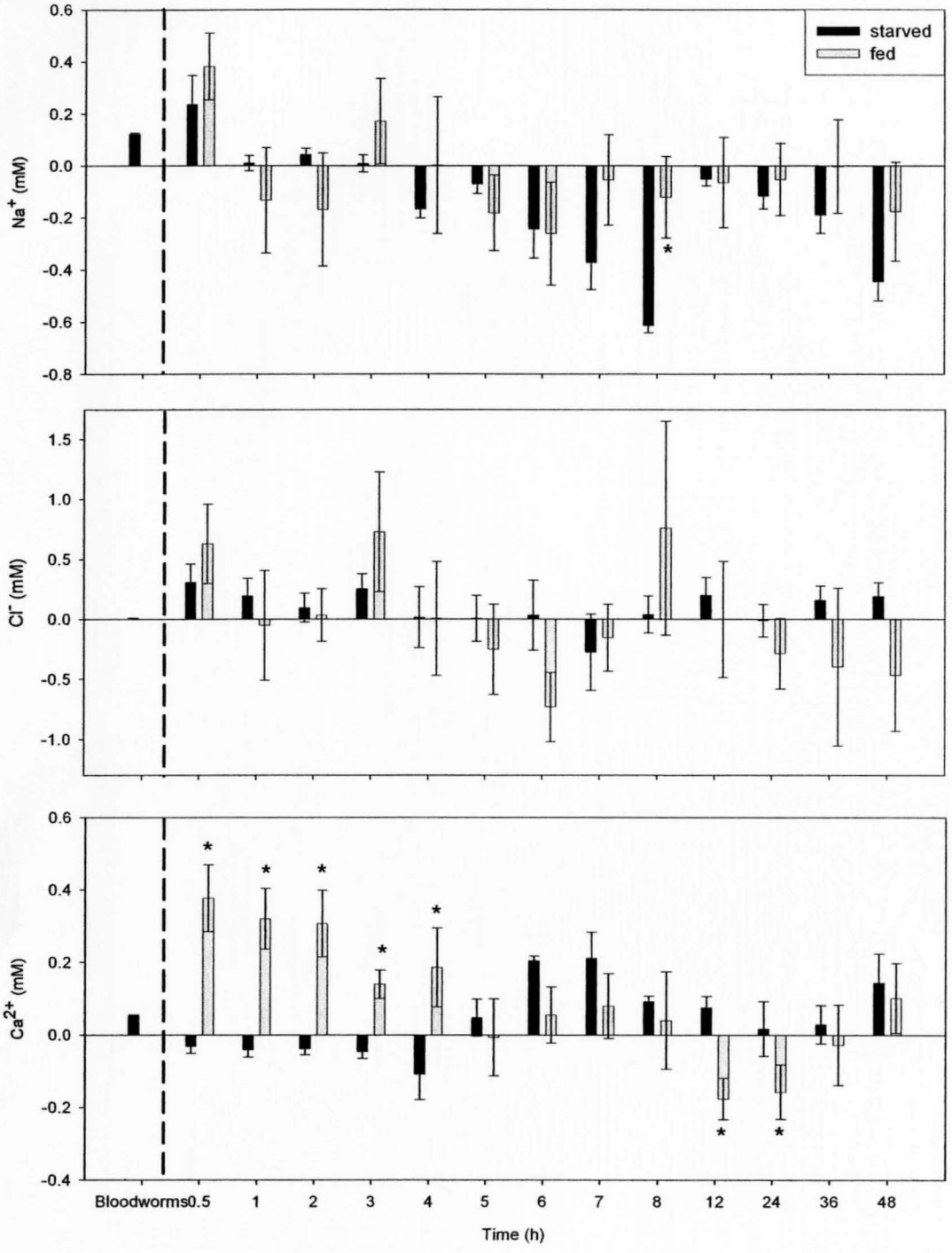

Starved
Fed
Na^+ (mM)
Cl^- (mM)
Ca^{2+} (mM)
Bloodworms
0.5
1
2
3
4
5
6
7
8
12
24
36
48
Time (h)

Figure 5. Cumulative changes in ion concentrations relative to initial water composition over 48 hours following a feeding and non-feeding event for the *Protopterus annectens*. (N=6). 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 meal (5 g = 3% ration) were to be released to the 2 L water volume. ($P<0.05$; Paired t-test and one way ANOVA).

* indicates difference between starved and fed lungfish

Figure 5


starved
fed
Na^+ (mM)
Cl^- (mM)
Ca^{2+} (mM)
Bloodworms
0.5
1
2
3
4
5
6
7
8
12
24
36
48
Time (h)

Unidirectional Waterborne Ion Uptake Rates:

The results overall for unidirectional ion uptake rates from the water were quite variable and with the exception of Na^+ levels in the starved fish, there were no significant differences between the *Protopterus dolloi* and *Protopterus annectens* (Table 3, Fig. 6). For comparison, the maximum ion uptake rate that could be achieved from the bloodworms is also shown in Fig. 6, assuming that the total ionic content of the 3% ration meal was absorbed over the 48 h period. For both species, this analysis suggests that while unidirectional uptake rates are low (Table 3), the water and the diet are of comparable importance for Na^+ and Ca^{2+} acquisition respectively (Fig. 6), whereas waterborne unidirectional uptake predominates for Cl^- acquisition (Fig. 6), because Cl^- concentrations in bloodworms are so low (Table 2). Note however that the ions taken up from the food and the water are not completely retained by the lungfish (Figs. 4, 5), and there is some ion loss to the water through the gills/skin, urine and feces, an issue which will be dealt with subsequently in Table 6.

Ammonia-N excretion rates of a starved *Protopterus dolloi* and *Protopterus annectens* ranged from 30-100 μmol/kg/h and 15-150 μmol/kg/h respectively (Fig. 7). However, following feeding there was a substantial, significant increase in ammonia-N excretion for 12 hours in the *Protopterus dolloi* and for 24 hours in the *Protopterus annectens*. Urea-N excretion rates of starved *Protopterus dolloi* and *Protopterus annectens* were about 50 μmol/kg/h, and tended to decline over time (Figure 8). Following a feeding event it appears there was a significant increase in urea-N excretion in the *Protopterus dolloi* for the first hour of the experiment, whereas none of the differences in urea-N excretion were significant for *Protopterus annectens.* Overall,

integration under the excretion curves indicated that the difference between the two integration curves for extra ammonia-N excretion and extra urea-N excretion due to feeding in *Protopterus dolloi* over 48 h amounted to 6624 umol/kg and -177 umol/kg respectively. Comparable figures for *Protopterus annectens* were 11843 umol/kg and -1534 umol/kg.

Table 4. A comparison of ion uptake rates (μmol/kg/h) in the *Protopterus dolloi* and *Protopterus annectens* with ion uptake rates reported for various freshwater teleosts.

Name	Na	Cl	Ca	Reference
Lungfish (*Protopterus dolloi*)	35	26	31	*this study*
Lungfish (Protopterus annectens)	63	86	30	*this study*
Northern Killifish (*Fundulus heteroclitis*)	425			*Scott et al., 2004*
Southern Killifish (*Fundulus heteroclitis*)	375			*Scott et al., 2004*
Rainbow Trout (*oncorhynchus mykiss*)	944	1006	34	*Rogers et al., 2003*
zebrafish (*Danio rerio*)	525	75		*Boisen et al., 2003*
Tilapia *(Tilapia mossambica)*			27.9	*Flik et al., 1985*
Killifish (*Fundulus heteroclitis*)			32.5	*Pang et al., 1980*
Goldfish *(carassius auratus)*			16.3	*Berg 1978*
Smolts *(Salmo salar)*	680	760		*Potts et al., 1970*
Steelhead Trout *(salmo gairdneri)*	230			*Greenwald et al., 1974*
Killifish *(Fundulus kansae)*			27	*Fleming et al., 1973*
European eel *(Anguilla anguilla)*	480	30-50		*Motais, 1967*
Flounder *(Platichthys flesus)*	220	10-20		*Motais, 1967*
Goldfish *(carassius auratus)*	90			*Maetz et al., 1964*
Goldfish *(carassius auratus)*		130-210		*Maetz and Garcia Romeu, 1964*

Figure 6. Unidirectional ion uptake rates from the water for the *Protopterus dolloi* (black bars) (N=6) and the *Protopterus annectens* (light gray bars) (N=6). For comparison, the maximum ion uptake rate that could be achieved from the bloodworms (dark gray bars) is also shown, assuming that the total ionic content of the 3% ration meal was absorbed over the 48 h period.

Figure 6

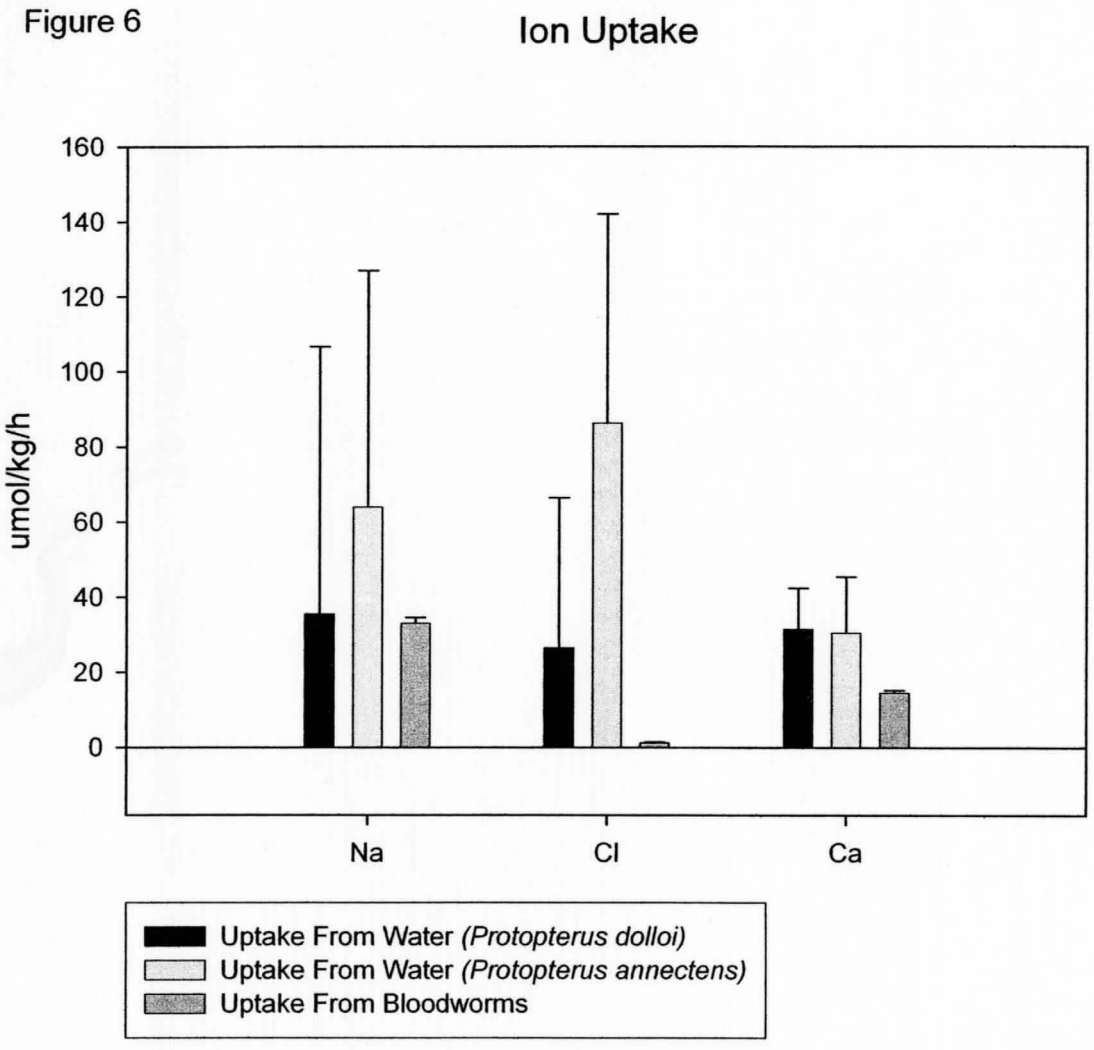

Ion Uptake
160
140
120
100
80
60
40
20
0
umol/kg/h
Na
Cl
Ca
Uptake From Water (Protopterus dolloi)
Uptake From Water (Protopterus annectens)
Uptake From Bloodworms

Figure 7. Ammonia excretion of the *Protopterus dolloi* (N=6) and *Protopterus annectens* (N=6) for 48 hours following a feeding and non-feeding event. ($P<0.05$; one way ANOVA plus Dunnett's test)

* indicates difference between starved and fed lungfish

Figure 7

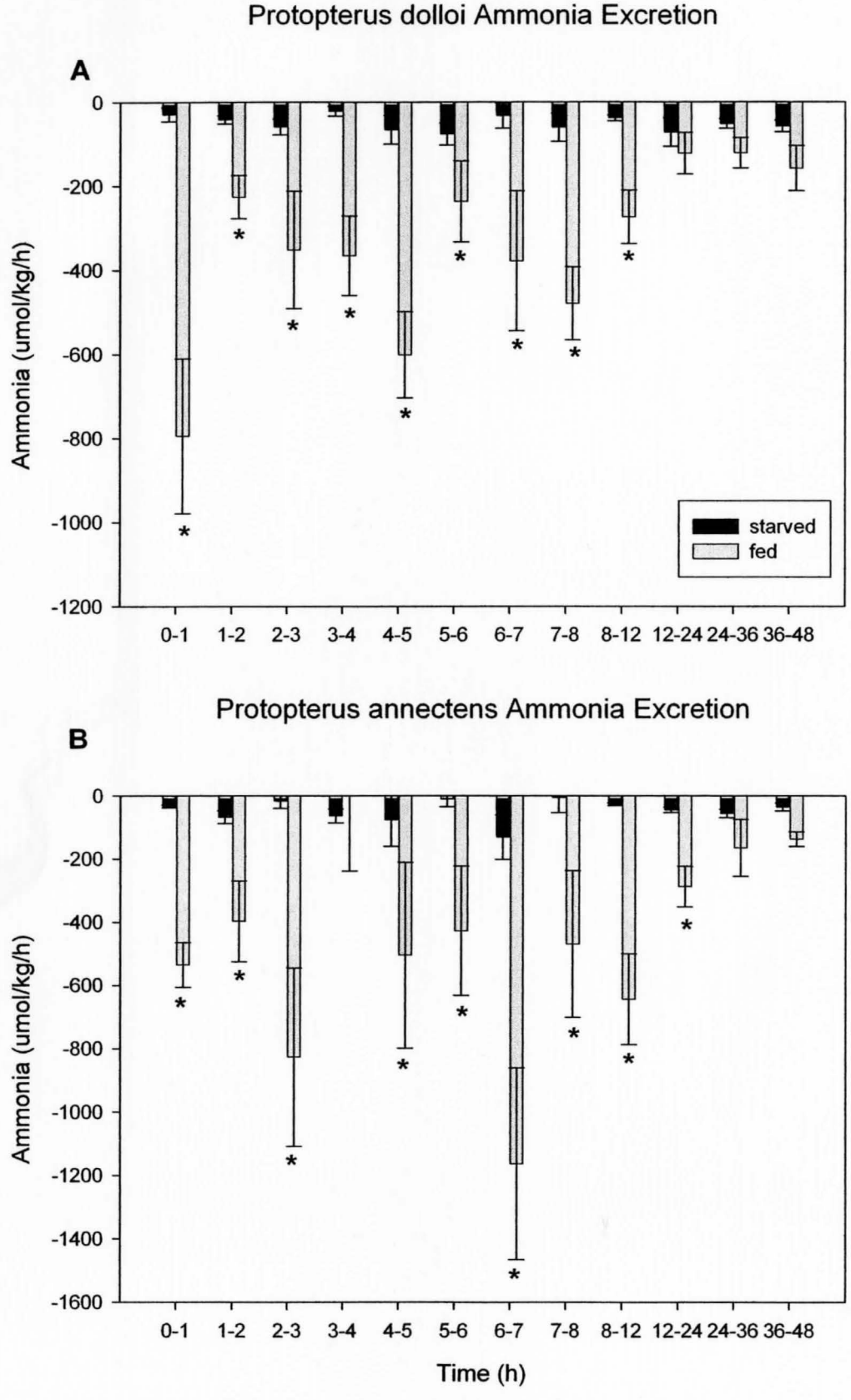
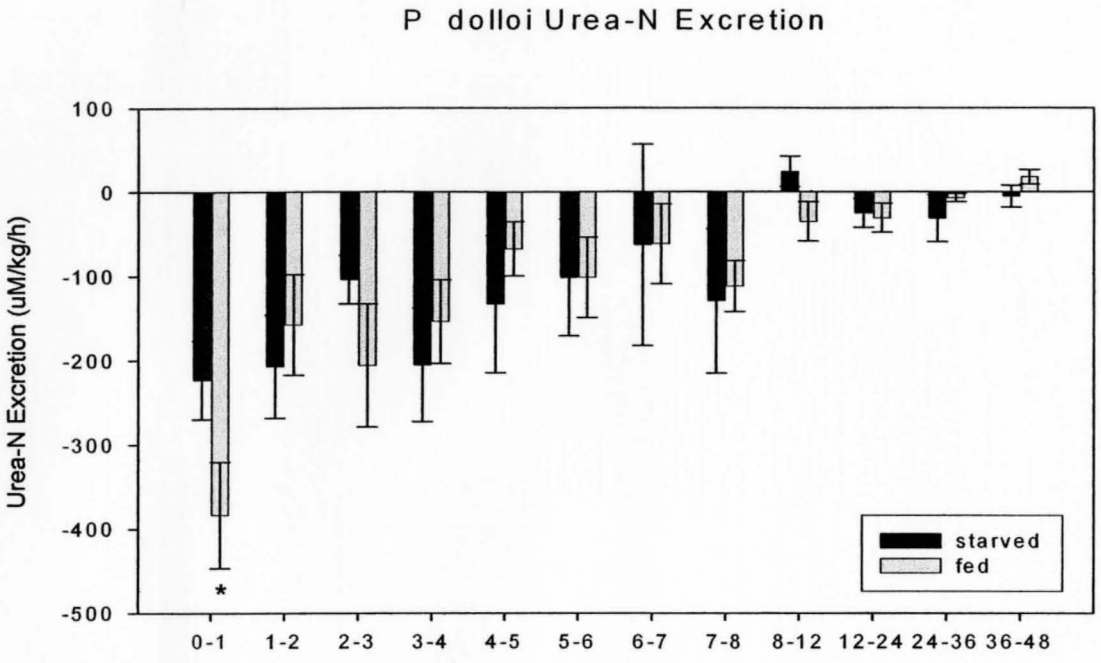
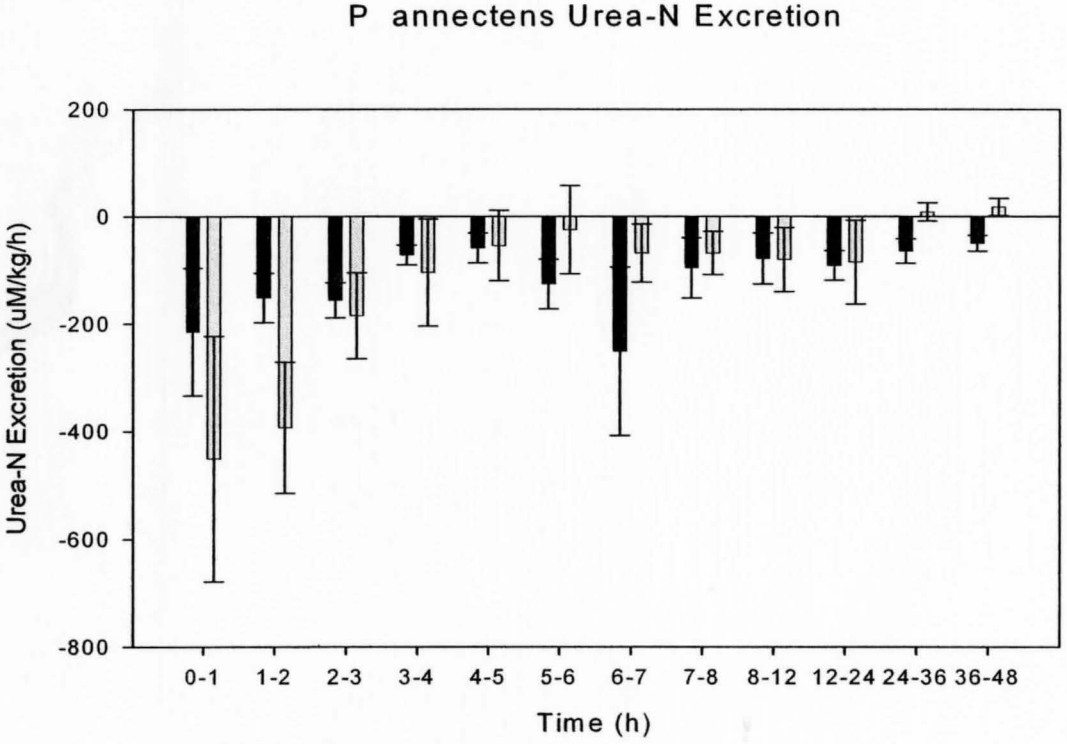

Protopterus dolloi Ammonia Excretion
A
Ammonia (umol/kg/h)
0
-200
-400
-600
-800
-1000
-1200
starved
fed
0-1
1-2
2-3
3-4
4-5
5-6
6-7
7-8
8-12
12-24
24-36
36-48
Protopterus annectens Ammonia Excretion
B
Ammonia (umol/kg/h)
0
-200
-400
-600
-800
-1000
-1200
-1400
-1600
0-1
1-2
2-3
3-4
4-5
5-6
6-7
7-8
8-12
12-24
24-36
36-48
Time (h)

Figure 8. Urea-N excretion of the *Protopterus dolloi* (N=6) and *Protopterus annectens* (N=6) for 48 hours following a feeding and non-feeding event. ($P<0.05$; one way ANOVA plus Dunnett's test)

* indicates difference between starved and fed lungfish


P dolloi Urea-N Excretion
Urea-N Excretion (uM/kg/h)
100
0
-100
-200
-300
-400
-500
*
starved
fed
0-1
1-2
2-3
3-4
4-5
5-6
6-7
7-8
8-12
12-24
24-36
36-48


P annectens Urea-N Excretion
Urea-N Excretion (uM/kg/h)
200
0
-200
-400
-600
-800
0-1
1-2
2-3
3-4
4-5
5-6
6-7
7-8
8-12
12-24
24-36
36-48
Time (h)

Diffusive Water Exchange:

Diffusive water efflux rates for adult *Protopterus annectens* and *Protopterus dolloi* were similar (Fig. 9); diffusive water exchange rates in juvenile *Protopterus dolloi* appeared to be somewhat lower, but the difference was not significant. Notably, in adult *Protopterus dolloi*, there was a significant decrease of approximately 70 % in water efflux following terrestrialization for 8 months (Fig. 10).

Following feeding there was a substantial increase in the rate constant and water efflux rate in both *Protopterus dolloi* and *Protopterus annectens* (Fig. 11). There were no differences between the two species of fish. The experiment was terminated early because the threshold for recycling was reached after only 4 h as opposed to diffusive water experiments in starved aquatic lungfish that lasted for 5 h.

The dental dam/bottle experiment which segregated water effluxes between head and tail ends revealed a greater water efflux in the anterior (gills) portion of the lungfish (Fig. 12). The overall division was approximately 70% into the anterior compartment and 30% into the posterior compartment. However there was no significant difference between the *Protopterus annectens* and *Protopterus dolloi*. Notably however, the total water efflux rates (80 – 120 ml/kg/h) recorded in these experiments (Fig. 12) still tended to be lower than in unrestrained fish (see Figs. 9, 10, 11) (100 – 300 ml/kg/h) suggesting that the restrained fish were stressed.

Figure 9. Mean rate constants (A) and water efflux rates (B) for the *Protopterus annectens* (N=6) and *Protopterus dolloi* adult (N=7) and juvenile (N=6). There were no significant differences.

Figure 9

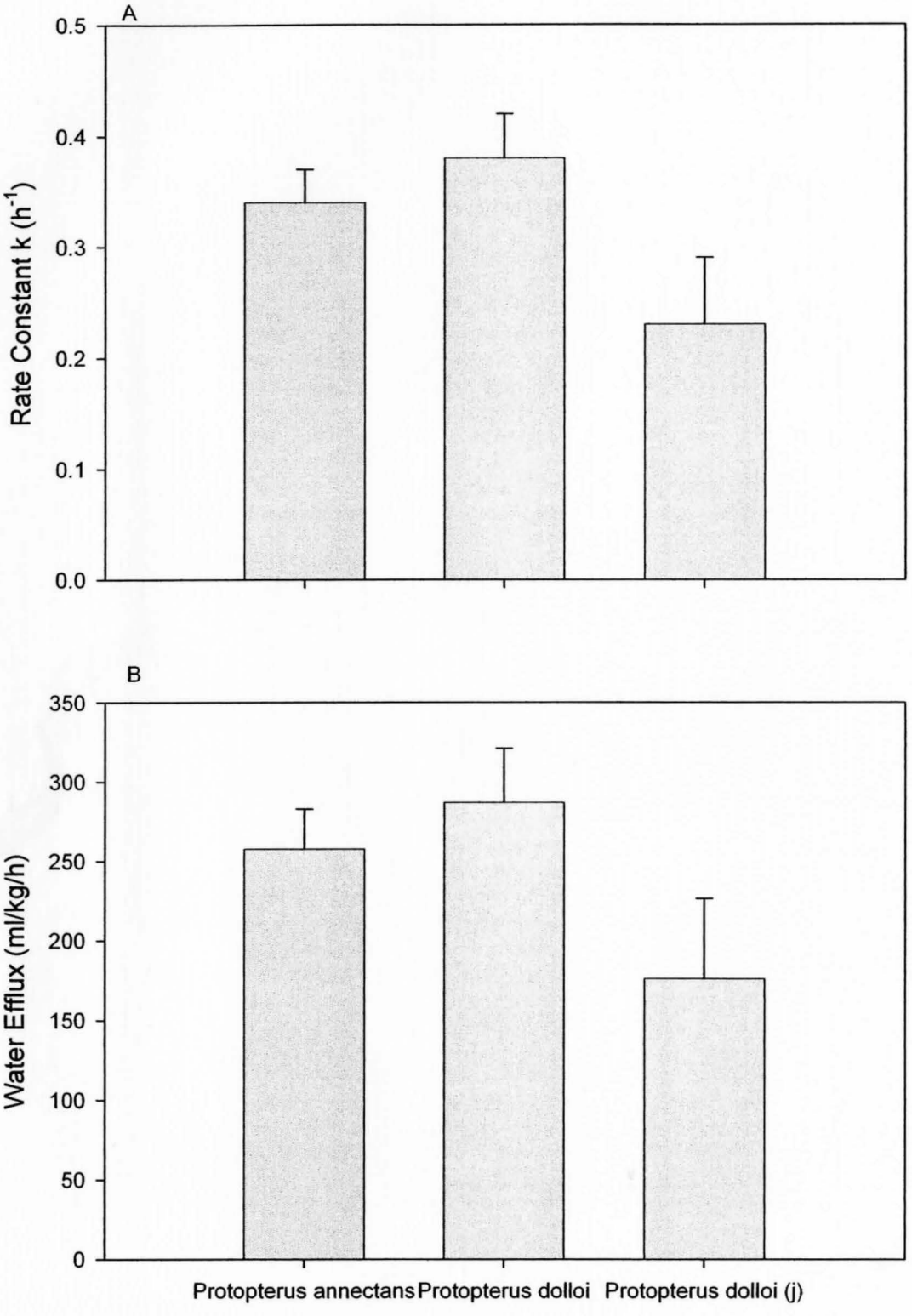
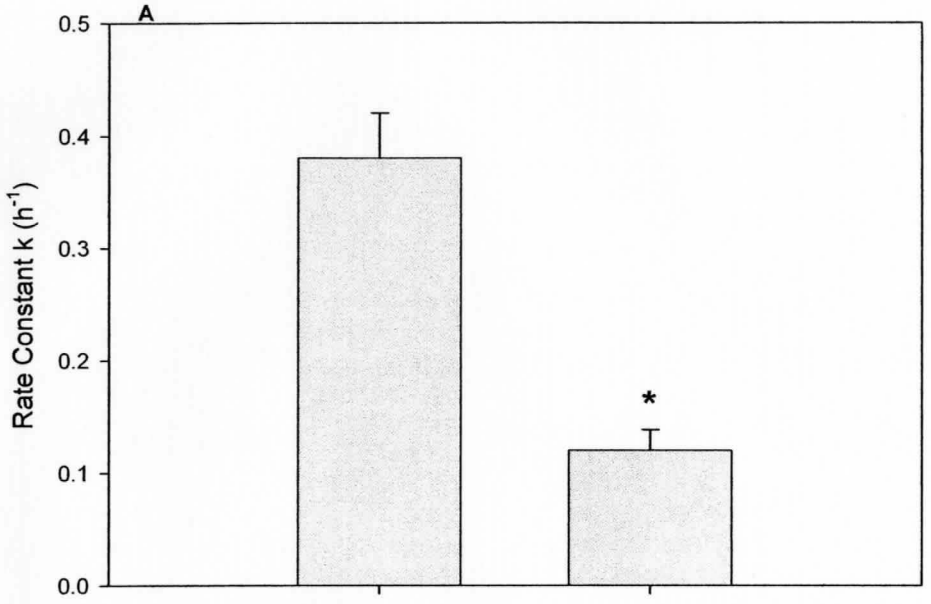

A
0.5
0.4
0.3
0.2
0.1
0.0
Rate Constant k (h-1)
B
350
300
250
200
150
100
50
0
Water Efflux (ml/kg/h)
Protopterus annectans
Protopterus dolloi
Protopterus dolloi (j)

Figure 10. Mean rate constants (A) and water efflux rates (B) for the aquatic *Protopterus dolloi* (N=7) and the *Protopterus dolloi* immediately after reimmersion following an 8 month terrestrialization period (N=5). ($P<0.05$; Paired t-test)

* indicates difference between aquatic and terrestrialized fish


A
0.5
0.4
0.3
0.2
0.1
0.0
Rate Constant k (h-1)
*

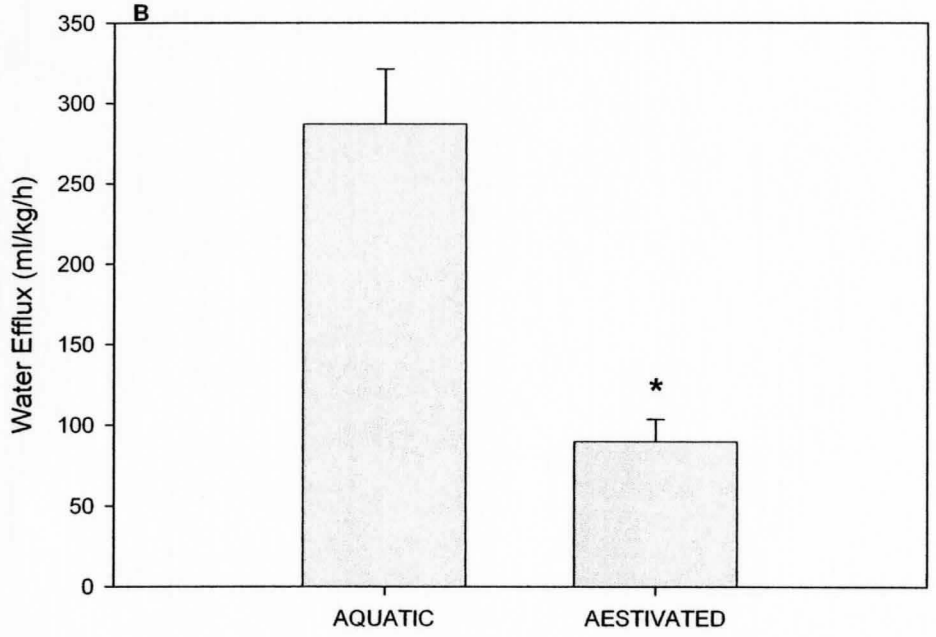

B
350
300
250
200
150
100
50
0
Water Efflux (ml/kg/h)
*
AQUATIC
AESTIVATED

Figure 11. Rate constants (A) and water efflux rates (B) for the *Protopterus dolloi* (black bars) and the *Protopterus annectens* (gray bars) following a feeding event N=6.

* indicates difference from pre-fed lungfish

Figure 11

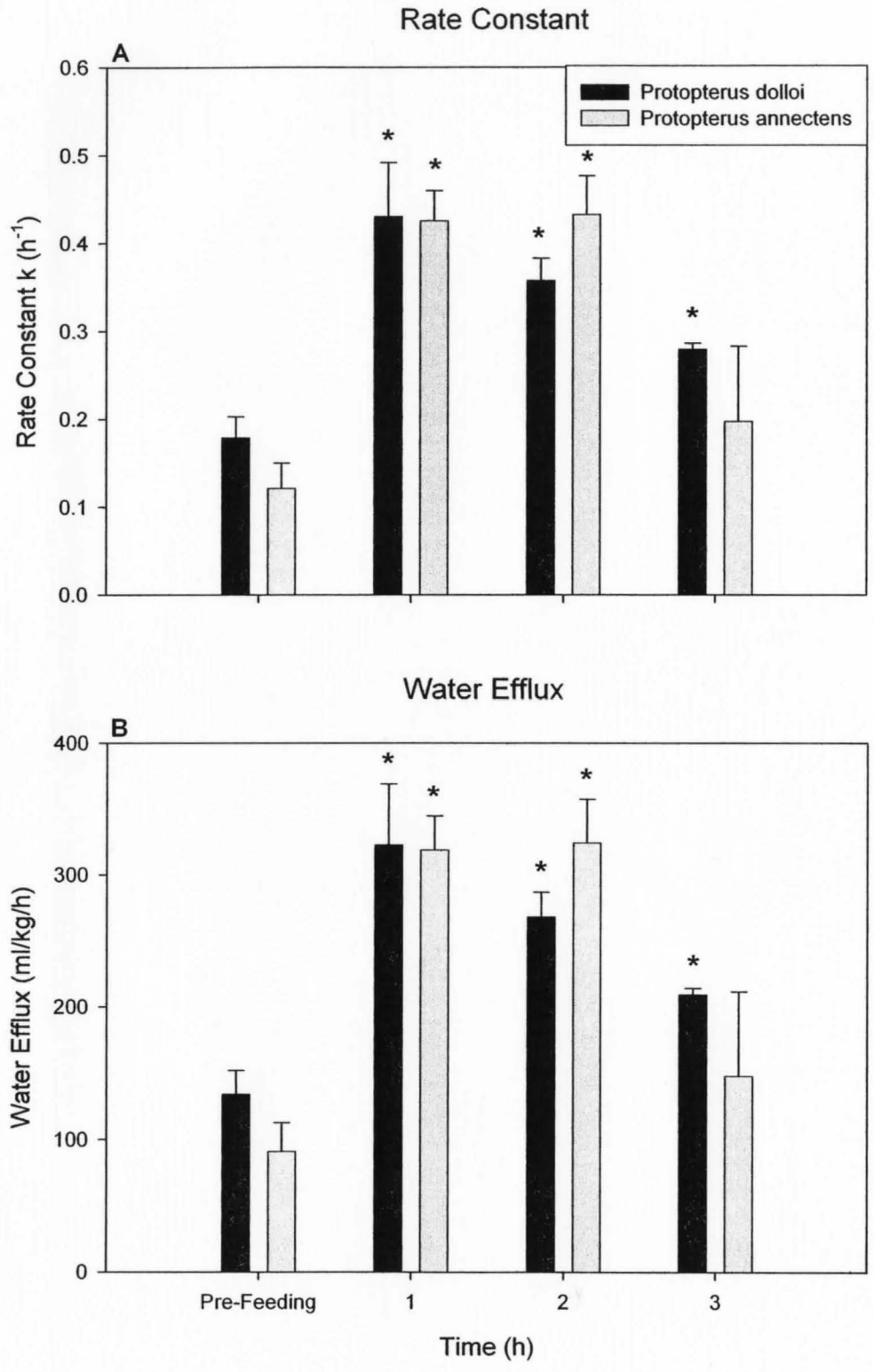
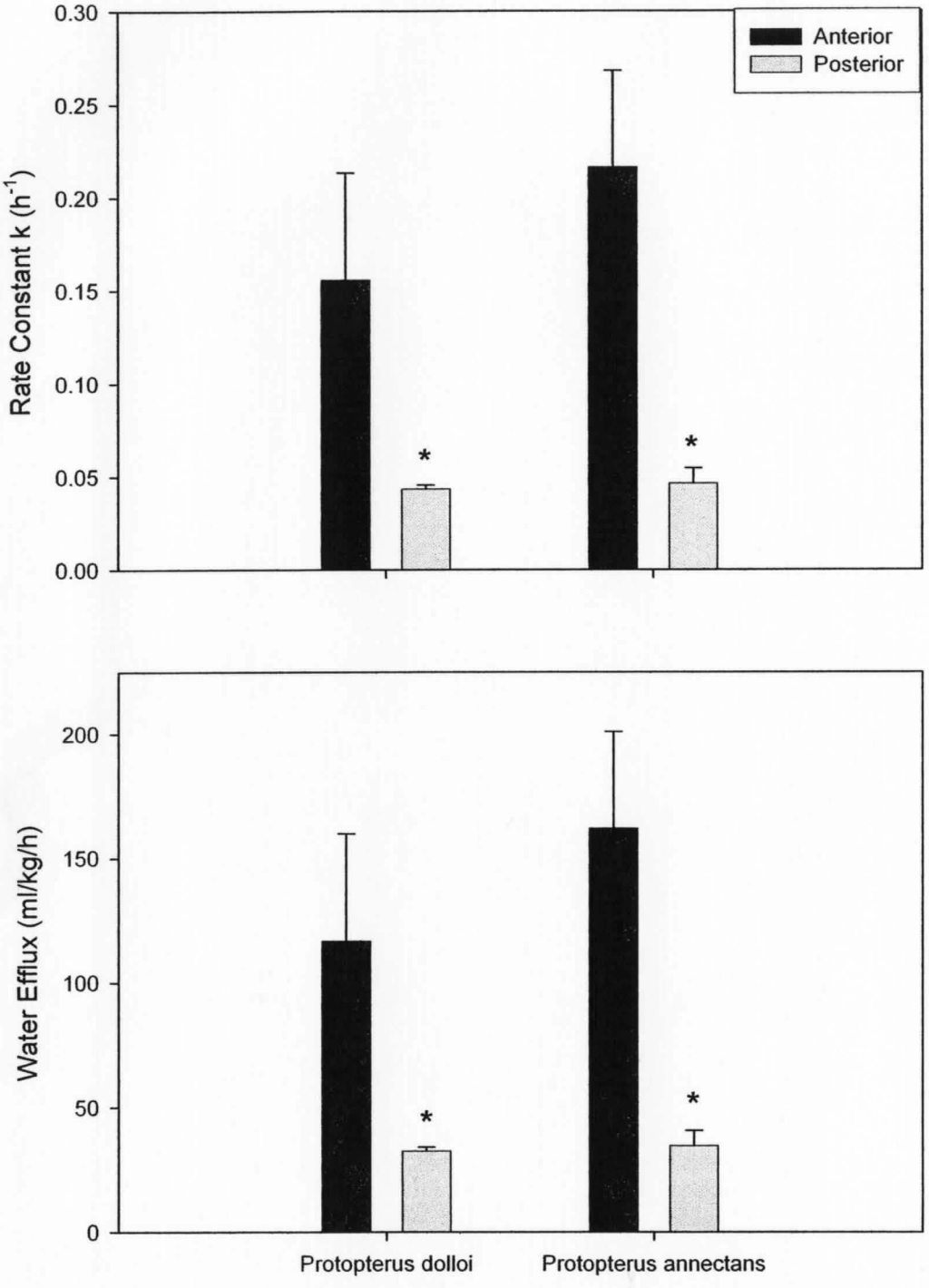

Rate Constant
A
Protopterus dolloi
Protopterus annectens
Rate Constant k (h-1)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
Water Efflux
B
Water Efflux (ml/kg/h)
0
100
200
300
400
Pre-Feeding
1
2
3
Time (h)

Figure 12. Mean rate constants (A) and water efflux rates (B) for the anterior and posterior regions of the adult *Protopterus dolloi* (N=6) and *Protopterus annectens* (N=6). ($P<0.05$; Paired t-test). There were no significant differences between the two species.

* indicates difference between anterior and posterior regions

Figure 12


Anterior
Posterior
Rate Constant k (h-1)
0.30
0.25
0.20
0.15
0.10
0.05
0.00
*
*
Water Efflux (ml/kg/h)
200
150
100
50
0
*
*
Protopterus dolloi
Protopterus annectans

Ion and Waste Excretion:

Analysis of fecal ion content indicated that in both species, Na^+ was very similar to that in the ingested blood worms, whereas Cl^- and Ca^{2+} concentrations (especially Ca^{2+}) were considerably higher. However the weight of the feces (~4g/kg) were only a small fraction of the weight of the ingested bloodworms (30 g/kg lungfish for a 3% ration). There were no differences in fecal ion content between the *Protopterus dolloi* and *Protopterus annectens* (Table 5, Fig. 13). There was a higher Ca^{2+} excretion rate in the feces than in the urine. Na^+ and Cl^- excretion in the feces was small. Overall, the ion loss through the feces is low compared to what could have been taken up by the food and the water (Table 6).

Based on the various measurements in this study, Table 6 presents a balance sheet for ion uptake (+) and loss rates (-) (umol/kg/h) over a 48 h period for *Protopterus dolloi* and the *Protopterus annectens* fed a standard bloodworm meal (3% ration) at 0 h. Uptake from bloodworms assumes 100% assimilation of bloodworm ion content as in Fig. 6. Uptake from the water is based on unidirectional ion uptake rates measured with radiotracers in Fig. 6. Ion excretion rates through waste such as feces were estimated in Fig. 13 and via the urine were measured in Fig. 14. Estimated rates of net gain from/loss to the water, assuming steady state conditions, are based on the difference: [uptake rate from bloodworms + unidirectional uptake rate from water] - [excretion rate through feces + excretion rate through urine]. Actual rates of net gain/loss to the water are calculated from the data of Figs. 4 and 5 (in Table 3). Any discrepancy between the two (actual – estimated) should represent the unidirectional efflux rate across the body surface (gills, skin) which was not measured. Note that in Table 6, 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 *Protopterus annectens* , may be explained by data variability, 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^{2+} 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 feces. There do not appear to be substantive differences in this regard between *Protopterus dolloi* and the *Protopterus annectens*.

Renal Function

Protopterus dolloi and the *Protopterus annectens* had similar glomerular filtration rates (Fig. 15) and urine flow rates (Fig. 16) which did not vary significantly over the 72 h experimental period. Although, following 12 h from the start of the experiment, GFR tended to be higher and UFR lower in *Protopterus dolloi*, there were no significant differences between the two species. Based on results reported by Patel et al. (2006) for the rainbow trout (*Oncorhynchus mykiss*) which are also illustrated in these Figures, UFR and GFR values were also similar to values in a model freshwater teleost.

Urine ion excretion rates were similar in the *Protopterus dolloi* and the *Protopterus annectens* (Fig. 14). The ion levels in the urine did not change over the course of the 72 hours. Urine ion loss of Na^+ and Cl^- were greater than via the feces, though the opposite was true for Ca^{2+} (Table 6). Overall, there was minimal ion loss via the urine compared to what could have been taken up by the food and the water (Table 6).

Also, it should be noted that these urinary ion levels may be lower due to the fact that the fish were starved prior to and during the experiment.

The clearance rate analysis of Table 8 showed low clearance rates of Na^+ and Cl^-. There were no significant differences in clearance rate between the *Protopterus dolloi* and *Protopterus annectens* (Table 8). Based on the calculated clearance ratios, there was virtually complete reabsorption (98-99%) of the filtered Na^+ and Cl^- (Table 8). Ca^{2+} was about 78-79% reabsorbed over the 72 hour time period (Table 8). Mean water clearance ratio values for the *Protopterus dolloi* and *Protopterus annectens* were 0.44 and 0.77 respectively, indicating 56% and 23% reabsorption of the filtered water load respectively. These values were significantly different between the two species.

Table 5. A comparison of fecal ion concentration (mmol/kg) in the *Protopterus dolloi* and (N=7) *Protopterus annectens* (N=7). There were no significant differences between the *Protopterus dolloi* and the *Protopterus annectens*.

Name	Na	Cl	Ca
Protopterus dolloi	47.24 ± 6.18	20.00 ± 2.64	76.58 ± 14.71
Protopterus annectens	40.87 ± 4.67	14.63 ± 1.95	78.94 ± 11.72

Table 6. A balance sheet for ion uptake (+) and loss rates (-) (umol/kg/h) over a 48 h period for *Protopterus dolloi* and the *Protopterus annectens* fed a standard bloodworm meal (3% ration) at 0 h. Uptake from bloodworms assumes 100% assimilation of bloodworm ion content as in Fig. 6. Uptake from the water is based on unidirectional ion uptake rates measured with radiotracers in Fig. 6. Ion excretion rates through waste such as feces were estimated in Fig. 13 and via the urine were measured in Fig. 14. Actual rates of net gain/loss to the water are calculated from the data of Figs. 4 and 5 (in Table 3). Estimated rates of net gain from/loss to the water, assuming steady state conditions, are based on the difference: [uptake rate from bloodworms + unidirectional uptake rate from water] - [excretion rate through feces + excretion rate through urine]. Any discrepancy between the two (actual – estimated) should represent the unidirectional efflux rate across the body surface (gills, skin) which was not measured..

Protopterus dolloi								
	ion uptake	ion uptake		net uptake/loss	net uptake/loss	difference	ion excretion	ion excretion
	Bloodworms	**Water**		**Water (estimated)**	**Water (actual)**	**estimated-actual**	**Urine**	**Feces**
Na^+ (uM/kg/h)	33	36		59	38	21	-6	-4
Cl^- (uM/kg/h)	1	26		11	-75	86	-14	-2
Ca^{2+} (uM/kg/h)	15	32		40	-4	44	-2	-5
Protopterus annectens								
	ion uptake	ion uptake		net uptake/loss	net uptake/loss	difference	ion excretion	ion excretion
	Bloodworms	**Water**		**Water (estimated)**	**Water (actual)**	**estimated-actual**	**Urine**	**Feces**
Na^+ (uM/kg/h)	34	64		88	44	44	-6	-4
Cl^- (uM/kg/h)	1	86		75	100	-25	-11	-1
Ca^{2+} (uM/kg/h)	15	30		34	-21	55	-2	-9

Table 7. Urine ion concentrations (mM/L).

	Na	Ca	Cl
Protopterus dolloi	1.71 ± 0.75	0.51 ± 0.11	3.58 ± 0.79
Protopterus annectens	1.24 ± 0.35	0.35 ± 0.06	2.54 ± 0.58

Table 8. Clearance rate (μmol kg^{-1} h^{-1}) and clearance ratios of various ions in the *Protopterus dolloi* (N=5) and *Protopterus annectens* (N=5). Calculations are based on using mean plasma values from Fig. 1, and mean GFR and UFR values from Figs. 15 and 16 respectively.

* indicates difference between the two species.

Substance Clearance Rate				
	Na+	**Cl-**	**Ca2+**	**Water**
Protopterus dolloi	0.055	0.132	1.914	3.879
Protopterus annectens	0.054	0.103	1.342	5.188
Substance Clearance Ratio				
	Na+	**Cl-**	**Ca2+**	**Water**
Protopterus dolloi	0.006	0.015	0.218	0.436
Protopterus annectens	0.009	0.017	0.213	0.770*

Figure 13. Fecal ion excretion rates in the *Protopterus dolloi* (N=6) and the *Protopterus annectens* (N=6). There were no significant differences between the two species.

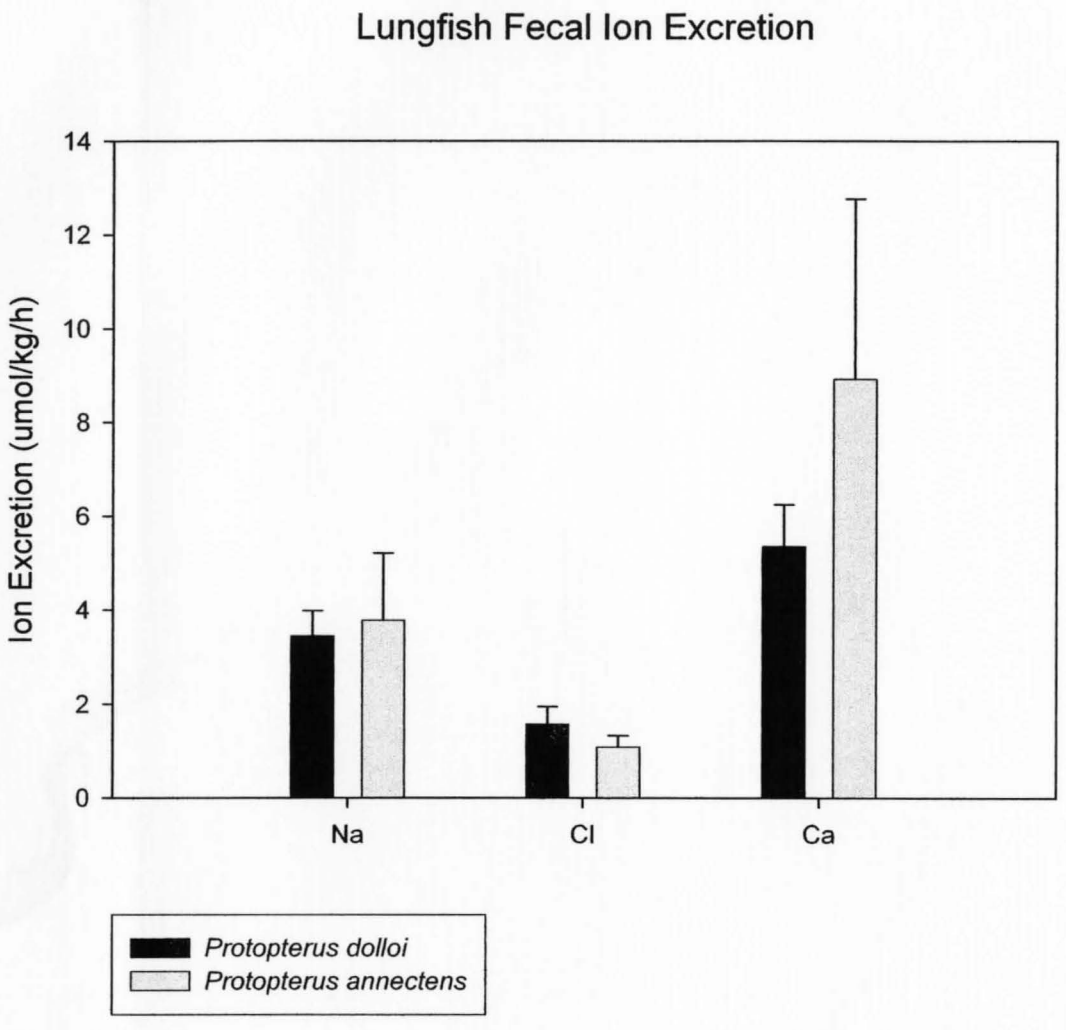

Lungfish Fecal Ion Excretion
Ion Excretion (umol/kg/h)
14
12
10
8
6
4
2
0
Na
Cl
Ca
Protopterus dolloi
Protopterus annectens

Figure 14. A comparison of urine ion excretion rates in the *Protopterus dolloi* (N=5) and the *Protopterus annectens* (N=6) with the rainbow trout *Oncorhyncus mykiss.* (Values obtained from Patel *et al.*, (2006).

* indicates difference between the two species.

Figure 14

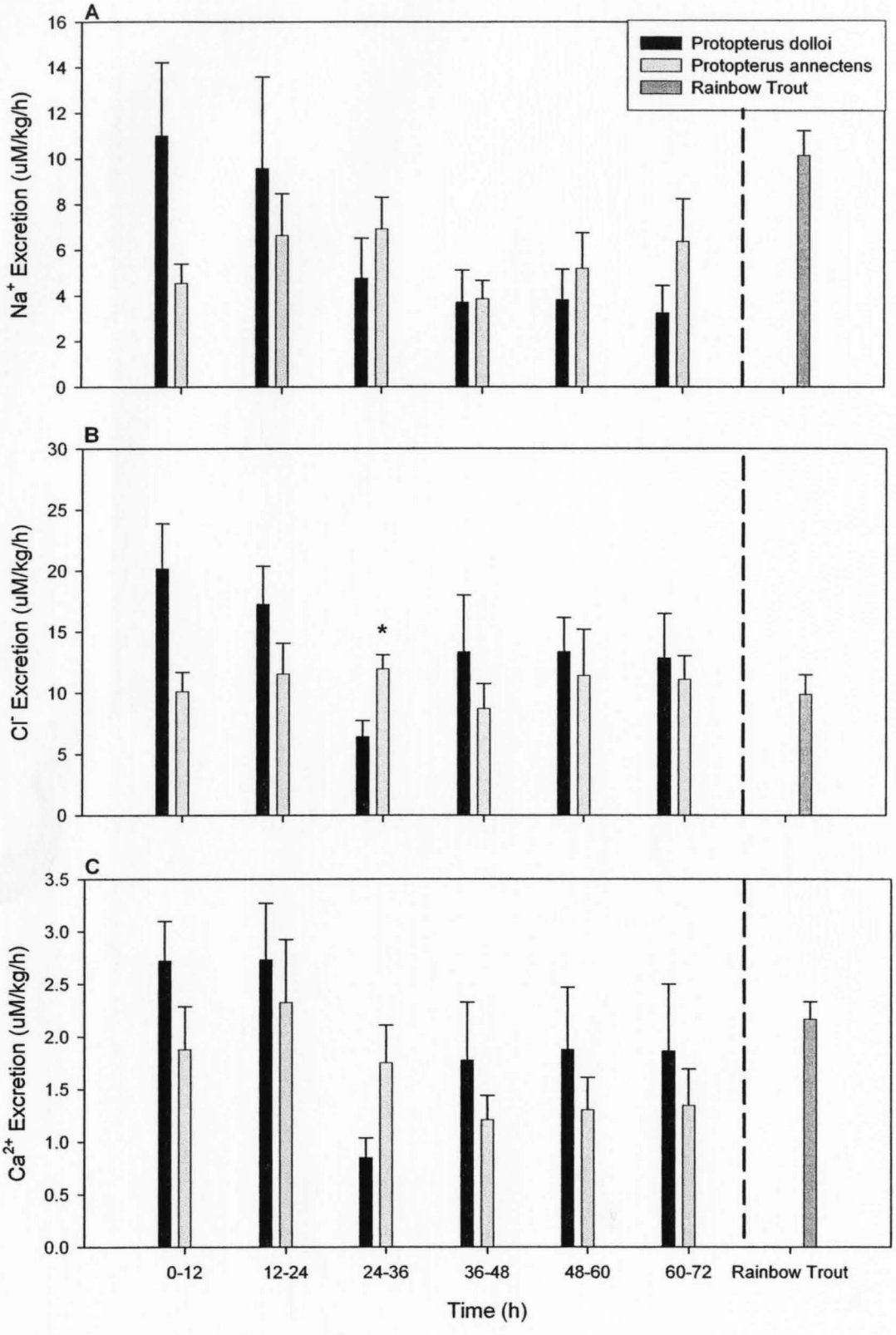

A
B
C
Protopterus dolloi
Protopterus annectens
Rainbow Trout
Na+ Excretion (uM/kg/h)
Cl- Excretion (uM/kg/h)
Ca2+ Excretion (uM/kg/h)
*
0-12
12-24
24-36
36-48
48-60
60-72
Rainbow Trout
Time (h)

Figure 15. Glomerular filtration rate for the *Protopterus dolloi* (N=5) and *Protopterus annectens* (N=6) over a 72 hour catheter experiment. There were no significant differences between the two species.

Figure 15

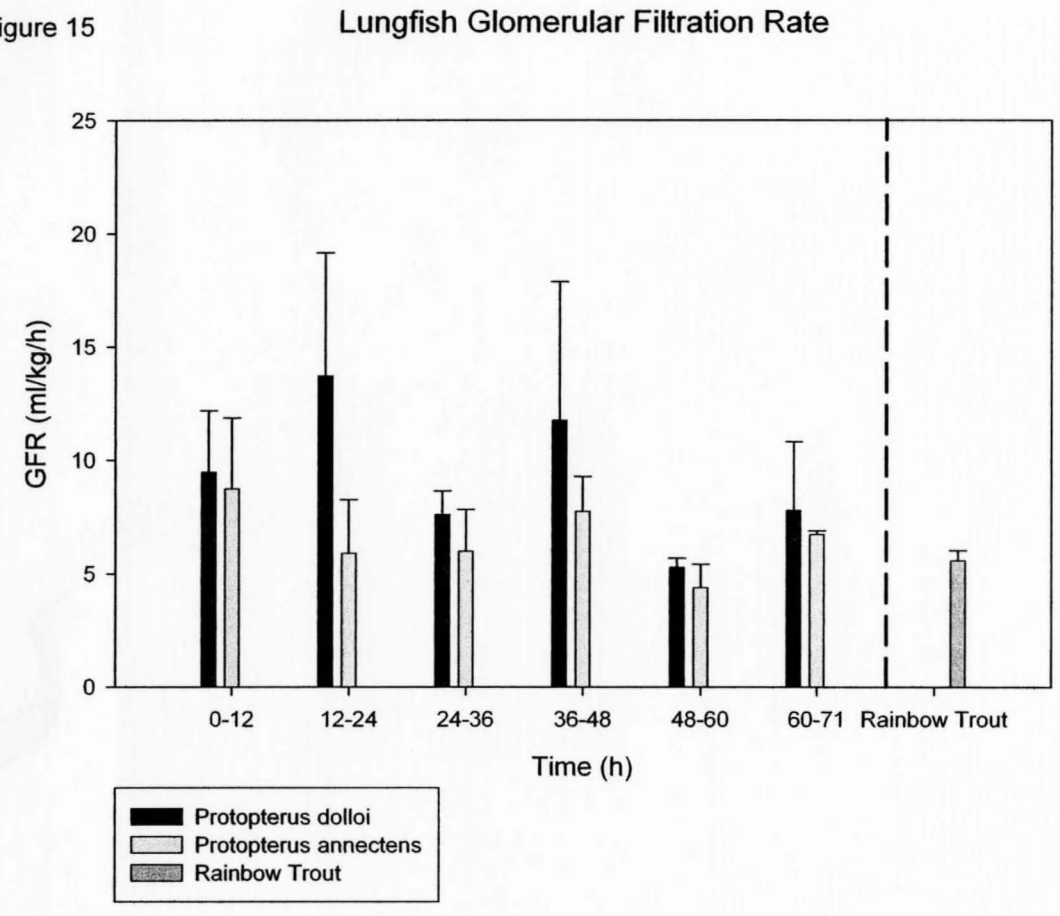
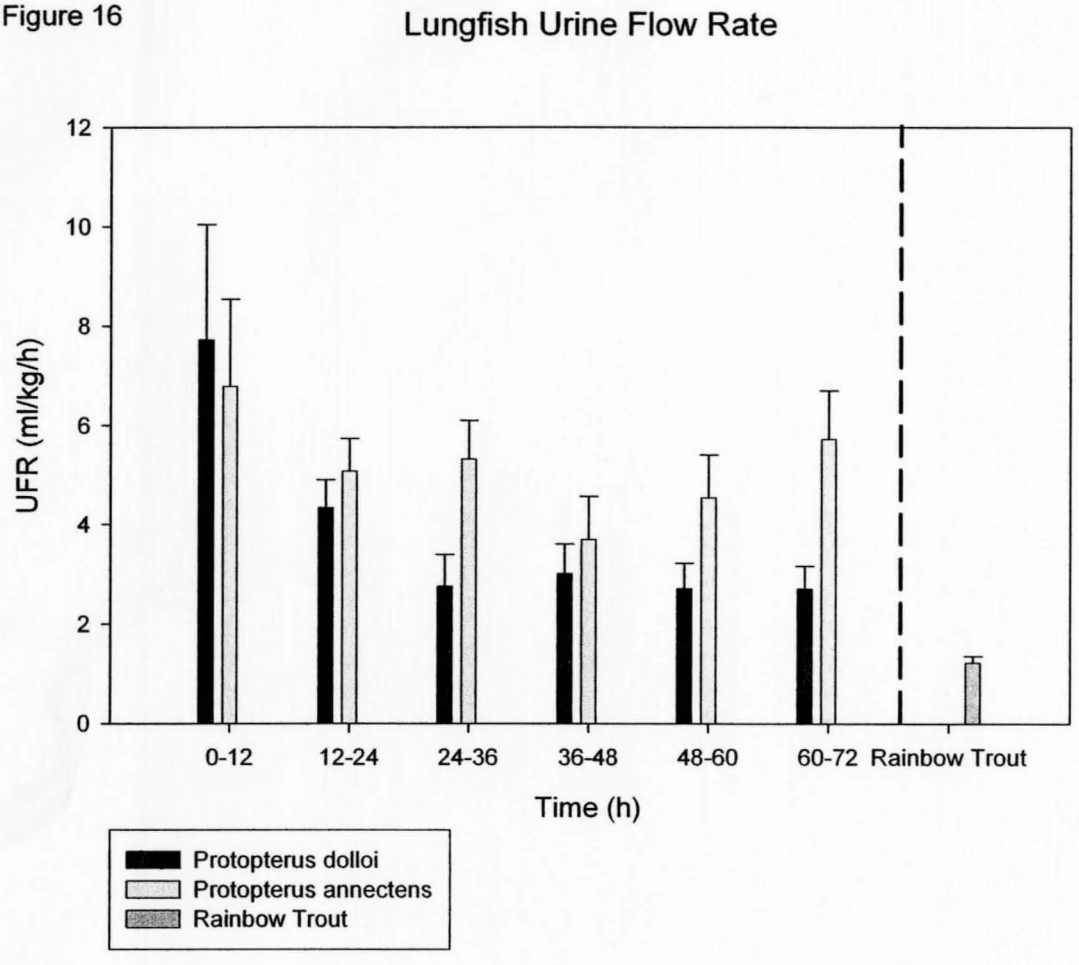

Lungfish Glomerular Filtration Rate
GFR (ml/kg/h)
25
20
15
10
5
0
0-12
12-24
24-36
36-48
48-60
60-71
Rainbow Trout
Time (h)
Protopterus dolloi
Protopterus annectens
Rainbow Trout

Figure 16. Urine flow rate for the *Protopterus dolloi* (N=5) and *Protopterus annectens* (N=6) over a 72 hour catheter experiment. T here were no significant differences between the two species.

Figure 16


Lungfish Urine Flow Rate
UFR (ml/kg/h)
12
10
8
6
4
2
0
0-12
12-24
24-36
36-48
48-60
60-72
Rainbow Trout
Time (h)
Protopterus dolloi
Protopterus annectens
Rainbow Trout

Discussion:

The goal of this study was to illustrate differences in ion and water balance both between the two species of lungfish and to freshwater teleost. Also, to show that overall lower metabolic rate observed in lungfish is further illustrated by lower ion uptake and water exchange rates.

There were no significant differences between the *Protopterus annectens* and *Protopterus dolloi* in terms of Na^+, Cl^-, and Ca^{2+} in the plasma (Fig 1). Urist *et al.* (1972) reported similar plasma composition results for Na^+ and Ca^{2+} and slightly lower Cl^- results for the *Protopterus annectens*. The plasma composition of the *Protopterus aethiopicus* reported by DeLaney *et al.* (1977) showed slightly lower Na^+ and Cl^- levels and higher Ca^{2+} levels. These Na^+ and Cl^- values are somewhat lower than those typically reported for freshwater teleost fish, while the Ca^{2+} values appear to be very low (Table 9).

Terrestrialization is the last resort of the African lungfish to water shortage prior to aestivation as it causes considerable physiological changes including slowing of metabolic rate to reduce energy expenditure and accommodate their starved conditions (Smith, 1930; Laurent *et al.*, 1978; Chew *et al.*, 2004). Also, there is reduced blood flow to the gills as mucus builds up on the them and renders them incapable of ion and gas exchange (Laurent *et al.*, 1978; Fishman *et al.*, 1986; Sturla *et al.*, 2001). As a result of this, oxygen consumption also decreases, partly due to loss of gill function so that the lungfish relies only on aerial respiration, and partly due to the decreased metabolic rate which does not require as much oxygen as for a lungfish in water (Smith, 1931; Fishman *et al.*, 1986; Wilkie *et al.*, 2007). Also, the excretion of ions is reduced as the lungfish's

urine and fecal excretion ceases (Smith, 1930; Laurent *et al.*, 1978; Chew *et al.*, 2004; Wilkie *et al.*, 2007). During this time the lungfish, which is normally ammoniotelic, starts to produce and store urea in its body which is excreted in the first 48 hours after re-immersion (Wood *et al.* 2005). With respect to different tissues in the lungfish and changes in their ion composition following a 1 month and 5 month terrestrialization period, there were only a few clear increases or decreases in specific tissues. Overall, plasma Na^+ increases when a lungfish is aestivated (Delaney *et al.*, 1977), but does not change during terrestrialization (Wilkie *et al.*, 2007). Similarly, tissues such as the muscle, liver, kidney, and heart did not exhibit any increase in Na^+ concentrations. However, there was a significant increase in Na^+ levels in intestinal tissue following a 1 month terrestrialization period and this increase was maintained at 5 months (Fig. 2A). This increase could be a result of undigested bloodworms remaining in the tract as there is a considerable amount of Na^+ in the bloodworms (Table 2). There was also a significant decrease in kidney Cl^- concentration following one month of terrestrialization but this was not evident after 5 months of terrestrialization (Fig. 2B). This initial loss of Cl^- from the kidney could be the result of diversion of blood flow away from a non-working kidney, resulting in less trapped extracellular fluid in the tissue. This is also associated with an increase in plasma bicarbonate following only 2 weeks of terrestrialization (Delaney *et al.* 1977). There was also an initial increase in liver Ca^{2+} which was not evident after 5 months at which point kidney Ca^{2+} became significantly elevated (Fig. 2C), suggesting possible internal redistribution of Ca^{2+} stores.

Table 9. A comparison of plasma ion levels in the *Protopterus dolloi* and *Protopterus annectens* with plasma ion levels reported for various freshwater teleosts.

Name	Na^+ (mM)	Cl^- (mM)	Ca^{2+} (mM)	Reference
Lungfish *(Protopterus dolloi)*	110	105	1.03	present study
Lungfish *(Protopterus annectens)*	103	105	1.22	present study
Rainbow trout (*Oncorhynchus mykiss*)	156	128	2.7	Rogers *et al.* (2003)
Brown trout (*Salmo trutta*)	155	124	5.3	Gordon (1959)
European eel (*Anguilla anguilla)*	150	105	2.8	Sharratt *et al.* (1964)
Goldfish (*Carassius auratus*)	130	115.8	2.5	Houston and Koss (1982)
Carp (*Cyprinus carpio*)	130	125	2.1	Houston and Madden (1960)
Smallmouth Bass (*Micropterus dolomieu*)	128	111	3.4	Shell (1959)

It is probable that re-immersion is a physiological challenge to the lungfish. However, even during terrestrialization the *Protopterus dolloi* and *Protopterus annectens* exhibit water and ion exchange across their ventral body surface (Wilkie *et al.* 2007). Thus, there is a substantial increase in muscle, liver, intestine and kidney water content after 5 months of terrestrialization (Fig.3). Wilkie et al. (2007) demonstrated that this tissue hydration (rather than the expected dehydration) was associated with a substantial accumulation of urea in the tissues. By maintaining osmotic balance during terrestrialization, this may help recovery when the lungfish are re-immersed in water.

The lungfish, under natural aquatic conditions is very calm and normally only moves to obtain a breath of air from the surface a few times per hour (Iftikar et al., submitted). However, during a feeding event the lungfish is quite vigorous and this activity is energetically costly, and in itself could disturb ion and water balance. Certainly, diffusive water exchange was elevated after feeding (Fig. 11), a time when O_2 consumption from both water and air was markedly elevated (Iftikar et al., submitted). In natural environments the lungfish has been reported to eat small invertebrates including crustaceans and molluscs, and in some cases they also eat some small fishes (Smith, 1935). Although the lungfish is predatory, in the lab setting they are accustomed to a frozen bloodworm diet. The bloodworm ion composition showed a substantial amount of Na^+, about half that of Ca^{2+} and only a very small amount of Cl^- (Table 2). The lungfish are put on a scheduled feeding every second day which immediately follows a water change, to which they are accustomed, but again there may be some disturbance which affects ion and water balance. Following feeding there were fluctuations in ion uptake and excretion for the fed and starved fish of both species (Figs. 4, 5).

Despite the high levels of Na^+ in the diet of lungfish, the fed *Protopterus dolloi* exhibited a net uptake of Na^+ from the water (Table 3). This could have been due to the initial excretion of Na^+ that was observed in the first 3 hours following feeding (Fig. 4). This trend of net Na^+ uptake was also observed in the fed and starved *Protopterus annectens* (Table 3). There was a significant difference in Na^+ uptake between the two starved species (Table 3). When comparing this finding to urine ion excretion (Fig. 14), which showed no differences between species, it is evident that this difference is not due to renal handling and thus requires further study. With the exception of the fed *Protopterus annectens* which showed a net uptake of Cl^- (perhaps an artifact of data variability, Fig. 5), all other experimental fish groups showed a net excretion of Cl^- (Table 3). The net uptake observed for the fed *Protopterus annectens* was not significantly different from the starved fish which exhibited a net excretion of Cl^- (Fig. 5). Overall, the fish showed no significant differences in Cl^- uptake/excretion from the initial values (Fig. 4 and 5). Although all fish showed a net excretion of Ca^{2+} to the water, when averaged over 48 h (Table 3,8), the *Protopterus annectens* showed a significant Ca^{2+} excretion for the first 4 hours following feeding when compared to the starved fish (Fig. 5); this was far more than could be explained by dispersal of the Ca^{2+} content of the blood worms into the water. Perhaps in some way, this transitory disturbance could have been associated with feeding activity.

The unidirectional ion uptake rates from the water measured for both *Protopterus dolloi* and *Protopterus annectens* provide further indirect evidence for the importance of feeding in ionic homeostasis. The rates found for this study are slightly higher than those found in a similar study conducted by Wilkie *et al.* (2007), and the influx rates for

individual fish were quite variable (Fig. 8). Overall, unidirectional Na^+ and Cl^- uptake rates from the water appear to be relatively low compared to teleost fish (Table 4). Influx rates of Ca^{2+} from water are typical of teleost fish despite the low plasma Ca^{2+} concentrations (Tables 1, 5). Clearly, the potential availability of Na^+ and Ca^{2+} from the food is comparable to the unidirectional uptake of these ions from the water, and likely plays a valuable supplementary role (Fig. 6, Table 6), whereas the water is the main source for Cl^- uptake. The budget calculations of Table 6 also suggest that unidirectional efflux of Na^+, Cl^-, and probably Ca^{2+} is the major route of loss for these electrolytes.

In a recent study by Sturla et al. (2001), it was shown that mitochondria rich (or "chloride") cells are abundant in the skin and gills of the *Protopterus annectens*. There are two different kinds of chloride cells, α and β cells, which have commonly been associated with Ca^{2+} and Cl^- uptake and Na^+ uptake respectfully, at least in teleost fish (Galvez et al., 2002; Galvez et al., 2006). Both α and β cells are found on the gill area suggesting the gills are the site for Na^+, Ca^{2+} and Cl^- exchange. However, only α cells are present on the skin of the *Protopterus annectens* and thus this may be a major site of trans-epithelial Ca^{2+} uptake, by analogy to studies on the skin of other fish species (Marshall *et al.*, 1992; McCormick *et al.*, 1992). Furthermore, Ca^{2+} -ATPase antibody was found to strongly immunostain all of the mitochondria rich cells suggesting that the skin is the most probable site of Ca^{2+} uptake from the water (Sturla *et al.*, 2001). Overall, there was little fluctuation in Cl^- uptake following feeding which reflects on the limited amount of Cl^- available in the lungfish diet (Figure 4 and 5; Table 2). This shows the importance of water uptake as a source of Cl^- acquisition. As noted earlier, the budget calculations of Table 6 demonstrate that unidirectional ion efflux across the body surface

(gills, skin) rather than in waste products such as urine and feces was the major route of loss of Na^+, Cl^-, and Ca^{2+}. (Table 6). Urine ion composition is typical of that seen in freshwater teleosts such as the rainbow trout (*Onchorynchus mykiss*) (Patel *et al.* 2006). However, it is likely that the lungfish exhibited lower urinary ion concentrations due to the starvation period before the experiment. With that said, clearance ratios showed high rates of Na^+ and Cl^- reabsorption (Table 8), a situation which is typical of other primitive fish (Wright, 2007).

African lungfish have been predominately shown to be ammonotelic (Wood *et al.*, 2005). Many studies have looked at ammonia and urea excretion in the lungfish, but few have looked at nitrogen excretion following a feeding event (Smith, 1930; Janssens and Cohen, 1967; Wood *et al.*, 2005). The normal ammonia excretion of a starved *Protopterus dolloi* or *Protopterus annectens* ranges from 130-180 umol/kg/h (Wood *et al.*, 2005; Iftikar et al., submitted). Urea excretion of a starved *Protopterus dolloi* or *Protopterus annecten* is about 50 umol/kg/h (Wood *et al.*, 2005; Iftikar et al. submitted) However following a feeding event both the *Protopterus dolloi* and *Protopterus annectens* significantly increased ammonia production in comparison to starved fish. This increase is probably related to an increase in metabolic energy required to process the food, a trend referred to as specific dynamic action (SDA) and likely results from amino acid deamination and oxidation in the liver (Jobling, 1981). Our results showed that urea excretion in both the starved and fed lungfish tended to decrease over time during the interval between normal feeding events. Furthermore, contrary to our findings, which showed a significant increase of urea-N excretion only in the first hour following feeding in the fed *Protopterus dolloi* but no other significant increase in urea

production between the fed and starved lungfish, a study by Lim et al. (2004) showed a significant and prolonged increase in urea production in the *Protopterus dolloi* following feeding. In our study the similar pattern of in urea excretion in both fed and starved fish could have been the result of anticipation of feeding as the lungfish are trained to be fed every 2 days and immediately following a water change. Furthermore, a recent study conducted by Iftikar et al. (submitted) did not show a significant increase in urea production in the *Protopterus annectens* until 36 hours following a feeding event.

Diffusive water exchange rates for adult *Protopterus annectens* and *Protopterus dolloi* were similar, but rates in juvenile *Protopterus dolloi* appear to be slightly lower, though the difference was not significant (Fig. 11). Overall, there were no significant differences between the two species or between mature and juvenile fish. The rate constants and associated diffusive water exchange rates obtained from this study are slightly higher than those obtained by Wilkie et al. (2007) in a recent study on *Protopterus dolloi* conducted under similar conditions. The difference may be methodological: the present study injected 1% of the body weight of tritiated water (as isotonic saline) whereas the study conducted by Wilkie et al. (2007) used 0.15% of the body weight. Furthermore, the rate constants obtained in this study are similar to rate constants found in a study conducted by Oduleye (1977), who reported a rate constant of 0.44 h^{-1} for *Protopterus annectens*. However, the rate constants were quite low compared to those measured for many freshwater teleosts (Table 10) (Potts and Fleming, 1970; Evans, 1969; Lotan, 1969; Potts et al., 1967). The lower rate constants in the *Protopterus dolloi* and *Protopterus annectens* are probably attributable to the under-developed and reduced gills when compared to freshwater teleosts (Laurent *et al.*, 1978).

Since the lungfish are capable of using their skin as a site of water exchange as shown during terrestrialization (Wilkie *et al.* 2007), it was interesting to find that the divided chamber study showed a water efflux via the body which was significant (approximately 25%), but less than the 75% across the anterior (gills) portion of the lungfish (Fig. 12). The results did not show a significant difference between *Protopterus annectens* and *Protopterus dolloi*. However, the rate constant and water efflux rate values obtained for this study were generally lower than data obtained for unrestrained fish in the other protocols of the present investigation. The protocol of the divided chamber study did not allow for full movement of the lungfish as the posterior end was in a polyethelene bottle. Thus, metabolism of the lungfish may have been substantially reduced resulting in lower water exchange rates. Following an 8 month period of terrestrialization and re-immersion, the *Protopterus dolloi* substantially reduce their water efflux (Fig. 10). This decrease is most likely due to the lungfish recovering from the period of water shortage and thus a slower metabolic rate is initially exhibited (Delaney *et al.*, 1976; Wilkie *et al.*, 2007). Also, during terrestrialization, the use of the gills as a site of water and ion exchange is abolished and thus upon re-immersion physiological adjustments may take some time to restore normal gill function (Delaney *et al.*, 1976; Wilkie *et al.*, 2007).

Glomerular filtration rate (GFR) and urine flow rate (UFR) were similar in the *Protopterus dolloi* and *Protopterus annectens* (Fig. 15 and 16). However, the water exchange rates of these lungfish were lower than in most freshwater teleost fish whereas UFR was typical of other freshwater fish including the rainbow trout (*Oncorhynchus mykiss*) (Table 9; Fig. 16). This difference in part can be explained by the fact that the

Table 10. A comparison of rate constants k (h^{-1}) for diffusive water exchange in the *Protopterus dolloi* and *Protopterus annectens* with rate constants k (h^{-1}) reported for various freshwater teleosts.

Name	Rate Constant k (h^{-1})	Reference
Lungfish *(Protopterus dolloi)*	0.38	Present Study
Lungfish *(Protopterus annectens)*	0.34	Present Study
Tilapia *(Tilapia mossambica)*	1.86	Potts *et al.* (1967)
Minnow *(Phoxinus phoxinus)*	1.39	Evans (1969)
Killifish (*Fundulus kansae*)	1.38	Potts and Fleming (1970)
Goldfish *(Carassius auratus)*	0.92	Evans (1969)
Toothcarp (*Aphanius dispar)*	0.68	Lotan (1969)
Atlantic Salmon (*Salmo salar)*	0.48	Potts *et al.* (1970)

water exchange studies looked at unidirectional diffusive water exchange whereas the UFR reflects on net water fluxes driven by osmotic and hydrostatic gradients. It is probable that the diffusive exchange mainly occurs through the cell membrane (i.e. transcellular pathway) and the junctions between the cells (i.e. paracellular pathway) while the osmotic permeability is seen predominantly to use the latter pathway. Secondly, during the catheter experiment the fish had been starved before starting the experiment and then had an external catheter sewn onto their cloaca. The water volume had been reduced to only 2 L and the container in which they were held was smaller than their aquaria. These factors could have stressed the fish at which time it could have increased the rate of osmotic water flux. This would have resulted in an increase in urine flow rate. The low ion clearance ratios (i.e. high ion reabsorption efficiencies) found for the *Protopterus dolloi* and the *Protopterus annectens* were consistent with clearance ratios observed in other primitive fish (Wright, 2007). Although ion clearance ratios were similar in both species of lungfish, water clearance ratios were significantly higher in the *Protopterus annectens,* reflecting the tendency for lower GFR and higher UFR in this species (Table 8). Thus *Protopterus annectens* reabsorbed less water from the glomerular filtrate, thereby excreting water more effectively via the urine.

Despite physiological differences between freshwater teleosts and African lungfish, it seems that water and ion balance is maintained similarly. Overall, the lower metabolic rate seems to be associated with lower ion uptake and water exchange rates exhibited by the lungfish. The reduced gill area of the lungfish for ion acquisition is compensated by uptake of ions through the skin and by lower clearance ratios by the kidneys. Furthermore, water balance during terrestrialization.is maintained by the uptake

of water via the ventral body surface (Wilkie et al., 2007). In future studies, it would be of interest to find out what happens to kidney function during terrestrialization.

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