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ORIGINAL PAPER

# Nitrogen metabolism in tambaqui (*Colossoma macropomum*), a neotropical model teleost: hypoxia, temperature, exercise, feeding, fasting, and high environmental ammonia

**Chris M. Wood[1,2,3,4] · José Gadelha de Souza Netto[4] · Jonathan M. Wilson[4,5,6] · Rafael M. Duarte[4,7] · Adalberto Luis Val[4]**

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**Abstract** The total rate of N-waste excretion ($M_N$) in juvenile tambaqui living in ion-poor Amazonian water comprised 85 % ammonia-N ($M_{Amm\text{-}N}$) and 15 % urea-N ($M_{Urea\text{-}N}$). Both occurred mainly across the gills with only ~5 % of $M_{Amm\text{-}N}$ and ~39 % of $M_{Urea\text{-}N}$ via the urine. Tambaqui were not especially tolerant to high environmental ammonia (HEA), despite their great resistance to other environmental factors. Nevertheless, they were able to maintain a continued elevation of $M_{Amm\text{-}N}$ during and after 48-h exposure to 2.5 mmol $L^{-1}$ HEA. The normally negative transepithelial potential (−18 mV) increased to −9 mV during the HEA period, which would help to reduce branchial $NH_4^+$ entry. During 3 h of acute environmental hypoxia (30 % saturation), $M_{Amm\text{-}N}$ declined, and recovered thereafter, similar to the response seen in other hypoxia-tolerant teleosts; $M_{Urea\text{-}N}$ did not change. However, during gradual hypoxia, $M_{Amm\text{-}N}$ remained constant, but $M_{Urea\text{-}N}$ eventually fell. The acute temperature sensitivities of $M_{Amm\text{-}N}$ and $M_N$ were low from 28 °C (acclimation) to 33 °C (Q10 ~1.5), but high (~3.8) from 33 to 38 °C, relative to $M_{O_2}$ (~1.9 throughout). In contrast, $M_{Urea\text{-}N}$ exhibited a different pattern over these temperature ranges (Q10 2.6 and 2.1, respectively). The nitrogen quotient (NQ = 0.16–0.23) was high at all temperatures, indicating a 60–85 % reliance on protein to fuel aerobic metabolism in these fasting animals. During steady-state aerobic exercise, $M_{O_2}$ and $M_{Urea\text{-}N}$ increased in parallel with velocity (up to 3.45 body lengths $s^{-1}$), but $M_{Amm}$ (and thus $M_N$) remained approximately constant. Therefore, the NQ fell progressively, indicating a decreasing reliance on protein-based fuels, as work load increased. In group feeding trials using 45 % protein commercial pellets, tambaqui excreted 82 % (range 39–170 %) of the dietary N within 24 h; N-retention efficiency was inversely related to the

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✉ Chris M. Wood
woodcm@zoology.ubc.ca

José Gadelha de Souza Netto
netto_badpi@hotmail.com

Jonathan M. Wilson
jmwilson@wlu.ca

Rafael M. Duarte
rafaelmd@clp.unesp.br

Adalberto Luis Val
dalval@inpa.gov.br

[1] Department of Zoology, University of British Columbia, Vancouver, BC V6T 1Z4, Canada

[2] Department of Biology, McMaster University, Hamilton, ON, Canada

[3] Rosenstiel School of Marine and Atmospheric Science, University of Miami, Coral Gables, FL, USA

[4] Laboratório de Ecofisiologia e Evolução Molecular, Instituto Nacional de Pesquisas da Amazônia (INPA), Manaus, Amazonas, Brazil

[5] Laboratório de Fisologia Molecular, Centro Interdisciplinar de Investigação Marinha e Ambiental, Porto, Portugal

[6] Department of Biology, Wilfrid Laurier University, Waterloo, ON, Canada

[7] Instituto de Biociências, Universidade do Estado de São Paulo, Campus do Litoral Paulista, Sao Vicente, SP, Brazil

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ration voluntarily consumed. $M_{Amm\text{-}N}$ peaked at 4–6 h, and $M_{Urea\text{-}N}$ at 6–9-h post-feeding, with an additional peak in $M_{Amm\text{-}N}$ only at 21 h. During subsequent fasting, $M_N$ stabilized at a high endogenous rate from 2 through 8 days post-feeding. Possible reasons for the high wasting of protein-N during both fasting and feeding are discussed.

**Keywords** Ammonia · Urea · Protein · Endogenous fraction · Exogenous fraction · Oxygen consumption · Nitrogen quotient · Transepithelial potential

## Introduction

From both socioeconomic and ecological perspectives, the tambaqui (*Colossoma macropomum*), a serrasalmid teleost, is one of the most important fish species in the Amazon basin. It is exploited in intense artisanal and commercial fishing, and is heavily farmed in aquaculture, both in South America and in tropical and subtropical areas of other continents. It is omnivorous in aquaculture, and readily eats a variety of plant and animal foods, including high-protein commercial pellets (Val and Honczaryk 1995; Araújo-Lima and Goulding 1997). In the wild, it undertakes annual migrations between circumneutral, moderately soft "white-water" and acidic, and extremely soft "blackwater", and is a major consumer of fruits, playing a critical role in the dispersal of seeds during the annual flooding cycle of the jungle (Goulding 1980; Goulding and Carvalho 1982; Roubach and Saint-Paul 1994; Val and Almeida-Val 1995; Anderson et al. 2009). Physiologically, it is generally considered to be a very sturdy fish, thriving in ion-poor waters, and surviving episodes of very low water pH, extreme hypercarbia, and extreme hypoxia. During the latter, it is renowned for growing a "lip" to facilitate aquatic surface respiration (Braum and Junk 1982; Florindo et al. 2006; Sundin et al. 2000). The basic physiology of this species has been fairly well studied to the point, where the tambaqui is becoming a neotropical model species. For example, there have been investigations on the ionoregulatory and acid–base responses to low water pH (Gonzalez et al. 1998; Wood et al. 1998; Wilson et al. 1999), the cardiorespiratory responses to hypoxia and hypercarbia (Saint-Paul 1984; Val et al. 1998; Sundin et al. 2000; Milsom et al. 2002; Florindo et al. 2004, 2006; Reid et al. 2005; Gilmour et al. 2005), and the transport physiology of the digestive tract (Pelster et al. 2015).

However, nitrogen (N) metabolism and N-waste excretion comprise one area, in which basic physiological knowledge is still scarce. Indeed, the "tambaqui bible" (Araújo-Lima and Goulding 1997), a detailed synthesis which has become a handbook for the ecology, conservation, and aquaculture of this species, provides no mention of this topic. Yet, it is well documented that management of N-metabolism is critically important to successful aquaculture both to maximize the conversion of dietary protein to fish protein (e.g., Wood 2001; Conceição et al. 2012), and to avoid ammonia toxicity from the buildup of ammonia in the environment as an unincorporated metabolic waste product (Randall and Tsui 2002; Chew et al. 2005; Martinez et al. 2006; Ip and Chew 2010; US EPA 2013). Moreover, ammonia discharges of anthropogenic origin are increasing in the Amazon basin, because of inputs of raw sewage from a growing urban population (e.g., Perz 2000; Couceiro et al. 2007).

The aim of this study was to address this critical knowledge gap. We first examined the basic routes of ammonia-N and urea-N excretion, hypothesizing that as in most fish, branchial excretion would dominate for both wastes (Smith 1929; Wood 1993). We then tested the tolerance of tambaqui to high environmental ammonia (HEA) and their ability to excrete against an unfavourable gradient. In light of their exceptional tolerance to ion-poor water, low pH, hypoxia, and hypercarbia, we hypothesized that tambaqui would also be unusually resistant to HEA, and would activate excretion against the gradient, and/or start to excrete excess nitrogen in the form of urea-N, as seen in some other resistant teleosts (Wood 1993; Wilkie 2002; Ip and Chew 2010). Increases in transepithelial potential (TEP) have been identified as an adaptive response to HEA in some fish which serves to decrease the electrochemical gradient for $NH_4^+$ entry (Wright and Wood 2012), so we hypothesized that these would also occur in the tambaqui. We also examined the responses of ammonia-N and urea-N excretion to both gradual and acute hypoxia, hypothesizing that the rates would decrease as in the oscar (*Astronotus ocellatus*). The oscar is a cichlid rather than a serrasalmid but is similarly hypoxia-tolerant teleost and lives in the same frequently hypoxic habitat as the tambaqui (Wood et al. 2007, 2009; De Boeck et al. 2013). The effects on N-waste excretion of acute increases in water temperature, another common stressor in this tropical environment, were evaluated. The influence of progressive aerobic swimming was also assessed, to understand how N-wastes might be managed during migration, as well as in aquacultural operations, where the fish are raised in a continuous current. For both temperature and exercise tests, we hypothesized that sensitivity to these natural stressors would be relatively low in this resilient species. Rates of $O_2$ consumption ($M_{O_2}$) were measured simultaneously during the temperature and swimming trials, all of which were performed on fasted fish. The results yielded unusually high nitrogen quotients (NQ; van den Thillart and Kesbeke 1978; van Waarde 1983; Lauff and Wood 1996a), indicating that a high proportion of aerobic metabolic rate was fueled by protein oxidation. Finally, to further investigate this possible "protein

wastage", which would be a serious economic problem in aquaculture, we investigated the quantitative utilization of dietary protein during feeding and fasting under conditions, where the fish were held in groups and fed a commercial high-protein diet, as would occur in fish farming.

## Materials and methods

### Experimental animals and water

Juvenile tambaqui [*Colossoma macropomum* (Cuvier 1818)] were obtained from a commercial aquaculture farm (Sítio dos Rodrigues, Km 35, Rod. AM-010, Brazil) and moved to the Ecophysiology and Molecular Evolution Laboratory of the Brazilian Institute for Research of the Amazon (Instituto Nacional de Pesquisas da Amazônia; INPA), at least 2 weeks prior to use. During this time, they were held in 500-L flow-through tanks at ~28 °C with external filtration and recirculation. The holding and experimental water was typical Amazonian soft water obtained from a well on the INPA campus ($[Na^+]$ = 35 μM, $[Cl^-]$ = 36 μM, $[Ca^{2+}]$ = 18 μM, $[Mg^{2+}]$ = 4 μM, $[K^+]$ = 16 μM; [titratable alkalinity] = 80 μM; pH 6.0–7.0). As this groundwater has a high $CO_2$ content, it was vigorously aerated for 24 h prior to use in any experiments. Fish were fed daily to satiation with commercial pellets (Nutripiscis-Presence® AL 45, SP Rações, São Paulo, SP, Brazil; 45 % protein) and fasted 48–72 h prior to experimentation. In most series, fish in the 10–25-g (designated as "small"; Series 5, 6, 7, and 8) or 30–50-g weight range (designated as "intermediate"; Series 2, 3) were used, depending on availability. However, in Series 1 and 4, larger fish (100–150 g) were employed (designated as "large"), as these involved surgical implantation of catheters. All fish were weighed at the end of the experiments, except in Series 1, 4, and 8, where they were weighed at the start. Experimental work was approved by the Ethics Committee on Animal Experiments of INPA under registration number 047/2012, and conformed to national animal care regulations.

### Experimental series

*Series 1—branchial versus renal N-waste excretion*

Large tambaqui were anaesthetized in 0.1 g $L^{-1}$ MS-222 (Syndel Laboratories, Vancouver, BC, Canada), pH-corrected with NaOH. Indwelling urinary bladder catheters filled with distilled water was implanted exactly as described by Wood and Patrick (1994). Catheters were constructed of Clay-Adams PE-50 tubing with a PE160 sleeve (Becton, Dickinson and Co., Franklin Lakes, NJ, USA), and secured to the ventral body wall with silk sutures. After weighing and recovery from anaesthesia, the fish were moved to darkened plastic chambers served with aeration and flow-through water from a 1000-L external re-circulating system. Urine collection was started using a siphon head of 3 cm. The fish were allowed to settle overnight during which time, the patency of the urinary catheter was checked. The next morning, a fresh urine collection was started, water flow to the chamber was suspended, while aeration continued, and the volume was set to 3.4 L. The initial and final 10-mL samples were taken over two subsequent 8-h periods, with simultaneous urine collections; the water in the chamber was renewed in between the two flux periods. Urine flow rate was measured gravimetrically. Water and urine samples were immediately frozen (−20 °C) for later analysis of ammonia-N and urea-N concentrations. Data were obtained from seven fish with working catheters.

*Series 2—ammonia toxicity*

The goal of this experiment was to obtain an estimate of the 48-h EC50. Intermediate-sized tambaqui were transferred to individual darkened 2.5-L plastic chambers served with aeration, and allowed to settle overnight. The next morning, the water was renewed and sufficient $NH_4Cl$ stock was added to raise the water ammonia concentration to 1, 2.5, 5, 10, or 20 mmol $L^{-1}$ ($N = 4$ at each concentration). The fish were monitored over the following 48 h, with water renewal at 24 h. The endpoint was persistent loss of equilibrium. Fish that exhibited this syndrome were removed to clean water for recovery. Water pH, monitored with a hand-held YSI pH 100 m and electrode (Yellow Springs Instrument, Yellow Springs, Ohio, USA), tended to rise throughout due to the presence of the fish, and was kept at pH 7.0 (range 6.8–7.2) by the addition of small amounts of 0.1 N HCl.

*Series 3—N-waste excretion in the presence of high environmental ammonia (HEA)*

Based on the results of Series 2, a concentration of 2.5 mmol $L^{-1}$ $NH_4Cl$ was selected as an HEA treatment which tambaqui could survive. Intermediate-sized tambaqui ($N = 10$) were again transferred to individual darkened 2.5-L plastic chambers served with aeration, and allowed to settle overnight. The next morning, the water was renewed and set to a volume of 2.0 L (time 0 h), and an initial 10-h control flux measurement was performed. The chamber was then flushed, the exposure water changed to HEA, and a new flux measurement was started from 10 to 24 h. Subsequent HEA flux measurements were made over 24–34, 34–48, and 48–58 h, followed by a return to control

conditions, with recovery flux measurements over 58–72, and 72–84 h. In between each flux period, the exposure water was renewed. Initial and final 10-mL samples were taken at the start and end of each flux period and immediately frozen for later analysis of ammonia-N and urea-N concentrations.

*Series 4—TEP changes during HEA exposure*

Large tambaqui ($N = 8$) were anaesthetized as in Series 1 and fitted with indwelling intraperitoneal catheters (PE50 with PE160 sleeves) filled with Cortland saline (Wolf 1963) as described for toadfish by Wood and Grosell (2008). They were then transferred to darkened 8-L plastic chambers served with aeration, and allowed to settle overnight. The larger chamber size than in Series 1 made it easier to measure TEP with minimal disturbance to the fish. The next morning, the water was renewed, control TEP measurements were made, and then, 2.5-mmol $L^{-1}$ $NH_4Cl$ was added to the water, marking the start of HEA exposure. TEP recordings were made immediately (0.05 h), and then again at 5, 24, 48, and 58 h of HEA, with water renewal after the 24-h and 48-h measurements. The chambers were then flushed with control water, and additional TEP measurements taken immediately (58.05 h) and at 72 h (i.e., 14-h recovery). The water pH was adjusted to 7.0 prior to each reading. TEP was measured using 3-M KCl-agar bridges, one connected to the external water and the other to the saline-filled catheter. The bridges were connected via Ag/AgCl electrodes (World Precision Instruments, Sarasoto, FL, USA) to a high-impedance voltmeter (Radiometer pHM84, Copenhagen, Denmark). TEP was expressed relative to the apical (water) side as 0 mV after correction for junction potential.

*Series 5—the responses of N-waste excretion to acute and gradual hypoxia*

Small tambaqui were transferred to the same 2-L chambers as in Series 3 and allowed to settle overnight in preparation for the hypoxia exposures. The next morning, the water was renewed and the volume set to 1.5 L. For the acute hypoxia exposure ($N = 12$), flux measurements were made over hourly intervals for 9 h. From 0 to 3 h, the water was bubbled with air, so as to keep $O_2$ saturation above 90 % (control), whereas from 3 to 6 h, the bubbling was changed to $N_2$, so as to keep water $O_2$ levels between 20 and 30 % (acute hypoxia), while from 6 to 9 h, 90 % saturation was restored (normoxic recovery). For the gradual hypoxia exposure ($N = 8$), flux measurements were again made over hourly intervals for 9 h. However, after 3 h at 90 % saturation (control), the air bubbling was turned off, so that water $O_2$ levels gradually declined due to consumption by the fish, reaching 21–54 % (mean = 33.5 %) by the final hour. Note that in both protocols, the water surface was freely exposed to the overlying air, so that aquatic surface respiration was possible. In both, water samples (10 mL) were taken every hour and immediately frozen for later analysis of ammonia-N and urea-N concentrations, thereby allowing calculations of flux rates over 1-h intervals. $O_2$ levels in each chamber were measured once per hour during normoxia, and twice per hour during hypoxia, using a hand-held Accumet® meter and polarographic electrode (Fisher Scientific, Toronto, ON, Canada).

*Series 6—the responses of N-waste excretion and oxygen consumption to acute increases in water temperature*

Custom-made 1.5-L respirometers were used to measure both $O_2$ consumption rates ($M_{O_2}$) and N-waste excretion rates in these experiments. These could be sealed without air bubbles and were submerged in a vigorously aerated constant temperature bath-containing 150 L of the same water. The respirometers themselves could also be aerated, and a sealable hole in each lid accommodated the electrode of the Accumet® meter. Small tambaqui ($N = 11$) were allowed to settle overnight at the acclimation temperature (28 °C) in these respirometers with aeration. A 12th respirometer was run as a blank, but there was no detectable $M_{O_2}$ in the blank. The next morning, aeration was stopped, the initial water samples (10 mL) and $O_2$ readings were taken, and the respirometers were sealed. Final $O_2$ readings were taken after 1.0 h and water samples after 1.5 h. The respirometers were then opened and allowed to flush with aerated bath water, and temperature was increased to 33 °C over a 1.5-h period. The measurement cycle was repeated, but the period for $O_2$ measurements was reduced to 0.75 h and that for water samples to 1.25 h. The respirometers were again flushed, the temperature was increased to 38 °C over 1.5 h, and a final measurement cycle was performed, using 0.5 h for $O_2$ measurements and 1.0 h for water samples. Water samples were frozen immediately for later analysis of ammonia-N and urea-N concentrations.

*Series 7—the responses of N-waste excretion and oxygen consumption to aerobic exercise*

Experiments were performed using two 3.2-L Blazka swimming respirometers identical to those described by Wilson et al. (1994). The respirometers were calibrated using an Onicon F-1100 flowmeter (Clearwater, FL, USA), and were submerged in the same 150-L constant temperature bath used in Series 6, which was set to the acclimation temperature (28 °C). Small tambaqui (fork length = 7.4–9.5 cm; $N = 10$) were transferred individually to the respirometers which were initially set on open

circuit, and the fish were allowed to settle for several hours at the minimum speed to which they would orient (about 4 cm $s^{-1}$) before the first measurements were taken. The goal was to measure $M_{O_2}$ and N-waste excretion rate at this speed, and at three higher speeds in steps of approximately 1 body length $sec^{-1}$ (BL $s^{-1}$). The actual mean speeds were 3.85 ± 0.08, 12.20 ± 0.56, 21.06 ± 1.77, and 28.21 ± 1.41 cm $s^{-1}$, or 0.47 ± 0.10, 1.49 ± 0.08, 2.57 ± 0.19, and 3.45 ± 0.18 BL $s^{-1}$ ($N = 10$). At each step, an initial water sample (50 mL) was taken, and the respirometer was then closed for 1.5 h. At the end, a second water sample was taken, and the respirometer was flushed on open circuit for 0.25 h during which the speed was increased to the next step. The cycle was then repeated. All fish swam successfully for 1.5 h at each of the four speeds. For each respirometer, one blank regime was run with all four measurement cycles, using water taken at the end of a step—i.e., that had been in contact with a fish for 1.5 h. The purpose of these blank regimes was to detect changes in water $O_2$ and N-waste concentrations that were not caused by the fish's metabolism—i.e., of microbial or other origin. There was no detectable change in water ammonia-N or urea-N concentrations in the blank runs, but for $M_{O_2}$, blank corrections up to 10 % of measured values were necessary. Water samples were discharged without air exposure into a tube which accommodated the electrode of the Accumet® meter. After $O_2$ concentration was read, 10 mL of the sample was immediately frozen for later analysis of ammonia-N and urea-N concentrations.

*Series 8—the daily cycle of N-waste excretion in response to feeding and fasting*

In our experience, tambaqui will only feed in groups, so four groups, each comprising 20 randomly chosen small fish of known weight, were established, and allowed to form stable feeding hierarchies. Each was housed in a square tank (44 cm × 64 cm × 30 cm deep) served with vigorous aeration and a constant level outlet to waste, which allowed the flow rate through the tank to be monitored. Inflow to each tank (~350 mL $min^{-1}$) was provided from a constant head reservoir served with inflowing fresh INPA well water. The head reservoir sat at the base of a marble-filled "stripping column" through which the water was pumped in a direction countercurrent to vigorous airflow. This was necessary to ensure that the excess $CO_2$ was removed from the groundwater, and that it became fully saturated with $O_2$ before entering the fish tanks.

Each day between 11:00 am and 12:00 noon, fish were manually offered commercial pellets (Nutripiscis- Presence® AL 45, SP Rações, São Paulo, SP, Brazil; 45 % protein) in small batches, the same food which they had been accustomed to eating previously. The feeder watched carefully to ensure that no more food was offered, once satiation was reached. The pellets for each tank were stored in a separate plastic sac which was weighed before and after the feeding bout, so as to yield the total amount of food consumed that day. This value could be corrected later for any uneaten food that was recovered, because the mean weight of a pellet was known. At about 15:00 pm, the tanks were siphoned to remove faeces and any uneaten pellets; the latter was counted.

Feeding patterns appeared to have stabilized by day 10 (see "Results"), so day 11 was the experimental day. Each of the four tanks was fed in the regular manner between 11:00 am and 12:00 noon, and the amount of food consumed was recorded. However, at 12:00 noon, the inflow to each tank was stopped, the volume was set to 79.6 L, and a 10-mL water sample was taken. Aeration ensured good mixing. Additional samples were taken at 1-h intervals for the next 24 h, so as to provide hourly flux measurements. The tanks were not siphoned, so as to avoid disturbance. However, immediately after the samples at 20:00 pm, and again after those at 04:00 am the next day, approximately half of the water volume in each tank was replaced with fresh water, so as to prevent water ammonia levels from rising too high, and a new sample was taken. All samples were immediately frozen (−20 °C) for later analysis of ammonia-N and urea-N concentrations.

At the end of the 24-h period, half of the water in each tank was replaced, and flow-through was re-established for 2 h. Then, flow was stopped again for a 4-h period (14:00–18:00 pm), for which the initial and final water samples were taken. This constituted the first flux measurement during fasting. At the end of this measurement, all the fish were weighed individually with a minimum of disturbance, and returned to their tanks; flow-through was re-established. The fish were not fed over the following 7 days (i.e., 8 days of fasting in total), but each afternoon, water flow was suspended and flux measurements were made over a 4-h period (14:00–18:00 pm). The fish were then weighed again individually at the end of the experiment.

## Analytical techniques and calculations

The colorimetric assays of Verdouw et al. (1978) and Rahmatullah and Boyde (1980) were used to measure ammonia-N and urea-N concentrations, respectively, in water and urine. In experiments where particularly high precision was required to detect small changes, and/or changes against high backgrounds (Series 3, 5, and 8 for ammonia-N, and Series 5, 6, 7, and 8 for urea-N), assays were read in 1-cm cuvettes rather than in microplates, and closely bracketing standards were employed.

Oxygen consumption rates ($M_{O_2}$), as well as ammonia-N ($M_{Amm\text{-}N}$) and urea-N ($M_{Urea\text{-}N}$) flux rates

(μmol $kg^{-1}$ $h^{-1}$), to the water were calculated in the standard fashion from changes in concentration (μmol $L^{-1}$), factored by the known fish weight (kg), volume (L), and time (h). In Series 1, 3, 5, 6, and 7, the weight was that of the individual fish, and volume was that of the chamber or respirometer. In Series 8, the weight was the total of all 20 fish, and the volume was that of the tank. Total N-excretion rate ($M_N$) was taken as the sum of $M_{Amm\text{-}N}$ + $M_{Urea\text{-}N}$, and the nitrogen quotient (NQ) was calculated as $[M_{Amm\text{-}N} + M_{Urea\text{-}N}]/M_{O_2}$. According to standard metabolic theory (cf. van den Thillart and Kesbeke 1978; van Waarde 1983; Lauff and Wood 1996a), an NQ of 0.27 represents an aerobic metabolism based entirely on the oxidation of protein (i.e., amino acids), so the percentage of aerobic metabolism fueled by protein was calculated as [NQ/0.27] × 100 %. In Series 6, temperature coefficients (Q10 values) for $M_{O_2}$, $M_{Amm\text{-}N}$, $M_{Urea\text{-}N}$, and $M_N$ were calculated in the standard fashion (cf. Clarke and Johnston 1999) based on group means.

In Series 1, the urine flow rate (UFR) was calculated by dividing the collected urine volume (mL) by body weight (kg) and time (h), and the urinary excretion rate of ammonia-N or urea-N (μmol $kg^{-1}$ $h^{-1}$) was calculated as the product of concentration (μmol $mL^{-1}$) times UFR (mL $kg^{-1}$ $h^{-1}$).

### Statistical analyses

Data have been expressed as means ± 1 SEM ($N$) where $N$ is number of fish (Series 1–7) or number of tank replicates (Series 8). In general, differences were evaluated by one-way repeated measures analysis of variance (ANOVA) followed by either Dunnett's test to evaluate differences from the pretreatment control, or Tukey's test to identify individual differences amongst means. In the time series analyses of Figs. 7 and 8, Fisher's LSD was used as the post-hoc test. Where necessary, data were log, natural log, or square root transformed to meet assumptions of normality or homogeneity of variance. Percentage data were arcsin transformed. In some cases, data were averaged over time for testing, as explained in "Results". Simple pairwise comparisons were made by Student's paired or unpaired two tailed $t$ test, as appropriate. A significance level of 0.05 was used throughout.

## Results

### Series 1—branchial versus renal N-waste excretion

Large juvenile tambaqui excreted the majority of their measured N-waste as ammonia (85 % as $M_{Amm\text{-}N}$, 15 % as $M_{Urea\text{-}N}$) and this occurred largely through the gills (Table 1). Branchial ammonia-N excretion was approximately 8.5-fold greater than branchial urea-N excretion. Urinary flow rate was relatively high (see "Discussion"), but urinary excretion rates of both ammonia-N and urea-N

**Table 1** Simultaneous measurements of branchial and urinary excretion rates of ammonia-N ($M_{Amm\text{-}N}$) and urea-N ($M_{Urea\text{-}N}$) and their totals, together with measurements of urine flow rate and urinary concentrations of ammonia-N and urea-N in large tambaqui of Series 1

| | |
|---|---|
| Total $M_{Amm\text{-}N}$ (μmol $kg^{-1}$ $h^{-1}$) | 324.13 ± 12.15 |
| Total $M_{Urea\text{-}N}$ (μmol $kg^{-1}$ $h^{-1}$) | 58.29 ± 5.46 |
| Branchial $M_{Amm\text{-}N}$ (μmol $kg^{-1}$ $h^{-1}$) | 309.22 ± 12.56 |
| Branchial $M_{Urea\text{-}N}$ (μmol $kg^{-1}$ $h^{-1}$) | 36.73 ± 7.25 |
| Urine flow rate (mL $kg^{-1}$ $h^{-1}$) | 11.48 ± 1.71 |
| Urinary [ammonia-N] (μmol $ml^{-1}$) | 1.46 ± 0.18 |
| Urinary [urea-N] (μmol $mL^{-1}$) | 2.06 ± 0.39 |
| Urinary $M_{Amm\text{-}N}$ (μmol $kg^{-1}$ $h^{-1}$) | 14.91 ± 1.50 |
| Urinary $M_{Urea\text{-}N}$ (μmol $kg^{-1}$ $h^{-1}$) | 21.56 ± 4.01 |

Means ± 1 SEM ($N = 7$)

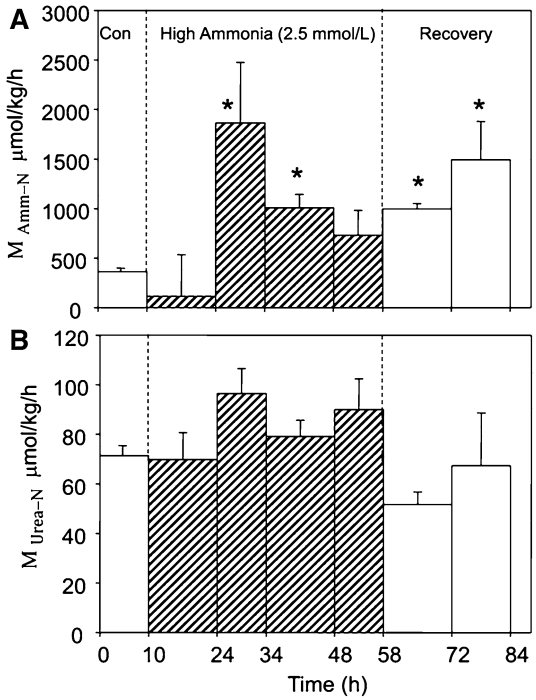


**Fig. 1** Influence of exposure to high environmental ammonia (HEA; 2500 μmol $L^{-1}$ $NH_4Cl$, pH 7.0) on **a** ammonia-N excretion ($M_{Amm\text{-}N}$) and **b** urea-N excretion ($M_{Urea\text{-}N}$) rates in intermediate-sized tambaqui of Series 3. The 0–10-h period represents the pre-exposure control (*open bar*). HEA exposure (*hatched bars*) lasted from 10 to 58 h, followed by recovery under control conditions from 58 to 72 h (*open bars*). Means ± 1 SEM ($N = 10$). *Asterisks* represent means which are significantly different ($P < 0.05$) from the pre-exposure control mean

were both low. Notably, they differed from branchial fluxes in being approximately equivalent to one another, reflecting the similar concentrations of these two N-wastes in the urine. Therefore, urinary ammonia-N excretion accounted for only 4.7 ± 0.5 % (7) of total $M_{\text{Amm-N}}$, while urinary urea-N excretion accounted for 39.4 ± 9.2 % (7) of total $M_{\text{Urea-N}}$, a significant difference.

### Series 2—ammonia toxicity

All tambaqui (intermediate-sized) exposed to $NH_4Cl$ concentrations of 10 and 20 mmol $L^{-1}$ exhibited persistent loss of equilibrium within the first 12 h, whereas two of four fish showed the same response with 48 h at 5 mmol $L^{-1}$, and none exhibited this response at 1 and 2.5 mmol $L^{-1}$. Thus, the approximate 48-h EC50 was 5 mmol $L^{-1}$ as total ammonia at pH 7.0, 28 °C, or 35 µmol $L^{-1}$ as $NH_3$, using the USEPA (2013) EPA conversion calculator.

### Series 3—N-waste excretion in the presence of high environmental ammonia (HEA)

Prior to HEA exposure, control rates of total $M_{\text{Amm-N}}$ and $M_{\text{Urea-N}}$ (Fig. 1) in these intermediate-sized tambaqui were comparable to those measured in the larger fish of Series 1 (see Table 1). Upon exposure to 2.5 mmol $L^{-1}$ $NH_4Cl$, selected as a tolerable HEA treatment based on the results of Series 2, $M_{\text{Amm-N}}$ became highly variable during the first 14-h period, with several fish exhibiting negative values—i.e., net ammonia uptake (Fig. 1a). However, overall, there was no significant change at this time, but by 24–34 h, $M_{\text{Amm-N}}$ had increased by approximately fourfold above the control rate $M_{\text{Amm-N}}$ remained elevated at 34–48 h of HEA exposure, but the increase at 48–58 h was not significant. $M_{\text{Amm-N}}$ was also significantly elevated during two successive recovery periods (58–72, 72–84 h) in ammonia-free water, so the net stimulation of excretion was far greater than any ammonia-N uptake that occurred initially during HEA. $M_{\text{Urea-N}}$ exhibited no significant change over the whole experiment (Fig. 1b).

### Series 4—TEP changes during HEA exposure

Under control conditions, the TEP in large juvenile tambaqui was negative, approximately −18 mV (inside relative to the outside water as 0 mV; Fig. 2). Within 5 min of exposure to HEA (2.5 mmol $L^{-1}$ $NH_4Cl$), the TEP had become significantly less negative (−13 mV), and from 5 to 58 h, it remained significantly less negative at about −9 mV. Upon return to ammonia-free water, TEP was statistically unchanged in the first 5 min, but, by 72 h, had recovered to a level not significantly different from the original control value.

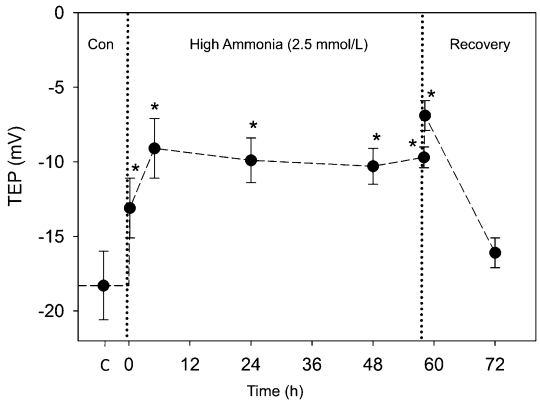


**Fig. 2** Influence of exposure to high environmental ammonia (HEA; 2500 µmol $L^{-1}$ $NH_4Cl$, pH 7.0) on transepithelial potential (TEP) in large tambaqui of Series 4. "*C*" represents the pre-exposure control measurement in ammonia-free water. HEA exposure lasted from 10 to 58 h, followed by recovery under control conditions from 58 to 72 h (*open bars*). TEP was measured relative to the apical (water) side as 0 mV. Means ± 1 SEM ($N = 10$). *Asterisks* represent means which are significantly different ($P < 0.05$) from the pre-exposure control mean

### Series 5—the responses of N-waste excretion to acute and gradual hypoxia

These and the following series were performed with small tambaqui; N-waste excretion rates, particularly $M_{\text{Amm-N}}$, tended to be higher than those of the preceding series with fish of large and intermediate size.

Fish were exposed to acute hypoxia (20–30 % saturation; Fig. 3a) for 3 h, after an initial 3-h period under normoxia (>90 %). $M_{\text{Amm-N}}$ tended to fall, a decrease which reached 55 % and became significant in the third hour of acute hypoxia (Fig. 3). There was an immediate return to control $M_{\text{Amm-N}}$ values in the first hour of normoxic recovery, with a slight overshoot. When the data were analyzed by averaging 3-h blocks, the decline in $M_{\text{Amm-N}}$ was significant for the whole hypoxic period, and the recovery rate was significantly greater than the hypoxic rate, but not different from the normoxic control rate (Fig. 3b). There were no significant changes in $M_{\text{Urea-N}}$ throughout the experiment (Fig. 3c).

When tambaqui were gradually exposed to progressive hypoxia (Fig. 4a), different patterns were seen. In this experiment, the first 3 h served as the normoxic control period. $M_{\text{Amm-N}}$ did not change (Fig. 4b), even at the final lowest level (approximately 35 % saturation), but $M_{\text{Urea-N}}$ fell significantly by about 50 % in the final 2 h of progressive hypoxia (Fig. 4b). Interestingly, most of the fish had started to exhibit the extended lip response (see "Introduction", as well as photographs in Sundin et al. (2000) by the end of the 6 h of progressive hypoxia, whereas this was never seen during the 3 h of acute hypoxia.

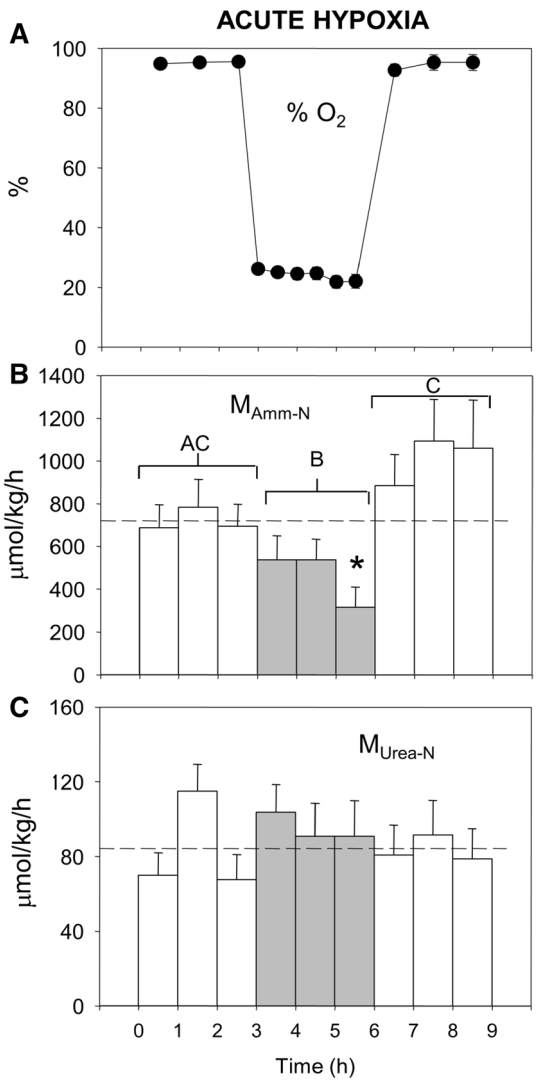


**Fig. 3** Influence of acute exposure to environmental hypoxia (20–30 % saturation), as shown in **a** on **b** ammonia-N excretion ($M_{Amm-N}$) and **c** urea-N excretion ($M_{Urea-N}$) rates in small tambaqui of Series 5. From 0 to 3 h, the water was bubbled with air, so as to keep $O_2$ saturation >90 % (control), whereas from 3 to 6 h, the bubbling was changed to $N_2$, so as to keep water $O_2$ levels between 20 and 30 % (acute hypoxia, *shaded bars*), while from 6 to 9 h, >90 % saturation was restored (normoxic recovery). Fluxes were measured on an hourly basis. Means ± 1 SEM ($N = 12$). *Asterisks* represent hourly means which are significantly different ($P < 0.05$) from the normoxic control mean, which is the average of the first three 1-h periods, as indicated by the *dashed line*. The data were also analyzed as means in 3-h blocks; *blocks sharing the same letter* are not significantly different ($P > 0.05$)

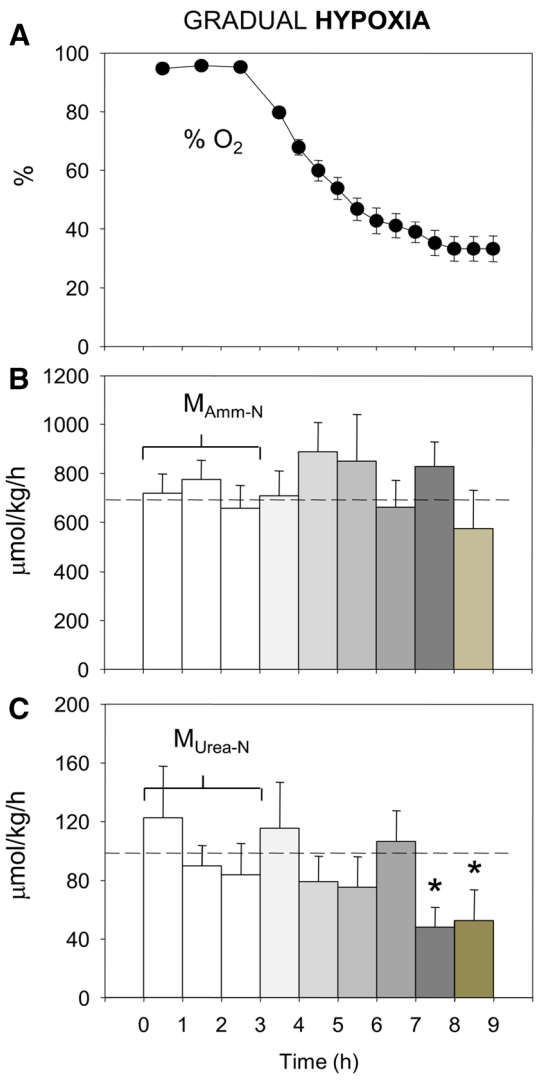


**Fig. 4** Influence of gradual exposure to progressive hypoxia, as shown in **a**, on **b** ammonia-N excretion ($M_{Amm-N}$) and **c** urea-N excretion ($M_{Urea-N}$) rates in small tambaqui of Series 5. From 0 to 3 h, the water was bubbled with air, so as to keep $O_2$ saturation >90 % (control), but thereafter, the air bubbling was turned off, so that water $O_2$ levels gradually declined due to consumption by the fish, as indicated by progressively *darker shaded bars*. Fluxes were measured on an hourly basis. Means ± 1 SEM ($N = 8$). *Asterisks* represent hourly means which are significantly different ($P < 0.05$) from the normoxic control mean, which is the average of the first three 1-h periods, as indicated by the *dashed line*

## Series 6—the responses of N-waste excretion and oxygen consumption to acute increases in water temperature

Acute increases in temperature, from the acclimation temperature of 28 °C, to first 33 °C, and then 38 °C, had differential effects on $M_{O_2}$, $M_{Amm-N}$, and $M_{Urea-N}$ in small tambaqui (Fig. 5). $M_{O_2}$ increased with temperature in a steady fashion, with moderate Q10 values (~1.9) over both intervals (Fig. 5a). In contrast, $M_{Amm-N}$ was relatively insensitive to temperature over the 28–33 °C interval (Q10 = 1.46), but increased greatly over the 33–38 °C range (Q10 = 3.91) (Fig. 5b). $M_{Urea-N}$, on the other hand, had a higher Q10 over the lower interval than over the higher range (2.65 versus 2.12) (Fig. 5b). The Q10 values

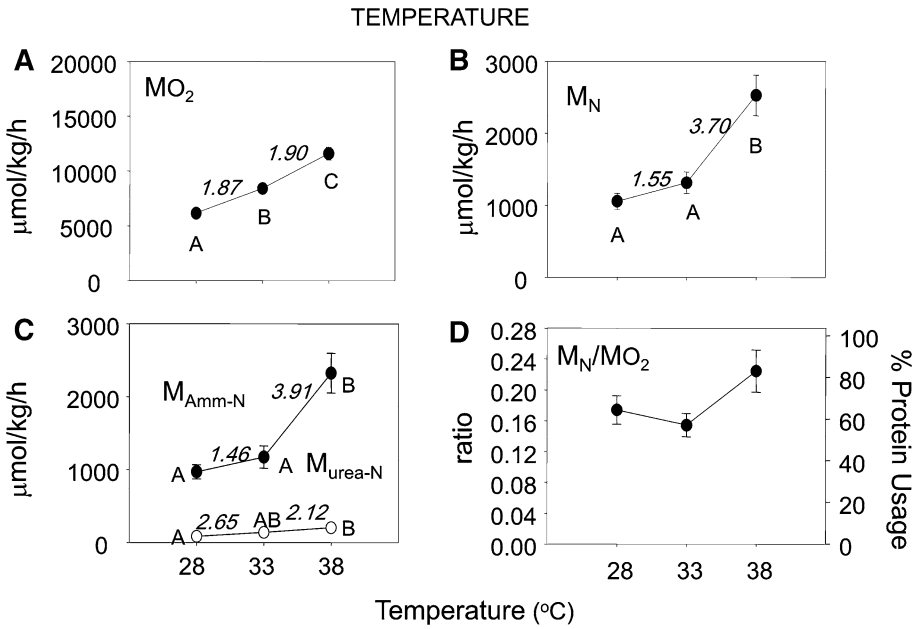


**Fig. 5** Influence of acute increases in temperature on **a** oxygen consumption ($M_{O_2}$) and **b** total N-waste excretion ($M_N$) rates in small tambaqui of Series 6. The latter is calculated as the sum of **c** ammonia-N excretion ($M_{Amm\text{-}N}$) and urea-N excretion ($M_{Urea\text{-}N}$) rates. **d** shows the nitrogen quotient (NQ) calculated as $M_N/M_{O_2}$, together with the percentage of aerobic metabolism fueled by protein (see text for details). The Q10 values over the relevant temperature interval are shown in *italics* for all rates. Means ± 1 SEM ($N = 11$). Within a parameter, *means sharing the same letter* are not significantly different ($P > 0.05$)

for total N-waste excretion ($M_N$; Fig. 5c) paralleled those for $M_{Amm\text{-}N}$, because ammonia-N excretion rates were about tenfold greater than urea-N excretion rates on an absolute basis (Fig. 5c). The NQ was high (0.16–0.23) with no significant variation among the three temperatures (Fig. 5d). This translated to a 60–85 % reliance on protein to fuel aerobic metabolism.

### Series 7—the responses of N-waste excretion and oxygen consumption to aerobic exercise

Small tambaqui proved to be good swimmers, and all completed the 6.75-h exercise regime at 28 °C. There were dramatic differences in the responses of $M_{O_2}$, $M_{Amm\text{-}N}$, and $M_{Urea\text{-}N}$, as swimming speed was progressively increased from 0.47 to 3.45 BL $s^{-1}$ (Fig. 6). While both $M_{O_2}$ (Fig. 6a) and $M_{Urea\text{-}N}$ (Fig. 6b) increased in a linear fashion with speed, $M_{Amm\text{-}N}$ remained completely unchanged (Fig. 6b). In consequence, $M_N$ also remained statistically unchanged (Fig. 6c), reflecting the dominant influence of $M_{Amm\text{-}N}$, though it is notable that the contribution of $M_{Urea\text{-}N}$ increased from 8 to 19 % of $M_N$. The NQ fell progressively from 0.20 at the lowest speed to 0.09 at the highest speed. Therefore, the reliance on protein to fuel aerobic metabolism dropped from about 75–33 %, as swimming velocity progressively increased.

### Series 8—the daily cycle of N-waste excretion in response to feeding and fasting

After the four groups of small tambaqui were first formed, they would not eat at all on the following day 1 (Fig. 7). However, food consumption started on day 2 and gradually increased on subsequent days, reaching a stable daily ration of about 1.3 % [(dry pellets/wet fish body mass) × 100 %] by days 8–10.

On the day of the experiment (day 11), two groups (tanks 2 and 4) ate their regular ration (~1.4 %), while two other groups consumed far less (tank 2 = 0.36 %, tank 4 = 0.78 %), so individual tank data are shown for $M_{Amm\text{-}N}$ and $M_{Urea\text{-}N}$ in Supplementary Figs. S1 and S2, respectively, and mean data in Fig. 8. The mean ration consumed was 1.01 ± 0.27 % ($N = 4$). There was considerable variability in patterns between the four groups. However, in all four, $M_{Amm\text{-}N}$ increased progressively over the first 4–6 h, in some cases by many-fold, and then declined or stabilized thereafter (Fig. 8a). Furthermore, all four tanks showed a coincident second peak in $M_{Amm\text{-}N}$ at 08:00–09:00, 21 h after feeding (Fig. S1; Fig. 8a). Note that in Fig. 8a, significant differences are illustrated in the figure legend, rather than on the figure itself.

Changes in water urea-N concentration were too small to permit reliable resolution of urea-N fluxes over 1-h intervals, so $M_{Urea\text{-}N}$ was calculated over 3-h intervals. As with $M_{Amm\text{-}N}$, $M_{Urea\text{-}N}$ exhibited considerable variability among groups, but in all four, it increased after feeding (Fig. S2). Note, however, that the peak $M_{Urea\text{-}N}$ occurred at 6–9 h after feeding (Fig. S2; Fig. 8b), whereas $M_{Amm\text{-}N}$ had already started to decline by this time (Fig. 8a). Furthermore, there was no late peak in urea-N excretion around 21-h post-feeding.

When feeding was stopped, $M_{Amm\text{-}N}$ remained high (~1100 µmol $kg^{-1}$ $h^{-1}$) in the first day (measurements taken 26–30 h after the last feeding), but thereafter dropped

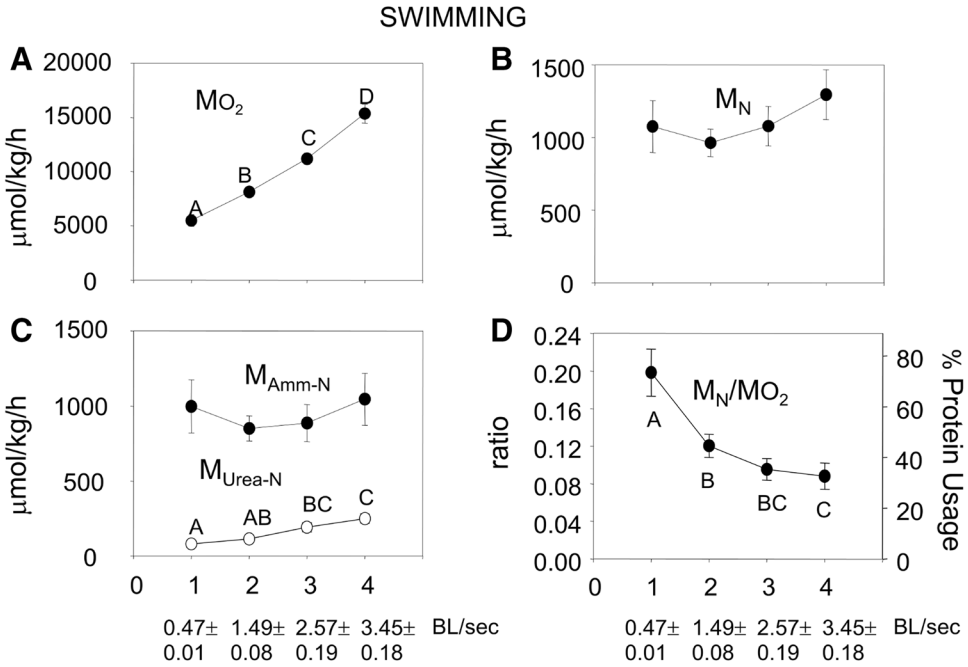


**Fig. 6** Influence of progressive increases in swimming speed on **a** oxygen consumption ($M_{O_2}$) and **b** total N-waste excretion ($M_N$) rates in small tambaqui of Series 7. The latter is calculated as the sum of **c** ammonia-N excretion ($M_{Amm-N}$) and urea-N excretion ($M_{Urea-N}$) rates. **d** shows the nitrogen quotient (NQ) calculated as $M_N/M_{O_2}$, together with the percentage of aerobic metabolism fueled by protein (see text for details). Means ± 1 SEM ($N = 10$). Within a parameter, *means sharing the same letter* are not significantly different ($P > 0.05$)

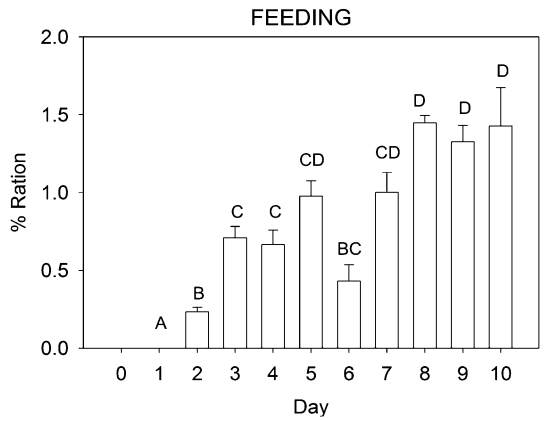


**Fig. 7** Voluntary daily ration (amount of food consumed on a single feeding to satiation) by groups of 20 small tambaqui, housed in single tanks, of Series 8. The groups were formed on day 0, and food was first offered on day 1. See text for details. Means ± 1 SEM ($N = 4$ groups, with 20 fish in each group). *Means sharing the same letter* are not significantly different ($P > 0.05$)

to a stable level (~600 µmol $kg^{-1}$ $h^{-1}$) throughout the subsequent 7 days (Fig. 9a). $M_{Urea-N}$ was variable, but did not change significantly during the entire fasting period (Fig. 9b).

Mean body weight fell slightly (−4.2 %) over the 11-day feeding regime, but the change was not significant (Table 2). However, after 8 days of fasting, there was a further, highly significant loss of body weight (−9.3 %) (Table 2).

## Discussion

### Branchial versus renal N-waste excretion

In accord with our first hypothesis, tambaqui excreted most of their N-wastes in the form of ammonia-N through the gills, with only a small amount appearing in the urine. Urea-N was a relatively minor component, but of this, 39 % appeared in the urine (Table 1). These patterns are fairly typical of most freshwater teleosts, although the urinary urea-N percentage is high (Smith 1929; Wood 1993; Wilkie 2002). However, there are two important caveats to these conclusions. First, a small percentage of the N-excretion attributed to the gills may actually have occurred across the skin (Zimmer et al. 2014). Second, like other teleosts (e.g., carp—Smith 1929; trout—Olson and Fromm 1971; De Boeck et al. 2001), tambaqui may additionally excrete unidentified N-compounds (i.e., other than ammonia-N and urea-N) which could only be detected by total N-analysis. As discussed by Wood (2001), these would most likely be unoxidized moieties, such as amino acids and mucoproteins, and, therefore, would contribute as losses to the total N-budget, but not to respirometric measures of metabolic fuel utilization.

A notable feature of the Series 1 data was the relatively high UFR (~11.5 mL $kg^{-1}$ $h^{-1}$; Table 1), which was twofold to threefold greater than values recorded in most freshwater teleosts [see summary Tables in Hickman and Trump (1969) and Wood and Patrick (1994)], including even some other

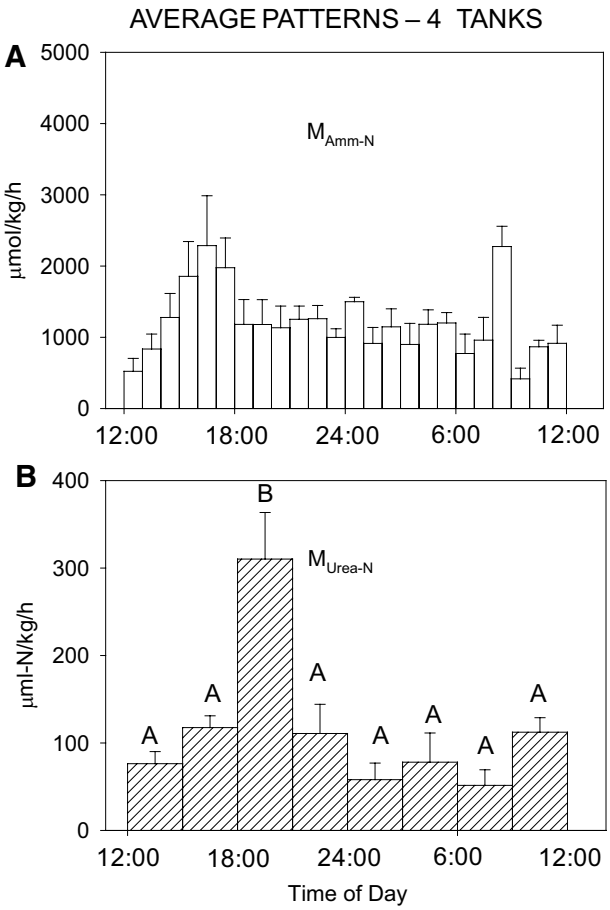


**Fig. 8** Rates of **a** ammonia-N excretion ($M_{Amm\text{-}N}$) and **b** urea-N excretion ($M_{Urea\text{-}N}$) for 24 h after voluntary feeding to satiation in groups of 20 small tambaqui in single tanks of Series 8. $M_{Amm\text{-}N}$ was measured on an hourly basis, whereas $M_{Urea\text{-}N}$ could only be resolved over 3-h periods. Food was offered between 11:00 and 12:00 pm (noon) on day 11 (see Fig. 7), and measurements started immediately thereafter. See text for additional details. Means ± 1 SEM ($N = 4$ groups, with 20 fish in each group). In **b**, *means sharing the same letter* are not significantly different ($P > 0.05$). In **a**, there are too many comparisons to portray on the figure, so in the list below, *lines underscore* time points (*a* am, *p* pm) which are not significantly different ($P > 0.05$); *13p* represents the first hour post-feeding, and *12p* represents the twenty-fourth hour post-feeding
9a 17p 18p 16p 1a 23p 22p 15p 6a 5a 3a 19p 20p 21p 24p 8a 2a 12p 11a 4a 14p 7a 13p 10a

species living at comparable high temperature in the very dilute Amazonian waters (Cameron and Wood 1978; Wood et al. 2009). However, the armoured catfish (*Liposarcus* sp.) had a similarly high UFR (10.6 mL $kg^{-1}$ $h^{-1}$; Randall et al. 1996). Perhaps, the very low calcium concentrations of the waters are a factor, increasing osmotic permeability of the gills (Hunn 1985). The high UFR in tambaqui did not appear to be a function of incomplete post-operative recovery, because it continued at this level in two fish monitored for several more days. This observation suggests that glomerular filtration rate (GFR) must also be high in tambaqui, perhaps, explaining the higher urea-N in the urine.

## Ammonia toxicity

Contrary to our second hypothesis, tambaqui in Series 2 were not exceptionally tolerant to high environmental ammonia, in contrast to their extreme tolerance to hypoxia, hypercarbia, ion-poor water, and low pH (see "Introduction"). We measured an approximate 48-h EC50 of 5 mmol $L^{-1}$ as total ammonia at pH 7.0, 28 °C, or 35 µmol $L^{-1}$ as $NH_3$. In a more rigorous study using death rather than loss of equilibrium as an endpoint, Souza-Bastos et al. (2016) have recently reported a slightly higher 96-h LC50 (7.8 mmol $L^{-1}$ as total ammonia, 64 µmol $L^{-1}$

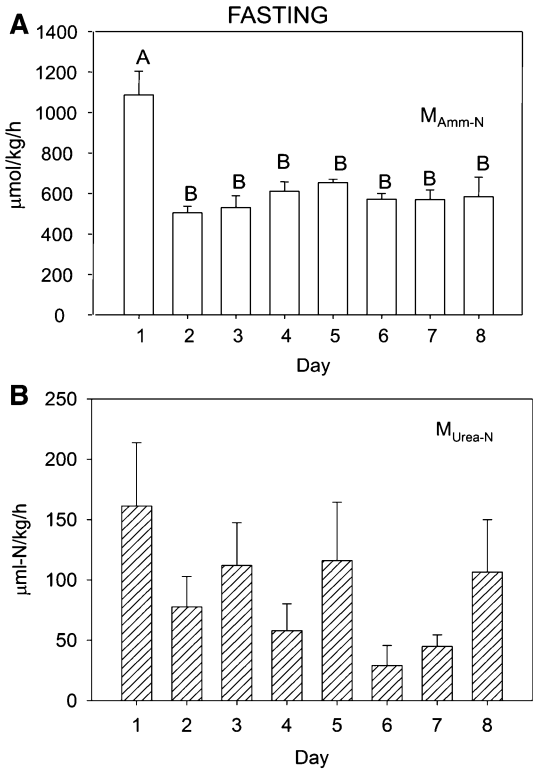


**Fig. 9** Rates of **a** ammonia-N excretion ($M_{Amm\text{-}N}$) and **b** urea-N excretion ($M_{Urea\text{-}N}$) measured daily over 4-h periods during 8 days of fasting (days 12–19; see Fig. 7) following the feeding experiment on day 11 (see Fig. 8) in groups of 20 small tambaqui in single tanks of Series 8. $M_{Amm}$. Day 12 is the first day of fasting. Means ± 1 SEM ($N = 4$ groups, with 20 fish in each group). *Means sharing the same letter* are not significantly different ($P > 0.05$)

as $NH_3$) under similar test conditions in tambaqui tenfold smaller than those used in this study (3.4 versus ~35 g). This sensitivity lies in the midrange for 11 species of Amazonian fish tested, and Souza-Bastos et al. (2016) concluded that, in general, Amazonian species are less tolerant of ammonia than most other teleosts, perhaps because they evolved in generally acidic waters, where ammonia would be less toxic.

### N-waste excretion in the presence of high environmental ammonia (HEA)

Very clearly, tambaqui did not elevate $M_{Urea\text{-}N}$ in the face of ammonia loading (Fig. 1b), in contrast to some ammonia-tolerant teleosts (Wood 1993; Wilkie 2002; Ip and Chew 2010; Sinha et al. 2013). Nevertheless, despite their limited tolerance and in partial accord with our initial prediction, tambaqui were able to maintain ammonia-N excretion, and, indeed, to establish a sustained elevation of $M_{Amm\text{-}N}$ above control rates during and after HEA exposure (2.5 mmol $L^{-1}$; Fig. 1a). This has been seen previously

**Table 2** Changes in body weight in groups of 20 small tambaqui in single tanks of Series 8 for 11 days after establishment of the groups on day 0, and after 8 days of fasting (days 12–19) following the feeding experiment on day 11

| | Day 0, weight (g) | Day 11, weight (g) | Day 19, weight (g) |
|---|---|---|---|
| Tank 1 | 24.72 ± 1.44 | 23.03 ± 1.48 | 21.64 ± 1.45 |
| Tank 2 | 18.20 ± 1.39 | 17.78 ± 1.22 | 16.57 ± 1.25 |
| Tank 3 | 21.98 ± 1.24 | 20.78 ± 1.19 | 19.34 ± 1.14 |
| Tank 4 | 19.85 ± 1.37 | 19.62 ± 1.31 | 18.35 ± 1.27 |
| Grand mean ± SEM | 21.19 ± 1.40$^{A}$ | 20.30 ± 1.10$^{A}$ | 18.97 ± 1.06$^{B}$ |

Means ± 1 SEM ($N = 4$ groups for grand mean, with $N = 20$ fish in each group mean). Grand means sharing the same letter are not significantly different ($P > 0.05$)

in other teleosts (carp, goldfish) and may reflect either an elevation in metabolic rate and/or an elevation in cortisol secretion which tends to drive metabolism towards proteolysis and enhanced amino-acid oxidation (Sinha et al. 2013). Furthermore, we now know that these and some other teleost species (e.g., trout, fugu) are able to activate a "$Na^+$/$NH_4^+$ exchange complex" consisting of several membrane transporters working together (Rh protein, v-type $H^+$-ATPase, $Na^+/H^+$ exchanger, carbonic anhydrase), so as to excrete ammonia across the gills against diffusive and electrochemical gradients—i.e., by active transport—during HEA exposure (Nawata et al. 2007, 2010; Weihrauch et al. 2009; Wright and Wood 2009, 2012; Sinha et al. 2013). It remains to be seen whether this mechanism is present in the tambaqui, and whether the observed increase in $M_{Amm\text{-}N}$ is really active excretion. Under control conditions, blood pH in tambaqui is about 7.8, plasma total ammonia is about 0.14 mmol $L^{-1}$, and TEP is about −20 mV (Wood et al. 1998), the latter confirmed in this study (Fig. 2). From this, we can calculate that diffusive gradients [see Wood and Nawata (2011), for calculation details] for both $NH_3$ ($PNH_3$ gradient) and $NH_4^+$ (electrochemical gradient) would be inwardly directed during the initial phase of HEA exposure, thereby explaining the non-significant decline (indeed reversal in some fish) of $M_{Amm\text{-}N}$ during the first 14 h (Fig. 1a). However, in the absence of measurements of blood pH and plasma total ammonia during the subsequent periods of $M_{Amm\text{-}N}$ elevation, it is problematical whether net excretion occurred against both gradients. These are important areas for future investigation.

### TEP changes during HEA exposure

In accord with our hypothesis, the tambaqui proved to be the fourth species of freshwater fish, in which a pronounced increase in TEP has been observed during HEA exposure

(Fig. 2), the others being rainbow trout, common carp, and goldfish (Wood and Nawata 2011; Liew et al. 2013). As discussed by Wright and Wood (2012), the mechanism behind the effect remains unknown, but its adaptive significance is clear. In general, the cell membranes of ammonotelic teleosts are much more permeable to $NH_4^+$ than those of higher vertebrates (Wood 1993), so during HEA exposure, the diffusive entry of $NH_4^+$ ions at the gills from the water along a steep electrochemical gradient into the fish plasma (which is negative relative to the water by almost −20 mV) is probably a major pathway of ammonia loading. By allowing the internal TEP to rise by about 9 mV (Fig. 2), this will attenuate the net electrochemical driving force on $NH_4^+$ by 9 mV, which is a substantial amount [see Wood and Nawata (2011) for detailed calculations]. In the rainbow trout, for example, a 15-mV rise in TEP during HEA exposure reduced the net electrochemical driving force for $NH_4^+$ entry by 40 % (Wood and Nawata 2011). However, as noted earlier, in the absence of measurements of blood pH and plasma total ammonia during HEA exposure, it is impossible to make a similar quantitative assessment in tambaqui. Nevertheless, the observed 9-mV rise in TEP would clearly be one factor facilitating the increase in net ammonia excretion (Fig. 1a) during prolonged HEA exposure.

### The responses of N-waste excretion to acute and gradual hypoxia

During acute exposure to hypoxia (30 % saturation for 3 h), tambaqui exhibited a significant reduction in $M_{\text{Amm-N}}$ (Fig. 3b), in accordance with original predictions. In this regard, the tambaqui proved to be similar to the oscar (*A. ocellatus*) another extremely tolerant Amazonian fish which occupies a similar habitat (Wood et al. 2007, 2009; De Boeck et al. 2013). Robertson et al. (2015) recently surveyed the responses of 12 tropical and temperate teleosts to a comparable hypoxic treatment (20–30 % saturation for 2 h), and found that most exhibited either no change or an increase in $M_{\text{Amm-N}}$. However, the tambaqui, the oscar, and the temperate pumpkinseed, all of which regularly experience hypoxic environments, and the rosaceu tetra (*Hyphessobrycon bentosi rosaceus*) another Amazonian species that inhabits a more stable environment, exhibited significant decreases in $M_{\text{Amm-N}}$. In the oscar, the response appeared to be linked to a rapid decrease in gill permeability, rather than a reduction in ammonia production rate during hypoxia (Wood et al. 2007, 2009), though there is certainly precedence for the latter in other species (van Waarde 1983). However, the tambaqui response differed from that of the oscars in that it did not occur during gradual hypoxia (Fig. 4b) and it was not accompanied by a reduction in $M_{\text{Urea-N}}$ during acute hypoxia (Fig. 3c), though, curiously, the latter occurred during gradual hypoxia (Fig. 4c). These may just be discrepancies in threshold $O_2$ levels, or they may result from more substantial metabolic differences between the two species. A possible explanation for the differences in the responses to acute versus gradual hypoxia in the tambaqui is that aquatic surface respiration probably occurred during gradual hypoxia, but not during acute hypoxia, because growth of the surface-skimming lip (Braum and Junk 1982; Val and Almeida-Val 1995; Sundin et al. 2000; Florindo et al. 2006) was observed only in the former.

### The responses of N-waste excretion and oxygen consumption to acute increases in water temperature

In general agreement with our prediction of low temperature sensitivity, Q10 values for $M_{O_2}$ (~1.9) over the entire 28–38 °C range (Fig. 5a) were lower than for teleost species, in general (~2.4; cf. Clarke and Johnston 1999). More notably, the Q10 values for $M_{\text{Amm-N}}$ (Fig. 5c) and, therefore, for $M_{\text{N}}$ (Fig. 5b) were particularly low (~1.5) between 28 and 33 °C, indicating that N-metabolism is well regulated over an environmentally realistic temperature range. This contrasts with most teleosts, where $M_{\text{Amm-N}}$ and $M_{\text{N}}$ generally exhibit much higher temperature sensitivity than $M_{O_2}$ (Wood 2001)—e.g., goldfish (Maetz 1972), trout (Kieffer et al. 1998), and tilapia (Alsop et al. 1999). However, with a further elevation to 38 °C, a temperature which is unlikely to occur in natural Amazonian waters (Caraballo et al. 2014), the regulation of N-metabolism appeared to break down as the Q10 values for $M_{\text{Amm-N}}$ and $M_{\text{N}}$ increased to about 3.8 (Fig. 5b, c). In agreement with one previous report on fasted tambaqui (Pelster et al. 2015), the NQ ratios were exceptionally high (Fig. 5d) relative to the previous determinations in teleost fish [see summaries in Van Waarde (1983) and Wood (2001)]. This analysis indicated that reliance on protein oxidation to support aerobic metabolism, which was already 60–70 % at the lower temperatures, increased to about 85 % at 38 °C, though the change was not significant (Fig. 5d). Interestingly, $M_{\text{Urea-N}}$ exhibited the opposite trend, a lower temperature sensitivity at the higher temperature range (Fig. 5c). These discrepancies in pattern and absolute Q10 values suggest that the pathway for urea-N production, which is probably mainly uricolysis in these fasted fish (Wood 1993), has different temperature sensitivity than that for general protein oxidation. Similar discrepancies have been reported in tilapia (Alsop et al. 1999).

### The responses of N-waste excretion and oxygen consumption to aerobic exercise

Small tambaqui swam easily in the respirometers; critical swimming speed ($U_{\text{Crit}}$) would clearly be greater than the

**Table 3** N-budgets on four groups of 20 small tambaqui of Series 8 for 24 h after voluntary feeding in single tanks

| | Ration[a] (g $kg^{-1}$) | Protein-N intake[a] (μmol $kg^{-1}$) | 24-h excretion | | | N-wastage[b] (%) |
|---|---|---|---|---|---|---|
| | | | Ammonia-N (μmol $kg^{-1}$) | Urea-N μmol $kg^{-1}$) | Total N (μmol/$kg^{-1}$) | |
| 1 | 13.7 | 70,400 | 36,087 | 3135 | 39,222 | 55.7 |
| 2 | 3.6 | 18,499 | 28,859 | 2535 | 31,394 | 169.7 |
| 3 | 7.8 | 40,100 | 22,677 | 3505 | 25,182 | 62.8 |
| 4 | 15.2 | 78,100 | 27,587 | 2795 | 30,386 | 38.9 |
| Mean ± SEM | 10.1 ± 0.27 | 51,775 ± 13,795 | 28,803 ± 2770 | 2993 ± 210 | 31,546 ± 2898 | 81.8 ± 29.7 |

See text for details

[a] The analysis employed the known protein content (45 %) of the diet and an assumed standard protein composition of 0.16 g N/g of protein

[b] N-wastage was greater than 100 % when total N excretion over 24 h exceeded protein-N intake

highest tested (~3.45 BL $s^{-1}$; Fig. 6). The pattern of steady increase in $M_{O_2}$ with swimming speed at 28 °C (Fig. 6a) was very similar to that for comparably sized tilapia swimming at 30 °C, where $U_{Crit}$ was 5.63 BL $s^{-1}$ (Alsop et al. 1999). However, in contrast to tilapia which excreted moderately more ammonia as exercise level increased, tambaqui did not exhibit any increase in $M_{Amm\text{-}N}$ (Fig. 6c) or in $M_N$ (Fig. 6b) at higher swimming speeds. In this respect, they behaved in accordance with our hypothesis, and were similar to catfish (Sukumaran and Kutty 1977) and trout (Lauff and Wood 1996b; Alsop and Wood 1997; Kieffer et al. 1998). Tambaqui also demonstrated a progressive increase in $M_{Urea\text{-}N}$, in contrast to $M_{Amm\text{-}N}$, but in parallel to $M_{O_2}$, as exercise workload increased (Fig. 6c). While the absolute contribution of $M_{Urea\text{-}N}$ to $M_N$ increased, it remained small overall. Again, this has been seen in at least three previous exercise studies (Lauff and Wood 1996b; Alsop and Wood 1997; Kieffer et al. 1998). The mechanism remains unknown; but Lauff and Wood (1996b) suggested that there could be a greater adenylate turnover at higher speeds, a byproduct of which would be greater urea-N production by uricolysis.

Based on these and other more anecdotal studies, Wood (2001) concluded that, in general, fish rely less on protein oxidation and more on other fuels when they exercise. This conclusion is congruent with a large literature showing that exercise actually promotes protein synthesis and growth (i.e., N-retention) in fish (e.g., Houlihan and Laurent 1987; Davison 1997). In this study, the marked decline in NQ as swimming speed increased (Fig. 6d), indicating a drop in protein oxidation from 75 to 33 %, is in accordance with this interpretation. Indeed, continuous exercise may be treatment worth investigating to increase protein growth and reduce N-wasting in tambaqui aquaculture. This phenomenon may also help tambaqui to conserve muscle protein during their annual migrations (see "Introduction"). Migrations occur both upstream for the purpose of spawning at the beginning of the flood season, as well as downstream from nursery habitats during the dry season. Post-spawning, tambaqui also migrate long distances into flooded forests to feed. Overall, these migrations may cover hundreds of kilometers, and in headwater, streams may occur against considerable currents (Goulding 1980; Goulding and Carvalho 1982).

### The daily cycle of N-waste excretion in response to feeding and fasting

The NQ measurements of the previous two Series 6 and 7 suggest that the heavy reliance on protein oxidation (60–80 %) by tambaqui (Figs. 5d, 6d) results in considerable N-wasting in fasted fish. The previous measurements on other species have uniformly indicated a much lower percentage (15–40 %) in fasted or post-absorptive individuals (van Waarde 1983; Wood 2001). The feeding trials of Series 8 were designed to evaluate whether similar N-wasting occurs in fed animals by quantifying the amount of N excreted after a single meal. Note that in these trials, only $M_{Amm\text{-}N}$ and $M_{Urea\text{-}N}$ were measured. $M_{O_2}$ was not recorded, both because it is practically difficult to do so without disturbing the fish, and because the respirometric approach to fuel utilization becomes problematic under the non-steady-state conditions of feeding (Lauff and Wood 1996a).

Although the four replicate groups did not all consume the same ration, they all exhibited a marked elevation in $M_{Amm\text{-}N}$, peaking at 4–6 h after the meal (Fig. 7a), and an asynchronous later elevation in $M_{Urea\text{-}N}$, peaking at 6–9 h after the meal (Fig. 7b). A second, short-lasting peak in $M_{Amm\text{-}N}$ at 21 h in all groups may have resulted from earlier re-ingestion of the faeces. Ismino-Orbe et al. (2003) reported several such delayed $M_{Amm\text{-}N}$ peaks in one previous tambaqui feeding study. We have anecdotally observed the occurrence of coprophagy in this species, though specific observations were not made in the present experiment, so as to avoid disturbance.

As first shown by Brett and Zala (1975) in juvenile coho salmon, a post-prandial increase in N-excretion has been reported almost universally in feeding studies, with differences in species physiology and water temperature affecting the exact timing and pattern (reviewed by Wood 2001). While all species showed the increases in $M_{Amm}$, only about half of them also showed the increase in $M_{Urea\text{-}N}$ seen in the tambaqui; for example, coho salmon did not (Brett and Zala 1975). The difference probably reflects the fact that some species, such as tambaqui, may use arginolysis to rapidly metabolize the N in dietary arginine into urea-N, a faster process than channeling the N through purine and pyrimidine rings and eventual production of urea-N by uricolysis.

The post-prandial increment in N-waste excretion is traditionally considered to represent the "exogenous" fraction—the amount of ingested nitrogen from the diet which is not retained as growth. This is superimposed on the "endogenous fraction"—the unavoidable nitrogen loss which results from the normal turnover of proteins, nucleic acids, adenylates, etc., as seen in the fasting state. In the tambaqui, the endogenous rate stabilized after 2 days of fasting (Fig. 8).

As the exact ration was recorded for each replicate group and the protein content (45 %) of the diet was known, it was possible to construct 24-h N-budgets (Table 3) assuming a standard protein composition, 0.16 g N/g of protein (Cho 1990). This analysis clearly indicates that tambaqui also waste a great deal of N during feeding. On average, 82 % of the dietary N consumed was excreted within 24 h rather than retained for growth, with individual group values ranging from 39 to 170 % (Table 3). N-wastage values greater than 100 % occurred when total N excretion over 24 h exceeded protein-N intake.

While these N-wastage values seem very high, losses of 62–72 % over 170 days can be calculated from the data of Arbelaez-Rojas et al. (2002) for tambaqui in two systems of experimental aquaculture. Interestingly, in the present study, the total N-excretion was actually quite uniform amongst groups, whereas the ration consumed was highly variable, so the % N-wastage was inversely related to the ration and, therefore, to the amount of protein-N consumed. Given these data and the slowly increasing pattern of voluntary food consumption during the 10-day pre-experimental period (Fig. 7), it is not surprising that the fish failed to gain weight over this interval (Table 2). Whole-body protein content in tambaqui is typically about 15 % of wet weight (Arbelaez-Rojas et al. 2002), so based on the endogenous N-excretion rate of ~750 μmol $kg^{-1}$ $h^{-1}$ during the 8-day period of subsequent fasting (Fig. 8), a weight loss of 8.4 % would be predicted, close to the observed value of 9.3 % (Table 2).

It seems counterintuitive that such a highly successful species should be so wasteful of protein-N during both fasting (i.e., high endogenous component) and feeding (i.e., high exogenous component). We speculate that there may be at least three possible explanations, which are interrelated and not mutually exclusive. First, in the wild, tambaqui are omnivorous with a large component of fruits and nuts in the diet, with a high lipid and carbohydrate content (Goulding 1980; Goulding and Carvalho 1982; Roubach and Saint-Paul 1994; Val and Honczaryk 1995; Araújo-Lima and Goulding 1997; Anderson et al. 2009). Their physiology may not be set up to efficiently process a very high-protein commercial diet into protein growth. Second, because the fish in this study had been entrained on a high-protein commercial diet, they may have re-organized their physiology as much as possible to oxidize protein, a pattern which may have continued during fasting resulting in the elevated oxidation of somatic protein. And finally, it may be that the high-protein commercial diet used in this study was unbalanced in amino-acid composition, such that N could not be efficiently incorporated into protein growth (Conceição et al. 2012). In future investigations, it will be of interest to repeat these trials using natural diets during both rearing and experimentation.

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