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ORIGINAL PAPER

Gut transport characteristics in herbivorous and carnivorous serrasalmid fish from ion-poor Rio Negro water

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Abstract Three closely related characids, Tambaqui (omnivore), black Piranha (carnivore), and Pacu (herbivore), all Serrasalmidae, inhabit the ion-poor, acidic Rio Negro. We compared O_2-consumption and N excretion rates in vivo, and sodium, chloride, glucose, and ammonia transport characteristics of gut sac preparations in vitro. The Pacu had a significantly higher weight-specific oxygen consumption, and a lower *N*/*Q* ratio than the omnivorous Tambaqui, and a significantly lower urea-N excretion rate than the carnivorous black Piranha, suggesting N-limitation in the herbivorous Pacu. With a value of 2.62 ± 0.15, gut to fork length ratio in the Pacu was about 2.5 times higher than in the black Piranha, and 2.0 times higher than in the Tambaqui. Anterior intestinal activities of three enzymes involved in N-fixation for amino acid synthesis (glutamate dehydrogenase, glutamate–oxaloacetate transferase, and glutamate–pyruvate transferase) were generally greatest in the carnivore and lowest in the herbivore species. In all three species, sodium, chloride, glucose, and ammonia were taken up at high rates from the intestine, resulting in an isosmotic fluid flux. Comparing the area-specific fluid flux of the anterior, mid, and posterior gut sections, no difference was detected between the three sections of the Pacu, while in the Tambaqui, it was highest in the anterior section, and in the black Piranha highest in the middle section. Overall, the area-specific uptake rates for sodium, chloride, glucose, and ammonia of anterior, mid, and posterior sections were similar in all three species, indicating that there is no difference in the area-specific transport rates associated with trophic position. The net ammonia uptake flux from gut interior was not significantly different from the net ammonia efflux to the serosal fluid, so that the ammonia removed from the intestine by the mucosal epithelium was quantitatively transferred through the tissue to the serosal side in all three species. Thus, metabolic activity of gut tissue did not significantly influence the net ammonia transfer. Due to the much higher gut to fork length ratio, the overall transport capacity of the gut of the herbivorous Pacu by far exceeded the transport capacity of their carnivorous and omnivorous relatives, thus compensating for the lower digestibility and the low

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B. Pelster and C. M. Wood contributed equally to this paper.

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B. Pelster (✉)
Institut für Zoologie, Leopold-Franzens-Universität Innsbruck, Technikerstr. 25, 6020 Innsbruck, Austria
e-mail: bernd.pelster@uibk.ac.at

B. Pelster
Center for Molecular Biosciences, University Innsbruck, Innsbruck, Austria

C. M. Wood
Department of Biology, McMaster University, Hamilton, ON L8S 4K1, Canada

C. M. Wood
Department of Zoology, University of British Columbia, Vancouver, BC V6T 1Z4, Canada

B. Speers-Roesch · W. R. Driedzic
Department of Ocean Sciences, Memorial University of Newfoundland, St. John's, NL A1C 5S7, Canada

V. Almeida-Val · A. Val
Instituto Nacional de Pesquisas da Amazonia, Manaus, Brazil

Na^+, Cl^-, and N-content of the plant diet. Accordingly, in order to cope with the more difficult digestible plant material and the very low nitrogen content of plants, herbivorous fish have not evolved more effective area-specific transport capacities, but rather have increased the length of the gut.

Keywords Ion exchange · Gut function · Osmotic water flux · Ion regulation · Freshwater fish · Rio Negro

Introduction

The Amazon basin is well known for its richness in freshwater fish. The high variability in physicochemical conditions found in the different regions of the Amazon certainly contributed significantly to the development of the fish fauna in this freshwater system. Freshwater-adapted teleosts are hyperosmotic to their environment and therefore face a constant osmotic water influx and an ion loss. This is compensated by the production of copious amounts of dilute urine and by the active uptake of ions (i.e., Na^+ and Cl^-) at the gills, as described in excellent reviews (Evans et al. 2005; Evans 2008; Hwang and Lee 2007). As first suggested by Smith et al. (1989), several studies have convincingly documented that this sort of traditional view, which is based on many experimental studies mostly performed with unfed fish in order to get standardized conditions, needs to be modified because the gut may significantly contribute to ion uptake (reviewed by Wood and Bucking 2011). In fed fish, ions contained in the food, either in plant material or in prey items, are effectively taken up from the different sections of the gut. In fact, ion uptake from the gut may even be similar or exceed the ion uptake recorded in the gills of freshwater fish.

Freshwater conditions typically means a near-neutral pH and a conductivity of 100 $\mu S\ cm^{-1}$ or more, but the Rio Negro as part of the Amazon basin is known for the extremely low pH (close to 4.0) and low conductivity (about 10–20 $\mu S\ cm^{-1}$) of its "black water" (Küchler et al. 2000). The low ion content combined with very acidic conditions appears to be toxic for 'normal' freshwater fishes such as salmonids or cyprinids, and acute exposure may result in death within a few hours (Gonzalez et al. 2005). While Na^+ and Cl^- uptake from freshwater in the fish gills typically requires the involvement of an ATP-consuming proton pump (Dymowska et al. 2012; Evans 2008; Lin et al. 1994), coping with this extremely ion-poor water combined with a very acidic situation appears to be particularly challenging. The low pH, i.e., the high proton concentration in the environmental water, renders the secretion of protons to the outside, which is believed to facilitate the energetically unfavorable sodium uptake at the gill cells (Dymowska et al. 2012; Evans 2008), even more difficult. Gonzalez et al. (2005) pointed out that the theoretical lower limit for active uptake of sodium with support of the H^+-ATPase would be pH 4.5–5.5. Accordingly, in this situation, the gut may gain special importance in order to maintain ionic homeostasis.

Considering gut function another interesting aspect is the comparison of herbivorous and carnivorous species. Plants use carbohydrates for building blocks, and therefore, plant material contains copious amounts of cellulose, the digestion of which in most animals is only possible thanks to an intestinal fauna of microbes and bacteria. Microbial cellulase breaks down the polymers into glucose and disaccharides, and herbivores therefore often have a much longer gut to facilitate the more difficult extraction of nutrients and the digestion processes (Clements and Raubenheimer 2005; Horn 1997; Wilson and Castro 2011). Eukaryotic animals, however, use protein for building blocks and therefore have a significantly higher N-content per g dry weight than plants (Mattson 1980). In addition, the high turn-over rates of certain tissues, such as the gut epithelium, for example, require a constantly active protein metabolism. Consequently, animals have a significantly higher nitrogen excretion rate than plants (Mattson 1980). Accordingly, herbivorous species often are nitrogen limited. Interestingly, several recent studies have indicated that even carnivorous salmonids absorb ammonia produced by digestive processes in the chyme, rather than "wasting" this nitrogen source by elimination in the feces (Bucking and Wood 2012; Karlsson et al. 2006; Rubino et al. 2014).

In the present study, we therefore analyzed the role of the gut in ion regulation and in glucose and ammonia transport in three species of Serrasalmidae of the Amazon basin, living in the extremely ion-poor and acidic Rio Negro. We also examined some key intestinal enzymes involved in the "trapping" of ammonia-N for amino acid synthesis. The black Piranha (*Serrasalmus rhombeus*) was chosen as a carnivorous species and the Tambaqui (*Colossoma macropomum*) as an omnivorous species, as young fish (<10 cm) eating up to 20 % zooplankton and 4 % insects, and then turning more to a herbivorous diet. The Pacu (*Myleus lobatus*) was chosen as a mostly herbivorous fish eating some insects as an admixture only during the low water season (Araujo-Lima and Goulding 1998; Santos et al. 2006). We hypothesized that the herbivorous species would have the longest relative gut length of these three species, the lowest relative whole animal N excretion rate, and the greatest capacities for intestinal ammonia and glucose absorption as a result of the N-limitation and high cellulose content of the plant diet, with reciprocal patterns in the carnivorous species. Furthermore, we postulated that the high capacity for ammonia absorption would be supported by high activity levels of N-fixing enzymes in the intestine. We also hypothesized that all three species in this extremely ion-poor and acidic environment would have higher intestinal ion (Na^+ and Cl^-) transport capacities relative to rates reported in fish living in more "normal," ion-rich freshwaters, and that

Table 1 Body mass, fork length, gut length, and gut/fork length ratio of black Piranha (*Serrasalmus rhombeus*), Tambaqui (*Colossoma macropomum*), and Pacu (*Myleus lobatus*)

	Body mass (g)	Fork length (cm)	Gut length (cm)	Gut/fork length ratio
Black Piranha (9)	540.0 ± 55.2^{a}	27.17 ± 0.80^{a}	29.89 ± 2.05^{a}	1.07 ± 0.06^{a}
Tambaqui (17)	120.9 ± 7.77^{b}	17.70 ± 0.36^{b}	22.94 ± 0.81^{b}	1.30 ± 0.04^{b}
Pacu (6)	96.17 ± 20.70^{b}	14.98 ± 1.26^{c}	38.5 ± 2.37^{a}	2.62 ± 0.15^{c}

Number in brackets indicates the number of animals analyzed, and values not sharing the same letter are significantly different ($p < 0.05$)

absorption of Na^+ and Cl^- would be hyperosmotic rather than isotonic to minimize water absorption through the gut.

Materials and methods

All experiments were performed in December 2013 on board a research vessel (the Ana Clara, from Manaus) during an expedition to the Anavilhanas Archipelago of the Rio Negro, approximately 110 km upstream from Manaus. All procedures were in compliance with Brazilian national and INPA animal care regulations.

Fish used for this study were the carnivorous black Piranha (*Serrasalmus rhombeus*), the omnivorous Tambaqui (*Colossoma macropomum*), and the herbivorous Pacu (*Myleus lobatus*). black Piranha and Pacu were caught in the local area by INPA fishermen, whereas Tambaqui had been purchased from a hatchery in Manaus. All three species were held on board in large tanks served with flowing "black water" pumped directly from the Rio Negro (temperature = 30–35 °C, pH = 4.0–4.5). The fish were not fed during this 12-day expedition, and were allowed to recover at least overnight after capture before experimentation.

Metabolic activity of the fish

Oxygen consumption and nitrogen excretion rates of fish were determined by closed respirometry. Table 1 reports body weights and other metrics on these fish. Fish were transferred from the holding tanks to the respirometer chamber (chamber volume 5 l for Tambaqui and Pacu; 25 l for black Piranha), and the respirometer was flushed with Rio Negro water for 1–2 h to let the fish settle down. It is our experience that these wild-caught fish do not hold well in captivity, particularly when confined in the small volume of the respirometers. Therefore, using 1–2 h of settling time was a compromise between allowing some time to settle versus avoiding undue stress on the fish-associated deterioration. At time $T = 0$, a water sample was taken for initial ammonia and urea-N determinations, and the PO_2 was recorded with an oxygen electrode (WTW Oxi325 Oximeter, Weilheim, Germany). The respirometer chamber was closed for 40–60 min. The final PO_2 was recorded, and a second water sample was taken for determination of final ammonia and urea-N concentrations. Final PO_2 was never allowed to go below 4 kPa.

Weight-specific oxygen consumption (MO_2) was calculated as

$$MO_2 = \alpha O_2 \times PO_2 \times V \times T^{-1} \times BM^{-1}, \quad (1)$$

where αO_2 is the physical solubility of oxygen in water (Boutilier et al. 1984), V is respirometer volume, T represents the time of closing the respirometer, and BM is body mass.

Similarly, the rates of ammonia and urea-N excretion were calculated as

$$M_{\text{ammonia; urea}} = (C_F - C_I) \times V \times T^{-1} \times BM^{-1}, \quad (2)$$

where C_F and C_I represent the final and the initial concentrations of ammonia or urea-N, respectively.

After completion of respirometry, the fish was killed with an overdose of tricaine methanesulfonate (MS222). A blood sample was taken by caudal puncture for determination of plasma ammonia and glucose concentrations. Blood samples were centrifuged at 13,000 rpm for 5 min at 4 °C, and the plasma was removed. Plasma samples were deproteinized by adding 70 µl 8 % PCA to 20 µl plasma. After centrifugation, the sample was neutralized using 1 mol l^{-1} KOH; the salt was precipitated by further centrifugation, and the supernatant was used for determination of plasma ammonia and glucose concentrations.

The fish was then weighed, and fork length was measured. The gut was quickly excised, and the various coils were carefully teased apart so that the gut could be laid out in a straight line for determination of total gut length (pyloric sphincter to anus). In a few cases, it was possible to obtain an uncontaminated chyme sample for analysis from the anterior part of the intestine (from black Piranha and Pacu) at this stage. These samples were centrifuged as for plasma samples, and the supernatant fluid was frozen for later analysis. The gut was then kept on ice while being made into gut sac preparations.

Gut sac preparations

Gut sac experiments were performed to quantify Na^+, Cl^-, glucose, ammonia, and fluid fluxes from the mucosal to the

serosal side in anterior, middle, and posterior gut sections. A common protocol was used across all treatments. Gut sections were excised, cleaned of excess connective tissue, and placed in an ice-cold Cortland saline bath (in mmol l^{-1}: NaCl 124, $NaHCO_3$ 11.9, KCl 5.0, $CaCl_2$ 1.4, $MgSO_4 \cdot 7 H_2O$ 1.9, Na_2HPO_4 2.9, glucose 5.0, pH = 7.4). The internal saline subsequently used for filling the gut sacs constituted the mucosal medium, and was the standard Cortland saline (already containing 5 mM glucose) but supplemented with 10.0 mM NH_4Cl. The external saline used for incubating the gut sacs constituted the serosal medium, and was of identical composition except that 10 mM NH_4Cl was not present but replaced with 20 mM mannitol so as to balance osmotic pressure. Thus, transport of glucose from mucosal to serosal solutions was measured under symmetrical conditions, whereas transport of ammonia was measured under asymmetrical conditions. These experimental conditions were selected based on the normal presence of similar levels of glucose in both chyme and plasma, but only very low levels of ammonia in plasma relative to chyme in vivo (see "Results"). Starting samples of internal and external saline were frozen for analysis.

Anterior, middle, and posterior gut sections were then separated and rinsed internally with Cortland saline to remove chyme and feces. Each section was then tied off at one end using 2–0 silk thread. Through the open end, a flared polyethylene (Intramedic Clay-Adams PE-60; Becton–Dickinson and Company, Sparks, MD, USA) tube was inserted and secured in place using silk thread. Many of the fish carried nematodes which perforated the gut wall, so care was taken to avoid sections which contained these parasites. The gut sac preparation (typical length approximately 4 cm) was then filled with internal saline until taut, and the PE tube was sealed. The gut sac was gently dried by blotting, weighed, and then immersed into 15 ml of external saline in a 20-ml glass scintillation vial which was standing in a bath of flowing river water to maintain temperature at 30–32 °C. In the vial, the saline was continuously bubbled with a precision gas mixture containing 99.7 % O_2 and 0.3 % CO_2 to assure sufficient oxygen supply and normal acid-base conditions for the tissues of the gut sac preparation. Following a 1-h flux period, the gut sac preparation was removed from the vial, and a 5-ml sample was taken from the bathing Cortland saline for each gut section. The gut sac preparation was carefully blotted dry and reweighed to determine the amount of fluid that was transported out of the gut sac during the 1-h incubation. The amount of fluid removed (V_R) was calculated as

$$V_R = \text{Initial weight} - \text{Final weight}. \quad (3)$$

The gut sac preparation was carefully opened, and the internal fluid was completely removed and frozen for further analysis. The empty gut sac was blotted and weighed again to determine the amount of fluid that was inside the gut sac preparation after the 1-h flux experiment (V_F):

$$V_F = \text{Final weight} - \text{Empty weight}. \quad (4)$$

The initial fluid volume (V_I) inside the gut sac at the start of the incubation was calculated as

$$V_I = V_R + V_F. \quad (5)$$

Surface area of the tissue constituting the gut sac was measured using a standard technique for gut sacs first outlined by (Grosell and Jensen 1999) wherein individual gut sections were cut in half, and the area was traced onto 0.5-mm graph paper. This also allowed calculation of the area of the gut section per unit length (cm) of the gut (see below).

Analytical procedures

Ammonia concentrations in salines, plasma samples, and chyme extracts were determined using a Raichem commercial assay (Cliniqa Corporation, San Marcos, CA, USA). Ammonia concentrations in water and urea-N concentrations in water were measured by the colorimetric methods of Verdouw et al. (1978) and Rahmatullah and Boyde (1980), respectively. Glucose concentrations were measured using the Infinity™ Glucose Hexokinase Liquid Stable Reagent from Thermo Fisher Scientific Inc. (Burlington, ON, Canada). Na^+ concentrations were measured with a 910 Digital Flame Photometer (Instrumentação Analítica São Paulo, SP, Brazil) and Cl^- concentrations by colorimetric assay (Zall et al. 1956).

Measurement of intestinal enzyme activities

For preparation of gut tissue samples for the analysis of enzyme activities, fish were killed with an overdose of MS222. Body mass of black Piranha used for these experiments was 432 ± 99 g ($N = 8$), of Tambaqui 108 ± 12 g ($N = 6$), and of Pacu 128 ± 18 g ($N = 8$). Some of these fish were the same as used in the respirometry and gut sac experiments.

An approximately 2-cm long section of anterior intestine was rapidly excised from each fish. If chyme was present, it was gently extruded with forceps, and the tissue was rinsed with Cortland saline. The intestine was then frozen in liquid N_2 and stored in dry ice. The segments of frozen intestine were homogenized on ice in 0.4–0.5 ml of ice-cold homogenization buffer (50 mM HEPES, 1 mM disodium EDTA, 0.1 % Triton X, pH 7.4 at 25 °C) using a motorized homogenizer (Fisher Powergen 125, Fisher Scientific, Ottawa, Canada). Homogenates were centrifuged at 5,000 rpm for 5 min at 4 °C, and dilutions of the supernatant were used for the enzyme and protein assays.

Maximal enzyme activities were measured using a SpectraMax 384Plus microplate spectrophotometer (Molecular Devices, Sunnyvale, CA, USA) with temperature control at 33 ± 0.1 °C. Activities of glutamate–pyruvate transferase (GPT, also known as alanine aminotransferase), glutamate–oxaloacetate transferase (GOT, also known as aspartate amino transferase), and glutamate dehydrogenase (GDH) were ascertained by measuring the oxidation of NADH at 340 nm (millimolar extinction coefficient ε_{340}, 3.67). Extinction coefficients were calculated for the final microplate well volume of 200 µl following the method of Brooks (1994). Assay conditions followed well-established protocols for fish tissues (Suarez et al. 1986; Treberg et al. 2002), and our preliminary measurements confirmed that reaction rates were linear with time and homogenate added for all species. Enzyme activities were measured in duplicate with a simultaneous, separate control reaction where substrate was omitted. Enzyme activities were measured in 50 mM HEPES buffer, pH 7.4, and with specific conditions as follows: GDH (EC 1.4.1.3): 0.2 mM NADH, 250 mM ammonium acetate, 2 mM ADP, 1 mM KCN, 10 mM alpha-ketoglutarate (omitted for control); GOT (EC 2.6.1.1): 0.2 mM NADH, 10 mM alpha-ketoglutarate, 8 U/ml malate dehydrogenase, 1 mM KCN, 40 mM aspartate (omitted for control); GPT (EC 2.6.1.2): 0.2 mM NADH, 10 mM alpha-ketoglutarate, 10 U/ml lactate dehydrogenase, 1 mM KCN, 200 mM alanine (omitted for control). Activities are presented as nmol substrate converted to product per minute per mg protein (nmol × min^{-1} × mg protein^{-1}). Protein was measured using the Bradford standard assay. All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Calculations

Fluid flux rate (F_f) per cm^2 was calculated from the amount of fluid removed from the gut sac (V_R), the area of the respective gut sac preparation and the incubation time (T_i):

$$F_f = V_R \times \text{area}^{-1} \times T_i^{-1}. \quad (6)$$

Incubation time was set to 1 h, and by using an initial and a final reading, linearity of flux rate over this time was assumed.

Flux rates (F) of glucose, ammonia, Na^+, and Cl^- ($=F_x$) were calculated from the concentration differences and the volumes of the respective samples, divided by the area of the respective preparation and incubation time:

$$F_x = (V_I \times C_I - V_F \times C_F) \times \text{area}^{-1} \times T_i^{-1}, \quad (7)$$

where C_I and C_F represent the initial and final concentrations of the substance in the mucosal saline, and V_I and V_F are the volumes of the mucosal fluid at the start of the incubation and at the end of the incubation, respectively.

Flux rates of ammonia (F_{samm}) to the external (serosal) fluid were calculated from the concentration differences and the volumes of the external fluid, divided by the area of the respective gut preparation and incubation time:

$$F_{samm} = (V_{Is} \times C_{Is} - V_{Fs} \times C_{Fs}) \times \text{area}^{-1} \times T_i^{-1}, \quad (8)$$

where C_{Is} and C_{Fs} represent the initial and final concentrations of ammonia in the external saline, and V_{Is} and V_{Fs} are the volumes of the external fluid at the start of the incubation and at the end of the incubation, respectively. Due to the large volume of the serosal fluid (=15 ml) compared to the fluid fluxes observed within the 1-h incubation, V_{Is} was usually equal to V_{Fs}.

The contribution of the gut tissue to ammonia production or consumption (T_{metab}) was determined by calculating the difference between ammonia flux rate from the internal fluid (F_{amm}) and the ammonia flux rate to the external fluid (F_{samm}):

$$T_{metab} = F_{amm} - F_{samm}. \quad (9)$$

Thus, a negative value indicated a net secretion of ammonia by the gut tissue, a positive value indicated that ammonia taken up from the mucosal side was not transferred to the serosal side, but remained in the tissue, and a value of zero would indicate that all the ammonia removed from the internal gut fluid was quantitatively transferred to the serosal side.

For calculation of the overall weight specific transport capacity of the gut (T_{cap}), the fractional contribution of the three gut sections to total gut length was determined from representative photographs of linearized guts. In black Piranha, the anterior, middle, and posterior sections of the gut contributed 27, 40, and 33 % to total gut length, respectively; in Tambaqui, the sections contributed 27, 31, and 42 %, respectively, and in Pacu, 25, 44, and 31 %, respectively. Based on these values and total gut length, lengths of the anterior, middle, and posterior sections (G_L) and also the lengths of each section normalized to body mass were calculated for each fish. The area of the gut section per unit length (cm) of the gut (A_L) was determined using 0.5 mm graph paper and the technique outlined by Grosell and Jensen (1999).

Total transport capacity (T_{cap}) for glucose, ammonia, Na^+, Cl^- (in µmol kg^{-1} h^{-1}), and fluid (in ml kg^{-1} h^{-1}) of each gut section was then calculated as

$$T_{cap} = F_x \times A_L \times G_L \times \text{BM}^{-1}, \quad (10)$$

where F_x is the fluid flux per cm^2, A_L is the gut section area per cm gut, G_L is total length of the respective gut section, and BM is body mass. Transport capacity of the whole gut

was determined as the sum of the transport capacities of the three gut sections:

$$T_{\mathrm{cap_{mid}}} = T_{\mathrm{cap_{ant}}} + T_{\mathrm{cap_{mid}}} + T_{\mathrm{cap_{post}}}. \quad (11)$$

Statistics

Data have been expressed as means ±1 sem., with *N* giving the number of animals for whole animal studies, or the number of individual gut sac preparations. For each animal, one preparation of each of the three gut sections was analyzed. For statistical analysis of metabolic activities of whole animals and for comparison of enzyme activities, one-way Anova was used. For the comparison of flux rates of the different gut sections, two-way Anova, followed by Holm–Sidak multiple comparison procedures or Kruskal–Wallis, One-Way analysis of variance on ranks was used. In rare cases, the normality test or equal variance test failed using the original data. In this case, the data were log transformed prior to statistical analysis. The statistical analysis was performed using SigmaStat 12.0. Statistical differences between values were accepted for $p < 0.05$.

Results

In vivo experiments

Table 1 summarizes the data for body mass, fork length, gut length, and gut length to fork length ratio for the black Piranha (*Serrasalmus rhombeus*), Tambaqui (*Colossoma macropomum*), and Pacu (*Myleus lobatus*). The lowest gut/fork length ratio was recorded for the carnivorous black Piranha (1.07 ± 0.06), while the herbivorous Pacu had by far the highest value (2.62 ± 0.15). The omnivorous Tambaqui was intermediate (1.30 ± 0.04), and all values were significantly different from each other.

Plasma glucose concentration was highest in the Tambaqui (12.77 ± 1.00 mmol l^{-1}), about 2.7-fold greater than in the other two species (Table 2). In the herbivorous Pacu, plasma ammonia concentration was significantly lower than in the omnivorous Tambaqui with a value of 0.198 ± 0.063 mmol l^{-1}, compared to 0.757 ± 0.199 mmol l^{-1}, respectively. In the carnivorous black Piranha, an intermediate value was observed (Table 2).

The guts of the Tambaqui were always empty, whereas in some individuals of the other two species, the guts contained chyme. In black Piranha, mainly fin material was recognizable in the chyme, whereas in Pacu, seeds and plant matter predominated. Table 3 summarizes the mean concentrations of ions as well as of glucose and ammonia measured in these samples. Chyme glucose concentrations were 2.5 ± 0.4 mM in black Piranha, and 11.3 ± 5.4 mM in Pacu, while chyme ammonia concentrations were 5.7 ± 4.5 mM in black Piranha, and 10.7 mM were measured in a single chyme sample of Pacu. Chyme Na^+ concentrations were 160.8 ± 8.7 mM in black Piranha, and 188.2 ± 16.3 mM in Pacu, while chyme Cl^- concentrations were 138.6 ± 19.1 mM, in black Piranha, and 156.9 ± 45.9 mM in Pacu.

Table 2 Plasma glucose and ammonia concentrations in black Piranha (*Serrasalmus rhombeus*), Tambaqui (*Colossoma macropomum*), and Pacu (*Myleus lobatus*)

	Glucose (mmol l^{-1})	Ammonia (mmol l^{-1})
Black Piranha (6)	4.78 ± 0.70[a]	0.440 ± 0.038[a,b]
Tambaqui (8)	12.77 ± 1.00[b]	0.757 ± 0.199[a]
Pacu (6)	4.48 ± 0.86[a]	0.198 ± 0.063[b]

Number in brackets indicates the number of animals analyzed, and values not sharing the same letter are significantly different ($p < 0.05$)

Weight specific-oxygen consumption rate (Fig. 1a) of the Pacu (8,311 ± 740.7 μmol kg^{-1} h^{-1}) was significantly higher than that of the Tambaqui (4,637 ± 342.1 μmol kg^{-1} h^{-1}). Ammonia excretion rates were similar in all three species (Fig. 1b), but urea-N excretion rate in the Pacu (45.58 ± 8.84 μmol kg^{-1} h^{-1}) was significantly lower than in the black Piranha (155.7 ± 38.6 μmol kg^{-1} h^{-1}) (Fig. 1c). The *N*/*Q* ratio was significantly higher in the Tambaqui (0.227 ± 0.026), as compared to the other two species (Fig. 1d). The lowest N/Q ratio was calculated for the Pacu with 0.100 ± 0.017.

In vitro experiments

Gut sac preparations were used to analyze the transport properties of the different gut sections. Figure 2 summarizes the Na^+ and Cl^- concentrations of saline solutions measured at the beginning of the incubation and at the end of the incubation time in the three species and the three different gut sections tested for each species. In all of the preparations, the Na^+ and Cl^- concentrations of the intestinal fluid remained constant over the course of the incubation. However, the volume of the intestinal fluid decreased significantly as fluid was absorbed from mucosal to serosal compartments. In Pacu, no difference in area-specific fluid flux rate of the three gut sections could be detected, but in Tambaqui, fluid flux of the anterior section was significantly higher than the area-specific flux rate recorded in the mid and posterior gut sections (Fig. 3c). In black Piranha, fluid flux measured in the mid gut section was significantly higher than the flux rate recorded in the posterior section.

In all three species, the general pattern of Na^+ fluxes calculated for the three gut sections basically followed the patterns of the fluid fluxes, but there were no significant

Table 3 Concentrations of glucose, ammonia, Na^+ and Cl^- measured in chyme samples collected from the anterior gut of black Piranha (*Serrasalmus rhombeus*) and Pacu (*Myleus lobatus*)

	Black Piranha		Pacu	
	Mean ± se	Range	Mean ± se	Range
Glucose (mM)	2.5 ± 0.4 (4)	2.0–3.8	11.3 ± 5.4 (5)	3.2–32.6
Ammonia (mM)	5.7 ± 4.5 (4)	0.4–19.1	10.7 (1)	
Na^+ (mM)	160.8 ± 8.7 (3)	144.9–175.0	188.2 ± 16.3 (6)	150.0–258.3
Cl^- (mM)	138.6 ± 19.1 (3)	100.6–160.9	156.9 ± 45.9 (6)	58.0–361.9

The gut of Tambaqui (*Colossoma macropomum*) was always empty, so that no samples could be taken. Numbers in brackets indicate number of samples analyzed

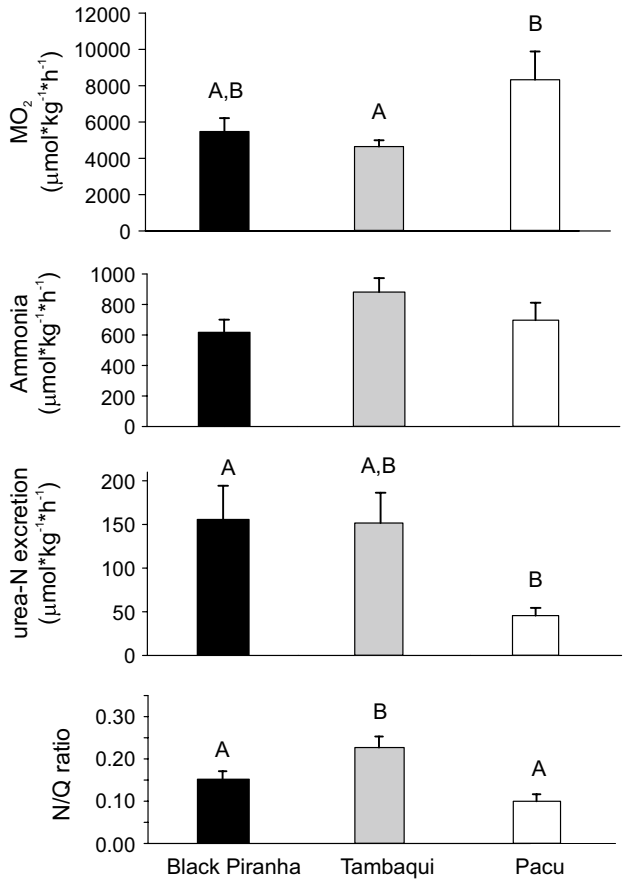

Fig. 1 Weight-specific oxygen consumption, ammonia excretion, urea-N excretion, and the ratio of total nitrogen excretion over oxygen consumption (*N*/*Q* ratio) of the black Piranha ($N = 7$), the Tambaqui ($N = 7$), and the Pacu ($N = 6$) measured in Rio Negro 'black water' at a temperature of 30–32 °C. *Bars sharing the same capital letter* are not different from each other ($p > 0.05$). *Absence of a letter* indicates no significant difference

differences between the flux rates in the different sections (Fig. 3a). The same was true for Cl^- fluxes in black Piranha and Pacu. Only in Tambaqui area-specific Cl^- flux rates in the anterior section were significantly higher than in the middle section (Fig. 3b).

While Na^+ and Cl^- concentrations remained unchanged in internal (intestinal) fluid of the gut sac preparations, glucose and ammonia concentrations severely decreased (Fig. 4a). Glucose concentration of the saline initially was close to 5 mmol l^{-1}, and within the 1-h incubation, about 70–80 % of the glucose was removed from the intestinal fluid. Ammonia concentrations were set to about 10 mmol l^{-1} and declined even more (Fig. 4b). In Tambaqui, in particular, ammonia concentrations were reduced by more than 90 % within 1 h of incubation. Note that while ammonia was moving down a concentration gradient from mucosal to serosal compartments, glucose was being removed against a gradient. Area-specific glucose uptake rates calculated from these data in the Tambaqui were significantly lower than in black Piranha and Pacu (Fig. 5a). A comparison of the area-specific glucose flux rates of the three different gut sections within a species did not reveal any difference at all. The same was observed for area-specific ammonia flux rates. There was no difference in the flux rates of the three different gut sections of a species, and there was no difference in the area-specific flux rates calculated for the three different species (Fig. 5b).

Ammonia concentration was also measured in the external (i.e., serosal) fluid. Therefore, the area-specific ammonia flux rate to the serosal side (Fig. 5b) could be calculated and compared with the area-specific flux rate calculated for the mucosal side (Fig. 5c). For all three species, the area-specific ammonia flux rates to the serosal side were similar to the flux rates calculated for the mucosal side. A direct comparison revealed no difference in these flux rates, so that the amount of ammonia removed from the internal fluid was not significantly different from the amount of ammonia that appeared on the serosal side of the gut sacs in all three species and in all three sites of the gut. Consequently, the metabolic activity of the gut tissue did not release a significant amount of ammonia to the serosal side, nor did it use some of the ammonia removed from the gut interior for metabolism (Fig. 6).

To account for the difference in total absorptive area associated with the different trophic position of the three

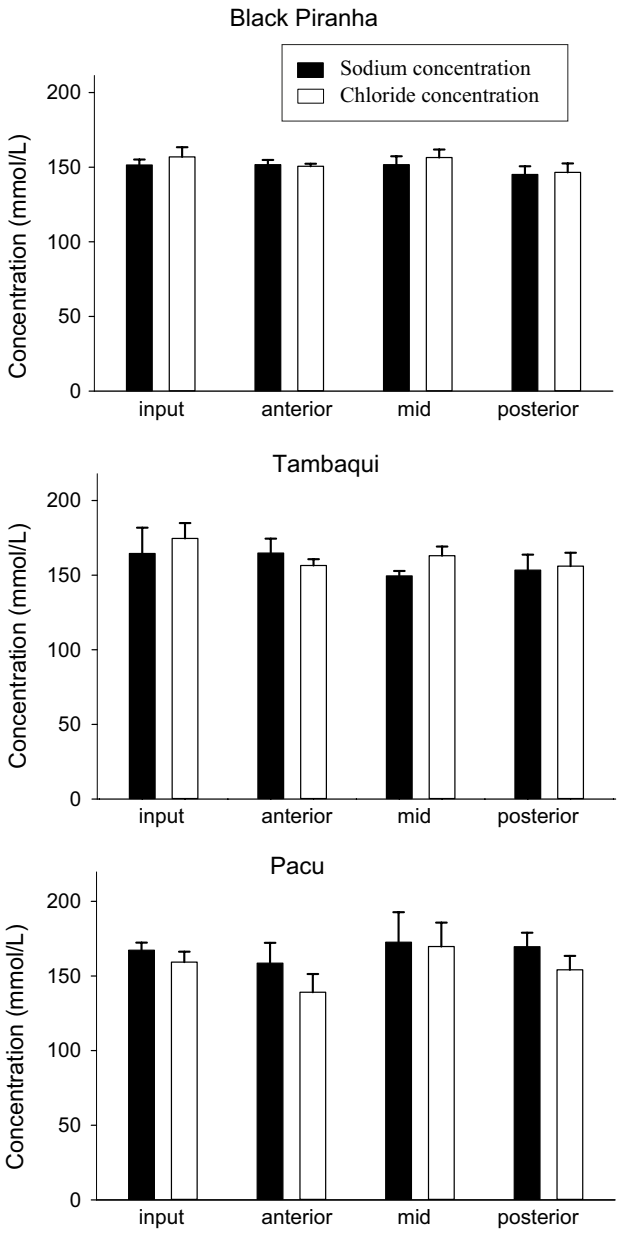

Fig. 2 Na^+ and Cl^- concentrations of saline solutions measured at the beginning of the incubation (input) and at the end of the incubation time in anterior, mid, and posterior gut sac preparations of the black Piranha, the Tambaqui, and the Pacu. $N = 6$; no significant differences between initial and final ion concentrations were detected ($p > 0.05$)

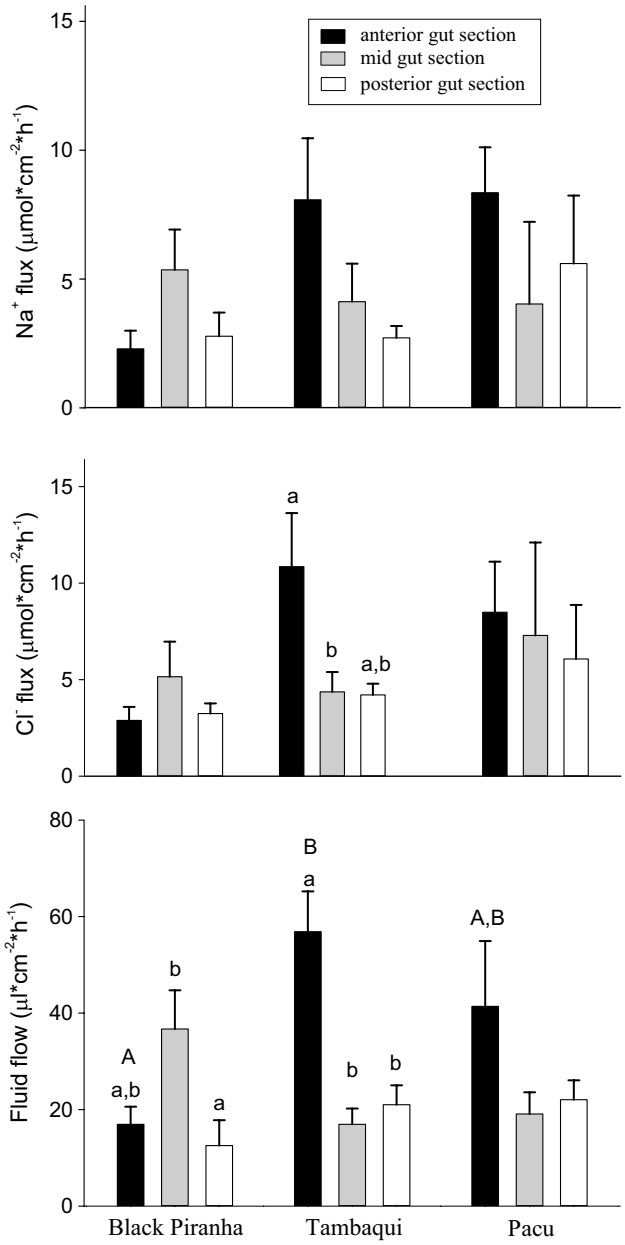

Fig. 3 Na^+ and Cl^--flux rates calculated per cm^2 h^1 and the fluid flow in anterior, mid, and posterior gut sac preparations of the black Piranha, the Tambaqui, and the Pacu. Na^+ and Cl^- flux: $N = 6$. Fluid flux: BP, $N = 7$; T, $N = 12$; P, $N = 6$. *Bars not sharing the same letter* are different from each other ($p < 0.05$), *capital letters* denote section differences between species, and *lower-case letters* denote differences between different sections of one species. *Absence of a letter* indicates no significant difference

species, the lengths of the anterior, middle, and posterior gut sections and the areas of these sections were measured (Table 4). While the differences in gut length between the herbivorous Pacu and the other two species were not so obvious when comparing the different sections of the gut of the three species, massive differences became apparent when the length of the three sections was normalized to body mass. Calculated per kg body mass, the gut sections of the herbivorous Pacu were about 8.5–10 times longer than the sections of the carnivorous black Piranha, and 2–4 times longer than the gut sections of the Tambaqui (Table 4). Surprisingly, the surface area per cm of gut was largest in the black Piranha (Table 4), meaning that the diameter of the gut of the black Piranha was significantly greater than the diameter of the gut of the Pacu.

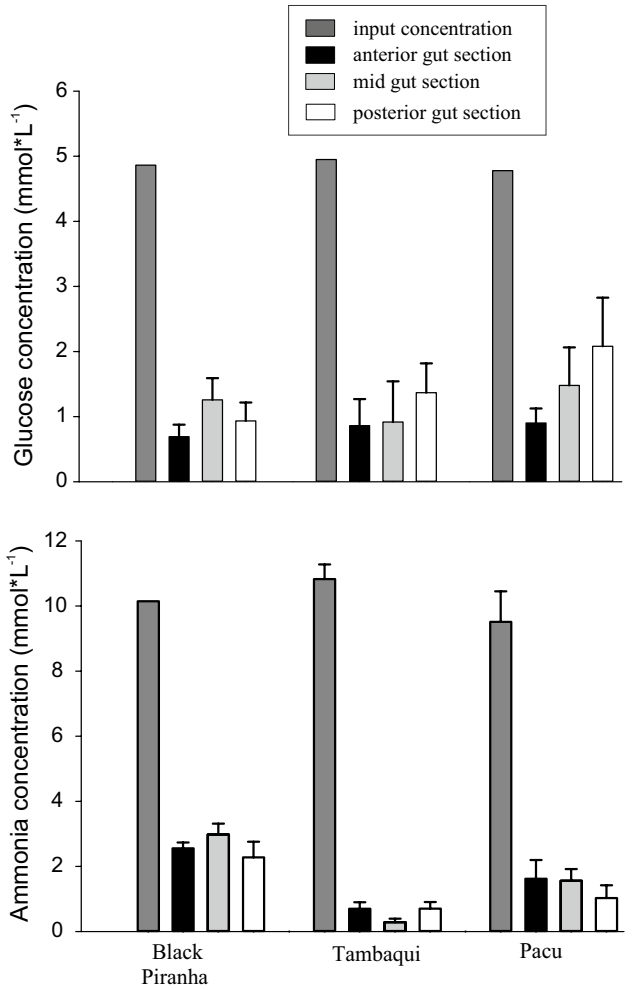

Fig. 4 Glucose and ammonia concentrations of saline solutions measured at the beginning of the incubation (input) and at the end of the incubation time in anterior, mid, and posterior gut sac preparations of the black Piranha, the Tambaqui, and the Pacu. Glucose, $N = 6$; ammonia, $N = 7$ for BP and T, $N = 6$ for P. *Absence of a letter* indicates no significant difference

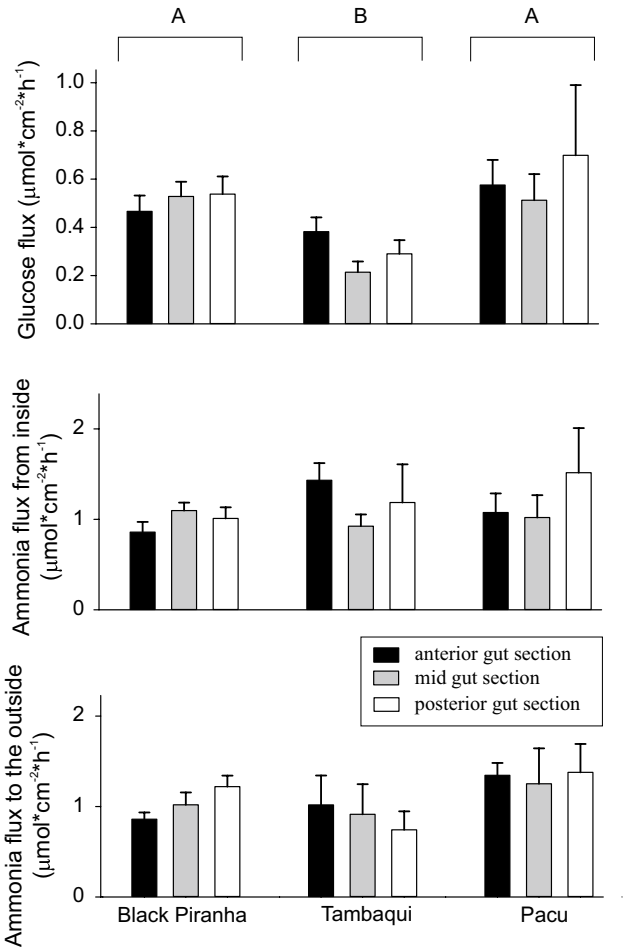

Fig. 5 Glucose and ammonia flux rates calculated per cm^2 per h in anterior, mid, and posterior gut sac preparations of the black Piranha, the Tambaqui, and the Pacu. Glucose, $N = 6$ for BP and P; $N = 12$ for T; ammonia, $N = 7$ for BP and T; $N = 6$ for P. *Bars not sharing the same letter* are significantly different from each other ($p < 0.05$). *Capital letters* denote differences between species, and absence of a letter indicates no significant difference

Based on these data, the transport capacity of the whole gut normalized to body mass could be calculated (transport capacity per kg fish). Transport capacities for fluid, Na^+, and Cl^- showed a very similar pattern for all three species. Na^+ transport capacity of Pacu with a value of 1,714 ± 786 µmol kg^{-1} h^{-1} significantly exceeded the value calculated for Tambaqui (384 ± 98 µmol kg^{-1} h^{-1}), which in turn was significantly higher than the Na^+ transport capacity in black Piranha (204 ± 53 µmol kg^{-1} h^{-1}) (Fig. 7a). Very similar results were obtained for Cl^- transport capacity (Fig. 7b). Cl^- transport capacity in Pacu was more than 4 times higher than in Tambaqui and more than 10 times higher than in black Piranha. The transport capacities for Na^+ and Cl^- in turn were reflective of the total fluid transport capacity calculated for the

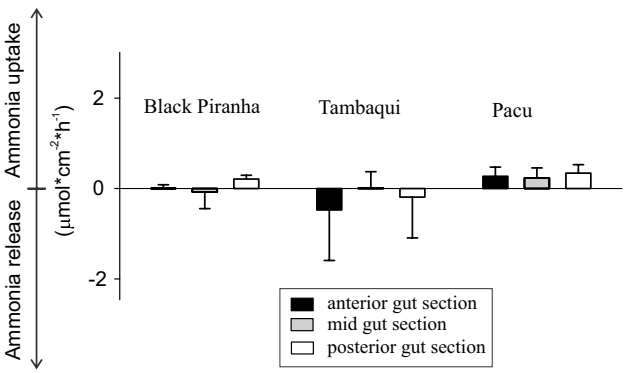

Fig. 6 Ammonia release and ammonia uptake rates of the gut tissues calculated from the differences in ammonia absorption at the mucosal side and the ammonia release at the serosal side. None of the obtained uptake and release values was significantly different from zero ($p > 0.05$)

Table 4 Total length of anterior, middle, and posterior gut sections (cm), length of the gut sections normalized to body mass (cm kg^{-1}), and the area per cm gut length (cm^2 cm^{-1}) in black Piranha (*Serrasalmus rhombeus*), Tambaqui (*Colossoma macropomum*), and Pacu (*Myleus lobatus*)

	N-value	Anterior			Middle			posterior		
		Length	Length/kg	Area/cm	Length	Length/kg	Area/cm^2	Length	Length/kg	Area/cm
Black Piranha	7	$8.07 \pm 0.56^{a,A}$	$15.5 \pm 1.5^{a,A}$	$1.14 \pm 0.10^{a,A}$	$11.96 \pm 0.82^{b,A}$	$23.0 \pm 1.5^{b,A}$	$1.00 \pm 0.05^{a,A}$	$9.86 \pm 0.67^{c,A}$	$19.0 \pm 1.3^{a,b,A}$	$0.78 \pm 0.03^{b,A}$
Tambaqui	17	$6.19 \pm 0.22^{a,B}$	$54.3 \pm 4.4^{a,B}$	0.48 ± 0.02^{B}	$7.11 \pm 0.25^{a,B}$	$62.4 \pm 5.1^{a,B}$	0.41 ± 0.03^{B}	$9.63 \pm 0.34^{b,A}$	$84.5 \pm 6.9^{b,B}$	0.43 ± 0.03^{B}
Pacu	6	$9.63 \pm 0.59^{a,A}$	$134.7 \pm 36.4^{a,C}$	0.58 ± 0.06^{B}	$16.94 \pm 1.04^{b,C}$	$237.0 \pm 64.0^{b,C}$	0.56 ± 0.07^{C}	$11.93 \pm 0.73^{c,B}$	$167.0 \pm 45.1^{a,b,C}$	0.58 ± 0.09^{C}

Values not sharing the same letter are significantly different ($p < 0.05$); small letters indicate significant differences within one species; capital letters indicate significant differences between species

three species (Fig. 7c). In Pacu, a fluid transport capacity of 7.59 ± 2.17 ml kg^{-1} h^{-1} was calculated, which by far exceeded the fluid transport capacity of Tambaqui with 2.06 ± 0.20 ml kg^{-1} h^{-1} and of black Piranha with 1.06 ± 0.29 ml kg^{-1} h^{-1}, and all values were significantly different from each other.

A comparison of the Na^+ and Cl^- transport capacities calculated for the different gut sections within a species revealed no difference at all between the sections (one-way ANOVA, data not shown). The pattern obtained for the fluid transport capacity within a species (suppl. data Fig. 1) was similar to the one obtained for the area-specific fluxes (cf. Fig. 3c). In black Piranha, the highest area-specific fluid flux was calculated for the mid gut section (suppl. data Fig. 1a), while in Tambaqui, the anterior section had the highest fluid flux rate (suppl. data Fig. 1b). In Pacu, no difference between the different gut sections was detected (suppl. data Fig. 1c).

As expected based on its longer gut, the herbivorous Pacu also showed a significantly higher whole gut ammonia transport capacity (Fig. 8a) than the other two species. Whole gut ammonia transport capacity amounted to 302.7 ± 75.9 μmol kg^{-1} h^{-1} in Pacu, which was about three times higher than in Tambaqui with 102.6 ± 25.96 μmol kg^{-1} h^{-1}, and about 5 times higher than in black Piranha with 58.0 ± 7.6 μmol kg^{-1} h^{-1}. Whole gut glucose transport capacity in turn was not different between black Piranha and Tambaqui (Fig. 8b). Compared to these two species, whole gut glucose transport capacity in Pacu was more than 5 times higher with a value of 153.5 ± 30.8 μmol kg^{-1} h^{-1}.

Activities of enzymes involved in N-fixation were determined in tissue samples taken from the anterior gut section of all three species (Fig. 9). In the carnivorous black Piranha, GDH and GOT activities were significantly higher than in the herbivorous Pacu, with the latter value being similar to that in the omnivorous Tambaqui. Glutamate–pyruvate transferase (GPT) activity was also highest in the black Piranha, but this difference was not significant. The comparison of enzyme activities of Tambaqui and Pacu revealed no significant differences.

Discussion

Nitrogen excretion and metabolic activity

Analysis of metabolic rate and rates of ammonia and urea-N excretion of the three different species of characid fish from the Rio Negro revealed overall N excretion rates comparable to those previously reported for unfed marble goby *Oxyeleotris marmorata* (Tng et al. 2008), and similar to rainbow trout when differences in acclimatization

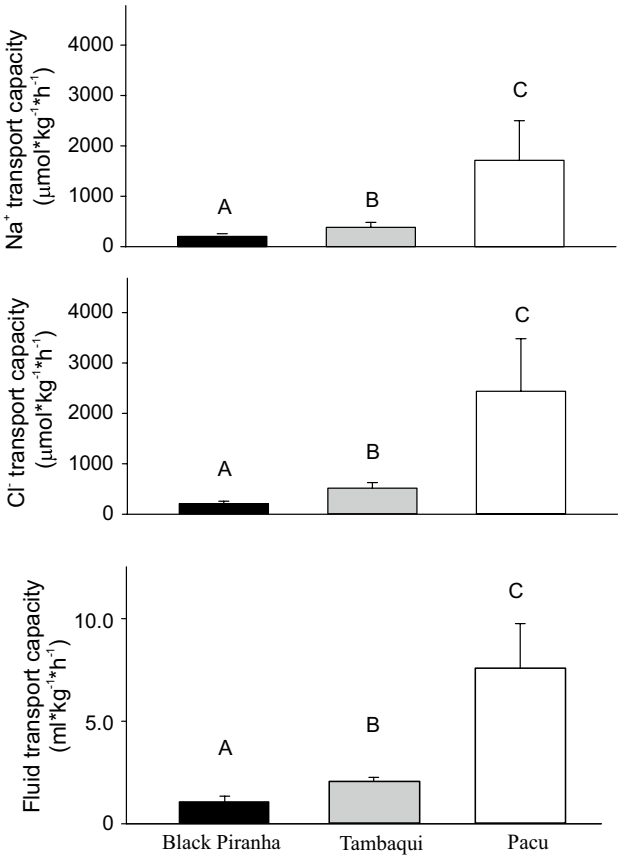

Fig. 7 Na^+ and Cl^- transport capacity as well as the fluid transport capacity of the whole gut of a 1 kg fish within 1 h. Na^+ and Cl^- transport capacity: $N = 5$ for T and P; $N = 6$ for BP; Fluid transport capacity: BP and P, $N = 6$; T, $N = 9$. *Bars not sharing the same letter* are significantly different from each other ($p < 0.05$)

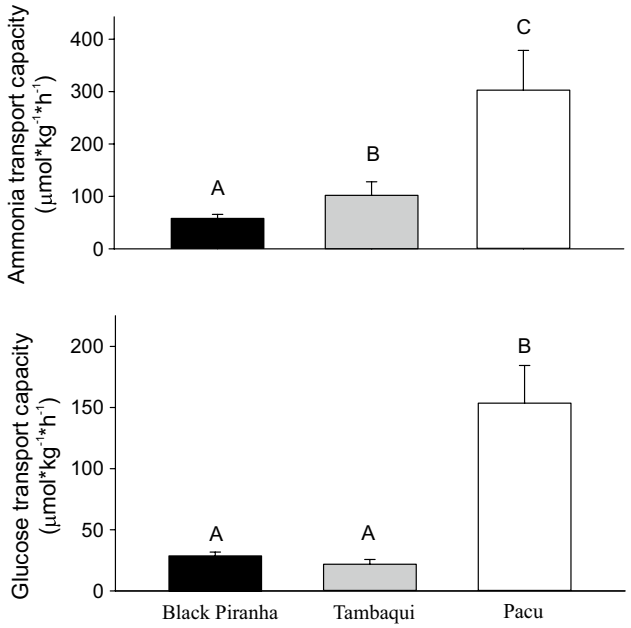

Fig. 8 Glucose and ammonia transport capacity of the whole gut of a 1 kg fish within 1 h. Ammonia transport capacity: $N = 6$ for BP and P; $N = 5$ for T. *Bars not sharing the same letter* are significantly different from each other ($p < 0.05$)

temperature are taken into account (assuming $Q_{10} = 2$) (Wilson et al. 1994). For the Oscar, another Amazonian species, significantly lower ammonia excretion rates have been reported, although the oxygen consumption rates were comparable to our values (DeBoeck et al. 2013).

In this study, the highest rate of oxygen consumption was recorded for the herbivore Pacu, significantly higher than oxygen consumption of the Tambaqui. The Pacu was the smallest fish among the three species, and its body mass was significantly lower than body mass of the black Piranha, but was not significantly lower than the body mass of the omnivorous Tambaqui. Therefore, this difference in oxygen consumption cannot be attributed to differences in body mass. While the ammonia excretion rate was similar in all three species, urea-N excretion rate was lowest in the Pacu, and it was significantly lower than in the carnivore black Piranha. Accordingly, compared to the Tambaqui, the *N*/*Q* ratio was significantly lower in the Pacu, suggesting that in the herbivorous Pacu, protein use as a fuel was much lower than in the other two species (Lauff and Wood 1996).

This observation also supported the hypothesis of N-limitation in the herbivore species, although data were not as clear cut as expected.

Analysis of gut performance

In recent years, the role of the gut for osmotic and ionic homeostasis in fish has gained increasing attention, but in particular for freshwater fish, many aspects remain unresolved so far. Analysis of gut function is complicated by the fact that after ingestion of food, secretory activity of the stomach as well as of the various intestinal sections and their associated digestive glands (particularly in the anterior section) increases greatly. These secretions add ions, fluid, and digestive enzymes to the food, while at the same time, there is also reabsorption of ions and fluid. In fact, several studies have demonstrated that in fish, all intestinal segments absorb nutrients (Bucking and Wood 2006b; Dabrowski 1986; Ferraris and Ahearn 1984; Smith 1969). To avoid this difficulty and to focus on the absorbing capacity of the intestine, we used isolated gut sac preparations. Artificial saline including glucose (5 mM) and ammonia (10 mM) was used as the internal (mucosal) fluid. Determination of ammonia concentration in the chyme revealed concentrations up to 10.7 mM in Pacu and up to 19.7 mM in the black Piranha, which is high compared with levels of up to 2 mM reported in trout and spiny dogfish (Bucking and Wood 2012; Wood et al. 2009). Similarly, glucose

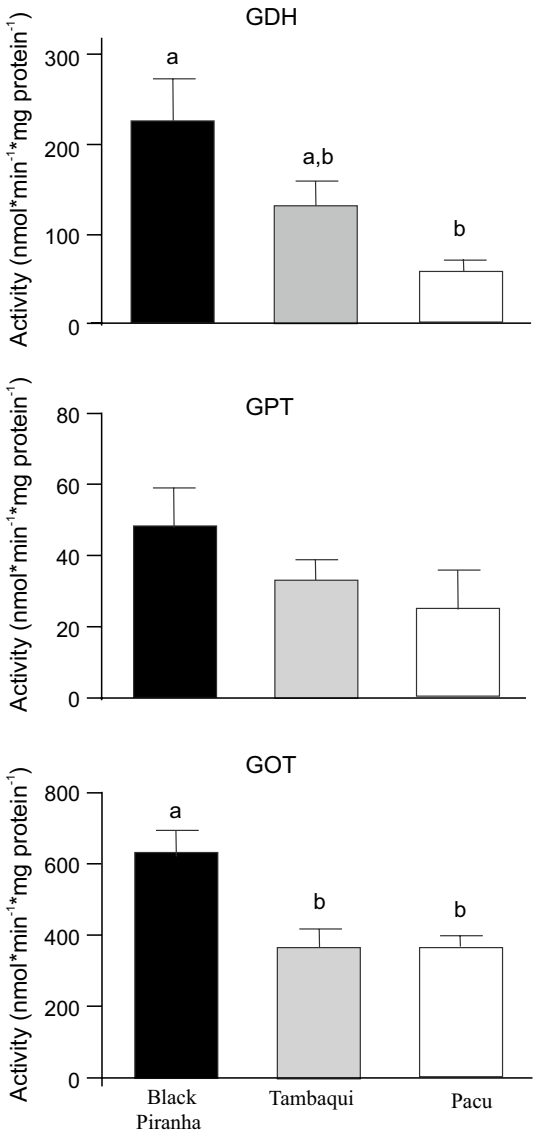

Fig. 9 Enzyme activities measured in homogenates of anterior gut sections of the black Piranha ($N = 8$), the Tambaqui ($N = 6$) and the Pacu ($N = 8$) measured at 33 ± 0.1 °C. *Bars not sharing the same letter* are significantly different from each other ($p < 0.05$). *Absence of a letter* indicates no significant difference

concentrations measured in chyme samples were in the mM range. In fish caught from the wild, the actual concentrations of these metabolites in the gut certainly are highly variable depending on the time of last feeding and the digestive activity (Day et al. 2014), but the values recorded confirm that our experimental conditions were within the physiological range of concentrations to be expected. This extends also to the ion concentration using isosmotic saline, because after water is ingested in freshwater fish, its composition in the gut is rapidly adjusted to the composition found in body fluids (Scott et al. 2006), as indicated by the present measurements (130–180 mM) of Na^+ and Cl^- in the chyme of wild fish eating natural foods, although in trout, the chyme remained hyperosmotic for many hours after a meal of dry, commercial pellets (Bucking and Wood 2006b).

In black Piranha and in Pacu, plasma ammonia concentration was with 0.44 and 0.20 mM at a level to be expected for resting fish (Bucking and Wood 2012; Karlsson et al. 2006; Wood et al. 1998, 2009) and much lower than the values measured in the chyme of fish. In trout and dogfish, ammonia concentrations in the chyme also by far exceeded the values recorded in plasma (Bucking and Wood 2012; Wood et al. 2009).

Plasma glucose concentration was just below 5 mM in black Piranha and Pacu, but in Tambaqui, plasma glucose concentration was higher than expected. Wood et al. (1998) observed an increase in blood glucose concentration of Tambaqui in acidic water at pH 3, and this was interpreted as a stress response. In fish, stress results not only in an elevated level of stress hormones such as catecholamines and cortisol, but also in an increase in blood glucose concentrations (Iwama 2006; Mommsen et al. 1999). Our fish, however, were kept at pH 4 for several days, and in the study of Wood et al. (1998), incubation at pH 4 did not result in an increase of blood glucose concentration or of the cortisol level, a typical stress indicator. Tambaqui used in this study was farmed fish, and they were transferred from the farm to the lab 10 days prior to the start of the field excursion and then kept on boat for up to 12 days. Incubations on board started 4–5 days after departure, and therefore, the time for acclimation should have been sufficient for the fish to settle to the new environment. Nevertheless, it cannot be excluded that this contributed to the elevated glucose levels measured in plasma of Tambaqui, but it does not appear very likely. Therefore, the reason for the elevated plasma glucose concentration in the Tambaqui is not obvious.

Absorption of ions and fluid

The guts of all three species effectively resorbed Na^+ and Cl^- from the mucosal fluid, and both ions were resorbed at a similar rate. Similarly, in rainbow trout, Na^+, K^+, and Cl^- are absorbed from the chyme on a net basis (Bucking and Wood 2006a). In killifish, however, Cl^- was taken up from the diet at higher rates than Na^+, and this was especially pronounced in freshwater killifish (Bucking et al. 2013b). In a number of fish, Cl^- uptake at the gills is lower than Na^+ uptake, and in some species, active Cl^- uptake in the gills appears to be missing (Tomasso and Grosell 2004). This suggests that in fish with a low capacity for Cl^- uptake at the gills, this is compensated by an elevated capacity of the gut to resorb Cl^- from the chyme. Following these considerations, we conclude that in the three species analyzed in the present study, Cl^- uptake at the gills is established at a

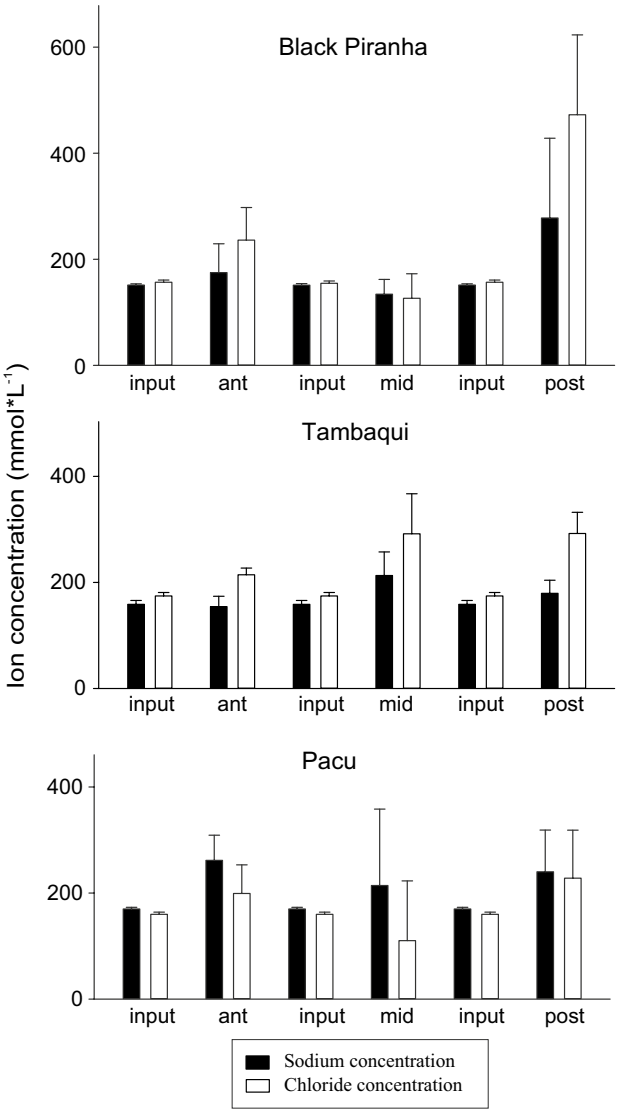

Fig. 10 Ion concentration in the fluid removed from the gut interior as calculated from the ion flux rates divided by the fluid flux in the different sections of the gut in the black Piranha, the Tambaqui, and the Pacu plotted together with the respective concentrations of the input fluid at the start of the incubation. $N = 6$; no significant differences between concentration of the input fluid and the concentration of the fluid removed from the different gut sections in any of the three species were detected (t test, $p > 0.05$)

similar rate than Na^+ uptake, so that there is no need for a compensation of a possible disequilibrium in the gut.

For killifish intestine, a linear correlation between bulk water absorption and net strong ion ($Na^+ + Cl^-$) absorption has been shown (Scott et al. 2006), indicating that the ion uptake in the gut of freshwater fish is isosmotic. Osmotic water transport has also been demonstrated for the gut of eel and trout (Boge et al. 1988; Bucking and Wood 2006b; Skadhauge 1974), and it is assumed that in most freshwater fish, fluid absorption in the gut is isosmotic (Wilson 2011). We hypothesized that in the special situation of very acidic water combined with extremely low ion content, the gut may be able to take up ions with reduced water flow, as seen for Cl^- absorption in freshwater killifish (versus seawater killifish), especially after long-term fasting (Wood et al. 2010). However, this was not the case. In all three species, Na^+ and Cl^- were effectively removed from the intestinal fluid in all three sections of the gut, but this ion uptake closely mimicked the absorption of fluid (Fig. 2), suggesting that the absorption of fluid was isosmotic. To verify this, we used the ion flux rates divided by the fluid flux to calculate to concentration of the fluid removed from the gut sacs (Fig. 10). The results show that the Na^+ and Cl^- concentrations of the fluid removed were always close to the input concentration, and no significant difference for any of these values could be detected.

Water flux in the different gut sections typically was about 20–40 $\mu l\ cm^{-2}\ h^{-1}$ in all three species, with the highest rates in the anterior section (Tambaqui and Pacu) or in the middle section (black Piranha). In comparison, water flux is only 4 $\mu l\ cm^{-2}\ h^{-1}$ in freshwater trout (Nadella et al. 2006, 2007) and 4–12 $\mu l\ cm^{-2}\ h^{-1}$ in freshwater killifish (Scott et al. 2006; Wood et al. 2010; Wood and Grosell 2012). The latter data are consistent with a lower Na^+ flux rate, recorded in freshwater killifish. Intestinal Na^+ influx 12 h after freshwater transfer amounted to 4.7 $\mu l\ cm^{-2}\ h^{-1}$, which is very close to the values recorded in the present study. However, after 3 days in freshwater, the Na^+ and Cl^- flux rates decreased to less than half (Scott et al. 2006), and Wood and Grosell (2012) reported an absorptive Cl^- flux of 1.8 $\mu mol\ cm^{-2}\ h^{-1}$ in this species. Because our fish originated from Rio Negro or were acclimated to the Rio Negro water for more than a week, they are most comparable with the lower values of freshwater-acclimated killifish. Thus, the Na^+ and Cl^- flux rates measured in black Piranha, Tambaqui, and Pacu are about 2–3 times higher than the values recorded in acclimated killifish. In agreement with our hypothesis, we conclude that intestinal net Na^+ and Cl^- transport rates are relatively high in our study species that live in the ion-poor, acidic Rio Negro water, where branchial ion uptake is very challenging.

Interestingly, although fluid absorption was, depending on the species, slightly elevated in the anterior (Tambaqui) or the middle (black Piranha) section, the overall picture suggests that there are no striking differences with respect to Na^+, Cl^-, and fluid fluxes between the three gut sections. Thus, although anatomically the three sections can be clearly identified, the functional separation with respect to ion and fluid absorption appears to be limited. On the other hand, based on the distribution of ion transport proteins (Grosell 2007) or on enzyme activities (Mommsen et al. 2003), a functional zonation of the gut has been postulated previously. However, because Na^+, for example,

may be transported by multiple carrier proteins, it may well be that the involvement of different carrier proteins obscures this regional zonation. A recent study on Na^+ uptake mechanisms in the different gut sections of rainbow trout indeed showed that Na^+ uptake rates were similar in anterior, mid, and posterior gut sections, but pharmacological interference with different carrier proteins revealed that $Na^+/K^+/2Cl^-$-cotransport could be inhibited in the anterior section, but not in mid and posterior sections. Na^+/Cl^--cotransport in turn could be inhibited in mid and posterior sections, but not in the anterior section (Nadella et al. 2014). Alternatively, in fish exposed to the extreme situation of ion-poor and acidic Rio Negro water, this zonation may not be as pronounced as it was observed in trout or tilapia, for example.

Absorption of ammonia and glucose

The guts of all three species very effectively took up glucose and ammonia, and within the 1-h incubation, about 80 % of the glucose and even up to 90 % of the ammonia (Tambaqui) were absorbed in all gut sections. As observed for the uptake of Na^+ and Cl^-, there was no difference in the area-specific uptake rate between the three gut sections. In the herbivorous grass carp *Ctenopharyngodon idella* and in the omnivorous channel catfish *Ictalurus puctatus*, glucose uptake per mg gut tissue appeared to be higher in the proximal part of the gut compared to the distal part, but unfortunately, the actual surface area of the 1 cm gut sleeves used for this study was not reported, and the difference was not statistically verified (Day et al. 2014), which complicates a comparison with our data set.

Ammonia flux rates (starting with a luminal concentration of 10 mM) were close to 1 $\mu mol\ cm^{-2}\ h^{-1}$ for all three species and all gut sections tested, which are about 4–5 times higher than the ammonia fluxes recorded in freshwater rainbow trout at a luminal concentration of 1 $mmol\ l^{-1}$ (Rubino et al. 2014). Although in trout, the ammonia flux appears to increase at higher luminal ammonia concentrations, our data suggest that the ammonia flux in the Rio Negro fish is higher than in trout. As already observed with respect to ion movements, we could not detect significant differences in the ammonia flux rates of the three different gut sections, and this is in line with the data reported for trout, where slightly lower flux rates were determined only for the posterior gut section (Rubino et al. 2014).

Concomitant with the removal of ammonia from the luminal fluid, we found the release of ammonia to the serosal side. The comparison of these two values revealed that all the ammonia taken up from the gut interior was effectively transferred to the serosal side. Accordingly, the metabolism of gut tissue itself neither released additional ammonia from internal protein degradation, nor did it consume or detoxify ammonia taken up from the gut. Following a 2-h incubation, a low rate of ammonia release in the range of 0.05–0.1 $\mu mol\ cm^{-2}\ h^{-1}$ has been observed in trout gut sac preparations, and the rate of ammonia production by the tissue was dependent on the feeding status or the ammonia concentration on the mucosal side (Rubino et al. 2014). Using a 3-h incubation, ammonia production at rates below 0.05 $\mu mol\ cm^{-2}\ h^{-1}$ has been reported for gut tissue of fasted plainfin midshipman *Porichthys notatus* (Bucking et al. 2013a). The gut epithelium is known for its high turn-over rate, and therefore, a low rate of N-release is expected, while high rates of N-release may be a hint to tissue decay. For Tambaqui guts after 1 h of incubation, we also observed a release of ammonia from the tissue of anterior and posterior gut sacs at a very low rate, but this was not significantly different from zero. Thus, with prolonged incubation times, we may have been able to quantify the metabolic activity of the gut tissue itself. Nevertheless, the low rate of ammonia production in our experiments clearly indicated that the incubation conditions were adequate, and the tissue was in good condition.

On the other hand, high ammonia concentrations impair cell metabolism, and a luminal concentration of 2 mM in the gut has been considered as potentially toxic on a local scale (Bucking et al. 2013a). Accordingly, in the plainfin midshipman at high luminal ammonia concentrations, ammonia entered the enterocytes and was partially converted to urea for detoxification via the ornithine-urea cycle (Bucking et al. 2013a). In trout, however, this detoxification reaction could not be initiated in enterocytes (Bucking et al. 2013a), and our data suggest that in the three Rio Negro species analyzed in our study, this detoxification reaction also does not occur. Ammonia taken up at the mucosal side was completely transferred to the serosal side of the gut tissue.

This also suggests that ammonia taken up by the enterocytes is not immediately exploited for amino acid metabolism. Dietary protein is a major source of amino acids in fish, and in the intestine of juvenile marble goby *Oxyeleotris marmorata*, glutamate appears to be the major amino acid accumulated in the gut and liver after feeding, associated with an increase in GDH activity in the intestine (Tng et al. 2008). Karlsson et al. (2006) proposed that in rainbow trout intestine, amino acids are deaminated for N-retention and somatic growth. In line with these data in our study, the highest GDH activity has been found in the anterior gut samples of the carnivorous black Piranha, and consistent with this observation, the activities of the two transaminases GOT and GPT, which connect additional amino acids (aspartate and alanine, respectively,) to glutamate metabolism, were highest in this species. Nevertheless, in this species as well as in the other two, no ammonia retention in the gut epithelium was detected: ammonia taken up from

the intestinal fluid was quantitatively transferred to the serosal side. This suggests that in the carnivorous black Piranha, the capacity for an active conversion of amino acids for anabolic metabolism is highest, but it is not activated if just ammonia is absorbed.

Given the large concentration gradient between the luminal and serosal side in our setup, diffusion along the concentration gradient probably contributed to this transfer (Ip and Chew 2010), but the rapid and almost complete removal of ammonia from the mucosal side suggests that transport via Rhesus glycoproteins is also involved (Weihrauch et al. 2009; Wright and Wood 2009; Bucking and Wood 2012), and that in these species, high ammonia concentrations do not harm the gut tissue.

Overall transport capacities of the gut

An unexpected result of our study was that the flux rates of Na^+ and Cl^- as well as of glucose and ammonia were similar between the carnivore and the herbivore, when considered on the basis of transport rate per cm^2 of gut. Because ion and also metabolite transport depends on the presence of specific channels or carrier proteins, we conclude that the density of these channels and carriers is similar in all three species. However, the overall length of the gut and thus gut to fork length ratio differed significantly, with the herbivorous Pacu having the by far longest gut. Consequently, with respect to the overall transport capacity of the gut, i.e., with respect to the transport capacity per kg fish, the Pacu far exceeds the omnivore Tambaqui and the carnivore black Piranha for all of the moieties assessed. Accordingly, in order to cope with the more difficult digestible plant material requiring microbial fermentation, and the very low Na^+, Cl^-, and nitrogen content of plants, herbivorous fish have not evolved more effective area-specific transport capacities, but rather have increased the length of the gut.

Conclusions

The results show that all three species effectively use the gut to absorb Na^+ and Cl^- isosmotically, supporting the notion that the gut significantly contributes to osmoregulation in these freshwater fish in an extremely ion-poor and acidic situation. All three species very effectively removed glucose as well as ammonia from the gut, and the flux rates of ions and metabolites expressed per cm^2 of gut tissue were not different between the three species. In all three species, ammonia taken up from the intestinal fluid was quantitatively transferred to the serosal side, indicating that the gut tissues itself did not use ammonia for immediate amino acid metabolism or for conversion of ammonia to urea in order to avoid any toxic effects of the high ammonia concentrations. Gut length to fork length ratio was much higher in the herbivorous Pacu as compared to the omnivorous Tambaqui and in particular the carnivorous black Piranha. Consequently, the overall ion and metabolite transport capacity of the gut of the Pacu expressed as $\mu mol\ kg^{-1}\ h^{-1}$ by far exceeded the transport capacity of the gut of the omnivorous Tambaqui and the carnivorous black Piranha. Accordingly, the lower digestibility and the low N-content of plants are compensated by increasing the length of the gut in herbivorous fish, not by increasing the area-specific transport capacity.

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