The influence of feeding on aerial and aquatic oxygen consumption, nitrogenous waste excretion, and metabolic fuel usage in the African lungfish, *Protopterus annectens*

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Abstract: We studied the utilization of air versus water as a respiratory medium for O_2 consumption (M_{O_2}) in the bimodally breathing African lungfish, *Protopterus annectens* (Owen, 1839), (151.2 ± 3.7 g) at 26–28 °C. We also investigated the impact of a single meal on this respiratory allocation and nitrogenous waste excretion in lungfish entrained to a 48 h feeding cycle. Correction for the "microbial blank" was found to be critically important in assessing the aquatic component of M_{O_2}. After correction, total M_{O_2} was low (~1000 $\mu mol \cdot kg^{-1} \cdot h^{-1}$), and lungfish took about 40% of M_{O_2} from water and 60% from air. Following a meal of chironomid larvae (3.3% of body mass), M_{O_2} values from both air and water increased in proportion over the first 3 h and continued to increase to a peak at 5–8 h postfeeding, at which point total M_{O_2} (still 40% from water) was approximately 2.5-fold greater than the prefeeding level. When the same fish, entrained to the same 48 h feeding regime, were fasted, M_{O_2} declined then later increased prior to the next anticipated feeding. In fed fish, the elevation in M_{O_2} relative to fasted values was approximately 3-fold at 0–3 h and 9-fold at 5–8 h. This specific dynamic action (SDA) effect lasted until 23–26 h and amounted to only 9.5% of the oxycalorific content of the ingested meal. N-waste efflux was only slightly elevated after feeding, where there was a tendency for greater urea–N excretion (significant at 42–48 h); however, the lungfish remained ammoniotelic overall during the 48 h postfeeding period.

Résumé : Nous avons étudié l'utilisation de l'air plutôt que l'eau comme milieu respiratoire d'absorption d'O₂ (M_{O_2}) chez le dipneuste africain, *Protopterus annectens* (Owen, 1839), (151,2 ± 3,7 g) à respiration bimodale à 26–28 °C. Nous avons aussi examiné l'impact d'un repas unique sur cette allocation respiratoire et sur l'excrétion des déchets azotés chez des dipneustes habitués à un cycle alimentaire de 48 h. Une correction pour le « blanc microbien » est absolument nécessaire dans l'évaluation de la composante aquatique de M_{O_2}. Après correction, M_{O_2} est faible (~1000 $\mu mol \cdot kg^{-1} \cdot h^{-1}$) et les dipneustes tirent environ 40 % de M_{O_2} de l'eau et 60 % de l'air. Après un repas de larves de chironomes (3,3 % de la masse corporelle), les valeurs de M_{O_2}, tant de l'air que de l'eau, augmentent proportionnellement durant les 3 premières h et atteignent un pic à 5–8 h après le repas, au moment où M_{O_2} total (toujours 40 % provenant de l'eau) est d'environ 2,5 fois plus élevé qu'avant le repas. Lorsque le même poisson, toujours habitué au même régime alimentaire de 48 h, est gardé à jeun, M_{O_2} décline pour ensuite se relever en anticipation du prochain repas. Chez les poissons alimentés, l'élévation de M_{O_2} par rapport aux valeurs de jeûne est approximativement 3 fois plus grande à 0–3 h et 9 fois supérieure à 5–8 h. Cet effet d'action dynamique spécifique (SDA) dure jusqu'à 23–26 h et correspond à seulement 9,5% du contenu oxycalorifique du repas ingéré. L'élimination des déchets de N est seulement un peu plus élevée après le repas et il y a une tendance vers une excrétion accrue de N sous forme d'urée (particulièrement à 42–48 h); cependant, le dipneuste demeure en gros ammoniotélique durant la période de 48 h qui suit l'alimentation.

[Traduit par la Rédaction]

Introduction

The origin of terrestrial vertebrates from water-dependent "lobe-finned" fishes involved several important morphological and physiological modifications (Benton 1990). By providing possible models for this transition, air-breathing fishes have heuristic importance (Graham and Lee 2004). Among the more derived extant air-breathing fishes are several species that use a modified air bladder (normally used for buoyancy) to exchange oxygen and act as a "lung". One such group, the Dipnoi, are sarcopterygiian fish commonly referred to as lungfish (Benton 1990). The remarkable ability of lungfish to breathe both air and water makes them a key group in understanding the transition of vertebrates from water to land (Oduleye 1977). One family of lungfish (Protopteridae) is found in Africa and further

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contains four species: *Protopterus annectens* (Owen, 1839); *Protopterus aethiopicus* Heckel, 1851; *Protopterus dolloi* Boulenger, 1900; *Protopterus amphibius* (Peters, 1844) (Ip et al. 2005).

Protopterus annectens occur in shallow ponds in west and northeast Africa (Greenwood 1986). The high environmental temperatures and organic content of this habitat often result in very low dissolved oxygen concentration in the water, which presumably increases reliance on air-breathing. *Protopterus annectens* additionally spend part of the year in aestivation when the ponds evaporate during the dry season (Oduleye 1977). While many studies have focused on air-breathing, aestivation, and biochemical analysis of metabolism in *P. annectens* (e.g., Oduleye 1977; Babiker 1979; Morescalchi et al. 2002; Sturla et al. 2002; Loong et al. 2005), little is known about the utilization of air versus water as a respiratory medium, and the impact of feeding on this usage.

The literature is controversial as to whether lungfish of the size used in this study (150 g) utilize mainly the air or water for O_2 consumption ($\dot{M}_{O_2}$). According to Babiker (1979), *P. annectens* of this size primarily take up O_2 from the water, whereas Oduleye (1977) reported exactly the opposite — i.e., that the air phase dominated for O_2 uptake. Recent reports by Perry et al. (2005a, 2005b) on the closely related *P. dolloi* concluded that the air phase overwhelmingly dominates for O_2 uptake in these obligate air breathers. These papers also highlighted a problem that may have caused an erroneous conclusion by Babiker (1979), specifically that there is a high microbial $\dot{M}_{O_2}$ "blank" in the water in which lungfish have been living. Likely, this would have an increasing effect both over the time course of $\dot{M}_{O_2}$ measurement and as the size of the fish decreased, and therefore may have possibly led to an erroneous conclusion as to the importance of O_2 uptake from the water phase. Consequently, our first objective was to partition measured O_2 uptake between air and water in *P. annectens*, taking the microbial blank into account, with the goal of clarifying this issue.

Our other objectives were related to feeding. An increase in metabolic rate following ingestion of food was first demonstrated in homeothermic animals by Laplace and Lavoisier and the phenomenon was later termed the "specific dynamic effect" by Ruber (see Kleiber 1975). In poikilotherms, the effect is commonly observed as a postprandial increase in the rate of $\dot{M}_{O_2}$ (Fu et al. 2005) and is currently termed specific dynamic action (SDA), which refers to the heat increment or the calorigenic effect of feeding (for a review see Jobling 1981). This is typically the sum of the energy costs of swallowing food, digestion, absorption, and biochemical transformation of absorbed nutrients.

Our second objective was to determine the extent of the postprandial SDA in *P. annectens* in terms of $\dot{M}_{O_2}$ and the routes by which it occurs. The low metabolic rate and habitual stillness of the lungfish make it an excellent experimental subject for closed-system respirometry (Smith 1935a, 1935b). Indeed, lungfish appear to have one of the lowest resting metabolic rates of any fish (e.g., Smith 1935a, 1935b; Johansen and Lenfant 1967; Oduleye 1977; Perry et al. 2005a, 2005b). Therefore, we wished to determine

whether the postprandial increase in $\dot{M}_{O_2}$ was correspondingly small, or comparable with, the increase observed in other fish. We also analyzed the energy content of the food (protein, lipid, and carbohydrate) to determine what percentage of the potential energy gain was dissipated as SDA. Ever since Smith's (1935b) work, it has been known that SDA can be measured in lungfish, but we wished to determine whether the "additional" O_2 uptake was from water, from air, or from both phases. Oduleye (1977) reported that O_2 uptake by unfed *P. annectens* was actually higher when fish were forced to breathe exclusively from the water phase than solely from the air phase, suggesting that the capacity for exploiting the water as an additional resource might be greater than for exploiting the air.

Our third objective was to assess the effects of feeding on nitrogen waste (N-waste) excretion in *P. annectens* in light of a recent report that the closely related and normally ammoniotelic *P. dolloi* becomes ureotelic after feeding (Lim et al. 2004). Waste excretion in lungfish is by means of urea and ammonia–N production (Lim et al. 2004). In teleosts, N excretion increases greatly, mainly in the form of ammonia, after a meal (reviewed in Wood 2001), whereas in elasmobranchs (Kajimura et al. 2004; Wood et al. 2007), virtually all the extra N is retained. To learn whether N-waste excretion by *P. annectens* changes after feeding, we measured ammonia–N and urea–N excretions throughout these feeding trials and related it to the protein–N of the food to determine what percentage of the ingested N was retained.

Materials and methods

Experimental animals

Protopterus annectens ($n = 8$, mean (SE) mass 151.2 ± 3.7 g) were collected in Nigeria and Zaire and air-shipped to a commercial dealer in Singapore. They were then purchased, housed briefly at the University of Singapore, and then air-shipped to McMaster University, Hamilton, Ontario, where the experiments were carried out. Animals were cared for in accord with the principles of the Canadian Council on Animal Care and protocols were approved by the McMaster Animal Care Committee.

Fish were held at 26–28 °C under 12 h light : 12 h dark photoperiod for several weeks prior to experimentation. Lungfish were kept individually in small plastic aquaria containing ~3 L of dechlorinated tap water that was supplemented with seawater to achieve a NaCl level of ~2 mmol/L. *Protopterus annectens* diet comprised frozen chironomid larvae (Sally's bloodworms, San Francisco Bay Brand Inc., Newark, California) that were administered to individuals every 2 days (3.3% body mass per feeding). Water was changed on the day of feeding before administration of food. These conditions are found to keep the animals healthy, the slightly saline water being important in preventing fungal infections. Food was withdrawn 48 h prior to experiments, providing sufficient time for the gut to be emptied of all food and waste (see Wood et al. 2005a, 2005b).

Experimental protocol

The lungfish were transferred to individual 15 L cylindrical tanks (height = 40.0 cm, water surface area = 111.3 cm²),

each with a narrow neck at the top which could be sealed with a rubber bung so that the tank could serve as a respirometer. They were acclimated for 5 days and experienced two feeding cycles prior to the experiment; the water was changed at 2.5 h prior to each feeding event. Each lungfish ($n = 8$) was used in a 48 h feeding experiment, a 48 h nonfeeding control experiment, and then in a second 48 h feeding experiment. Several weeks intervened between each of the three experiments. The results of the first and second feeding experiments were not significantly different and were averaged for each fish. In the feeding experiments, fish were fed at time 0, which was 48 h since their last meal. At the time of feeding, a mass of chironomid larvae equivalent to 3.3% of the body mass was added to the chamber. Lungfish typically consumed the entire meal within 30 min.

Air and water samples were taken over a prefeeding 2 h control time period, and over 0–3, 5–8, 12–15, 23–26, 33–36, and 46–49 h postfeeding time periods. In the nonfeeding control experiment, the fish were treated identically (i.e., not fed for the prior 48 h), but no food was administered at time 0. Air and water samples were taken over the same time periods as in the feeding experiment.

Measuring $\dot{M}_{O_2}$ via closed respirometry

Closed-system respirometry was used to measure the relative $\dot{M}_{O_2}$ from air and from water in *P. annectens*. $\dot{M}_{O_2}$ was measured after the respirometer was filled with water (typical volume = 15 L) to leave a known volume of air space (typical volume = 0.3 L) at the top of the chamber into which the lungfish could surface for air-breathing. The respirometer was shielded with black plastic to minimize visual disturbances. Before the experiment, the water was changed, a stir bar was added, the respirometer was placed on a magnetic stirrer, and the top was sealed with a rubber bung. A system of sampling catheters and three-way stopcocks exiting through the rubber bung allowed both air and water samples to be withdrawn from the middle of the respective volumes.

A polagraphic O_2 microelectrode (E5046; Radiometer, Copenhagen, Denmark) kept at the experimental temperature in a thermostatted cuvette, with its output displayed on a Radiometer pHM71 blood gas meter (Radiometer, Copenhagen, Denmark), was used for all measurements of P_{O_2} in the water and gas phases. Calibration was performed with water-saturated N_2 (zero) and air, the latter at known barometric pressure. Air samples were drawn first, followed by water samples; the water volume was gently stirred for 30 s by means of a magnetic stirrer immediately prior to water sampling. For each air or water sample, 1 mL was first drawn into a syringe and discarded (dead space), and then a subsequent 1 mL sample was taken for analysis. Each 1 mL sample of air or water was replaced by 1 mL of water. Air and water P_{O_2} were measured every 30 min for a 3 h period (or 2 h in the control period) and then the sealing bung was removed. The tank was re-aerated with an air-stone until the next sampling time (i.e., from 3 to 5, 8 to 12, 15 to 23, 26 to 36, 39 to 46 h) where water P_{O_2} increased to typical levels (>130 mm Hg).

$\dot{M}_{O_2}$ was measured from the rate of decline of P_{O_2} over the 2–3 h period in each compartment (air or water) factored by the respective solubility coefficient of O_2 (αO_2) in the particular medium, compartment volume (v), and the lungfish mass (m). The volume of the water compartment was corrected for the lungfish mass. The rate of P_{O_2} decline ($\Delta P_{O_2}/t$) was calculated as the slope of the least-squares regression of P_{O_2} versus time fitted to the 5–7 measurements over 2–3 h. Therefore, absolute $\dot{M}_{O_2}$ was calculated by

$$\dot{M}_{O_2} = \left(\frac{\Delta P_{O_2}}{t \times \alpha O_2 \times v} \right) / m$$

where ΔP_{O_2} is in mm Hg, v is in L, m is in g, t is in h, and αO_2 ($\mu mol \cdot L^{-1} \cdot mm\ Hg^{-1}$) is the solubility constant for O_2 in air or water at the experimental temperature (Dejours 1975; Boutilier et al. 1984). Ambient P_{O_2} in the respirometer in both the air and the water phases was not allowed to fall below 100 mm Hg; if it did, the protocol was interrupted for 30 min to allow re-aeration. Temperature was maintained throughout the experiment at 26–28 °C by working in a constant-temperature room.

The area of the interface between the air phase (0.3 L) and the water phase (15 L) was 111.3 cm². We were concerned that significant diffusion of O_2 between air and water might occur during the 2–3 h period of closure during an experiment, thereby causing an artifact. Therefore several runs were performed with clean water in the absence of a lungfish (to avoid the microbial blank issue addressed below) in which either the air or water phase was set to a typical P_{O_2} level (~130 mm Hg) observed during a real experiment, while the other phase remained at air saturation P_{O_2}. Magnetic stirring for 30 s prior to sampling was used as in the actual experimental tests. There were never any significant changes over the measurement period, although this effect might be slightly underestimated because of the "lack of breaking of the surface by lungfish" in our blank tests.

Correcting for the microbial background $\dot{M}_{O_2}$

Perry et al. (2005a, 2005b) have recently reported that there is considerable background metabolism ("microbial blank") associated with aquatic respirometry in *P. dolloi*. To address this potential problem in *P. annectens*, blank trials were carried out in six separate experimental runs additional to the ones outlined above. In each run, the same lungfish ($n = 8$) was fed in the normal fashion at time 0. However, after sampling at the end of the 0–3, 5–8, 12–15, 23–26, 36–39, or 46–49 h period, the lungfish was removed, the system was resealed, and the water P_{O_2} was monitored at 30 min intervals over the next 2 h. These measurements revealed that there was a substantial microbial $\dot{M}_{O_2}$ in the water. Therefore the mean background $\dot{M}_{O_2}$ of the water (in $\mu mol \cdot kg^{-1} \cdot h^{-1}$) for individual lungfish after every measurement time was then subtracted from the measured aquatic $\dot{M}_{O_2}$ values of that same lungfish for each measurement time period.

Observational studies

Casual observations suggested that the air-breathing frequency of *P. annectens* increased during and after the feeding period. Therefore, the respiratory frequency from air (number of breaths·h⁻¹) and from water (number of breaths·min⁻¹ measured for 1 min every 0.5 h) was visually

quantified in *P. annectens*. For aerial (pulmonary breaths) respiratory frequency, one surfacing event was considered to represent one air breath. For branchial (water breaths) respiratory frequency, the rate of opercular movements was counted using a stop watch. Both aerial and branchial respiratory frequencies were quantified throughout the prefeeding control period (2 h), the feeding period (0–3 h), and the immediate postfeeding period (5–8 h).

Analysis of N-waste products

Ammonia–N and urea–N excretions in fed lungfish were measured using a 15 L water volume; water samples were taken every 6 h during a 48 h experimental period. Prior experiments indicated that urea-excretion levels into the water were at the limits of the urea assay in unfed fish at 15 L total volume. Therefore, a separate series was performed for unfed fish ($n = 8$) in an identical manner except that only 4 L of water was present in the 15 L respirometer tanks; the area of the water surface remained unchanged. Samples for analysis of ammonia–N and urea–N were taken at 0, 6, 12, 18, 24, 30, 36, 42, and 48 h in all series. The indophenol blue method of Ivancic and Degobbis (1984) was used to measure the concentrations of ammonia–N in water using freshly prepared NH_4Cl standards made up in the test water (Lim et al. 2004). The urea–N concentration in the water samples was determined by the diacetyl monoxime method of Rahmatullah and Boyd (1980). Total nitrogen flux was estimated by adding ammonia–N flux and urea–N flux for individual fish to give a mean total nitrogen flux.

Analysis of dietary components

Frozen chironomid larvae (~200 mg) were ground into a fine powder under liquid N_2 using an insulated mortar and pestle cooled with liquid N_2. Subsamples (50 mg) of this frozen powder were then used in assays to determine dietary components. Total protein concentration was determined by the method of Lowry et al. (1951) with bovine serum albumin (Sigma-Aldrich, Toronto, Ontario) as standards. Total lipid content was determined by the sulphophosphovanillin method (modified from Barnes and Blackstock 1973). Carbohydrate content was estimated as the sum of glucose, lactate, and glycogen. Frozen chironomid powder was freeze-dried (Lyph-Lock 6, LabConco, Kansas City, Missouri), deproteinized with 8% perchloric acid (PCA), and then centrifuged. The supernatant was assayed enzymatically for lactate by the reversible L-lactate dehydrogenase (EC 1.1.1.27) method (Bergmeyer 1983) (LKB UltralspecPlus 4053, LKB Ltd, Cambridge, UK). PCA-extracted supernatant was neutralized with 3 mol·(L $K_2CO_3)^{-1}$ prior to enzymatic analysis for glucose and glycogen content (hexokinase, EC 2.7.1.1; glucose-6-phosphate dehydrogenase, EC 1.1.1.49) using a commercial kit (Sigma-Aldrich, 301A).

Calculations

The magnitude of the whole SDA effect was determined by plotting the curve of total $\dot{M}_{O_2}$ (sum of $\dot{M}_{O_2}$ from air plus $\dot{M}_{O_2}$ from water, corrected for the bacterial blank) against time until the effect subsided, then integrating the area beneath the curve that lay above the comparable total $\dot{M}_{O_2}$ curve of fasted control fish (Jobling 1981). The magni-

tudes of the SDA components from air and from water were determined separately by comparable integration of the areas between the air $\dot{M}_{O_2}$ curves and the water $\dot{M}_{O_2}$ curves, respectively, for fed and fasted lungfish. The difference in total N excretion between fed and fasted fish was also determined in the same manner by integrating the area between the total N excretion curves for the two treatments.

Several calculations were performed based on the analysis of the chironomid larvae, assuming a meal size of 3.3% (wet/wet) of the body mass of the lungfish, so as to compare fuel intake with respirometry measurements. Protein–N content in the diet was taken as the standard value of 0.16 g N·(g protein)$^{-1}$ (Kajimura et al. 2004). While total protein and total lipid contents were measured directly, total carbohydrate content was estimated as the sum of glycogen plus glucose plus lactate. Oxycalorific values of dietary metabolites were calculated using energy equivalents for fish bioenergetics (carbohydrate 0.84 L $O_2·g^{-1}$; lipid 2.0 L $O_2·g^{-1}$; protein 0.96 L $O_2·g^{-1}$) (Schmidt-Nielsen 1990).

Statistical analysis

Data are reported as means ± SE (n is the number of fish), unless otherwise stated. Data were normally distributed, therefore parametric statistics were used in all analyses. Differences within an experimental group (e.g., fed and unfed) at different time periods were evaluated with a repeated measures analysis of variance (ANOVA) followed by a post hoc test (Tukey's). A paired *t* test was used to detect differences between treatments (fed vs. unfed) at the same time period. A χ^2 test was used to determine if % urea–N excretion exceeded 50% total N excretion in a given time period. The level of significance was set at $P < 0.05$. All statistical tests were run using SigmaStat® version 3.1 (Systat Software, Inc., San Jose, California).

Results

Energy and N content of the food

Based on the analysis of chironomid larvae and a 3.3% ration (Table 1), the energy content of the meal, in oxycalorific equivalents, was about 273 000 μmol O_2·(kg lungfish)$^{-1}$, assuming that all the nutrients were assimilated. The major component was fat (57.5%), reflecting its greatest abundance in the food and high energy density, followed by protein (39%), whereas carbohydrate contributed only 3.5%, reflecting its low abundance. The protein–N content of the meal was about 28 000 μmol N·(kg lungfish)$^{-1}$.

Oxygen uptake

The background $\dot{M}_{O_2}$ of the water ("microbial blank") varied significantly over time (Table 2) and among individual lungfish, and contributed considerably to the total measured aquatic $\dot{M}_{O_2}$. In the prefeeding control period, the microbial blank contributed about 25% of the total aquatic $\dot{M}_{O_2}$. This contribution by the microbial blank increased greatly at 12 h after feeding, where we found that nearly 70% of the aquatic $\dot{M}_{O_2}$ by lungfish during the 12–15 and 23–26 h time periods was due to microbial respiration. Therefore, aquatic $\dot{M}_{O_2}$ data for each individual fish were corrected for its characteristic blank at the appropriate time.

Table 1. Digestible energy (means ± SE) provided by individual dietary components calculated as oxycalorific value (μmol O_2) of dietary fuels in a meal consisting of 3.3% chironomid larvae ingested per 1 kg of African lungfish (*Protopterus annectens*).

Dietary fuel type (n)	Dietary fuel in chironomid larvae (g·(g wet mass)$^{-1}$)	Digestible energy (μmol O_2·(kg lungfish)$^{-1}$)
Carbohydrate (8)	0.0078 ± 0.0004	9 769 ± 644
Fat (4)	0.0527 ± 0.0050	156 798 ± 14 881
Protein (12)	0.0744 ± 0.0026	106 250 ± 3 651
Protein–N (12)	0.0119 ± 0.0004	28 313 ± 973*

*The digestible energy of protein–N is in mmol N·(kg lungfish)$^{-1}$.

Table 2. Microbial O_2 consumption from water (mean ± SE, n = 8) per 1 kg of African lungfish (*Protopterus annectens*) which had resided in that water.

Treatment or time period (h)	Water $\dot{M}_{O_2}$ (μmol·kg^{-1}·h^{-1})
Control	150.7±46.1a
0–3	423.1±62.5b
5–8	420.9±42.3b
12–15	469.1±43.8b
23–26	296.9±78.3ab
36–39	147.8±51.0a
46–49	213.4±100.0ab

Note: Means sharing the same letter are not significantly different from one another at $P < 0.05$.

Mean $\dot{M}_{O_2}$ (n = 8) of fed and fasted control fish, corrected for the microbial blank, are illustrated in Fig. 1, with the amounts of oxygen taken up from air (through the lungs) and from water (through the gills and skin) shown separately, whereas the two components are summed in Fig. 2. In the control period for both treatments, mean total $\dot{M}_{O_2}$ was about 1000 μmol·kg^{-1}·h^{-1}, with about 60% from air and 40% from water (Fig. 1A). $\dot{M}_{O_2}$ values from both the air and the water phases were significantly elevated over the first 3 h during and after feeding and continued to increase to a peak at 5–8 h postfeeding, at which point $\dot{M}_{O_2}$ was ~2.5-fold greater than the prefeeding level. This maximum postprandial $\dot{M}_{O_2}$ peak during the 5–8 h time period postfeeding was observed in both the air (1448.6 ± 44.4 μmol·kg^{-1}·h^{-1}) and the water (1115.9 ± 99.2 μmol·kg^{-1}·h^{-1}) phases. During the postprandial increase in $\dot{M}_{O_2}$, these obligate air breathers continued to obtain about 60% of their oxygen from the air phase. Subsequently, total $\dot{M}_{O_2}$ and medium-specific rates gradually declined, with total $\dot{M}_{O_2}$ falling below the prefeeding levels by 23–26 h, a situation that was maintained through 46–49 h owing to the continued depression of $\dot{M}_{O_2}$ from air. In contrast, $\dot{M}_{O_2}$ from water did not fall significantly below the prefeeding rate except for a slight depression in the 23–26 h time period.
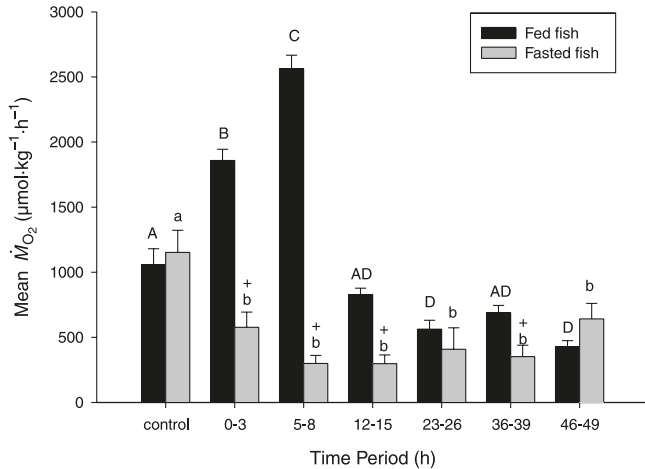
Comparison of the stimulatory effect of feeding on $\dot{M}_{O_2}$ (Figs. 1A, 2) to the pattern in the fasted animals (Figs. 1B, 2) reveals a more pronounced difference. When the fasted fish were not fed at their regular time, the $\dot{M}_{O_2}$ from both media fell; an effect that persisted right from 0–3 to 36–39 h, after which $\dot{M}_{O_2}$ rose back to its original value prior to

Fig. 1. Mean (SE) O_2 consumption by *Protopterus annectens* (n = 8) during a 48 h experimental period: (A) fed fish, with feeding occurring at 0 h; (B) fasted fish. Means sharing the same letter of the same case are not significantly different from one another at $P < 0.05$. The asterisk denotes a significant difference from the $\dot{M}_{O_2}$ of fed lungfish, while the plus sign denotes a significant difference between air and water $\dot{M}_{O_2}$ in a given time period at $P < 0.05$.
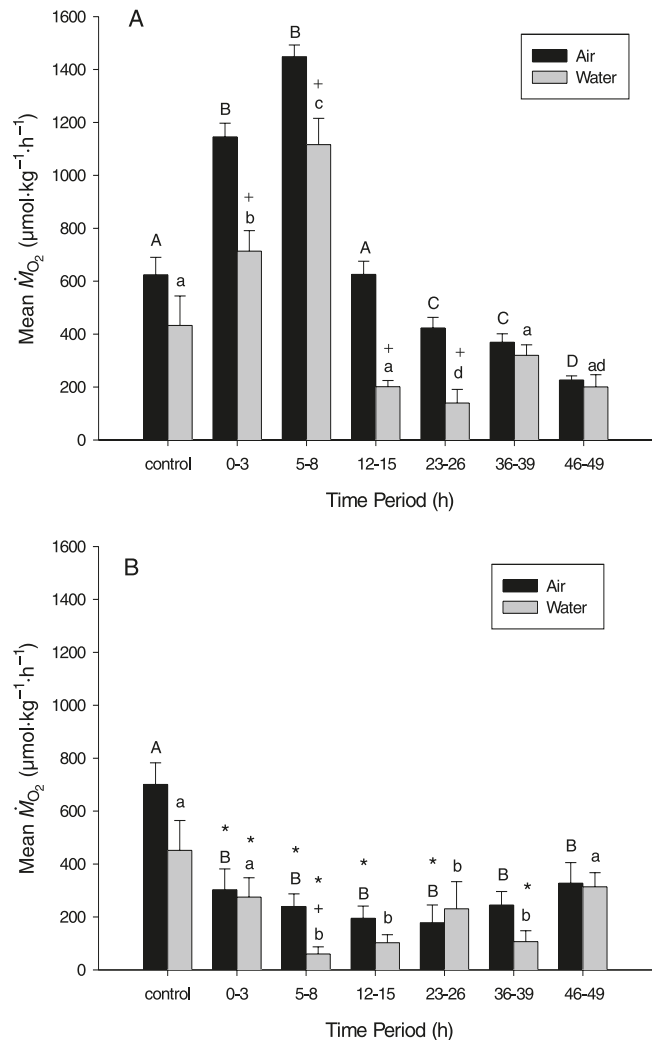
the next anticipated feeding at 46–49 h. Therefore, fed fish exhibited about a 3-fold increase in total $\dot{M}_{O_2}$ compared with that of fasted fish (1859 ± 86 vs. 578 ± 116 μmol·kg^{-1}·h^{-1},

Fig. 2. Comparison of total O_2 consumption from both air and water between fed and fasted *Protopterus annectens* ($n = 8$) during a 48 h experimental period, with feeding at 0 h. Means (SE) sharing the same letter of the same case are not significantly different from one another at $P < 0.05$. The plus sign denotes a significant difference between $\dot{M}_{O_2}$ values for fed and fasted fish in a given time period at $P < 0.05$.

Fig. 3. Mean (SE) ventilation rate of *Protopterus annectens* ($n = 8$) during an 8 h experimental period, with feeding at 0 h: (A) pulmonary (air-breathing) ventilation per hour; (B) branchial (water-breathing) ventilation per minute. Means sharing the same letter are not significantly different from one another at $P < 0.05$.

respectively) in the 0–3 h time period and almost a 9-fold increase (2564 ± 103 vs. 299 ± 62 μmol·kg^{-1}·h^{-1}, respectively) at 5–8 h. Overall, Fig. 2 shows that the SDA effect persisted through 12–15 h postfeeding in *P. annectens* but had disappeared by 23–26 h. Integration of the difference between the two curves (see Materials and methods) over the whole experimental period revealed a total SDA of 25 900 μmol O$_2$·(kg lungfish)$^{-1}$, which accounted for only 9.5% of the energy content (273 000 μmol O$_2$·(kg lungfish)$^{-1}$; Table 1) of the ingested meal. The SDA from the air phase was about 62% of the total SDA and that from the water phase was about 38% of the total SDA. The total $\dot{M}_{O_2}$ of the fed lungfish over the 48 h period was only 49 000 μmol O$_2$·(kg lungfish)$^{-1}$ or 18% of the oxycalorific content of the food ingested. The total $\dot{M}_{O_2}$ of the fasted lungfish over the 48 h period was 23 100 μmol O$_2$·(kg lungfish)$^{-1}$, so the overall ratio (fed/fasted = 49 000 / 23 100) was about 2.1 to 1.

Observational studies

Feeding caused more than a doubling of air-breathing frequency in the first hour, with a return to prefeeding rate after 3 h (Fig. 3A). Lungfish were visibly more active during the first 3 h postfeeding only, reflecting more frequent excursions to the surface. Water-breathing frequency increased dramatically (7-fold) during the actual feeding event but quickly declined thereafter. The notable elevation at 0 h during the actual feeding event could reflect the animal ingesting food by suction and releasing excess water via the gills. However, water-breathing frequency still remained significantly elevated by 50%–100% through 0.5–8 h postfeeding (Fig. 3B).

N-waste excretion

Nitrogen flux data were rather variable, as illustrated by the values for fed fish in Fig. 4. In general ammonia–N
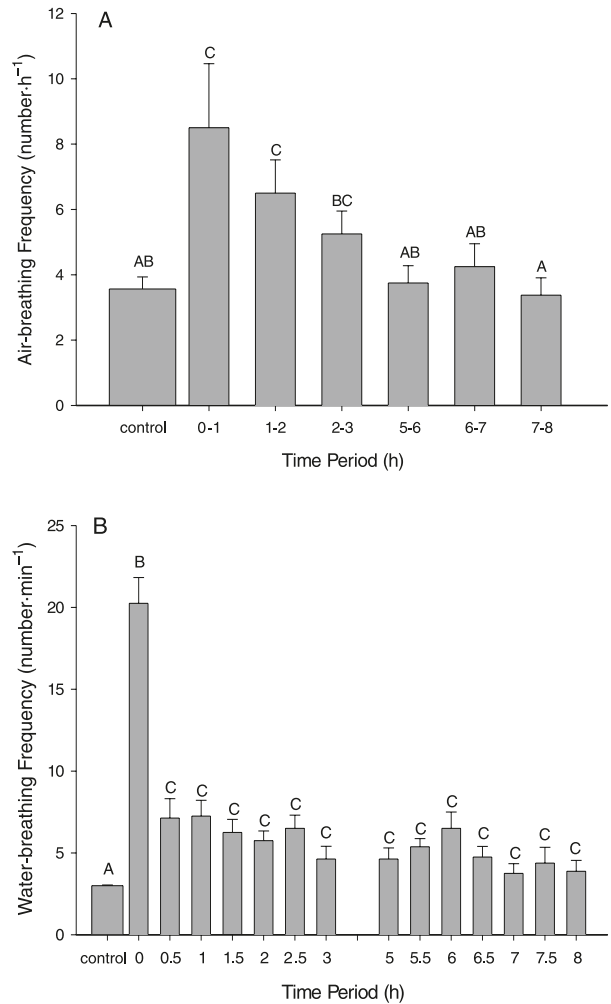
excretion was greater than urea–N excretion, although the differences were rarely significant. In fed lungfish, compared with prefeeding levels, ammonia–N efflux was lower and urea–N efflux was higher during the 42–48 h period. There were no significant variations in the fasted control fish (data not shown). When ammonia–N efflux plus urea–N efflux were summed to estimate total N excretion, values were higher in the fed fish over the 48 h period compared with the fasted fish, although none of the individual differences were significant (Fig. 5). By integration, the overall elevation owing to feeding in total N excretion over the experimental period was 4 525 μmol N·kg^{-1} or about 16% of the ingested protein–N (28 313 μmol N· kg^{-1}; Table 1). However, total N excretion in the fed fish over the 48 h period amounted to 13 275 μmol N·kg^{-1}, or about 47%, of the ingested protein–N. We also calculated the nitrogen quotient (NQ), which provides a quantitative measure of protein utilization by relating the excretion of waste nitrogen (M_N) to the consumption of oxygen ($\dot{M}_{O_2}$) (Kleiber 1992). In the fed
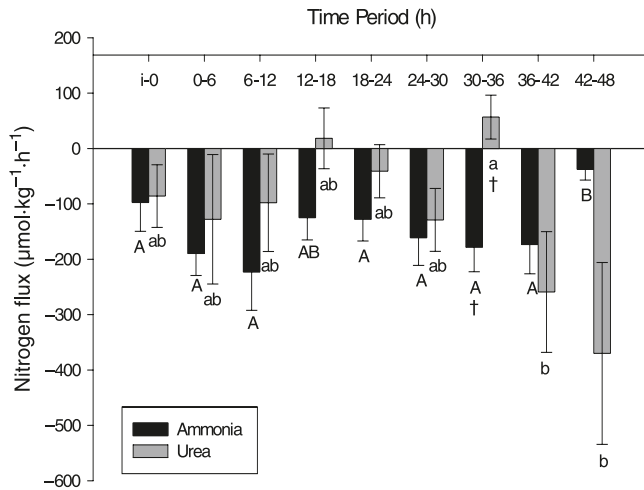
fish, the NQ (i.e., (ammonia–N + urea–N excretion)/$\dot{M}_{O_2}$) was 0.27. In fasted fish, the NQ was 0.38.

Discussion

The microbial blank

The modification of water P_{O_2} by microbial metabolism can be a major source of error in closed-system respirometry and must be taken into consideration when making conclusions (Kaufmann and Forstner 1989; Perry et al. 2005a). Clearly, the present study confirms the finding of Perry et al. (2005a, 2005b) that a substantial portion of the apparent O_2 uptake of the lungfish from water is due to microbial respiration, although the methods used to assess it and the conclusions as to its magnitude differ. In the present study on *P. annectens*, we used water blanks (with the fish absent) matched to the time and treatment of the individual fish and concluded that the microbial blank accounted for 25%–70% the $\dot{M}_{O_2}$ from water, depending on the treatment and time. In *P. dolloi*, Perry et al. (2005a, 2005b) euthanized the fish and measured the continued decline in water P_{O_2}, which was almost equal to that seen with a live fish, and concluded that the majority of the $\dot{M}_{O_2}$ from water was of microbial origin. The advantage of the euthanasia method is that it would include microbes living on the fish's surface in the blank measurement; the disadvantage is that true $\dot{M}_{O_2}$ of the lungfish by and through the skin (i.e., cutaneous metabolism) would likely continue for some time after euthanasia and would be included in the blank measurement. Our approach was therefore more conservative and it avoided sacrificing these valuable animals.

$\dot{M}_{O_2}$ from air versus water in *P. annectens*

Addressing our first objective in this study, we were able to partition O_2 uptake between air and water in *P. annectens*. A similar pattern for the relative proportions of $\dot{M}_{O_2}$ taken from air (~60%) versus water (~40%) was observed
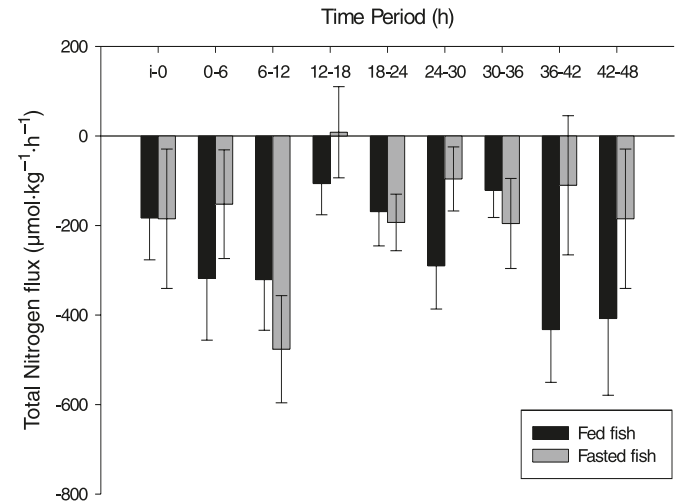
for both fed and fasted fish (Figs. 1A, 1B), using our matched water blanks method, so both components increased proportionally during the SDA effect. In agreement with the findings of Oduleye (1977), *P. annectens* clearly has the capacity to increase $\dot{M}_{O_2}$ from water, although how much of this is through the skin versus the gills is unclear. Certainly, the prolonged increase in water ventilation rate after feeding (Fig. 3B) suggests that the gills play an important role.

Previous studies have reported that *P. annectens* of the same mass range (up to 150 g) acquired more than 90% of their O_2 from either the air phase (Oduleye 1977) or more than 75% from the water phase (Babiker 1979) — i.e., clearly opposing conclusions. However, in support of the former conclusion, Perry et al. (2005a) reported over 90% of $\dot{M}_{O_2}$ from air in *P. dolloi* with a mean mass of 159 g, a value achieved by using the euthanasia blank method outlined above. Similarly, the lungs were shown to be the primary route of O_2 uptake in other lungfish, where *P. aethiopicus* obtained about 90% and *Lepidosiren* Fitzinger, 1837 obtained about 98% of their oxygen requirements by air-breathing (Johansen and Lenfant 1967, 1968). Our study on *P. annectens* with a mean mass of 150 g agrees with these findings of a higher $\dot{M}_{O_2}$ from the air than from the water (Figs. 1A, 1B), although not to the same extent (~60% vs. ≥90%). Specifically, we found that the gills and (or) skin of *P. annectens* in well-oxygenated water with access to air made a far greater contribution to $\dot{M}_{O_2}$ (~40%) than the 6.4% previously reported by Oduleye (1977). However, our data certainly do not support Babiker's (1979) observation that *P. annectens* of the same mass range obtained >75% of their O_2 by means of aquatic respiration. This over-representation of the gills and (or) skin as a source for O_2 uptake could be due to the "microbial blank" that was not accounted for in Babiker's study (1979). Notably, the total $\dot{M}_{O_2}$ values reported by Babiker (1979) were very high, in the range of 7000 µmol·kg^{-1}·h^{-1}, most of which came from the water, pointing to the likely

Table 3. Duration of the SDA effect and maximum rates of O_2 consumption induced by feeding in relation to low routine or standard rates in fish.

	Duration of SDA (h)	Maximum rate of $\dot{M}_{O_2}$ in relation to resting metabolic rate	Temperature (°C)	Reference
Smooth blenny, *Blennius pholis* L., 1758	20	1.75	8	Vahl and Davenport 1979
Plaice, *Pleuronectes platessa* L., 1758	24–72	2.00	10	Jobling and Spencer Davies 1980
Aholehole, *Kuhlia sandvicensis* (Steindachner, 1876)	60	2.60	10	Muir and Niimi 1972
Largemouth bass, *Micropterus salmoides* (Lacepède, 1802)	30	1.78	25	Beamish 1974
Atlantic cod, *Gadus morhua* L., 1758	12–18	2.0	9–11	Lyndon et al. 1992
Southern catfish, *Silurus meridionalis* Chen, 1977	16	1.5	27.5	Fu et al. 2005
Nile tilapia, *Oreochromis niloticus* (L., 1758)	10	2.4	28	Ross et al. 1992
Spiny dogfish, *Squalus acanthias* L., 1758	75+	1.7	12	Wood et al. 2007
Small-spotted catshark, *Scyliorhinus canicula* (L., 1758)	72	2.8	15	Sims and Davies 1994
Lungfish *Protopterus aethiopicus* Heckel, 1851	24	1.5	25	Smith 1935b
African lungfish, *Protopterus annectens* (Owens, 1839)	23–26	2.5	26–28	Present study

presence of a high microbial blank (although much higher water-breathing frequencies were also reported as discussed below). By way of contrast, much lower rates in the range of 300–2500 μmol·kg^{-1}·h^{-1} were reported in lungfish by all the other studies, including the present one (Fig. 2; see also Smith 1935a, 1935b; Seifert and Chapman 2006). These $\dot{M}_{O_2}$ values are extremely low relative to teleost fish of similar size and at similar temperature, which typically exhibit resting $\dot{M}_{O_2}$ values in the range of 6000–9000 μmol·kg^{-1}·h^{-1} (for a review see Clarke and Johnston 1999).

Protopterus annectens of the present study had much lower control ventilatory rates both from the air (4 breaths·h^{-1}; Fig. 3A) and water (3 breaths·min^{-1}; Fig. 3B) compared with the data of Babiker (1979) for the same species (6–22 air breaths·h^{-1} and 50–70 water breaths·min^{-1}, respectively). In *P. dolloi*, Perry et al. (2005a, 2005b) also reported much higher control pulmonary breathing frequencies (16–19 air breaths·h^{-1}), but they noted that "casual observation suggested that air-breathing frequency tended to be higher in fish held in respirometers compared to their holding aquaria". Both Babiker (1979) and Perry et al. (2005a, 2005b) used small flow-through respirometry chambers that held a volume of only ~2 L. The lungfish were subjected to a constricted space and the notably higher air-breathing frequency could be attributed to a stress response. In comparison, we believe our respirometers reflected a more natural condition that *P. annectens* experience in their habitat. These west African freshwater lungfishes live in shallow lakes and swamps that experience seasonal drought (Greenwood 1986). In the much larger respirometers (15 L) of the present study, *P. annectens* were subjected to a depth of ~40 cm, which is probably closer to the depth that they live in nature. Therefore, they were less spatially stressed, and the air- and water-breathing frequencies of *P. annectens* probably more closely reflect their natural behaviour.

Postprandial $\dot{M}_{O_2}$ in *P. annectens*

Addressing our second objective of the study, despite the low values of resting $\dot{M}_{O_2}$, *P. annectens* exhibited a marked SDA effect (Figs. 1, 2). The stimulatory influence of feeding on $\dot{M}_{O_2}$ in other fish is well documented, with most studies

demonstrating significant increases in metabolic rate and breathing rates following a meal (e.g., Jobling and Spencer Davies 1980; Lyndon et al. 1992). Similarly, lungfish fed a meal of chironomid larvae exhibited marked and prolonged increases in the rates of $\dot{M}_{O_2}$ (Figs. 1A, 1B, 2), as well as both water- and air-breathing frequencies. Since increased swimming activity by the lungfish (primarily more frequent surfacing for air-breathing; Fig. 3) was visually observed only during the first 3 h postfeeding, whereas $\dot{M}_{O_2}$ peaked at 5–8 h and remained elevated at 12–15 h (Figs. 1, 2), increased activity alone cannot explain the SDA effect. While the bases of calculation differ among studies, a survey (Table 3) of the literature on teleosts and elasmobranchs indicates that typical peak increases in $\dot{M}_{O_2}$ (relative to "resting", "standard", or "low routine" metabolic rates) are ~1.5- to 2.6-fold, with the duration of the SDA effect lasting anywhere from 15 to >75 h after the meal. In Table 3, we referenced the peak postprandial increase of $\dot{M}_{O_2}$ in *P. annectens* relative to the prefeeding value (i.e., 2.5-fold increase) rather than to the simultaneous rate in fasted lungfish (9-fold increase). Nevertheless, this summary indicates that relative SDA effects in *P. annectens* are comparable with or larger than in most fish and last a comparable period of time after the meal. However, since the absolute $\dot{M}_{O_2}$ values are exceptionally low in lungfish, the absolute magnitude of the SDA component is similarly low.

In *P. aethiopicus*, Smith (1935b) reported that $\dot{M}_{O_2}$ increased to a maximum value, the "feeding level of metabolism" that was independent of ration size. Thereafter, between feedings, the metabolic rate decreased in a regular manner until the next feeding period. To our knowledge, Smith's (1935b) observation on increased metabolic rate in fed lungfish is the only study to have investigated the SDA effect in air-breathing fishes, although Smith (1935b) did not use this term. His studies did not have the time resolution of the current investigation, but he clearly demonstrated that the maximum effect of feeding on lungfish metabolism was during the first 24 h after the ingestion of food, where *P. aethiopicus* increased their $\dot{M}_{O_2}$, on average, about 1.5-fold compared with fasting levels (Table 3)

Besides the SDA effect, the difference in $\dot{M}_{O_2}$ between fed and fasted lungfish (Fig. 1) could be associated with

depression of metabolism caused by fasting. However, whereas Smith (1935b) observed a steady decline in $\dot{M}_{O_2}$ until the next feeding in *P. aethiopicus*, fasted *P. annectens* of the present study exhibited a resting $\dot{M}_{O_2}$ that was depressed from 0 to 36 h (Fig. 1B), but thereafter increased back to the prefeeding control level during the 46–49 h time period. This elevation in $\dot{M}_{O_2}$ just before the "anticipated feeding event" was also observed in sockeye salmon (*Oncorhynchus nerka* (Walbaum in Artedi, 1792)) by Brett and Zala (1975), who attributed it to an entrained excitement effect associated with anticipation of the next feeding.

Lungfish were fed a meal of chironomid larvae that was 3.3% of their body mass, of which the oxycalorific value was about 273 000 μmol O_2·(kg lungfish)$^{-1}$ (Table 1). This agrees well with literature values (Pandian and Delvi 1973; Salonen et al. 1976; Ryan 1982) for bomb calorimetric measurements of total energy content in various chironomid species (all close to 20 000 J·(g dry mass)$^{-1}$, which would equate to about 221 000 μmol O_2·(kg lungfish)$^{-1}$ in the meal of chironomid larvae), suggesting that unmeasured components such as trehalose and glycerol were insignificant. The total $\dot{M}_{O_2}$ of the fed lungfish over the 48 h period was only 49 000 μmol O_2·(kg lungfish)$^{-1}$, or 18% of the oxycalorific content of the food ingested. *Protopterus annectens* utilized 25 900 μmol O_2·(kg lungfish)$^{-1}$ of this total $\dot{M}_{O_2}$ during the 24 h SDA effect. Therefore, they "waste" only 9.5% of energy ingested towards this metabolically costly event, which compares favourably with teleost values in the 15%–40% range (Jobling 1994). If similar amounts of energy are assimilated in lungfish and teleosts, relatively more energy would be available for growth in the former. This economic utilization of energy by *P. annectens* may also assist them in being able to survive the energetically demanding process of aestivation during which they do not feed (Wilkie et al. 2007).

N-waste excretion

Finally, we investigated the effects of feeding on N-waste excretion in *P. annectens*. To address concerns about variability in these data being due to microbial conversion or degradation of ammonia–N and urea–N, we analyzed the concentration of ammonia–N and urea–N in "lungfish water" from a typical experiment over a 6 h period following 48 h after feeding (the lungfish was removed at this point) when we would expect the bacterial metabolism to be at its highest. There was no significant loss of either ammonia or urea from the water, so the source of this variability remains unexplained.

In general, fed teleost fish have a much higher excretion of total nitrogen compared with fasted fish (Wood 2001). However, fed *P. annectens* had a total N efflux (13 275 μmol N·kg^{-1}) that was not significantly elevated compared with that of fasted lungfish (8 750 μmol N·kg^{-1}) (Fig. 4). In contrast, another African lungfish, *P. dolloi*, which is similarly ammonotelic under fasting and resting conditions (Chew et al. 2003; Lim et al. 2004; Wood et al. 2005b), increased total N excretion more than 4-fold after a meal and became ureotelic (>60% urea–N excretion) at this time (Lim et al. 2004). Integrated over the 48 h experimental period, *P. annectens* maintained slightly higher levels of ammonia–N excretion (7450 μmol·kg^{-1}) than urea–N excretion (6237 μmol·kg^{-1}) (Fig. 5) but increasingly excreted

urea–N towards the end of the experimental period. In the final 6 h (42–48 h), on average, % urea–N excretion significantly exceeded 50% of total N excretion, indicating that ureotely did occur in *P. annectens* at this time period.

Some authors have argued that protein catabolism is a major contributor to the total energy production in fish (Mommsen and Walsh 1992), while others have noted that many fish preferentially burn other fuels (lipid, carbohydrate) and conserve protein (Wood 2001; Kajimura et al. 2004). This does not appear to be the case in *P. annectens*. In contrast to very low $\dot{M}_{O_2}$ values (see above), rates of N excretion in the lungfish are not proportionately reduced, so there is a high reliance on protein (amino acids) as a fuel for aerobic metabolism. Indeed, by standard metabolic theory for fish (van den Thillart and Kesbeke 1978; Kleiber 1992), the postprandial NQ value of 0.27 indicates that their low rate of aerobic respiration is fueled 100% by protein, and their fasting NQ value of 0.38 suggests net protein wastage during starvation. Nevertheless, the drop in this value after feeding indicates a transition to anabolic processes. We measured the protein–N content of the meal (28 313 μmol N·(kg lungfish)$^{-1}$) and found that only 4 525 μmol N·(kg lungfish)$^{-1}$ or about 16% of the ingested protein–N was actually excreted above fasting rates. Over the 48 h intermeal interval, only 47% of the ingested protein–N was excreted in total, so more than half could be put towards muscle growth. This compares favourably with teleosts (Jobling 1994; Wood 2001), so despite the greater relative reliance on protein (amino acids) as a fuel source in the lungfish, a relatively large fraction of the high protein diet (Table 1) can be committed to muscle growth.

Acknowledgements

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