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REGULAR PAPER

Understanding the gastrointestinal physiology and responses to feeding in air-breathing Anabantiform fishes

Harriet R. Goodrich[1,2] | Mark Bayley[3] | Lina Birgersson[4] | William G. Davison[2] | Ora E. Johannsson[5] | Anne B. Kim[5] | Phuong Le My[6] | Tran H. Tinh[7] | Phuong N. Thanh[8] | Huong Do Thi Thanh[8] | Chris M. Wood[5]

[1]School of Biological Sciences, The University of Queensland, St Lucia, QLD, Australia

[2]College of Life and Environmental Sciences, The University of Exeter, Exeter, Devon, UK

[3]Department of Bioscience, Zoophysiology Aarhus University, Aarhus, Denmark

[4]Department of Biological and Environmental Sciences, University of Gothenburg, Göteborg, Sweden

[5]Department of Zoology, University of British Columbia, Vancouver, BC, Canada

[6]Department of Agriculture, Bac Lieu University, Bac Lieu, Vietnam

[7]Aquaculture and Fisheries, Department of Animal Sciences, Wageningen University and Research, Wageningen, The Netherlands

[8]College of Aquaculture and Fisheries, Can Tho University, Cần Thơ, Vietnam

Correspondence
Harriet R. Goodrich, School of Biological Sciences, The University of Queensland, St Lucia, QLD, Australia, 4067
Email: hg380@exeter.ac.uk

Abstract

The Mekong Delta is host to a large number of freshwater species, including a unique group of facultative air-breathing Anabantiforms. Of these, the striped snakehead (*Channa striata*), the climbing perch (*Anabas testudineus*), the giant gourami (*Osphronemus goramy*) and the snakeskin gourami (*Trichogaster pectoralis*) are major contributors to aquaculture production in Vietnam. The gastrointestinal responses to feeding in these four species are detailed here. Relative intestinal length was lowest in the snakehead, indicating carnivory, and 5.5-fold greater in the snakeskin, indicating herbivory; climbing perch and giant gourami were intermediate, indicating omnivory. N-waste excretion (ammonia-N + urea-N) was greatest in the carnivorous snakehead and least in the herbivorous snakeskin, whereas the opposite trend was observed for net K^+ excretion. Similarly, the more carnivorous species had a greater stomach acidity than the more herbivorous species. Measurements of acid–base flux to water indicated that the greatest postprandial alkaline tide occurred in the snakehead and a potential acidic tide in the snakeskin. Additional findings of interest were high levels of both PCO_2 (up to 40 mmHg) and HCO_3^- (up to 33 mM) in the intestinal chyme of all four of these air-breathing species. Using *in vitro* gut sac preparations of the climbing perch, it was shown that the intestinal net absorption of fluid, Na^+ and HCO_3^- was upregulated by feeding but not net Cl^- uptake, glucose uptake or K^+ secretion. Upregulated net absorption of HCO_3^- suggests that the high chyme (HCO_3^-) does not result from secretion by the intestinal epithelium. The possibility of ventilatory control of PCO_2 to regulate postprandial acid–base balance in these air-breathing fish is discussed.

KEYWORDS

acid–base regulation, ammonia excretion, feeding, gut, ion regulation, urea excretion

1 | INTRODUCTION

Vietnam is the fourth-largest aquaculture producer in the world. In 2017, Vietnam contributed ~3.8 million tonnes to aquaculture production (FAO, 2019). A main source of these products (~70%) comes from the Mekong River Delta. The Mekong Delta is a vast network of rivers, swamps and islands in southern Vietnam that encompasses almost 40,500 sq. km and is home to more than 850 freshwater fish species (Hortle, 2009). Of these, four members of the order Anabantiformes, all of which are facultative air breathers, have

enormous commercial and economic value to Southeast Asia – the striped snakehead (*Channa striata*), the climbing perch (*Anabas testudineus*), the giant gourami (*Osphronemus goramy*) and the snakeskin gourami (*Trichogaster pectoralis*). Collectively these species contribute almost half a billion US dollars to aquaculture each year (FAO, 2019). Unlike more well-known, commercially important fishes such as salmonids, very little is known about their biology, ecology and physiology. Understanding the gastrointestinal physiology and responses to feeding of these species can provide key insights to enhance their productivity in aquaculture.

Feeding and digestion are energetically expensive processes that are usually facilitated by the upregulation of gastrointestinal performance after the consumption of a meal (Jobling, 1994; McCue, 2006). Generally, there is a rapid increase in both the secretory and absorptive functions of the stomach and intestine during feeding. These secretions are important to add ions, acidic and basic equivalents, fluid and digestive enzymes to food, whereas elevated absorptive processes take up nutrients and reabsorb ions and fluid (Bucking and Wood, 2006a; Marshall *et al*., 2002; Wood *et al*., 2010, Wood & Bucking, 2011). Nonetheless, gastrointestinal responses to feeding can vary substantially among species and with diet. For example, herbivorous fishes are often nitrogen limited and in turn have greater retention of dietary N and lower rates of N excretion than carnivorous species (Pelster *et al*., 2015). The absorption of N and other nutrients for growth by herbivorous species is facilitated by a comparatively longer gut, which aids in the digestion, breakdown and uptake of nutrients from fibrous food of lower energetic content (Day *et al*., 2014). In contrast, the greater concentration and digestibility of nutrients from a carnivorous diet (*e.g*., greater protein and fat content) mean that carnivores tend to have a comparatively shorter gut and higher N excretion.

Irrespective of diet, many vertebrates experience an acid–base disturbance known as the postprandial alkaline tide (increase in blood pH and HCO_3^- after feeding) after the consumption and digestion of a meal (Brunton, 1933; Wang *et al*., 2001). In fish with true stomachs, the alkaline tide is inherently linked to gastric acid secretion in the stomach (Wood *et al*., 2009). The activation of the acid-secreting cells that line the stomach leads to an equimolar efflux of HCO_3^- to the bloodstream and a resultant alkalosis in the systemic bloodstream (Kopic *et al*., 2009; Niv & Fraser, 2002; Wood *et al*., 2005). In fish, this activation is often associated with an efflux of basic equivalents (*i.e*., HCO_3^-) across the gills to the external environment so as to mitigate the extent of the alkalosis (Bucking & Wood, 2008; Cooper & Wilson, 2008; Wood *et al*., 2007).

It is likely that carnivorous species that process their protein-rich food quickly with more acidic stomachs and a larger gastric HCl secretion will experience a greater alkaline tide and a greater net base excretion after feeding. Nonetheless, not all fish experience an alkaline tide (Taylor *et al*., 2007; Taylor & Grosell, 2006), and at least one agastric teleost, the omnivorous killifish (*Fundulus heteroclitus*), which has an alkaline digestive system, experiences an acidic tide in the systemic bloodstream after feeding (Wood *et al*., 2010). No comparable information on herbivorous species is available. Similarly, in air-breathing fish, there appears to be no information on the nature of acid–base disturbances after feeding and the mechanisms used to correct them.

Therefore, the overall aim of this study was to gain insights into the gastrointestinal physiology and responses to feeding in air-breathing fishes from the Mekong Delta. Here, the first investigation of the acid–base, N-balance and ionoregulatory responses to fasting and feeding in these species and the acid–base conditions in their digestive tracts is presented. To facilitate comparison, all four species were fed the same ration of commercial pellets, and the relative length of the intestine ("gut index") was used to diagnose the degree of carnivory (short) *vs*. herbivory (long) of each species. Specifically we hypothesized that (a) N-waste excretion would be minimized in the most herbivorous species and maximized in the most carnivorous; (b) the post-feeding alkaline tide would be greatest (greatest net base excretion after feeding) in the most carnivorous and least in the most herbivorous species; (c) the most carnivorous species would have the most acidic stomach chyme after feeding and the most herbivorous species would have the least acidic stomach chyme after feeding; and (d) absorptive processes throughout the tract would be upregulated after feeding, a hypothesis that was tested in one species, the climbing perch, using *in vitro* gut sac preparations (Nadella *et al*., 2014; Pelster *et al*., 2015; Wood & Bucking, 2011).

2 | METHODS

2.1 | Ethical approval and animal husbandry

All applicable international, national and/or institutional guidelines for the care and use of animals were followed.

Experiments were conducted at the College of Aquaculture and Fisheries, Can Tho University, in December 2018. Four air-breathing species of the family Anabantiformes [*A. testudineus* (climbing perch) (N = 29; body mass: 293 ± 25 g; total length 23.1 ± 0.3 cm), *O. goramy* (giant gourami) (N = 23; 357 ± 14 g, 26.8 ± 0.8 cm), *T. pectoralis* (snakeskin) (N = 18; 150 ± 13 g, 19.2 ± 0.6 cm) and *C. striata* (snakehead) (N = 20; 222 ± 19 g, 30.4 ± 0.5 cm)] were obtained from commercial fish farms in Vietnam and housed at the College of Aquaculture and Fisheries at Can Tho University. Fish were housed in eight static 1000 l stock tanks: four fasting tanks and four feeding tanks. Measured mean water chemistry included pH 7.65, (Na^+) 269, (K^+) 61, (Cl^-) 363, (Ca^{2+}) 630 and titratable alkalinity (to pH 4.0) 1087 μmol l^{-1}. Water changes were conducted every second day. The tanks were located in a shaded area outdoors under natural photoperiod and temperature (32–36°C). The fish in the feeding tanks were fed a 1% ration of commercially sourced fish pellet feed each day, which consisted of 40% protein; 10% crude fat; 5% crude fibre; 11% moisture; and 4600, 135, 635 and 186 mM kg^{-1} of dietary N, Na^+, Cl^- and K^+, respectively (Stella S7, Tomboy Aquafeed JSC, Skretting, Vietnam, Ho Chi Min).

2.2 | Laboratory experiments

All experiments were performed indoors at ambient temperature (32°C–34°C).

2.2.1 | Net flux of ammonia, urea, titratable alkalinity and acid-base-relevant ions in fasted and fed fish

To determine the net fluxes between the fish and their external environment, fish were transferred to individual, aerated, static chambers containing fresh water. To ensure the most accurate measurement of fluxes of ions and metabolic wastes to water by fish, minimal volumes of water were used for each species and determined as the volume at which fish were completely covered by water and appeared comfortable. Therefore, the snakeskin gourami, striped snakehead, climbing perch and giant gourami were placed in 4, 5, 7 and 10 l of fresh water, respectively. Fasted fish had not been fed for 5–8 days (sufficient time for fluxes associated with the most recent meal to be concluded). Fed fish were transferred to the flux chambers 1 h after their daily feeding at 9–10 a.m. Water samples were collected every hour for 5 h for fasted fish (n = 8 for all species) and every hour for 5 h plus overnight (~12 h) for fed fish (n = 8 for giant gourami and climbing perch, n = 4 for snakehead and snakeskin gourami). In the tanks containing fed fish, 80% of the water was replaced before starting the overnight flux period. After the completion of flux protocols, fish were measured and weighed. The concentrations of Cl^-, Na^+, K^+, ammonia and urea-N were measured using 20 ml water samples for each time point to determine the initial and final concentrations for each flux period. The titratable alkalinity (J_{Talk}) at the start and end of the first 5 h in fasted and fed fish was measured by acid titration run using 10 ml water samples (see Analytical Procedures later).

The net flux rates of Cl^-, Na^+, K^+, ammonia, urea and titratable alkalinity (J_{Talk}) were calculated using the following equation adapted from Cooper and Wilson (2008):

$$J_{net}X = \frac{\{[(X)_i - (X)_f] \times V\}}{(M \times t)}, \quad (1)$$

where X_i and X_f are the initial and final concentrations in each tank (μmol l^{-1}) for each flux period, V is the tank volume (l), M is the animal mass (kg) and t is the flux duration (h). Positive values indicate net uptake from the water by the fish, and negative values indicate net losses by the fish. The net acidic equivalent flux (J_H) over 5 h was calculated as the sum of J_{Amm} and J_{TA}, signs considered, as explained by McDonald and Wood (1981).

2.2.2 | Gut index, pH, PCO_2 and HCO_3^- concentrations in blood and along the length of the gut

To determine how the acid–base status changes along the length of the gut in fed fish, snakehead (n = 8), climbing perch (n = 13), giant gourami (n = 7) and snakeskin gourami (n = 6) were euthanized in benzocaine (100 mg l^{-1}) 1–4 h after the daily morning feeding of a 1% ration. After euthanasia, blood (0.5–1.0 ml) was immediately drawn into a 1 ml syringe through caudal puncture using a #22 needle. The dead space of the syringe was filled with 1000 i.u. of lithium heparin (Sigma-Aldrich, St Louis, MO, USA) in Cortland saline (Wolf, 1963). The blood pH was measured immediately. The sample was then centrifuged (5000*g*, 2 min), and the plasma was placed on ice in sealed 0.5 ml tubes for total CO_2 measurement (see Analytical Procedures for measurements of pH and total CO_2). Fish mass, total length and fork length were then recorded. The gut of each fish was dissected and measured for total length, pylorus-to-anus length and gut index, calculated as the ratio of the last two. The gut was then sectioned into the stomach and anterior-, mid- and posterior intestine. Contents (chyme) were gently squeezed out of each section and placed on ice in sealed 2 ml tubes until analysis. Chyme samples were centrifuged (5000*g*, 2 min), and the supernatants were analysed for pH and total CO_2. It is possible that some CO_2 was lost during the removal of chyme from each gut section. Considering this loss, these data may be underestimates of *in vivo* chyme total CO_2 and should be interpreted with caution. Plasma and chyme PCO_2 and HCO_3^- concentrations were calculated using the Henderson–Hasselbalch equation adapted from Wood *et al*. (1983):

$$PCO_2 = \frac{C_{CO_2}}{\alpha CO_2 \times (1 + \text{antilog}\, pH - pk')} \quad (2)$$

and

$$[HCO_3^-] = C_{CO_2} - (\alpha CO_2 \times PCO_2), \quad (3)$$

where PCO_2 is the CO_2 tension and C_{CO_2} is the total CO_2. Values for CO_2 solubility (αCO_2) and pk′ at the experimental temperature were determined from Boutilier *et al*. (1984).

2.2.3 | Section-specific fluxes and total fluid, ion and glucose transport capacities along the gut in fasted and fed climbing perch

In vitro gut sac experiments were performed on the gut of climbing perch (n = 6 for fed and n = 6 for fasted treatments) using methods adapted from Pelster *et al*. (2015). These trials quantified the flux of fluid, Na^+, Cl^-, HCO_3^-, K^+ and glucose into/out of the gastrointestinal lumens. Fasted fish (5–8 days of starvation) and fed fish (1–4 h after the daily morning feeding with 1% ration) were euthanized in benzocaine (100 mg l^{-1}). After euthanasia, the whole gut was dissected out, cleaned of external fat and connective tissue and gently flushed with Cortland saline (Wolf, 1963) to remove chyme and faeces. Total gut length was measured and then sectioned into the stomach and anterior-, mid- and posterior intestinal regions of the digestive tract. The length of each section was then measured. A gut sac (anterior gut sac preparation shown in Figure 1b) was made within each section by first suturing at one end using 2-0 silk thread (Ethicon, Johnson & Johnson, New Brunswick, NJ, USA). A short-flared polyethylene tube (Clay-Adams PE50, Becton and Dickinson Co., Franklin Lakes, NJ, USA) was inserted at the open end and secured with silk thread.

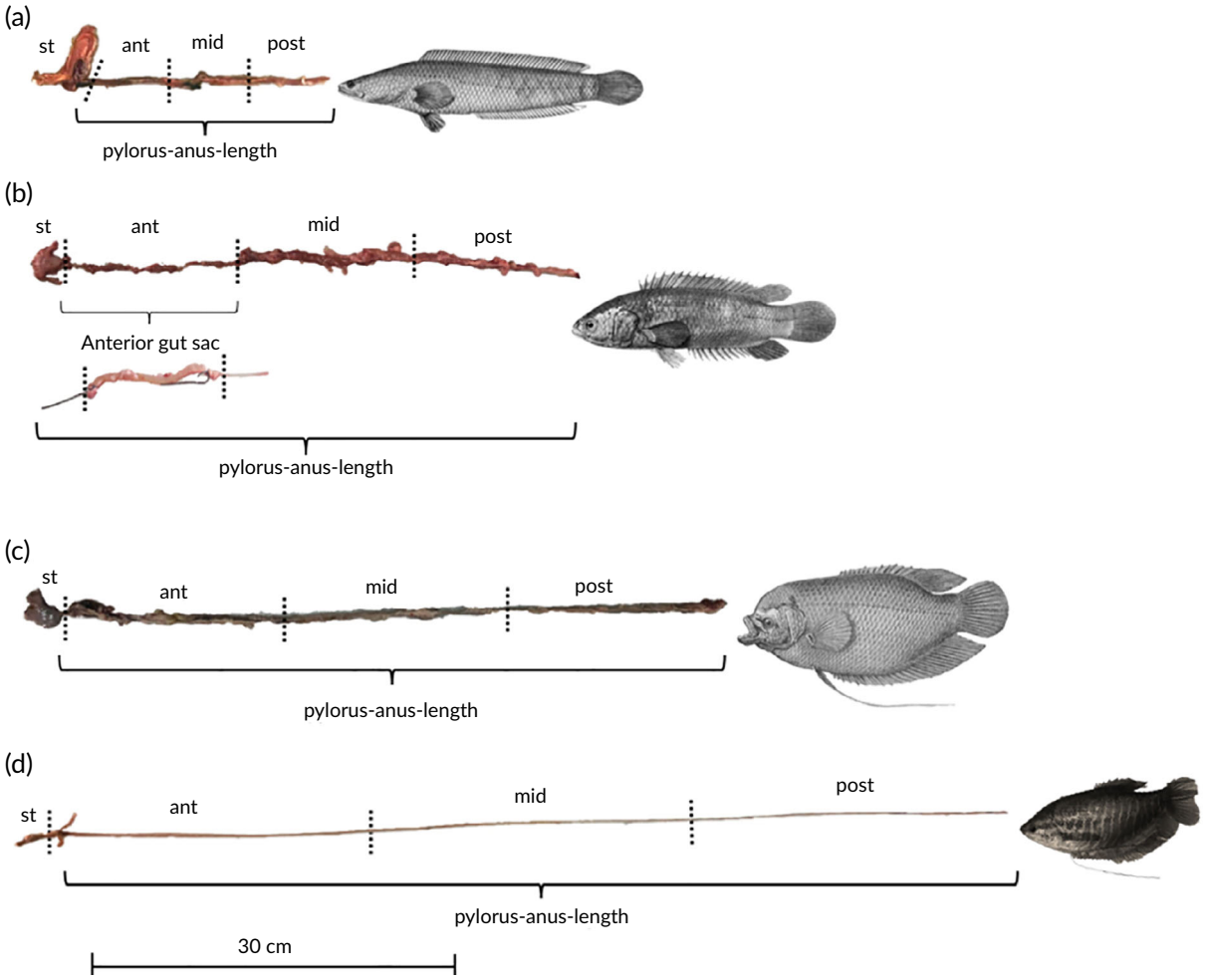

FIGURE 1 Photographs of the guts of the (a) snakehead, (b) climbing perch, (c) giant gourami and (d) snakeskin gourami showing the pylorus-to-anus length and the stomach (st), anterior- (ant), mid- (mid) and posterior (post) regions of the gut. Panel (b) also shows an anterior gut sac preparation from climbing perch. Gut to scale, fish diagram not to scale

The gut sac was then filled with Cortland saline through a syringe attached to the polyethylene tube and carefully checked for leaks, and the cannula was plugged with a pin. Typical gut sacs were 30%–60% of the full lengths of the various gut sections. The gut sac was blotted in a standardized fashion, the initial mass (g) was recorded and the preparation was immediately placed into an aerated vial filled with the same Cortland saline for a specific time (1.30 ± 0.05 h). Following this period, the gut sac was removed, blotted dry in the same fashion and weighed again. The difference between the initial and final gut sac mass (g) was used to determine the amount of fluid (ml) that was transported out of the gut sac during the incubation time period. The gut sac was then opened and the remaining fluid was removed; the pH was measured; and the sample was refrigerated for later analysis of final total Cl^-, Na^+, K^+, glucose and CO_2, for comparison with initial values of the same parameters; PCO_2 and HCO_3^- were calculated from pH and CO_2 measurements as described in the previous section. The mass of the empty sac (g), thoroughly blotted, was measured. Subtraction of this value from the initial and final mass (g) of the filled gut sac provided the initial and final volumes (ml), respectively, in the lumen of the sac. The total surface area (cm^2) and length (cm) of the gut sac were measured, allowing the calculation of surface area per unit length (cm^2 cm^{-1}). This calculation was accomplished using a 0.5 mm graph paper following a method adapted from Grosell and Jensen (1999).

The fluid flux rate (ml cm^{-2} h^{-1}; F_f), and flux rates of glucose, ammonia, Na^+, K^+, Cl^- and HCO_3^- (μmol cm^{-2} h^{-1}; F_x) were calculated using the following equations adapted from Pelster *et al.* (2015):

$$F_f = V_R \times \text{ area of gut sac}^{-1} \times T_i^{-1} \quad (4)$$

and

$$F_x = (V_I \times C_I - V_F \times C_F) \times \text{area of gut sac}^{-1} \times T_i^{-1}, \quad (5)$$

where V_R is the amount of fluid (ml) removed from the gut (*i.e.*, $V_I - V_F$), V_I and V_F are the initial and final volumes (ml) of the mucosal fluid at the start and end of the incubation time (T_i, *h*) and C_I and C_F represent the initial and final concentrations of the substances in the mucosal

saline at the start and end of the incubation time. The transport capacity (T_{cap}) of each gut section for fluid (ml kg^{-1} h^{-1}), glucose, ammonia, Na^+, K^+, Cl^- and HCO_3^- (μmol kg^{-1} h^{-1}) was calculated (Pelster *et al.*, 2015):

$$T_{cap} = F \times A_L \times G_L \times BM^{-1}, \quad (6)$$

where F is F_f or F_x that represents the flux per square centimetre as calculated in Equations 2 and 3, respectively, A_L is the gut section area per unit length (cm^2 cm^{-1}), G_L is the total length (cm) of the respective gut section and BM is the body mass (kg). Transport capacity of the whole gut was determined as the sum of the transport capacities of all gut sections:

$$T_{cap(whole\ gut)} = T_{cap(stomach)} + T_{cap(anterior)} + T_{cap(mid)} + T_{cap(posterior)}. \quad (7)$$

2.3 | Analytical procedures

Ammonia and urea-N concentrations in water samples were measured using the colorimetric methods described by Verdouw *et al.* (1978) and Rahmatullah and Boyde (1980) respectively. Na^+ and K^+ concentrations in water and gut sac fluids were measured using a model 420 flame photometer (Sherwood Scientific, Cambridge, UK) with lithium as an internal standard, whereas Cl^- concentrations in these same fluids were measured using a colorimetric assay adapted from Zall *et al.* (1956). Water-titratable alkalinity measurements were performed as described by McDonald and Wood (1981), by titrating 10 ml water samples to an endpoint of pH 4.0 with 0.02 N HCl (Sigma-Aldrich) using a micro-burette (Gilmont Instruments, Great Neck, NY, USA), whereas the sample was continually bubbled with N_2. WD-35801-00 epoxy body pH probes (Oakton Instruments, Vernon Hills, IL, USA) coupled to H160 portable meters (Hatch Co., Loveland, CO, USA) were used for the titrations. Glucose concentrations in gut sac fluids and plasma were measured using a clinical meter (Accu-Chek GT Compact Plus, Roche Diagnostics, Mannheim, Germany), calibrated with standards made up in Cortland saline. Blood, gut sac fluid and chyme pH at the experimental temperature were measured using a combination glass pH microelectrode (Biotrode, Hamilton, Reno, NV, USA) coupled to a hand-held Accumet combination meter (Fisher Scientific, Toronto, ON, Canada). The electrode was calibrated using precision buffers (Fisher Scientific and Radiometer-Copenhagen, Copenhagen, Denmark). Total CO_2 in plasma and gut sac fluids was measured using a Corning 965 Analyser (Ciba-Corning, Halstead, Essex, UK).

2.4 | Statistical analyses

All statistical analyses were performed in Prism Version 7.00 for Mac (GraphPad Software, La Jolla, CA, USA). Analyses were conducted following a D'Agostino and Pearson normality test or a Shapiro–Wilk and KS normality test when $n < 7$. A one-way analysis of variance (ANOVA) and Tukey's multiple comparisons test were performed to examine the differences among species and regions of the gut (where applicable) for hourly, total and overnight flux rates of ions, glucose, metabolic wastes and the acid–base flux, as well as species gut index, chyme pH, PCO_2 and HCO_3^-. A two-way ANOVA and Sidak multiple comparisons test were used to compare fluxes among regions of the gut and between fasted and fed gut sac preparations. Where applicable, an unpaired *t*-test was used to determine significant differences between feeding treatments for the same species. Finally, a general linear regression and Pearson's correlation analysis were conducted to examine the correlations between the species gut index and fluxes of Na^+, K^+, Cl^-, urea-N, ammonia-N and net acid–base equivalents in fasted and fed states (see Supplementary Table S1 and Supplementary Figure S1). Specific tests used in each experiment are reported in figure legends. Data have been expressed as mean ± S.E.M. (N = number of fish or preparations). Where applicable, outliers were identified and removed from data following a ROUT analysis (Q = 0.05). Significance was accepted at $P < 0.05$.

3 | RESULTS

3.1 | Gut index

As illustrated in Figure 1, the four species differed markedly in gut morphology; note that the intestine, which is normally folded or coiled in the coelomic cavity, has been dissected out and extended to its full length in these photographs. The lowest mean gut index (the ratio of gut length to fork length; Figure 2) was recorded for snakehead (0.66), indicating greatest carnivory, and the highest for snakeskin gourami

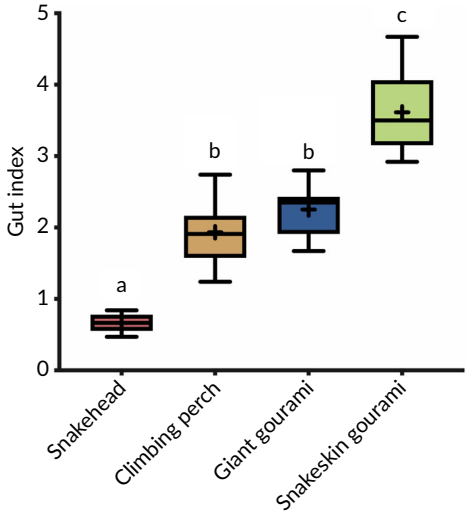

FIGURE 2 Gut index (ratio of the pylorus-to-anus length to the fork length) in the striped snakehead (n = 6), climbing perch (n = 13), giant gourami (n = 7) and snakeskin gourami (n = 6). Data are presented as mean (+), median (horizontal line), min and max. Different letters indicate significant differences among species at $P < 0.05$ after a one-way analysis of variance and Tukey's multiple comparisons test

(3.60), indicating greatest herbivory. Climbing perch and giant gourami were intermediate at 1.93 and 2.25, respectively.

3.2 | Flux rates of ammonia, urea-N and ions in fed and fasted fish

The hourly flux rates of ammonia to the external water over time were significantly different among species in fasted groups but not in fed groups (hourly flux data not shown). The two-way ANOVA revealed not only a significant effect of species for fasted groups but also an overall flux period effect. Nonetheless, there was no interaction, meaning the differences observed among species were consistent across all flux periods. For fasted groups, the average hourly ammonia flux rate over 5 h (Figure 3a) was greatest in the snakehead (−882 μmol kg^{-1} h^{-1}) and smallest in snakeskin gourami (−260 μmol kg^{-1} h^{-1}). Multiple comparisons show a significant difference between the snakehead, giant gourami and snakeskin gourami in fasted groups. In contrast, there were no significant differences among species in fed groups for the average hourly ammonia flux rate over 5 h (Figure 3c) though trends were similar. Snakeskin gourami were the only species to have a significantly greater flux of ammonia to the external water after feeding (Figure 3a,c).

Urea-N fluxes were too small to determine reliably within each 1 h period, so flux rates averaged over 5 h are shown (Figures 3b,d). In fasted groups, mean urea-N flux rate was greatest in the snakehead (−168 μmol-N kg^{-1} h^{-1}) and lowest in snakeskin gourami (−63 μmol-N kg^{-1} h^{-1}) (Figure 3e), *i.e.*, parallel to the ammonia flux pattern. There was a statistically significant difference only between the snakehead and snakeskin gourami. In fed groups, mean urea-N flux rate was greatest in giant gourami (−181 μmol-N kg^{-1} h^{-1}) and lowest in

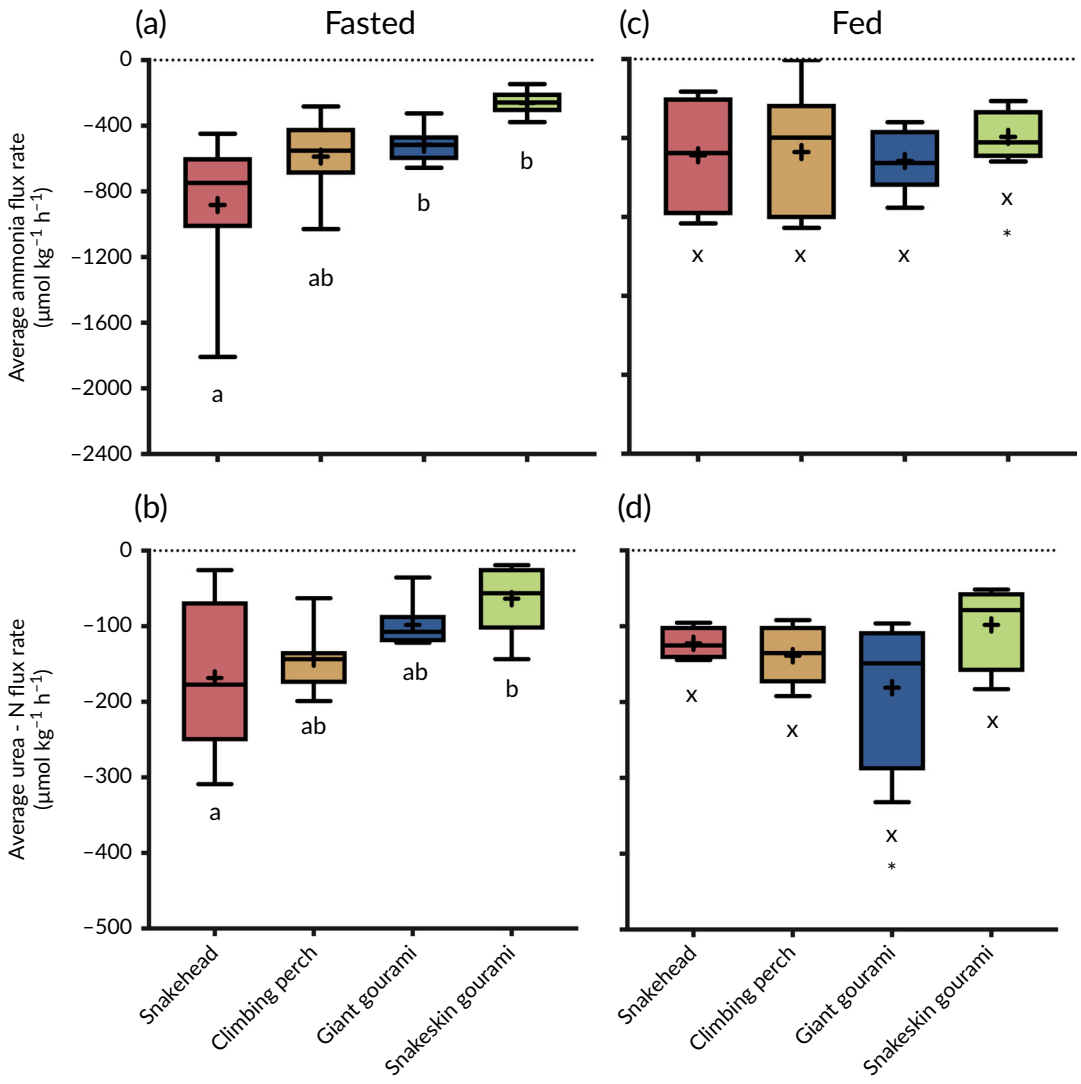

FIGURE 3 The average ammonia and urea-N flux rates over 5 h in fasted (a, b) and fed (c, d) striped snakehead (n = 4 fed, n = 8 fasted), climbing perch (n = 8 fasted and fed), giant gourami (n = 8 fasted and fed) and snakeskin gourami (n = 4 fed, n = 8 fasted). Data are presented as mean (+), median (horizontal line), min and max. Different letters indicate significant differences among species at $P < 0.05$ after a one-way analysis of variance and Tukey's multiple comparisons test. The abc comparisons are for fasted fish, and the xyz comparisons are for fed fish. Asterisks (*) indicate significant differences between fed and fasted groups from the same species at $P < 0.05$ after an unpaired t-test

snakeskin gourami (−98 μmol-N kg^{-1} h^{-1}). Nonetheless, there was no significant difference between species (Figure 3d) in fed fluxes of urea-N. In the giant gourami, urea-N fluxes were significantly greater (almost double) in fed groups, but feeding had no effect on the average urea-N flux for any other species.

The contribution of urea-N to total N excretion was between 19% and 26% in the four species, increasing to 26%–31% after feeding (Supplementary Table S2). None of the interspecies differences or increases were significant.

Excretion of urea-N and ammonia-N was positively correlated in a linear fashion, with increases in the gut index in fasted groups only, meaning with increasing herbivory, fluxes of ammonia and urea-N decreased linearly (see Supplementary Figure S1 and Table S1; Pearson's $r > 0.95$; $P < 0.05$ for both comparisons). There was no significant variation over time in the hourly flux rates of K^+, Na^+ and Cl^- in fed or fasted groups (data not shown), so flux rates averaged over 5 h are shown in Figure 4. The mean net loss rate of K^+ in fasted groups (Figure 4a) was greatest in the snakehead (−107 μmol kg^{-1} h^{-1}) and smallest in snakeskin gourami (−20 μmol kg^{-1} h^{-1}). The difference between snakehead and snakeskin gourami was significant, but there were no other significant differences among species. This pattern reversed after feeding (Figure 4d), with the greatest loss rate of K^+ in snakeskin gourami (−163 μmol kg^{-1} h^{-1}), which was eight-fold higher than that in the fasted state. The lowest flux rate of K^+ in fed groups was now observed in climbing perch (−31 μmol kg^{-1} h^{-1}), a significant difference from the snakeskin gourami. There were no other significant differences among species (Figure 4a,d). The change between fasted and fed states was significant only for snakeskin gourami (Figure 4a,d).

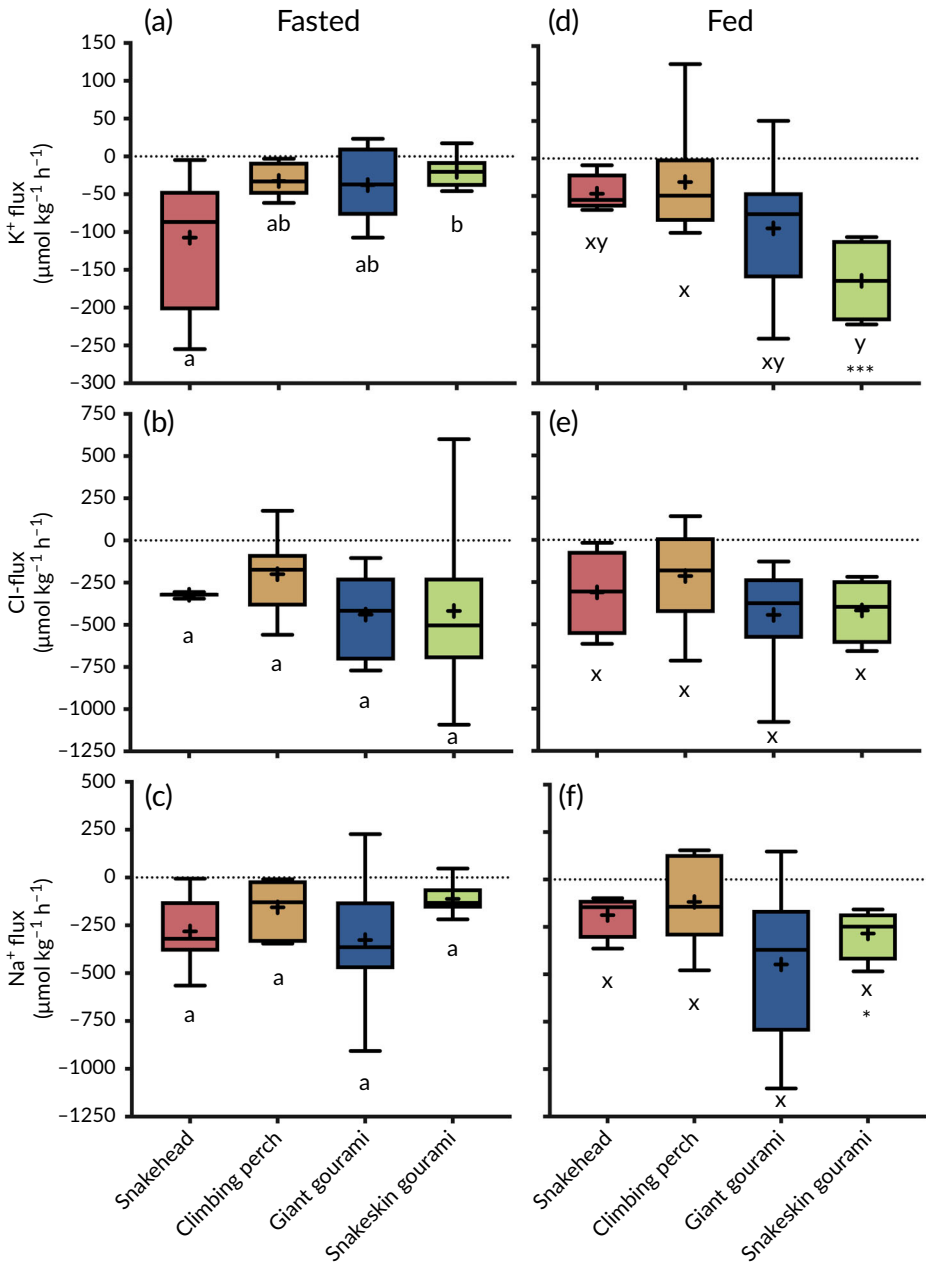

FIGURE 4 The average K^+, Cl^- and Na^+ flux rates over 5 h (μmol kg^{-1} h^{-1}) in fasted (a, b, c) and fed (d, e, f) striped snakehead ($n = 4$ fed, $n = 8$ fasted), climbing perch ($n = 8$ fed and fasted), giant gourami ($n = 8$ fasted and fed) and snakeskin gourami ($n = 4$ fed, $n = 8$ fasted). Data are presented as mean (+), median (horizontal line), min and max. Different letters indicate significant differences among species within feeding treatments at $P < 0.05$ after a one-way analysis of variance and Tukey's multiple comparisons test. The abc comparisons are for fasted fish, and the xyz comparisons are for fed fish. Asterisks (*) indicate significant differences between fed and fasted groups from the same species at $P < 0.05$ after an unpaired t-test

The mean net flux rates of Cl^- in fed and fasted treatments of all species were similar, ranging from −200 μmol kg^{-1} h^{-1} in fasted climbing perch (Figure 4b) to −444 μmol kg^{-1} h^{-1} in fed giant gourami (Figure 4e). There were no significant differences among species or feeding treatments. The situation was similar for the mean net flux rates of Na^+, ranging from −111 μmol kg^{-1} h^{-1} in fasted snakeskin gourami (Figure 4c) to −448 μmol kg^{-1} h^{-1} in fed giant gourami (Figure 4f). Again, there were no significant differences among species or feeding treatments. Nonetheless, Na^+ loss significantly increased after feeding for the snakeskin only (Figure 4f).

Flux rates of ammonia, urea-N and the three ions were observed overnight for another 12 h to determine if there were long-term effects of feeding (Supplementary Table S2). In general, the overnight loss rates were lower, but there were no significant differences relative to the 5 h values in the snakehead or climbing perch. In contrast, giant gourami had significantly lower mean flux rates of ammonia (−99 *vs.* −645 μmol kg^{-1} h^{-1}), Cl^- (−135 *vs.* −444 μmol kg^{-1} h^{-1}) and Na^+ (−30 *vs.* −448 μmol kg^{-1} h^{-1}) overnight than within the first 5 h. Similar significant attenuations of mean excretion rates over time were observed in snakeskin gourami, which actually changed to positive values overnight for Cl^- (+202 *vs.* −417 μmol kg^{-1} h^{-1}) and Na^+ (+161 *vs.* −284 μmol kg^{-1} h^{-1}).

There were no significant differences among species in the overnight flux rates of K^+ or urea-N. Similarly, the overnight flux rate of Cl^- did not vary among the snakehead, climbing perch or giant gourami, but the positive, overnight, net uptake rate of Cl^- in snakeskin gourami was significantly different in comparison to the negative overnight loss rates in all other species. Similarly, the positive, overnight, net uptake rate of Na^+ in snakeskin gourami was significantly different in comparison to the negative overnight loss rate in giant gourami but not in comparison to the negative rates of the other two species. The most pronounced and significant interspecies differences were observed in the overnight excretion rates of ammonia, which were greatest in climbing perch (−641 μmol kg^{-1} h^{-1}) and smallest in giant gourami (−135 μmol kg^{-1} h^{-1}).

3.3 | Net acid–base flux rates

Net acid–base flux rates (Figure 5) were calculated using the mean flux rates of titratable alkalinity (data not shown) and the simultaneously measured net flux rates of ammonia (Figures 3c,d) over 5 h. In both fasted and fed treatments, there were no significant differences among species. The data were also examined statistically for changes in net acid–base flux rates between fasted and fed treatments within each species. Nonetheless, none of these differences were significant. Further analysis did reveal that the net acid–base flux in fed animals was highly correlated with gut index (Pearson's r = 0.91), where more herbivorous fishes had a net acid flux and more carnivorous species had a greater net base excretion, but this relationship was not statistically significant (P = 0.09) (see Supplementary Table S1).

3.4 | The pH, PCO_2 and HCO_3^- concentration in the blood and chyme of fed fish

To better understand the profiles of pH, PCO_2 and HCO_3^- concentration along the gut in the four species, the chyme from all regions in

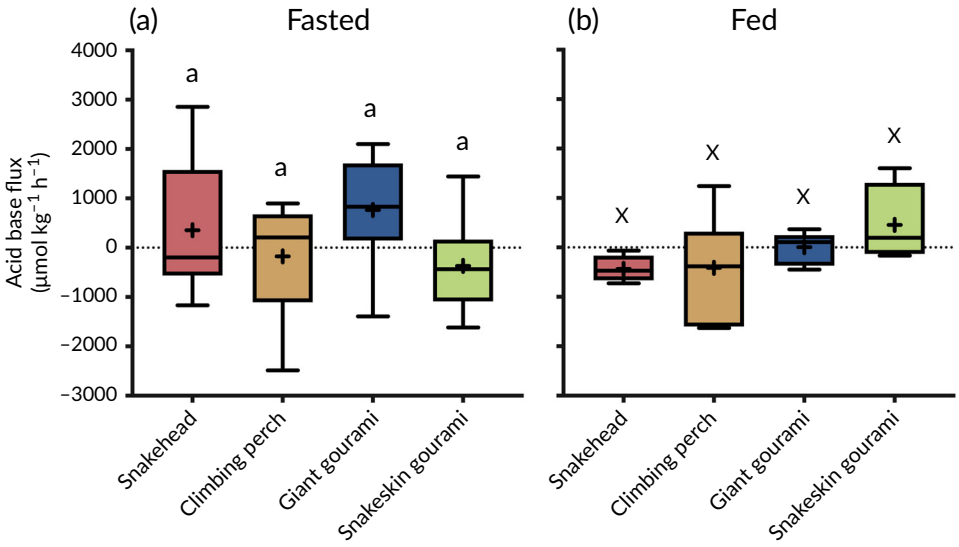

FIGURE 5 The average net acid–base excretion rates (μmol kg^{-1} h^{-1}) in fasted (a) and fed (b) striped snakehead (n = 8 fasted, n = 4 fed), climbing perch (n = 8 fasted, n = 7 fed), giant gourami (n = 8 fed and fasted) and snakeskin gourami (n = 6 fasted, n = 4 fed). For net acid–base excretion, positive values indicate a net base uptake (*i.e.*, acid excretion) and negative values indicate net base excretion (*i.e.*, acid uptake). Data are presented as mean (+), median (horizontal line), min and max. Different letters indicate statistical significance among species and within feeding treatments at P < 0.05 after a one-way analysis of variance. The abc comparisons are for fasted fish, and the xyz comparisons are for fed fish. Asterisks (*) indicate significant differences between fed and fasted groups from the same species at P < 0.05 after an unpaired t-test

fed fish was sampled. Blood pH, PCO_2 and HCO_3^- concentrations were also recorded in terminal caudal puncture samples from these anaesthetized fish. Mean blood pH was significantly greater in the snakehead (7.33) relative to the climbing perch (7.12), whereas values in the other two species were intermediate and not different among species (Figure 6). There were no significant differences among species in mean blood plasma (HCO_3^-), which ranged from 9.8 mM in climbing perch (Figure 6f) to 11.2 mM in giant gourami (Figure 6g), or in mean blood PCO_2, which was greatest in giant gourami (17.6 mmHg, Figure 6k) and lowest in the snakehead (13.5 mmHg, Figure 6i).

Chyme in the stomach was significantly more acidic in comparison to all other regions of the gut in three species but not in snakeskin gourami where there was only a difference between the pH of the mid- and posterior regions of the gut (Figure 6a–d). Climbing perch had the lowest average stomach pH at 3.89, with values as low as 2.20 in some individuals. Snakeskin gourami had the highest average stomach pH (6.78), whereas giant gourami (5.63) and snakehead (5.37) exhibited intermediate values. There were no significant differences among species in the pH of the chyme from the anterior-, mid- or posterior regions of the gut.

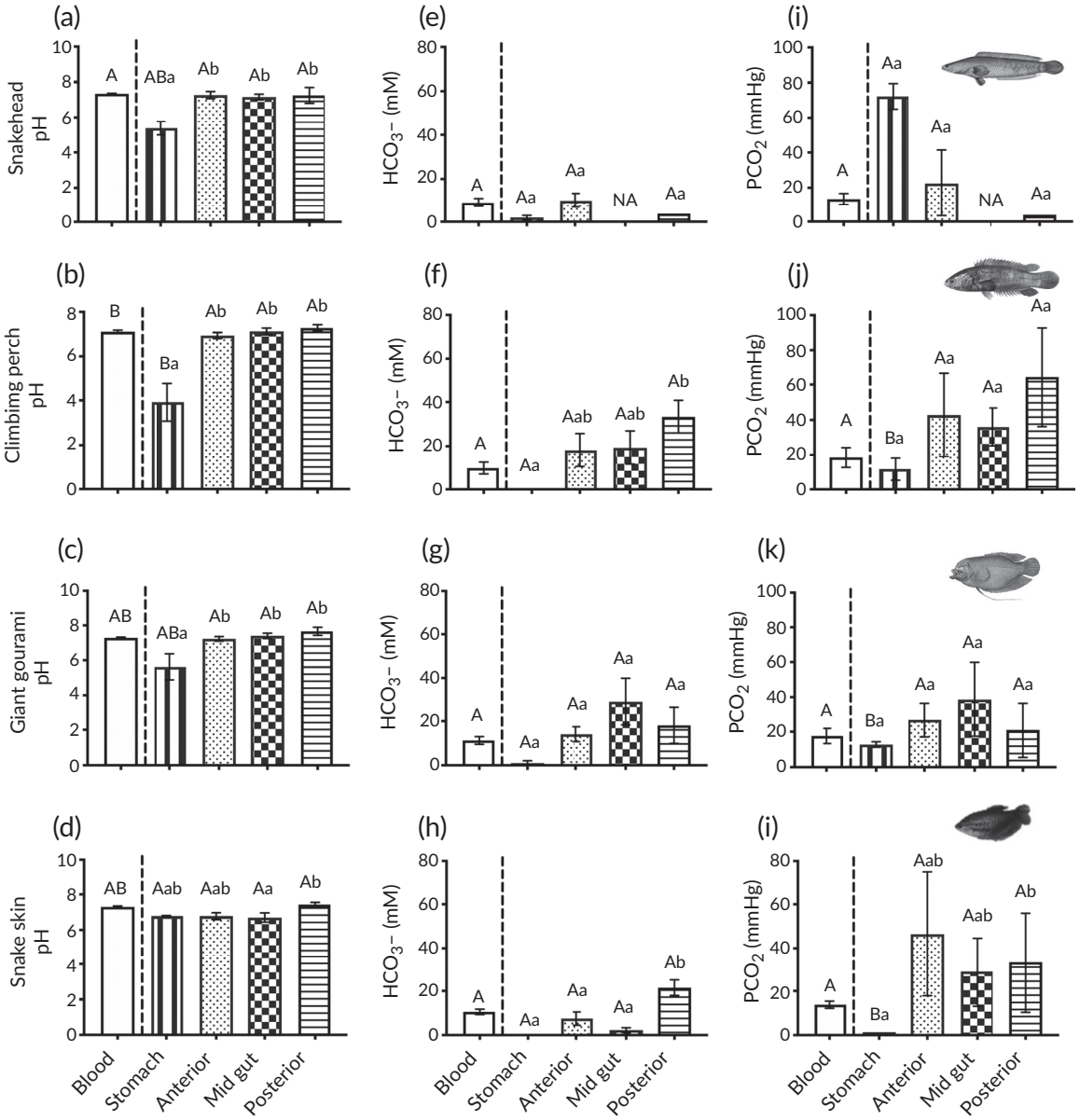

FIGURE 6 Blood and chyme pH, HCO_3^- (mM) and PCO_2 (mmHg) in the stomach and anterior-, mid- and posterior regions of the gut from the striped snakehead (n = 7) (a, e, i), climbing perch (n = 5) (b, f, j), giant gourami (n = 5) (c, g, k) and snakeskin gourami (n = 4) (d, h, l) fed on a 1% ration of commercial fish feed. Means ± S.E.M. Different letters indicate significant differences at $P < 0.05$ after a one-way analysis of variance and Tukey's multiple comparisons test: AB indicate significant differences between species, and ab indicate significant differences among regions of the gut within the same species. Blood data were not included in the analysis among sections of the gut. NA: not available. (▭) Blood, (▮) Stomach, (▤) Anterior, (▦) Midgut, (▬) Posterior

All species had low-to-zero concentrations of HCO_3^- in the stomach chyme, but this level increased substantially as chyme moved to the intestinal regions of the gut (Figure 6e–h). Mean intestinal HCO_3^- concentration ranged from 7.5 mM in the anterior gut of snakeskin gourami (Figure 6h) to 33.4 mM in the posterior intestine of the climbing perch (Figure 6f). Nonetheless, differences were not significant among regions of the gut for any species except for snakeskin where HCO_3^- level was highest in the posterior intestine. There were also no significant differences among species in the concentration of HCO_3^- in the stomach and anterior-, mid- or posterior regions of the gut.

The snakehead had a relatively high mean PCO_2 in the stomach (72 mmHg), significantly greater than that in all other species, and about 50-fold higher than that in the stomach of snakeskin gourami (1.3 mmHg, Figure 6i–l). Generally, high PCO_2 values (~15–70 mmHg) were also observed in other sections of the gut in all species, but there were no significant differences among species for any other region of the gut. Similarly, comparisons among gut sections for each species revealed no differences except between the stomach and posterior regions for snakeskin gourami (Figure 6l).

3.5 | Section-specific fluxes and total transport capacities in the gut of climbing perch

In vitro gut sac preparations were used to investigate the absorptive/secretory functions of each section of the gut and how they changed with feeding in climbing perch. The approach in this study (see Methods) allowed the recording of area-specific net flux rates in each section (stomach and anterior-, mid- and posterior intestine), the calculation of the total capacity (T_{cap}) of each section and the total capacity [$T_{cap(whole\ gut)}$] of the whole gut for the transport of each substance. Positive values indicate a net movement out of the lumen (*i.e.*, net absorption), whereas negative values indicate movement into the lumen (*i.e.*, net secretion). Blood glucose concentration was recorded in terminal blood samples taken before the gut sac preparations and was not significantly different (t = 0.62, P = 0.55) between fed 8.46 ± 1.65 mM l^{-1}, n = 6) and fasted (7.24 ± 0.6 mM l^{-1}, n = 6) individuals.

All fluid flux values were positive, indicating a net movement of fluid out of the lumen in both fed and fasted fish (Figure 7). For area-specific fluid absorption rates (Figure 7a), there were significant overall feeding and gut section effects but no significant interaction. Overall absorption rates were greater in preparations from fed individuals, though individual means were significantly different only in the anterior portion of the gut (0.056 *vs.* 0.009 ml cm^2 h^{-1}, a six-fold difference, Figure 7a). For mass-specific T_{cap} of fluid absorption in each section, there were significant effects of feeding and gut section though, again, no interaction (Figure 7b). The feeding difference was significant only in the midgut (2.42 *vs.* 0.34 *vs.* ml kg^{-1} h^{-1}, a seven-fold difference). There were no differences in T_{cap} among sections in fasted groups. Nevertheless, in fed groups, there were significantly greater T_{cap} values in the anterior and midgut regions relative to the stomach and posterior intestine (Figure 7a,b). The average whole gut $T_{cap(whole\ gut)}$ was significantly higher by five-fold in fed *vs.* fasted groups (4.2 *vs.* 0.8 ml kg^{-1} h^{-1}) (Figure 7c).

All glucose flux values were positive, indicating a net movement of glucose out of the lumen (Figure 8). There was no effect of feeding across sections of the gut for the area-specific glucose flux (Figure 8a) or mass-specific T_{cap} of glucose (Figure 8b). Nonetheless, an overall gut section effect was observed for both the area-specific flux rates and mass-specific T_{cap} of glucose, and significant differences were

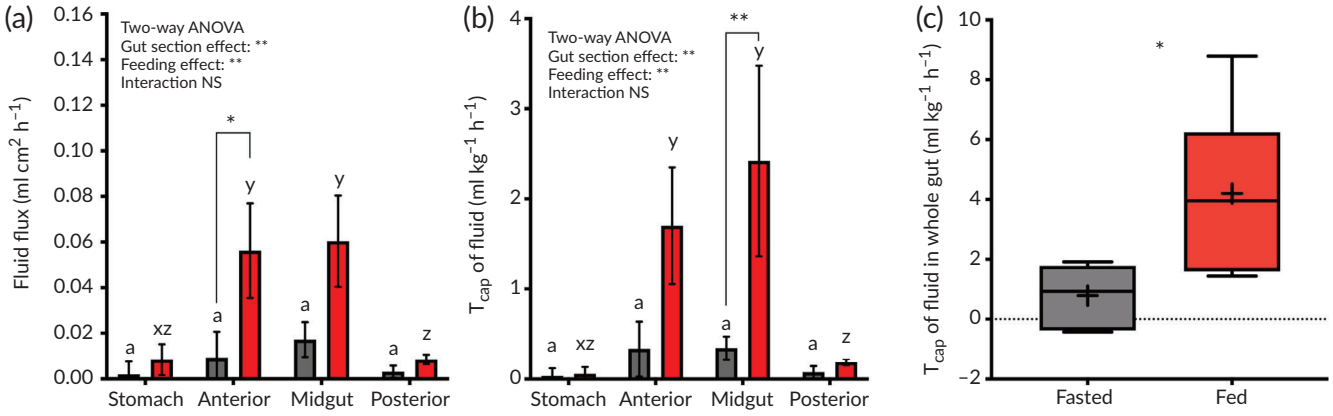

FIGURE 7 Average area-specific net fluid flux rates (ml cm^2 h^{-1}) (a) and mass-specific net fluid transport capacity (T_{cap}) (ml kg^{-1} h^{-1}) (b) in each section (stomach and anterior-, mid- and posterior regions) of the gut in fasted (n = 6) (grey bars) and fed (n = 6) (red bars) climbing perch (fed fish received a 1% ration of commercial fish feed 1–4 h before sampling). (c) The total capacity for fluid transport (T_{cap}) across the whole gut in fasted and fed climbing perch. Positive values represent net absorption, and negative values represent net secretion. Asterisks in panels (a) and (b) indicate significant differences between fasted and fed regions of the gut at P < 0.05 (*), P < 0.0022 (**) or P < 0.00021 (***), and different letters specify section-specific differences in each feeding treatment, where abc comparisons indicate differences in fasted groups and xyz comparisons indicate differences in fed groups at P < 0.05 after a two-way analysis of variance (ANOVA) and Sidak multiple comparisons. Two-way ANOVA results are shown in panels (a) and (b). Asterisk in (c) indicates a significant (P < 0.05) difference between the total T_{cap} of the whole gut in fed and fasted fish after an unpaired t-test. Data in (c) are presented as mean (+), median (horizontal line), min and max

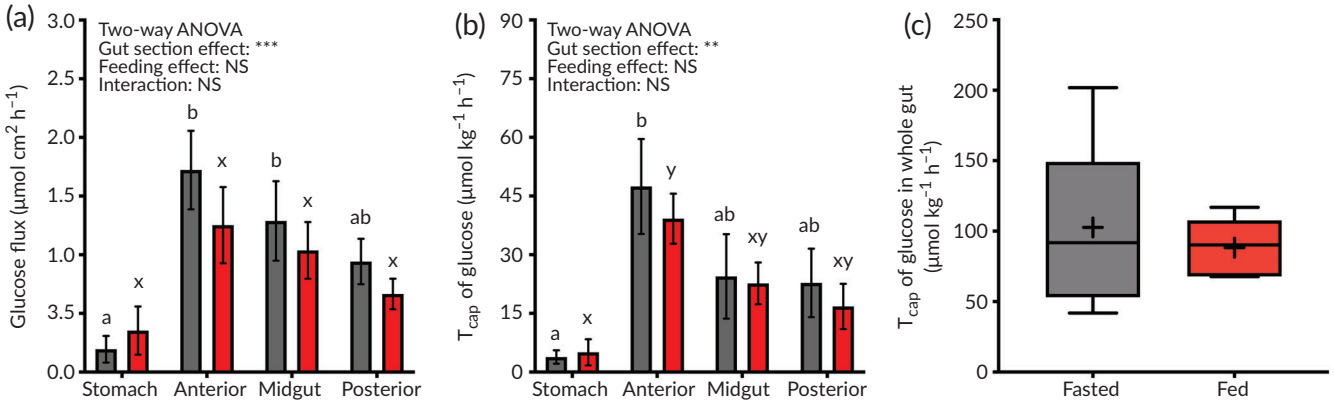

FIGURE 8 (a) Average area-specific net glucose flux rates (μmol cm^2 h^{-1}) and (b) mass-specific net glucose transport capacity (T_{cap}) (μmol kg^{-1} h^{-1}) (b) in each section (stomach and anterior-, mid- and posterior regions) of the gut in fasted (n = 6) (grey bars) and fed (n = 5) (red bars) climbing perch (fed fish received a 1% ration of commercial fish feed 1–4 h before sampling). Panel (c) shows the total capacity for glucose transport (T_{cap}) across the whole gut in fasted and fed climbing perch. All other details are as in legend of Figure 7

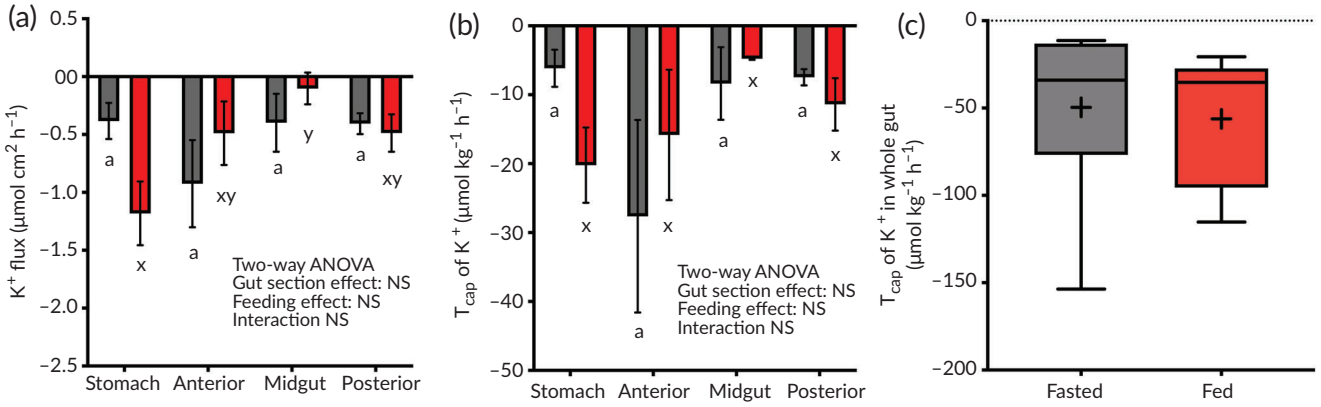

FIGURE 9 (a) Average area-specific net K^+ flux rates (μmol cm^2 h^{-1}) and (b) mass-specific net K^+ transport capacity (T_{cap}) (μmol kg^{-1} h^{-1}) in each section (stomach and anterior-, mid- and posterior regions) of the gut in fasted (n = 6) (grey bars) and fed (n = 5) (red bars) climbing perch (fed fish received a 1% ration of commercial fish feed 1–4 h before sampling). Panel (c) shows the total capacity for K^+ transport (T_{cap}) across the whole gut in fasted and fed climbing perch. All other details are as in the legend of Figure 7

observed among sections for fasted and fed preparations. Area-specific glucose absorption rates were greater in the anterior and mid-intestine than in the stomach for fasted preparations only (Figure 8a). Similar trends were observed in the mass-specific T_{cap} of glucose (Figure 8b); however, these differences were also significant in fed preparations. The $T_{cap(whole\ gut)}$ of glucose was similar (about 90 μmol kg^{-1} h^{-1}) in fed and fasted animals (Figure 8c).

All K^+ flux values were negative, indicating a net flux of K^+ into the lumen in both fed and fasted fish; however, there was no observed feeding effect (Figure 9). In both fed and fasted groups, the greatest net flux rates of K^+ were observed in the stomach and anterior regions of the gut. There were no significant differences in the area-specific flux or mass-specific T_{cap} of K^+ among sections of the gut for fasted preparations. Nonetheless, in fed preparations a significantly greater area-specific flux of K^+ between the stomach and midgut was observed, a difference of almost 12-fold (Figure 9a). The $T_{cap\ (whole\ gut)}$ of K^+ was ~−50 μmol kg^{-1} h^{-1} and did not differ between fed and fasting treatments.

The direction of net Na^+ movement into or out of the lumen was not consistent among sections or between fed and fasted groups – *e.g.*, negative in the stomach and posterior intestine but only in fasted preparations (Figure 10). There was a significant overall stimulatory effect of feeding on net Na^+ uptake, though no significant differences in individual sections. There were also no significant differences among sections of the gut for the area-specific Na^+ flux rates in both feeding treatments. In contrast, the mass-specific T_{cap} of Na^+ was significantly greater by about 55% in the fed treatment in the anterior intestine (Figure 10b). There was also a significant overall effect of feeding and gut section for T_{cap} of Na^+ (Figure 10b) but no interaction. There were differences among gut sac preparations only for the fed treatment, where T_{cap} of the anterior intestine was greater than that of the stomach (Figure 10b). The mean $T_{cap(whole\ gut)}$ of Na^+ was substantially higher by about eight-fold in fed preparations (501 μmol kg^{-1} h^{-1}) than in fasted preparations (62 μmol kg^{-1} h^{-1}) (Figure 10c).

Mean HCO_3^- flux rates were almost entirely positive, indicating net transport out of the lumen (Figure 11). There were no significant

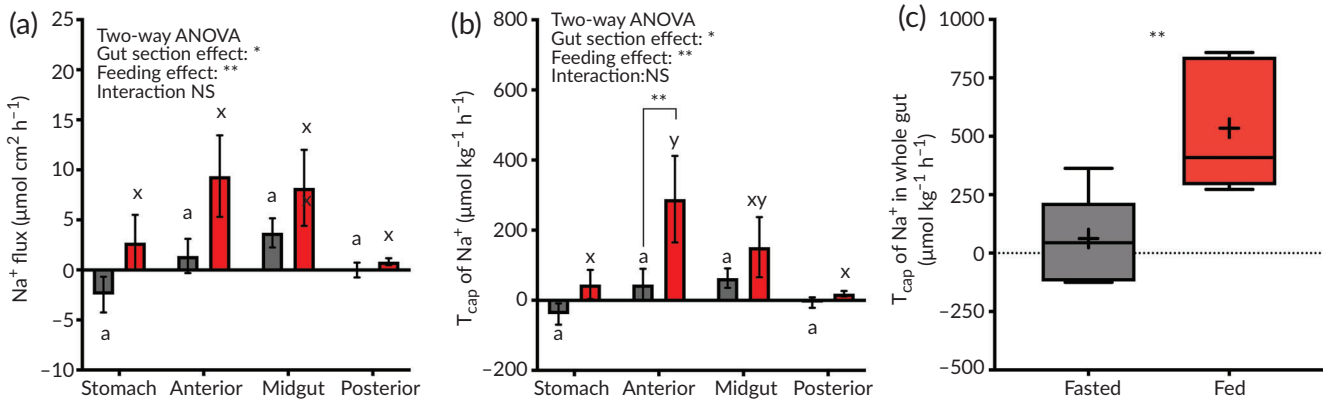

FIGURE 10 (a) Average area-specific net Na^+ flux rates (µmol cm^2 h^{-1}) and (b) mass-specific net Na^+ transport capacity (T_{cap}) (µmol kg^{-1} h^{-1}) in each section (stomach and anterior-, mid- and posterior regions) of the gut in fasted (n = 6) (grey bars) and fed (n = 5) (red bars) climbing perch (fed fish received a 1% ration of commercial fish feed 1–4 h before sampling). Panel (c) shows the total capacity for Na^+ transport (T_{cap}) across the whole gut in fasted and fed climbing perch. All other details are as in legend of Figure 7

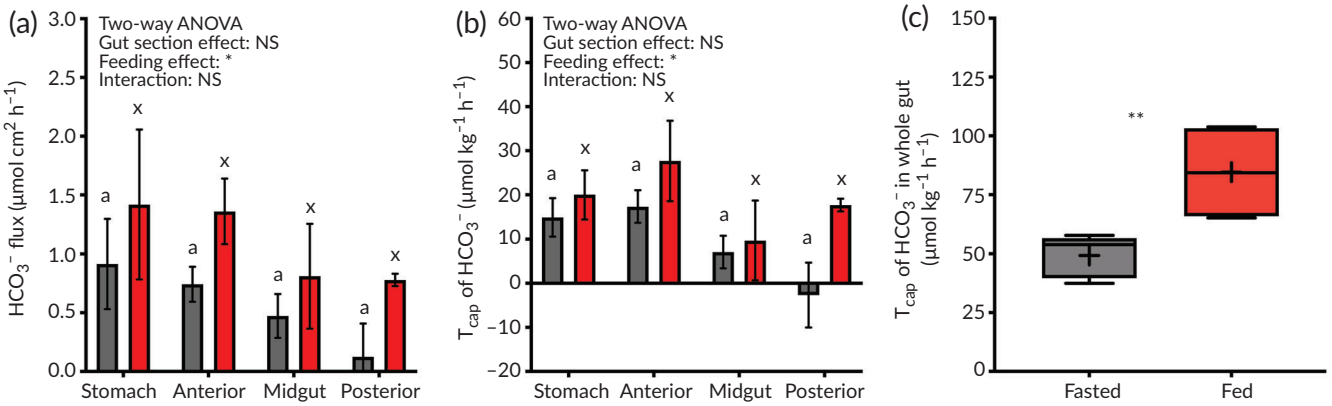

FIGURE 11 (a) Average area-specific net HCO_3^- flux rates (µmol cm^2 h^{-1}) and (b) mass-specific net HCO_3^- transport capacity (T_{cap}) (µmol kg^{-1} h^{-1}) in each section (stomach and anterior-, mid- and posterior regions) of the gut in fasted (n = 6) (grey bars) and fed (n = 5) (red bars) climbing perch (fed fish received a 1% ration of commercial fish feed 1–4 h before sampling). Panel (c) shows the total capacity for HCO_3^- transport (T_{cap}) across the whole gut in fasted and fed climbing perch. All other details are as in the legend of Figure 7

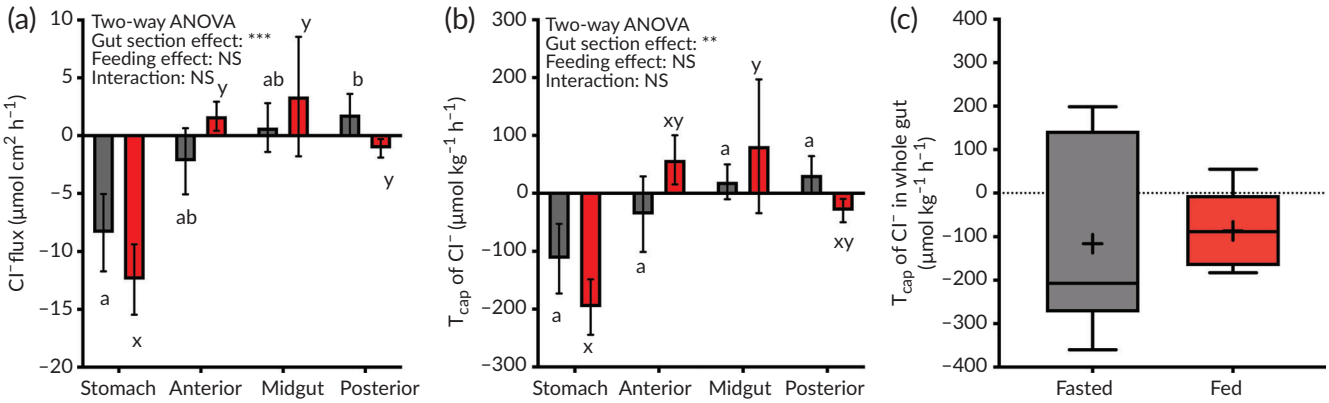

FIGURE 12 (a) Average area-specific net Cl^- flux rates (µmol cm^2 h^{-1}) and (b) mass-specific net Cl^- transport capacity (T_{cap}) (µmol kg^{-1} h^{-1}) in each section (stomach and anterior-, mid- and posterior regions) of the gut in fasted (n = 6) (grey bars) and fed (n = 5) (red bars) climbing perch (fed fish received a 1% ration of commercial fish feed 1–4 h before sampling). Panel (c) shows the total capacity for Cl^- transport (T_{cap}) across the whole gut in fasted and fed climbing perch. All other details are as in the legend of Figure 7

effects of feeding for the area-specific net flux rates (Figure 11a) or mass-specific T_{cap} of HCO_3^- (Figure 11b) in any region of the gut. There were also no significant differences among regions for the average mass or area-specific T_{cap} of HCO_3^- (Figure 11a,b). Nonetheless, the mean $T_{cap(whole\ gut)}$ of HCO_3^- was significantly greater by ~ 1.7-fold in fed preparations (84 μmol kg^{-1} h^{-1}) than in fasted preparations (49 μmol kg^{-1} h^{-1}) (Figure 11c).

The direction of Cl^- net movement into or out of the lumen was not consistent among sections or between fed and fasted groups (Figure 12), and it differed from that of Na^+ (*cf*. Figure 10). For both the area-specific net flux rates and T_{cap} of Cl^-, there was a significant effect of section but no significant effect of feeding. In the stomach, Cl^- flux rates were highly negative (*i.e*., into the gastric lumen) in both fasted and fed groups but only negative in the anterior intestine of fasted fish and in the posterior intestine of fed fish. There were more positive, area-specific flux rates of Cl^- in the anterior-, mid- and posterior regions of the gut of fasting fish; however, this comparison was only significant between the stomach and the posterior preparations. In fed preparations, the area-specific flux rates of Cl^- were all significantly different from those in the stomach (Figure 12a). In contrast, there were no significant differences among regions of the gut for the mass-specific T_{cap} of Cl^- in fasted preparations (Figure 12b), but a significant difference was observed for this parameter between the stomach and midgut only of fed fish. The mean $T_{cap(whole\ gut)}$ values of Cl^- were negative overall (~−100 μmol kg^{-1} h^{-1}) and not significantly different between fasted and fed groups (Figure 12c).

4 | DISCUSSION

4.1 | Overview

Given the gut morphology of the four species (Figures 1 and 2), the striped snakehead is probably the most carnivorous and the snakeskin gourami is probably the most herbivorous, whereas the giant gourami and climbing perch are likely omnivorous. With respect to the original hypotheses, the following were observed. (a) It was confirmed that N-waste excretion would be minimized in the most herbivorous species and maximized in the most carnivorous species. Similarly, K^+ excretion was greatest in the snakeskin, whose K^+-rich plant diet would normally supply K^+ more than required, but low in the most carnivorous snakehead. (b) Although the alkaline tide was not measured directly, measurements of changes in net acid–base flux to the water between fasted and fed fish indicated that the greatest postprandial alkaline tide occurred in the snakehead (carnivore) and a potential acidic tide in the snakeskin (herbivore), consistent with predictions. Despite a strong correlation, the changeover was not significant in any species, so no firm conclusions can be drawn. (c) This study's results support the prediction that carnivores would have more-acidic stomachs and herbivores the least-acidic stomachs after feeding, but again the differences between carnivores and herbivores were not significant. This result may be due to the variability in sampling time of chyme (ranged between 1 and 4 h post-feed). The increase in pH between the stomach and the rest of the tract was also least in the herbivorous snakeskin. Additional findings of interest were the unexpected high levels of both PCO_2 and HCO_3^- in the intestinal chyme of all four species. Finally, consistent with the hypothesis (d), *in vitro* experiments demonstrated that absorptive processes for fluid, Na^+ and HCO_3^- were upregulated in gut sac preparations for the climbing perch after feeding. The latter response indicates that high levels of chyme (HCO_3^-) in the intestine were not due to secretion by the intestinal epithelium. It is hoped that these data can provide new insights into the nutritional physiology of these study species and that these data could be used to improve productivity in aquaculture.

4.2 | Feeding habits

The link between the morphology of the gut and the likely feeding habit of teleost fishes is well recognized (German & Horn, 2006; Wilson & Castro, 2011). Herbivorous fishes tend to have a much longer intestine that is typically coiled and uniform, whereas carnivorous species have a much shorter and straighter gut morphology (Day *et al*., 2014; Ferraris & Ahearn, 1984; Kapoor *et al*., 1976; Kramer & Bryant, 1995). The longer intestine present in herbivores will increase exposure to digestive processes that are necessary for the effective digestion of a fibre-rich plant diet. By contrast, the greater nutrient availability (protein and fat) present in a carnivorous diet is more easily digested and absorbed, explaining the shorter intestine observed in carnivorous fishes (Karasov & del Rio, 2007).

4.3 | Excretion and net uptake from feed

The likely feeding habits of these four species may also explain the observed differences in the excretion of ions and metabolic wastes from feed. The most carnivorous species (snakehead) and the most herbivorous species (snakeskin) possessed different nutrient retentions. The carnivore excreted more nitrogen and Cl^- from the feed than the herbivore, whereas the herbivore excreted more K^+ and ingested more Na^+ from the feed than the carnivore (Table 1). In fasted conditions, these results were similar to those observed in a study conducted by Yanagitsuru *et al*. (2019). Here fluxes of Cl^- and K^+ by snakeskin gourami and giant gourami were similar to the results observed in these species from the current study. Nonetheless, in contrast, a much-reduced Na^+ loss by snakeskin and giant gourami was observed. It is clear that snakeskin gourami and giant gourami used throughout this study had a better ion balance than those studied by Yanagitsuru *et al*. (2019). This result may be, in part, due to the overall condition of the fish, as Yanagitsuru *et al*. (2019) suggested that the high Na^+ loss could be associated with stress from holding conditions.

The average excretion rates of ammonia and urea-N were significantly greater in fasted snakehead than in fasted snakeskin gourami (Figure 3a). Excretion of ammonia and urea-N in fasted fish was also highly correlated with gut index (see Supplementary Figure S1 and Table S1), where increases in the gut index (more herbivorous) led to

TABLE 1 The percentage of key dietary components present in the 1% ration meal excreted by each of four Anabantiformes species over 24 h as determined from flux experiments

Component	Diet composition (μmol kg^{-1} of fish)	Percentage excreted by species			
		Snakehead	Climbing perch	Giant gourami	Snakeskin
Nitrogen	46,000	27	38	16	21
K^+	1860	41	26	43	58
Na^+	1350	(12)	(23)	209	(121)
Cl^-	6350	43	17	64	(27)

Note: Numbers in brackets indicate uptake from the environment instead of excretion.

a reduction in the excretion of ammonia and urea. During digestion, the majority of ingested nitrogen is converted to protein growth, whereas the remainder is excreted from the gills as ammonia-N and to a lesser proportion as urea-N (Wood, 2001). Because herbivorous fishes are often nitrogen limited, the natural herbivorous diet of snakeskin gourami may explain why this species had a lesser excretion of ammonia-N and urea-N than the carnivorous snakehead in fasted and fed groups, although this difference was significant only in fasted groups ($P < 0.05$) (Figure 3a,c). Considering this limitation, it is surprising that snakeskin gourami had the highest stomach pH. The secretion of hydrochloric acid during digestion creates a low stomach pH that works to activate enzymes that facilitate the breakdown of protein and the absorption of dietary N (Smith *et al*., 2000; Stevens and Hume, 2004). Therefore, snakeskin gourami must depend on other mechanisms to obtain protein from their relatively protein-poor diet.

The high rate of K^+ excretion in snakeskin gourami may also be due to the relatively higher concentration of K^+ in the natural diet of herbivores. It is probable that the higher K^+ content of a herbivorous diet may lead to a lesser requirement for its absorption and/or a greater requirement for its excretion, leading to a greater excretion rate of K^+ in snakeskin than in the more carnivorous species (Figure 4a,d). The omnivores, climbing perch and giant gourami, possessed intermediate characteristics. The climbing perch excreted a high percentage of N (38%) but a low percentage of K^+ (26%) from the feed, whereas it was the reverse in giant gourami, conserving N (16% excretion) but not K^+ (43% excretion) (Table 1).

4.4 | Changes in pH, HCO_3^- and PCO_2 along the gut

In higher vertebrates, enzymatic digestion is facilitated by neutralizing chyme as it moves from the stomach to the intestine, and the major sources are pancreatic and biliary HCO_3^- secretions (Wang *et al*., 2001). Nonetheless, virtually nothing is known about the potential contributions of the pancreatic juice and the bile as sources of HCO_3^- in fish. In seawater teleosts, HCO_3^- secretion into the intestinal lumen for osmoregulatory needs originates from the intestinal epithelium itself, facilitated by Cl^-/HCO_3^- anion exchangers (Grosell, 2011a; Grosell, 2011b; Taylor & Grosell, 2006; Wilson *et al*., 1996). However, it is not clear whether this same mechanism is responsible for neutralizing acidic chyme after feeding. In freshwater fish, Cl^-/HCO_3^- exchange in the intestinal epithelium is not needed for osmoregulation, and nothing is known about the source(s) of HCO_3^- that neutralizes chyme from the stomach.

Bucking and Wood (2009), Day *et al*. (2014) and Wood and Eom (2019) observed substantial increases in pH as chyme moved from the stomach to the anterior-, mid- and posterior regions of the gut in several freshwater teleosts. This same trend was observed in each of the four species studied here; however, this effect was much reduced in snakeskin gourami, which had the most alkaline stomach chyme in comparison to all other species (Figure 6a–d). As expected, this increase in chyme pH along the tract was matched by a greater concentration of HCO_3^- in the intestine of all four species (Figure 6e–h). The present finding of high HCO_3^- (up to 33 mM) in the intestinal chyme of four air-breathing species complements the recent observations of Wood and Eom (2019) where HCO_3^- > 40 mM was observed in the intestinal chyme of two obligate water breathers (rainbow trout and goldfish) after feeding. Wood and Eom (2019) did not identify the source of the intestinal HCO_3^-. The present *in vitro* experiments on gut sacs of climbing perch indicate that it did not originate from secretory Cl^-/HCO_3^- exchange by the intestinal epithelium, as discussed subsequently. There is clearly a need to investigate potential pancreatic and biliary sources in future studies.

PCO_2 levels in the chyme, as calculated by the Henderson–Hasselbalch equation, were also very high (~15–70 mmHg) in the four species (Figure 6i–l). Again this observation is consistent with several reports of high gastrointestinal PCO_2, using the same indirect methods in water-breathing species (reviewed by Wood *et al*., 2010), and with recent direct PCO_2 measurements using fibre-optic PCO_2 sensors by Wood and Eom (2019) in trout and goldfish. In the stomach, the reaction of HCl (secreted through gastric H^+/K^+ ATPase and Cl^- channels in oxynticopeptic cells; Kopic *et al*., 2009; Wilson & Castro, 2011) with carbonates in the pellets was the likely source of this high PCO_2 as demonstrated by Wood and Eom (2019). The highest stomach PCO_2 was observed in the snakehead; this was likely due to a greater requirement for acid secretion resulting from the higher protein content in their natural diet. Despite this condition, the lowest stomach pH value was observed in climbing perch. Given this, the greatest stomach PCO_2 would be expected in this species. It is probable that the variation in gut sampling time (*i.e*., 1–4 h after feeding) may have contributed to the mismatch between stomach pH and PCO_2 in this species. Although the smaller stomach of climbing perch in comparison to the snakehead (Figure 1) may have meant that food

passed through the stomach more quickly, PCO_2 in this species did not increase to the same extent as in the snakehead.

Regardless, high PCO_2 levels, far above blood levels, in the intestine of mammals are well documented, and the two major sources of CO_2 are metabolic production by the microbial community and the reaction of pancreatic and biliary HCO_3^- with gastric HCl entering through the pylorus (Altman, 1986; Kurbel *et al*., 2006; Macfarlane *et al*., 1992; Steggerda, 1968). The same explanations for high intestinal PCO_2 (up to an average of 64 mmHg in the present study; Figure 6i–l) may apply to fish, though simultaneous H^+ and HCO_3^- secretion by intestinal tissue, which has been reported in marine teleosts (Grosell, 2011a; Grosell, 2011b), may also play a role. At present it is unclear whether these high gastrointestinal PCO_2 levels affect venous blood PCO_2 levels in water-breathing fish (Wood & Eom, 2019). In the present study, blood PCO_2 levels were relatively high (13–18 mmHg; Figure 6i–l), but, as noted later, they must be interpreted with caution because terminal samples were obtained by caudal puncture from anaesthetized fish and in any event may have reflected the natural air-breathing behaviour of the fish.

4.5 | Recovering from the post prandial alkaline tide

Although a handful of freshwater and saltwater fish species have been shown to experience a postprandial alkaline tide (*e.g*., Bucking & Wood, 2008; Cooper & Wilson, 2008; Li *et al*., 2010; Wood *et al*., 2005), much less is known about the compensatory mechanisms used by fish to combat such metabolic acid–base disturbances. In higher vertebrates, this blood alkalosis is balanced by reduced ventilation and the retention of CO_2 (*i.e*., compensatory respiratory acidosis: Busk, Overgaard, *et al*., 2000; Busk, Jensen, & Wang, 2000; Ou & Tenney, 1974; Wang *et al*., 1995; Wang *et al*., 2001). Because of the efficiency of fish gills, it has previously been suggested that it would be unlikely for water-breathing fish to compensate for the alkaline tide by retaining CO_2 (Gilmour, 2001; Perry & Gilmour, 2006). This theory has been supported by all studies to date on the alkaline tide in fish (Wood *et al*., 2005; Cooper & Wilson, 2008, Bucking & Wood, 2008; Bucking *et al*., 2009; Li *et al*., 2010), where arterial PCO_2 remained universally unchanged.

Although there were no marked differences in blood acid–base status (pH, PCO_2, HCO_3^-) among the four species, these data must be interpreted cautiously because the samples were obtained by caudal puncture of anaesthetized animals, a procedure that would likely have increased PCO_2 and decreased pH levels. Nevertheless, similar, high PCO_2 values have been observed among obligate air-breathing lungfish sampled by a catheter (Lenfant & Johansen, 1968; Perry *et al*., 2005). Gilmour *et al*. (2007) induced both metabolic acidosis and alkalosis in the African lungfish. Their findings indicate that this species was able to utilize both respiratory and metabolic compensation to restore arterial pH and that the lungfish is able to alter air ventilation to compensate for metabolic acid–base disturbances. Perhaps the present Anabantiform fishes can compensate for an alkaline or acidic tide in a similar way, at least in part. The review by Bayley *et al*. (2019) highlights the lack of representation of air-breathing fishes in the literature, and because of this lack, there is little evidence regarding whether other air-breathing fish are capable of respiratory pH control.

In freshwater trout, it is observed that individuals are able to recover from the alkaline tide through the excretion of excess HCO_3^- from the gills (Bucking & Wood, 2008; Cooper & Wilson, 2008; Goss & Perry, 1994; Perry & Gilmour, 2006) and, to a lesser extent, the kidney (Bucking *et al*., 2010; Wood *et al*., 1999). Because of this condition, a greater net base excretion in water by fish between 0 and 24 h post-feeding is observed (see Bucking & Wood, 2008; Cooper & Wilson, 2008; Wood *et al*., 2007). Nonetheless, not all fish follow this trend. Wood *et al*. (2010) described the presence of an apparent "acidic tide" in the freshwater killifish, an agastric teleost with no gastric H^+, K^+ATPase and an alkaline digestive tract that secretes HCO_3^-. In the killifish, there were decreased levels in blood pH and HCO_3^- after feeding. Interestingly, snakeskin gourami not only had the most alkaline digestive system in all study species (average stomach chyme pH 6.7) but also was the only species to have an apparent acid excretion after feeding (Figure 5b), indicating that this herbivorous species may also experience an acidic tide. The other three species trended towards increased base excretion after feeding, though none of the differences were significant (Figure 5). It is possible that these three air-breathing species experience a post-feeding alkaline tide but adopt a mammalian strategy to recover from it whereby they reduce air breathing to increase blood PCO_2, so that they rely less on base excretion to the water than do exclusively water-breathing fish (Bucking & Wood, 2008; Wood *et al*., 2007). To address this issue, future studies should measure arterial PCO_2 in blood samples taken using a catheter in fed and fasted, unanaesthetized Anabantiform fishes.

4.6 | Absorptive processes assessed *in vitro*

In most vertebrates, gastrointestinal performance rapidly increases in fed animals to meet the physiological and energetic demands of digestion and absorption. This has been particularly well documented in snakes (Ott & Secor, 2007; Secor & Diamond, 2000).

Similarly, transport processes in the gut of fish are typically upregulated after feeding (Bucking and Wood, 2006a,b; Day *et al*., 2014). Because of this condition, an apparent increase in both the secretory and absorptive functions of the gut is observed, which are important to add ions, fluid and enzymes to food and simultaneously reabsorb fluid and nutrients. Despite this, the present investigation on climbing perch is one of only a handful to clearly prove that intestinal processes are upregulated *in vitro* by feeding. Previously this was confirmed for the killifish (Wood *et al*., 2010), rainbow trout (Bucking *et al*., 2009) and toadfish (Taylor & Grosell, 2009). Here it is shown that intestinal processes are upregulated in climbing perch for net fluid, Na^+ and HCO_3^- absorption (Figures 7, 10 and 11) but not net glucose, K^+ or Cl^- transport (Figures 8, 9 and 12).

Interestingly, this species exhibited a strong reabsorption of HCO_3^- that significantly increased with feeding (Figure 11c). No other studies have reported intestinal reabsorption of HCO_3^- in fish. All previous

studies on water breathers (Bucking *et al*., 2009; Taylor & Grosell, 2009; Wood *et al*., 2010) have shown that intestinal HCO_3^- secretion, not reabsorption, increases after feeding. It is probable that this observation may be due to the need for HCO_3^- conservation in air-breathing fish that regulate pH by modifying respiration. As noted earlier, these data argue against the intestinal epithelium as a source of secreted HCO_3^- to neutralize the chyme. This result is even more surprising considering that a net absorption of Cl^- in the intestinal portions of the gut during feeding was observed (Figure 12a,b). Net Cl^- absorption would usually be coupled to a net secretion of HCO_3^- in the lumen, resulting from apical Cl^-/HCO_3^- exchangers. Nonetheless, as the net Cl^- absorption rates were several fold greater than the net HCO_3^- absorption rates (compare Figures 11 and 12), a small component of secretory Cl^- flux (*i.e.*, Cl^-/HCO_3^- reversal) may have been masked.

In most fish species, the intestine shows a high, net transmural flux of nutrients and can be considered a main site of absorption during feeding and digestion (Bakke *et al*., 2011; Ferraris & Ahearn, 1984). This may explain why there was a greater flux of most substances in the anterior- and mid-intestine than in the stomach. Consistent with other *in vitro* studies on water breathers (Nadella *et al*., 2014; Pelster *et al*., 2015; Wood *et al*., 2010; Wood & Grosell, 2012), a greater total capacity for Na^+ and fluid absorption in gut sac preparations from fed animals was observed, with the greatest net absorption rates in the anterior section of the intestine (Figures 7 and 10). *In vivo*, the situation may be more complex, because Bucking and Wood (2006a) and Bucking and Wood (2006b) showed a net secretion of water and Na^+, respectively, in the anterior intestine of the trout after feeding, presumably because of input from pancreatic and biliary secretions and elevated osmotic pressure in the chyme.

Overall, the anterior portion of the intestine would require the greatest fluid turnover to neutralize chyme from the stomach and simultaneously begin the absorption of nutrients and ions.

Notably, K^+ flux values were negative, indicating a net secretion of K^+ in the lumen in both fed and fasted fish (Figure 8). Nonetheless, there was a significantly greater net flux of K^+ in the stomach compared to that in the midgut (Figure 8a) in fed groups. This is likely due to the recycling of K^+ in the stomach and the opening of the K^+ leakage channels that fuel the H^+/K^+ ATPase to facilitate gastric HCl secretion. Similarly, a net flux of Cl^- in the stomach lumen was observed, which is likely due to the movement of Cl^- through apical Cl^- channels from acid-secreting oxynticopeptic cells (Wilson & Castro, 2011), similar to the parietal cells of mammals (Kopic *et al*., 2009).

4.7 | Future directions

Given the findings from this study and the known gaps in knowledge, a number of future studies on these economically important Anabantiform fishes are proposed:

1. In Anabantiform fish, post-feeding, (a) confirm the presence/absence of an alkaline tide in blood in species with acidic stomachs, (b) determine whether HCO_3^- is absorbed from the intestine after feeding in more species and (c) investigate the possible role of arterial PCO_2 and ventilatory regulation in the correction of any alkaline tide if found in these air-breathing fishes.
2. Assess whether a greater reliance on air breathing (*e.g.*, under aquatic hypoxia) could elicit changes in the acid–base, ionoregulatory and N-waste excretion responses to feeding.
3. Further elucidate the possible presence of a postprandial "acidic tide" in snakeskin gourami.
4. Investigate acid–base and ionoregulatory responses to feeding on diets representative of the natural diets of these species, so as to generate more effective species-specific diets for these aquaculturally important species. For example, it may be possible to minimize wasting of protein-N.
5. Track the blood gas and acid–base status in fed and fasted cannulated animals to understand the possible role of high-chyme PCO_2 after feeding on blood gas transport.

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AUTHOR CONTRIBUTIONS

H.R.G., L.B., W.G.D., O.E.J., A.B.K., P.L.M., T.H.T. and C.M.W. helped in data generation. H.R.G., C.M.W. and O.E.J. assisted in data analysis. H.R.G., C.M.W. and O.E.J. were involved in manuscript preparation.

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ORCID

Harriet R. Goodrich https://orcid.org/0000-0002-2568-0562
William G. Davison https://orcid.org/0000-0003-0501-8851
Ora E. Johannsson https://orcid.org/0000-0002-7944-379X
Chris M. Wood https://orcid.org/0000-0002-9542-2219

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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