



An in vitro study of urea and ammonia production and transport by the intestinal tract of fed and fasted rainbow trout: responses to luminal glutamine and ammonia loading

Ellen H. Jung¹ · Joanna Smich^{2,3} · Julian G. Rubino² · Chris M. Wood^{1,2}

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Abstract

Digestion of dietary protein in teleosts results in high ammonia levels within the intestinal chyme that may reach concentrations that are many-fold greater than blood plasma levels. We used in vitro gut sac preparations of the ammoniotelic rainbow trout (*Oncorhynchus mykiss*) to investigate the role of the intestine in producing and transporting ammonia and urea, with specific focus on feeding versus fasting, and on responses to loading of the lumen with 2 mmol L⁻¹ glutamine or 2 mmol L⁻¹ ammonia. Feeding increased not only ammonia production and both mucosal and serosal fluxes, but also increased urea production and serosal fluxes. Elevated urea production was accompanied by an increase in arginase activity but minimal CPS III activity, suggesting that urea may be produced by direct arginolysis. The ammonia production and serosal fluxes increased in fasted preparations with glutamine loading, indicating an ability of the intestinal tissue to deaminate glutamine and perhaps use it as an energy source. However, there was little evidence of urea production or transport resulting from the presence of glutamine. Furthermore, the intestinal tissues did not appear to convert surplus ammonia to urea as a detoxification mechanism, as urea production and serosal flux rates decreased in fed preparations, with minimal changes in fasted preparations. Nevertheless, there was indirect evidence of detoxification by another pathway, as ammonia production rate decreased with ammonia loading in fed preparations. Overall, our study suggests that intestinal tissues of rainbow trout have the ability to produce urea and detoxify ammonia, likely via arginolysis.

Keywords *Oncorhynchus mykiss* · Arginase · CPS III · Ornithine urea cycle · Prandial · Gastrointestinal tract

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✉ Ellen H. Jung
jung@zoology.ubc.ca

Joanna Smich
joannasmich@hotmail.com

Julian G. Rubino
rubinojg@gmail.com

Chris M. Wood
woodcm@zoology.ubc.ca

¹ Department of Zoology, University of British Columbia, 6270 University Boulevard, Vancouver, BC V6T 1Z4, Canada

² Department of Biology, McMaster University, 1280 Main St. West, Hamilton, ON L8S 4K1, Canada

³ Faculty of Veterinary Medicine, Warsaw University of Life Sciences, Nowoursynowska 166, 02-787 Warsaw, Poland

Introduction

In teleost fish, protein degradation during digestion produces amino acids (Cowe and Sargent 1972), which are mainly absorbed into the blood and transported to the liver to be catabolized (Collie and Ferraris 1995; Bakke-McKellep et al. 2000). Some deamination occurs within the intestinal lumen, such that ammonia levels in the chyme of teleosts may reach 1 mmol L⁻¹ or higher, concentrations that are many-fold greater than blood plasma levels (Buckling and Wood 2012; Buckling et al. 2013b; Rubino et al. 2014; Pelster et al. 2015; Wood et al. 2019). The intestine itself also catabolizes some of the absorbed amino acids from ingested food as endogenous energy sources, producing additional ammonia (Ballantyne 2001; Buckling and Wood 2012; Buckling et al. 2013b). Most teleosts, such as rainbow trout (*Oncorhynchus mykiss*), are ammoniotelic, excreting ammonia as the primary nitrogenous waste (Randall and Wright 1987; Anderson 2001; Wright and

Wood 2009). Following a meal, the intestine both absorbs ammonia from the chyme and produces ammonia by its catabolism of amino acids (Rubino et al. 2014), increasing systemic plasma ammonia concentration by up to two to threefold (Bucking and Wood 2008, 2012; Karlsson et al. 2006). The plasma ammonia level after feeding is particularly high in the hepatic portal vein (HPV) draining from the gastrointestinal tract (GIT) (Karlsson et al. 2006). The ammonia originating from the intestine is first passed by the HPV to the liver where it may be used for amino acid synthesis, or else it escapes to the ventral aortic blood perfusing the gills, where much of it is excreted out to the water (e.g. Brett and Zala 1975; Bucking et al. 2010; Bucking and Wood 2008; Zimmer et al. 2010), leaving lower, but still elevated, plasma ammonia level in the dorsal aorta (Karlsson et al. 2006). In the rainbow trout, (Rubino et al. 2014) it is estimated that approximately 47% of post-prandial ammonia excretion was produced in the intestine.

In contrast, most terrestrial species are ureotelic, producing nitrogenous waste in a less toxic form, urea. Typically in mammals, the amino groups released when amino acids are oxidized feed into the ornithine-urea cycle (OUC) in the liver and the resulting urea is excreted by the kidney as a component of urine (Schmidt-Nielsen 1958). Although urea production is more energetically costly, a few species of teleosts are ureotelic (e.g. Magadi tilapia and gulf toadfish) and/or may synthesize urea to detoxify ammonia depending on the environment, life stage, or physiological condition (Randall et al. 1989; Wood et al. 1995; Felskie et al. 1998; Julsrud et al. 1998; Kong et al. 1998). Indeed, many species in their early life stages express OUC enzymes, producing urea and minimizing ammonia accumulation within the embryo, but lose this ureagenic pathway as development proceeds (Dépêche et al. 1979; Chadwick and Wright 1999; Terjesen et al. 2002; Braun et al. 2009; Zimmer et al. 2017). Moreover, in the marine plainfin midshipman (*Porichthys notatus*, an ammoniotelic relative of the ureotelic gulf toadfish) (Bucking et al. 2013b) presented evidence for a model in which ammonia is transported from the intestinal lumen into the enterocytes, where it is detoxified by conversion to urea via the OUC, and then transported back into the lumen for use by ureolytic bacteria or later excretion via the anus. However, in typical adult ammoniotelic teleosts such as freshwater rainbow trout, little is known about the capacity of the intestinal cells to produce and/or transport urea. Interestingly, Karlsson et al. (2006) showed a significant increase in urea concentration in the HPV and dorsal aorta (DA) after feeding, implying a possible production and/or transport of urea by the intestinal cells. Additionally, Kajimura et al. (2004) provided indirect evidence for urea production by the intestine and excretion by the anus in trout. Thus, in this study, we used a similar in vitro gut sac approach to that

employed by Bucking et al. (2013b) and Rubino et al. (2014) to test whether the intestinal cells of rainbow trout are able to synthesize and transport urea.

With the gut sac preparation, we can test the production and direction of transport (to lumen or blood side) of the nitrogenous wastes in response to experimental treatments. If the intestinal cells have the ability to produce urea, we hypothesized that feeding would increase not only ammonia, but also urea production and flux rates. We also tested for two key enzymes involved in urea production (Julsrud et al. 1998; Lindley et al. 1999; Anderson et al. 2002; Kajimura et al. 2006; Bucking et al. 2013b). In the OUC of mammals and amphibians, carbamoyl phosphate synthetase (CPS) I catalyzes the ATP-dependent synthesis of carbamoyl phosphate from ammonia substrate. However, in most teleosts, CPS III prefers glutamine as the nitrogen-donating substrate (reviewed by Anderson 1995). Arginase is also involved in the OUC, converting L-arginine to L-ornithine, producing urea directly in the process. If the intestinal cells have the ability to produce urea, we hypothesized that there would be an increase in CPS III and arginase activities after feeding. Furthermore, the gut sac preparation allows us to examine the in vitro response of loading the lumen with potential substrates for urea production such as glutamine and ammonia. As glutamine is thought to be the immediate nitrogen-donating substrate for the OUC in fish (Anderson 1995), we hypothesized that a luminal glutamine supply would increase both ammonia and urea production and flux rates as the intestinal cells break down the amino acid and/or feed it into the OUC. In addition, if the intestinal cells have the ability to detoxify high luminal ammonia, we hypothesized that they would convert the surplus ammonia to urea and thus increase urea production and flux rates.

Methods

Experimental animals and holding

Freshwater rainbow trout (150–250 g) were obtained from Humber Springs Trout Hatchery, Ontario, Canada. Upon arrival, the trout were acclimated for 1–2 months to laboratory conditions in running, dechlorinated Hamilton tap-water with constant aeration (moderately hard water from Lake Ontario: $[\text{Na}^+] = 0.6$ mequiv L^{-1} , $[\text{Cl}^-] = 1.8$ mequiv L^{-1} , $[\text{Ca}^{2+}] = 0.8$ mequiv L^{-1} , $[\text{Mg}^{2+}] = 0.3$ mequiv L^{-1} , $[\text{K}^+] = 0.05$ mequiv L^{-1} ; titration alkalinity 2.1 mequiv L^{-1} , pH ~ 8.0; hardness ~ 140 mg L^{-1} as CaCO_3 equivalents; temperature 12.5–15 °C, water flow rate = 30 mL s^{-1} , background ammonia concentration ≤ 10 $\mu\text{mol L}^{-1}$). The trout were kept in aerated 500-L tanks with approximately 30 fish per tank. Scheduled feedings occurred three times per week (Martin Profishent Aquaculture Nutrition, Tavistock, ON,

Canada; crude protein 45%, crude fat 9%, crude fiber 3.5%). Ration size was 3% of body mass, which provided satiation. Experiments on fed fish occurred exactly 24 h following a meal and fasted experiments occurred 7 days following the last meal. Animal handling was in compliance with an approved McMaster Animal Care Committee Animal Utilization Protocol 12-12-45.

In vitro gut sac experiments and calculations

Gut sac experiments were performed to quantify the changes in urea-N and ammonia fluxes (J_s and J_m), as well as tissue ammonia and urea-N loads, based on the influence of the feeding state and varying composition of mucosal solutions used. Trout were euthanized using 0.07 g L^{-1} MS-222, neutralized with NaOH. The intestine was removed, thoroughly rinsed with Cortland saline (in mmol L^{-1} : NaCl 124, KCl 5.1, CaCl_2 1.6, MgSO_4 0.9, NaHCO_3 11.9, NaH_2PO_4 3, glucose 5.5, pH 7.4), then sectioned into the anterior, mid, and posterior regions. Each of these regions was tied off at one end with a 2-0 silk thread while the other end had a flared polyethylene tube (Intramedic Clay-Adams PE 60; Becton–Dickinson and Company, Sparks, MD, USA) inserted and held securely with another silk thread. Saline, which had been pre-equilibrated with a 99.7% O_2 : 0.3% CO_2 gas mix, was then injected into the gut sac through the open polyethylene tube using a syringe (termed mucosal saline hereafter). In the control group, the gut sacs were loaded with regular Cortland saline. In the high luminal glutamine treatment, the sacs were loaded with Cortland saline containing 2 mmol L^{-1} of L-glutamine (Sigma-Aldrich, St. Louis, MO, USA) and in the high luminal ammonia group with Cortland saline containing 2 mmol L^{-1} of NH_4Cl (Fisher Scientific, Toronto, ON, Canada). The saline was infused and then withdrawn multiple times for thorough mixing. On the final filling, the saline was injected until the intestinal region became taut, and the remainder of the saline was collected as the initial mucosal sample. The PE tube was then sealed, and the sac was thoroughly blotted dried, then weighed to 0.0001 g accuracy for initial weight (W_i). It was then inserted into a centrifuge tube that contained regular Cortland saline (termed serosal saline hereafter). Anterior intestine preparations were placed in a 50-mL centrifuge tubes while both mid and posterior intestine sacs were placed in 15-mL centrifuge tubes. The amount of serosal saline needed to fully immerse the preparations varied, and was exactly recorded, and an initial serosal sample was taken.

During a 2-h flux period, the saline was bubbled with 99.7% O_2 : 0.3% CO_2 gas mix to mimic physiological PCO_2 and to maximize O_2 supply in the preparation. Then, the gut sacs were removed, thoroughly blotted dry, and weighed again (W_f). Their internal contents were collected as the final mucosal sample. Finally, the drained gut sac was thoroughly

blotted again and weighed to yield the empty weight (W_e) of the preparation. In the equations below, the final mucosal volume (V_{m_f}) was calculated as $W_f - W_e$, and the initial mucosal volume (V_{m_i}) as $W_i - W_e$. The saline left in the centrifuge tubes was collected as the final serosal sample. The intestinal regions were traced onto 0.5-mm graph paper, which allowed each of their surface areas to be determined. This technique was first outlined by Grosell and Jensen (1999). All samples, including the initial ones and the gut sac tissue itself, were frozen in liquid N_2 , then stored at -80°C for later analysis of ammonia and urea-N concentrations. The following parameters were calculated.

Serosal urea ($J_{s_{\text{urea-N}}}$) flux rate

$$J_{s_{\text{urea-N}}} = \frac{[(T_{s_{\text{urea}}f} - T_{s_{\text{urea}}i}) \times V_s]}{SA \times t} \times 2, \quad (1)$$

where $J_{s_{\text{urea-N}}}$ is in $\mu\text{mol-N cm}^{-2} \text{ h}^{-1}$, $T_{s_{\text{urea}}f}$ and $T_{s_{\text{urea}}i}$ are the final and initial urea concentrations ($\mu\text{mol L}^{-1}$) in the serosal saline, V_s is the volume of serosal solution (L), SA is intestinal surface area (cm^2), and t is time (h). Note that Eqs. (1) and (2) are multiplied by 2 due to the presence of two nitrogen in urea. All $J_{s_{\text{urea-N}}}$ fluxes were positive, into the serosal saline.

Mucosal urea ($J_{m_{\text{urea-N}}}$) flux rate

$$J_{m_{\text{urea-N}}} = \frac{[(T_{m_{\text{urea}}i} \times V_{mi}) - (T_{m_{\text{urea}}f} \times V_{mf})]}{SA \times t} \times 2, \quad (2)$$

where $J_{m_{\text{urea-N}}}$ is in $\mu\text{mol-N cm}^{-2} \text{ h}^{-1}$, $T_{m_{\text{urea}}f}$ and $T_{m_{\text{urea}}i}$ are final and initial urea concentrations ($\mu\text{mol L}^{-1}$) in the mucosal saline, V_{mi} and V_{mf} are initial and final volumes of mucosal saline (L). Positive $J_{m_{\text{urea-N}}}$ fluxes were out of the mucosal saline; negative $J_{m_{\text{urea-N}}}$ fluxes were into the mucosal saline.

Serosal ammonia ($J_{s_{\text{amm}}}$) flux rate

$$J_{s_{\text{amm}}} = \frac{[(T_{s_{\text{amm}}f} - T_{s_{\text{amm}}i}) \times V_s]}{SA \times t}, \quad (3)$$

where $J_{s_{\text{amm}}}$ is in $\mu\text{mol cm}^{-2} \text{ h}^{-1}$, $T_{s_{\text{amm}}f}$ and $T_{s_{\text{amm}}i}$ are the final and initial ammonia concentrations ($\mu\text{mol L}^{-1}$) in the serosal saline, V_s is the volume of serosal solution (L), SA is intestinal surface area (cm^2), and t is time (h). All $J_{s_{\text{amm}}}$ fluxes were positive, into the serosal saline.

Mucosal ammonia ($J_{m_{\text{amm}}}$) flux rate

$$J_{m_{\text{amm}}} = \frac{[(T_{m_{\text{amm}}i} \times V_{mi}) - (T_{m_{\text{amm}}f} \times V_{mf})]}{SA \times t}, \quad (4)$$

where $J_{m_{amm}}$ is in $\mu\text{mol cm}^{-2} \text{h}^{-1}$, where $T_{m_{amm}^f}$ and $T_{m_{amm}^i}$ are final and initial ammonia concentrations ($\mu\text{mol L}^{-1}$) in the mucosal saline, V_{mi} and V_{mf} are initial and final volumes of mucosal saline (L). Positive $J_{m_{amm}}$ fluxes were out of the mucosal saline; negative $J_{m_{amm}}$ fluxes were into the mucosal saline.

Total tissue urea and ammonia production rates ($J_{t_{urea}}$ and $J_{t_{amm}}$)

$$J_t = J_s - J_m, \quad (5)$$

J_t ($\mu\text{mol cm}^{-2} \text{h}^{-1}$) represents the net rate of endogenous production of ammonia or urea-N by the intestinal tissue itself.

Total tissue urea and ammonia (T_{urea} and T_{amm})

$$T = \frac{[\text{urea} - \text{N or ammonia in tissue}]}{\text{weight of tissue}}, \quad (6)$$

T ($\mu\text{mol g}^{-1}$) represents the concentration of urea-N or ammonia found in the tissue itself.

Analytical methods

Mucosal samples were deproteinized using ice-cold 20% perchloric acid (PCA) before the ammonia assay was run to prevent potential protein interference with the assay, then spun at 13,000 rpm for 1 min. After the supernatant was collected, samples were pH-neutralized using 1 mmol L^{-1} KOH. Flash-frozen intestinal tissues were ground into fine powder using N_2 -cooled mortar and pestle, then deproteinized using a solution of 8% PCA and 1 mM ethylenediaminetetraacetic acid (EDTA), followed by pH neutralization using 1 mmol L^{-1} KOH. Serosal samples showed no protein interference with the ammonia assay, thus were not deproteinized. The ammonia assay was performed via a commercial kit (Raichem Cliniqa™; glutamate dehydrogenase method) and read at 340 nm. The urea assay employed the colorimetric method of Rahmatullah and Boyde (1980) and was read at 525 nm.

Enzymatic analysis

Intestine tissue was taken from randomly selected trout that had been subjected to either fasted or fed pre-treatment, prior to euthanization as described above. The intestine was sectioned, cleaned and immediately flash frozen in liquid N_2 . In some intestinal sections, the muscle layer was separated from the epithelial layer using a glass slide. This was done to further analyze enzymatic activity within these different constituents.

OUC enzyme CPS III activity was measured using a CPS assay similar to that employed by Bucking et al. (2013b) at 412 nm. The reaction involved in this assay proceeds in the forward direction. The reaction mixture contained 20 mmol L^{-1} ATP, 25 mmol L^{-1} MgCl_2 , 25 mmol L^{-1} *N*-acetyl glutamate (AGA), 2 mmol L^{-1} dithiothreitol, 5 mmol L^{-1} ornithine, 20 mmol L^{-1} glutamine, 25 mmol L^{-1} phospho(enol) pyruvate, and 50 mmol L^{-1} Hepes, pH 8.0. 20 mmol L^{-1} glutamine was used to determine the activity of CPS III or CPS II while 1.7 mmol L^{-1} UTP was used to inhibit CPS II. Arginase enzyme activity was analyzed using an arginase assay similar to that employed by Felskie et al. (1998). This reaction also proceeds in the forward direction. The reaction mixture contained 250 mmol L^{-1} arginine, 1 mmol L^{-1} MnCl_2 , and 50 mmol L^{-1} Hepes, pH 8.0. The enzyme was activated with mixture that contained 5 mmol L^{-1} MnCl_2 in 50 mol L^{-1} Hepes. Activity units of all enzymes were quantified as the micromolar appearance of reaction product in the measured solution per minute (U g^{-1} tissue).

Statistical analysis

All graphs were made and statistical analyses were performed using Graphpad Prism software (version 7.0a). Data have been expressed as means \pm SEM (N = number of fish). Two-way ANOVA and post hoc Tukey's multiple comparison tests were performed on the control group to look at the effect of feeding and the intestinal sections on flux rates, tissue urea or ammonia concentrations, and total tissue urea or ammonia levels, as well as on the CPS III and arginase activities. Comparisons between controls and either glutamine or ammonia treatment on all other flux rates, $J_{t_{urea/amm}}$ or $T_{urea/amm}$ were conducted using two-way ANOVA with intestinal section as the other factor, and post hoc Dunnett's multiple comparison tests. A significance level of $p < 0.05$ were used in all tests.

Results

Effects of feeding on urea and ammonia handling

In general, for both urea-N and ammonia, serosal flux rates (positive = appearance in the serosal fluid) were many-fold higher on an absolute basis than mucosal flux rates (negative = appearance in the mucosal fluid) (Table 1). There were significant overall effects of feeding on $J_{s_{urea-N}}$ ($p < 0.0001$), $J_{t_{urea-N}}$ ($p = 0.0015$), $J_{s_{amm}}$ ($p < 0.0001$), $J_{m_{amm}}$ ($p = 0.0034$), $J_{t_{amm}}$ ($p < 0.0001$), and T_{amm} ($p = 0.0063$) (Table 1). In all cases these effects were stimulatory, resulting in more positive serosal and total fluxes, more negative mucosal fluxes, and greater tissue contents. Feeding did not have a significant overall effect

Table 1 Comparison of serosal ($J_{s_{\text{urea-N}}}$) and mucosal ($J_{m_{\text{urea-N}}}$) urea-N flux rates, total tissue urea-N production rate ($J_{t_{\text{urea-N}}}$), tissue urea-N concentration ($T_{\text{urea-N}}$), ammonia flux rates ($J_{s_{\text{amm}}}$ and $J_{m_{\text{amm}}}$), total tissue ammonia production rate ($J_{t_{\text{amm}}}$), and tissue ammonia (T_{amm}) of fasted and fed fish

		Anterior intestine	Mid intestine	Posterior intestine
$J_{s_{\text{urea-N}}}$ ($\mu\text{mol urea-N cm}^{-2} \text{ h}^{-1}$) [†]	Fasted	0.086 ± 0.019 ^a	0.020 ± 0.002 ^b	0.051 ± 0.006 ^{ab}
	Fed [‡]	0.171 ± 0.027 ^{A*}	0.077 ± 0.010 ^B	0.083 ± 0.010 ^B
$J_{m_{\text{urea-N}}}$ ($\mu\text{mol urea-N cm}^{-2} \text{ h}^{-1}$)	Fasted	-0.019 ± 0.006	-0.006 ± 0.003	-0.029 ± 0.008
	Fed	-0.009 ± 0.007	-0.009 ± 0.003	-0.046 ± 0.034
$J_{t_{\text{urea-N}}}$ ($\mu\text{mol urea-N cm}^{-2} \text{ h}^{-1}$)	Fasted	0.104 ± 0.020	0.026 ± 0.003	0.079 ± 0.010
	Fed [‡]	0.180 ± 0.028	0.086 ± 0.011	0.129 ± 0.035
$T_{\text{urea-N}}$ ($\mu\text{mol urea-N g}^{-1} \text{ tissue}$)	Fasted	0.22 ± 0.02	0.29 ± 0.05	0.37 ± 0.02
	Fed	0.33 ± 0.02	0.27 ± 0.03	0.41 ± 0.05
$J_{s_{\text{amm}}}$ ($\mu\text{mol ammonia cm}^{-2} \text{ h}^{-1}$) [†]	Fasted	0.305 ± 0.034 ^a	0.136 ± 0.014 ^b	0.112 ± 0.018 ^b
	Fed [‡]	0.595 ± 0.038 ^{A*}	0.387 ± 0.052 ^{B*}	0.292 ± 0.038 ^{B*}
$J_{m_{\text{amm}}}$ ($\mu\text{mol ammonia cm}^{-2} \text{ h}^{-1}$) [†]	Fasted	-0.041 ± 0.010	-0.039 ± 0.014	-0.094 ± 0.018
	Fed [‡]	-0.071 ± 0.033 ^A	-0.088 ± 0.024 ^A	-0.287 ± 0.070 ^{B*}
T_{amm} ($\mu\text{mol ammonia g}^{-1} \text{ tissue}$) [†]	Fasted	2.19 ± 0.18	2.61 ± 0.49	2.45 ± 0.27
	Fed [‡]	2.56 ± 0.07 ^A	3.18 ± 0.15 ^{A^B}	4.39 ± 0.75 ^{B*}
$J_{t_{\text{amm}}}$ ($\mu\text{mol ammonia cm}^{-2} \text{ h}^{-1}$)	Fasted	0.346 ± 0.035	0.174 ± 0.020	0.206 ± 0.026
	Fed [‡]	0.666 ± 0.050 [*]	0.475 ± 0.058	0.579 ± 0.079 [*]

Single dagger (†) in the first column indicates a significant overall effect of intestinal section and double dagger (‡) in the second column indicates a significant overall effect of feeding ($p < 0.05$, two-way ANOVA)

Letters represent significant difference among GIT sections in each row, using small letters for fasted fish and capital letters for fed fish ($p < 0.05$). Asterisk (*) represents significant differences between fasted and fed fish within the same intestinal section ($p < 0.05$). Values are means ± SEM ($N = 5$)

on $J_{m_{\text{urea-N}}}$ ($p = 0.79$) and $T_{\text{urea-N}}$ ($p = 0.17$) (Table 1). Specifically, feeding significantly increased $J_{s_{\text{urea-N}}}$ only in the anterior intestine ($p = 0.0055$), but there were no differences of $J_{t_{\text{urea-N}}}$ between groups in each individual section, despite the significant overall difference. However, fed fish had more positive $J_{s_{\text{amm}}}$ in all intestinal sections ($p < 0.05$), and more negative $J_{m_{\text{amm}}}$ ($p = 0.0065$) and greater T_{amm} ($p = 0.0210$) in the posterior intestine than fasted fish. There were also significant effects of intestinal sections on $J_{s_{\text{urea-N}}}$ ($p < 0.0001$), $J_{s_{\text{amm}}}$ ($p < 0.0001$), both of which tended to be greater in the anterior section, and on $J_{m_{\text{amm}}}$ ($p = 0.0008$), and T_{amm} ($p = 0.0460$), both of which tended to be greater in the more posterior sections. There was no significant interaction between feeding and intestinal sections for any of the measurements ($p > 0.05$).

Table 2 CPS III activity ($\text{nmol min}^{-1} \text{ g}^{-1} \text{ tissue}$) of the anterior, mid, and posterior muscle and epithelial scrapings of fasted and fed fish

		Anterior intestine	Mid intestine	Posterior intestine
Muscle CPS III activity ($\text{nmol min}^{-1} \text{ g}^{-1} \text{ tissue}$)	Fasted	0.529 ± 0.152	0.111 ± 0.059	0.546 ± 0.102
	Fed [‡]	0.112 ± 0.019 [*]	0.358 ± 0.080	0.230 ± 0.035
Epithelial scraping CPS III activity ($\text{nmol min}^{-1} \text{ g}^{-1} \text{ tissue}$)	Fasted	0.355 ± 0.101	0.093 ± 0.037	0.091 ± 0.033
	Fed	0.074 ± 0.027 [*]	0.149 ± 0.073	0.130 ± 0.027

There were no significant overall effects of intestinal section, but double dagger (‡) in the second column indicates a significant overall effect of feeding; there were also significant interactive effects in both muscle and epithelial scrapings ($p < 0.05$, two-way ANOVA). Asterisk (*) represents significant difference of fed muscle or epithelial scraping in comparison to fasted muscle or epithelial scraping ($p < 0.05$). Values are means ± SEM ($N = 5$)

CPS III and arginase enzyme activity levels after feeding

Feeding had a significant overall effect on the muscle CPS III activity, but not the epithelial scraping CPS III activity (Table 2: $p = 0.031$ and 0.20, respectively). The anterior intestine, specifically, had a significant decrease in CPS III activity in the muscle ($p = 0.0251$) and epithelial scraping ($p = 0.0209$) after feeding. There was no significant overall effect of intestinal section on either the muscle or epithelial scraping CPS III activity ($p = 0.23$ and 0.15, respectively). In contrast, feeding had a significant overall effect on arginase activity in the epithelial scrapings, but not in the muscle (Table 3: $p = 0.005$ and 0.74, respectively). In fed fish, the arginase activity was significantly lower in the muscle of

Table 3 Arginase activity ($\mu\text{mol min}^{-1} \text{g}^{-1}$ tissue) of the anterior, mid, and posterior muscle and epithelial scrapings of fasted and fed fish

		Anterior intestine	Mid intestine	Posterior intestine
Muscle arginase activity ($\mu\text{mol min}^{-1} \text{g}^{-1}$ tissue) [†]	Fasted	0.417 ± 0.103	1.680 ± 0.349	2.397 ± 0.365
	Fed	0.873 ± 0.275	2.558 ± 0.380	0.821 ± 0.190*
Epithelial scraping arginase activity ($\mu\text{mol min}^{-1} \text{g}^{-1}$ tissue) [†]	Fasted	0.807 ± 0.212	0.821 ± 0.214	1.234 ± 0.247
	Fed [‡]	0.840 ± 0.247	3.175 ± 0.380*	1.035 ± 0.380

Single dagger (†) in the first column indicates a significant overall effect of intestinal section and double dagger (‡) in the second column indicates a significant overall effect of feeding; there were also significant interactive effects in both muscle and epithelial scrapings ($p < 0.05$, two-way ANOVA). Asterisk (*) represents significant difference of fed muscle or epithelial scraping in comparison to fasted muscle or epithelial scraping ($p < 0.05$). Values are means ± SEM ($N = 5$)

posterior intestine ($p = 0.0105$), but higher in the epithelial scraping of mid intestine ($p < 0.0001$). There was also a significant overall effect of intestinal section on the arginase activity in both muscle and epithelial scrapings ($p = 0.0002$ and 0.0013 , respectively). There were significant interactions between feeding and intestinal sections in both muscle and epithelial scraping on CPS III activity ($p = 0.0016$ and 0.0105 , respectively) and arginase activity ($p = 0.0007$ and 0.0003 , respectively).

High luminal glutamine treatment

In general, there were minimal effects of high luminal glutamine on intestinal urea-N handling. There was no significant overall effect of high luminal glutamine on either serosal or mucosal urea-N flux rates in either fasted or fed fish (Fig. 1: all $p = 0.32$ – 0.66). In both fasted and fed fish, there were also no overall effects of treatment on $J_{\text{urea-N}}$ (Fig. 2: $p = 0.31$ and 0.62 respectively) and $T_{\text{urea-N}}$ (Fig. 3: $p = 0.66$ and 0.06 respectively). When compared between treatment and control groups within intestinal sections, the glutamine treatment significantly increased $J_{\text{S}_{\text{urea-N}}}$ in the anterior intestine of fed fish only (Fig. 1b: $p = 0.0105$), which translated to higher $J_{\text{urea-N}}$ in the same anterior intestine (Fig. 2b: $p = 0.0275$).

However, in contrast to urea-N handling, ammonia handling was substantially altered by high glutamine in both fasted and fed fish. High glutamine had a significant overall effect ($p = 0.0111$) to increase $J_{\text{S}_{\text{amm}}}$ in fasted fish (Fig. 4a) with a significantly higher $J_{\text{S}_{\text{amm}}}$ in the anterior intestine only (Fig. 4a: $p = 0.0262$). There were no significant effects of glutamine on $J_{\text{S}_{\text{amm}}}$ in fed fish (Fig. 4b: $p = 0.31$). There were also significant overall effects of glutamine on $J_{\text{M}_{\text{amm}}}$ in both treatments (Fig. 4c, d: $p = 0.0159$ in fasted fish and $p = 0.0198$ in fed fish). However, these effects were different between fasted fish where $J_{\text{M}_{\text{amm}}}$ became more negative (Fig. 4c), and fed fish where $J_{\text{M}_{\text{amm}}}$ became less negative (Fig. 4d). The more negative $J_{\text{M}_{\text{amm}}}$ in the posterior intestine of fasted fish was significant (Fig. 4c: $p = 0.0456$), but none of the section-specific differences in $J_{\text{M}_{\text{amm}}}$ of fed fish

were significant (Fig. 4d). As a result, there were also significant effects on $J_{\text{T}_{\text{amm}}}$ (Fig. 5: $p = 0.0012$ in fasted fish and $p = 0.0245$ in fed fish), where $J_{\text{T}_{\text{amm}}}$ became greater in fasted fish (Fig. 5a: significant in all three intestinal sections, $p < 0.05$), but lower in fed fish, with no significant section-specific differences in the latter (Fig. 5b). There was also a significant overall effect of glutamine elevating T_{amm} in fasted fish (Fig. 6a: $p = 0.0060$), which was not seen in the fed fish (Fig. 6b: $p = 0.96$). There was no significant interaction between treatment and intestinal sections for any of the measurements ($p > 0.05$) except for $J_{\text{S}_{\text{urea-N}}}$ in fed fish ($p = 0.045$).

High luminal ammonia treatment

High luminal ammonia had minimal or negative effects on urea-N fluxes and production in both fasted and fed fish. There was a significant overall inhibitory effect of treatment on $J_{\text{S}_{\text{urea-N}}}$ in fed fish (Fig. 1b: $p = 0.0007$), but no effect on $J_{\text{S}_{\text{urea-N}}}$ in fasted fish (Fig. 1a: $p = 0.09$). In fed fish, ammonia treatment significantly decreased $J_{\text{S}_{\text{urea-N}}}$ in mid and posterior intestines (Fig. 1b: $p = 0.0404$ and 0.0314 respectively). There was no effect of high ammonia on $J_{\text{M}_{\text{urea-N}}}$ in either fasted or fed fish (Fig. 1c, d: $p = 0.59$ and 0.83 respectively). Thus, there were also no significant effects of treatment on $J_{\text{T}_{\text{urea-N}}}$ in fasted fish (Fig. 2a: $p = 0.22$), but a significant overall inhibitory effect on $J_{\text{T}_{\text{urea-N}}}$ in the fed fish ($p = 0.0067$), which was significant in the posterior intestine (Fig. 2b: $p = 0.0257$). In both fasted and fed fish, the high luminal ammonia treatment also had a significant overall inhibitory effect on urea-N content, $T_{\text{urea-N}}$ (Fig. 3a, b: $p = 0.0007$ and < 0.0001 respectively). With the exception of the anterior intestine of fasted fish (Fig. 3a: $p = 0.33$), these inhibitory effects on $T_{\text{urea-N}}$ were significant in all intestinal sections in both fasted and fed fish (Fig. 3a, b: $p = 0.0004$ – 0.03).

There were also marked effects of high luminal ammonia on ammonia fluxes and ammonia production rates. However, while there were no significant overall effects of high ammonia on $J_{\text{S}_{\text{amm}}}$ in either fasted or fed fish (Fig. 4a, b:

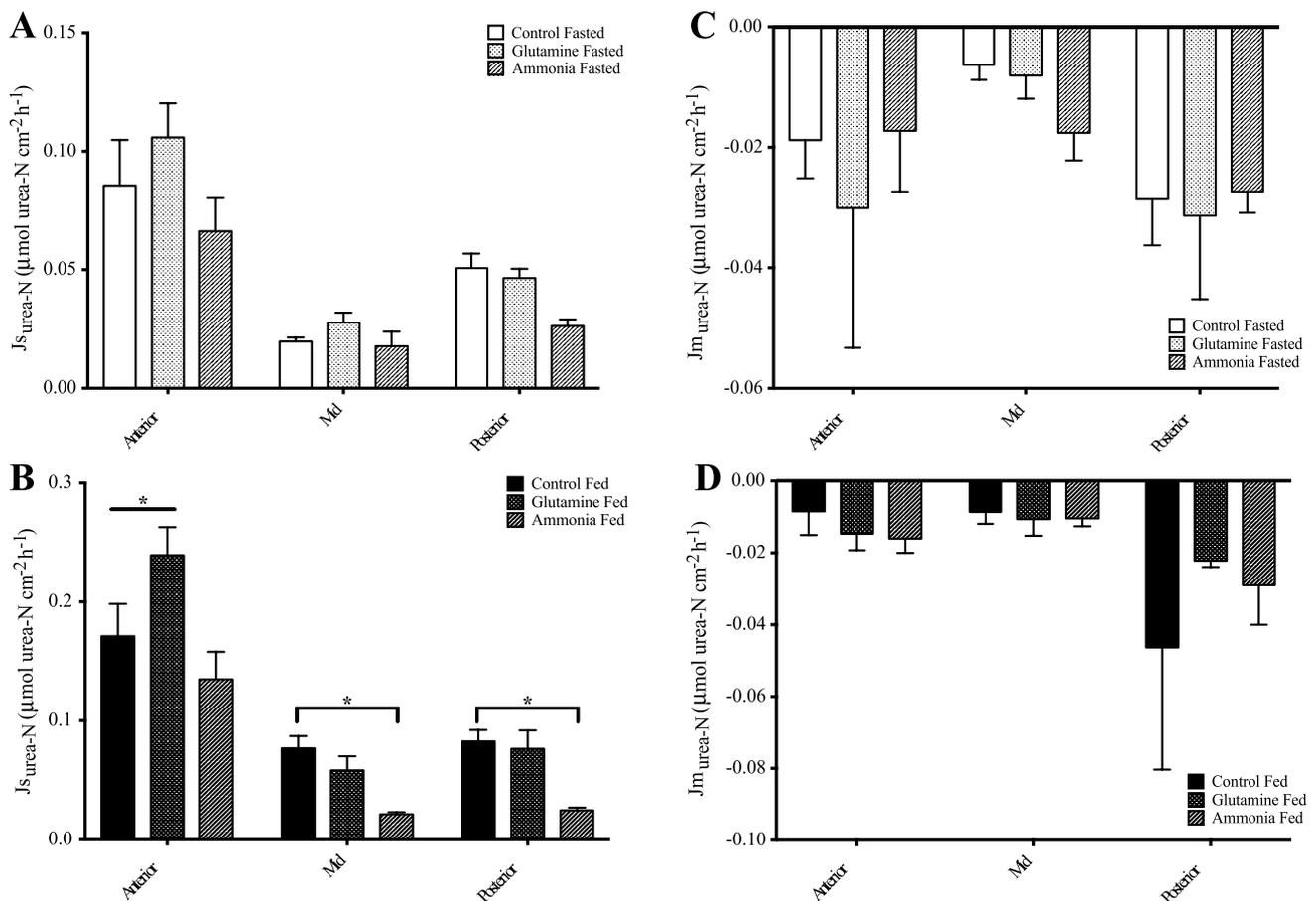


Fig. 1 Serosal (**a, b**; $J_{s_{urea}}$) and mucosal (**c, d**; $J_{m_{urea}}$) urea-N flux rates ($\mu\text{mol cm}^{-2} \text{h}^{-1}$) of the anterior, mid, and posterior intestines of fasted (**a, c**; white bars) and fed (**b, d**; filled bars) fish in the presence of either control saline (no pattern), high glutamine (dotted

pattern), or high ammonia (diagonal pattern) in the lumen. Asterisk (*) represents significant difference between high glutamine or high ammonia treated group relative to control group ($p < 0.05$). Values are means \pm SEM ($N = 5$)

$p = 0.19$ and 0.99 respectively), the treatment had contrasting effects depending on the intestinal sections. It increased anterior intestine $J_{s_{amm}}$ in both fasted and fed fish (Fig. 4a, b: $p = 0.0008$ and 0.0004 respectively), but decreased $J_{s_{amm}}$ in the mid intestine of fed fish (Fig. 4b: $p = 0.0339$). The high luminal ammonia treatment had a significant overall effect on $J_{m_{amm}}$ in both fasted and fed fish (Fig. 4c, d: $p = 0.0067$ and $p < 0.0001$ respectively), converting the normally negative flux rates (appearance in the lumen) to positive values (removal from the lumen), at least in the anterior and mid intestine, and reducing the negative values in the posterior intestine. These effects on $J_{m_{amm}}$ were significant in all three individual sections of fed fish (Fig. 4d: $p = 0.0009$, 0.0198 , and 0.0022 respectively). There was a significant interaction between treatment and intestinal sections on $J_{s_{amm}}$ in both fasted and fed fish ($p < 0.0001$). The net results were no significant overall effects of high ammonia on $J_{t_{amm}}$ of fasted fish (Fig. 5a: $p = 0.17$) but an overall reduction of $J_{t_{amm}}$ in fed fish (Fig. 5b: $p = 0.0010$), which was significant

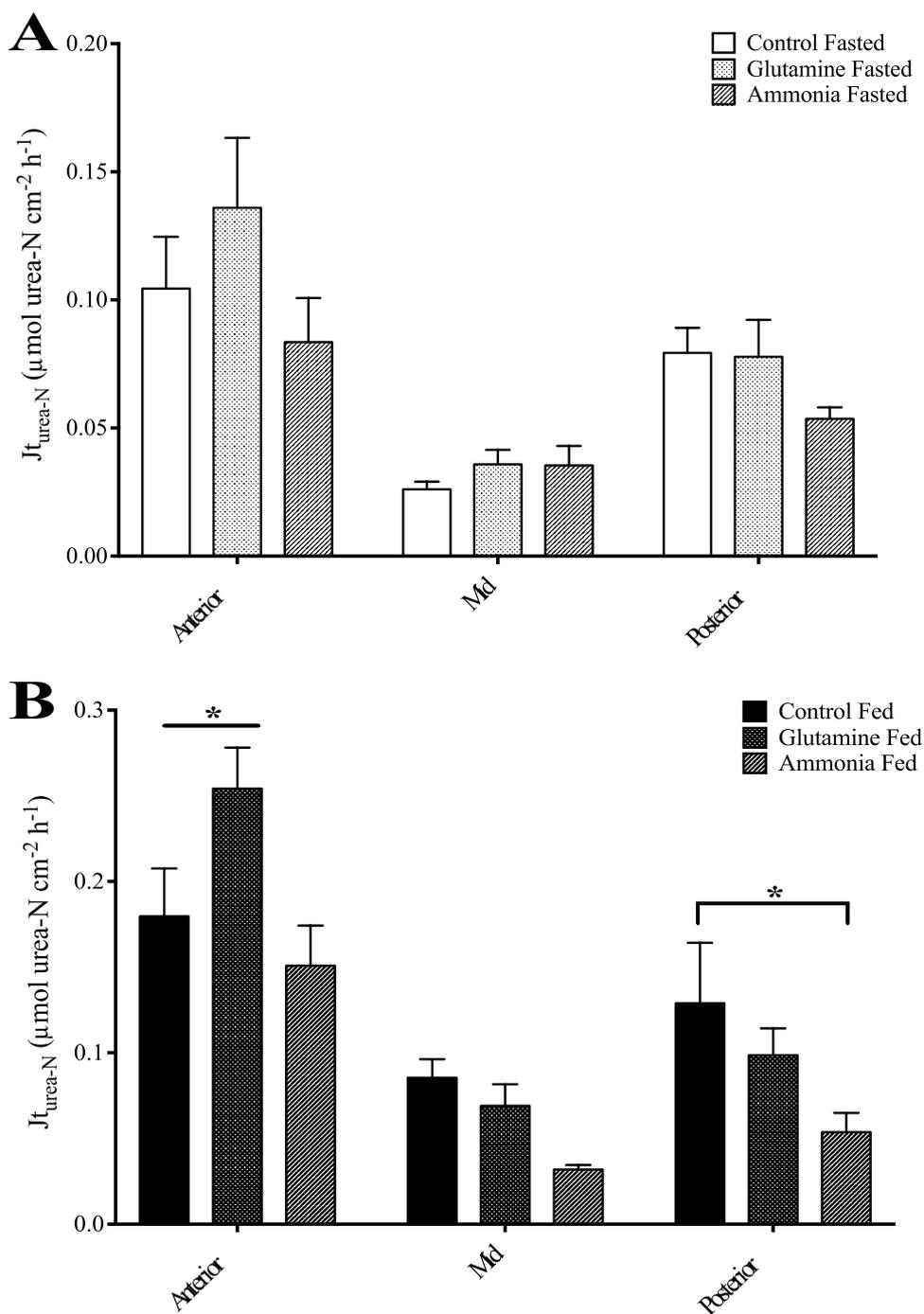
in the mid and posterior intestine (Fig. 5b: $p = 0.0017$ and 0.0018 respectively). There was also a significant interaction between treatment and intestinal sections in both fasted and fed fish ($p = 0.0014$ and 0.0104 respectively). High ammonia increased T_{amm} overall in fasted fish (Fig. 6a: $p = 0.0060$) but had no effect on T_{amm} of fed fish (Fig. 6b: $p = 0.96$).

Discussion

Key findings addressing our hypotheses

Our first hypothesis, that feeding would increase not only ammonia, but also urea production and flux rates in these trout gut preparations, was confirmed. While the finding on ammonia is confirmatory of previous trout studies (Rubino et al. 2014, 2019), the urea result is novel, and in accord with the findings of Bucking et al. (2013b) on similar in vitro preparations of the plainfin midshipman.

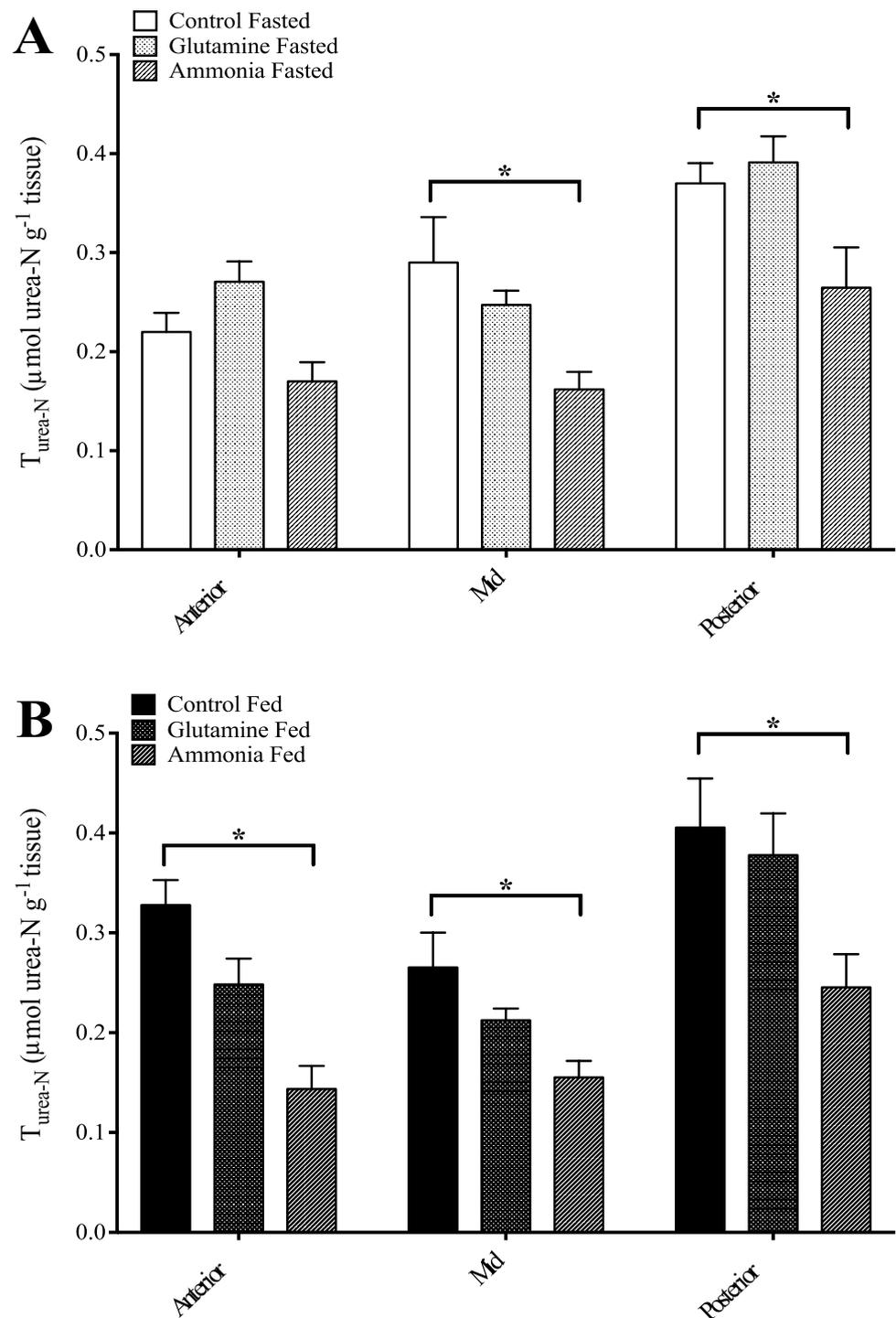
Fig. 2 Total tissue urea-N production rates ($J_{t_{\text{urea-N}}}$: $\mu\text{mol cm}^{-2} \text{h}^{-1}$) of the anterior, mid, and posterior intestine of **a** fasted and **b** fed fish in the presence of either control saline (no pattern), high glutamine (dotted pattern), or high ammonia (diagonal pattern) in the lumen. Values are calculated from Fig. 1 data. Asterisk (*) represents significant difference between high glutamine or high ammonia treated group relative to control group ($p < 0.05$). Values are means \pm SEM ($N=5$)



With respect to our second hypothesis, that the activities of both CPS III and arginase activities would increase if the OUC were involved in this urea production, our data do not provide support for OUC involvement, because CPS III activity did not increase with feeding. However, the observed increase in arginase activity suggests that an alternate pathway, direct arginolysis, may have contributed to elevated urea production. Our third hypothesis, that luminal glutamine supply would increase the production

and flux rates of both ammonia and urea, was supported in part by observations of elevated ammonia production and transport in preparations from fasted fish, but not from fed fish. This indicates an ability of the intestinal tissue to deaminate glutamine, and perhaps use it as an oxidative fuel, similar to the observations of Bucking et al. (2013b). However, with the exception of the anterior intestine of fed trout, there was no evidence of increased urea production or flux resulting from the presence of glutamine,

Fig. 3 Total tissue urea-N concentration ($T_{\text{urea-N}}$, $\mu\text{mol g}^{-1}$ tissue) of the anterior, mid and posterior intestines of **a** fasted and **b** fed fish in the presence of either control saline (no pattern), high glutamine (dotted pattern), or high ammonia (diagonal pattern) in the lumen. Asterisk (*) represents significant difference between high glutamine or high ammonia treated group relative to control group ($p < 0.05$). Values are means \pm SEM ($N = 5$)



again arguing against the presence of a functional OUC in the intestine. Our final hypothesis, that elevated luminal ammonia supply would result in elevated urea production and flux rates, reflecting a detoxification mechanism, was not supported. There were negligible or negative effects on urea-N fluxes and production in both fasted and fed fish, but indirect evidence of ammonia detoxification by another pathway.

The effect of feeding

This study provides further evidence (c.f. Rubino et al. 2014, 2019) that the intestinal cells of rainbow trout break down dietary amino acids, because feeding increased overall $J_{s_{\text{amm}}}$, $J_{m_{\text{amm}}}$, $J_{t_{\text{amm}}}$, and T_{amm} (Table 1), in the absence of the original chyme. Most of this increased intestinal production was transported to the serosal side (Table 1;

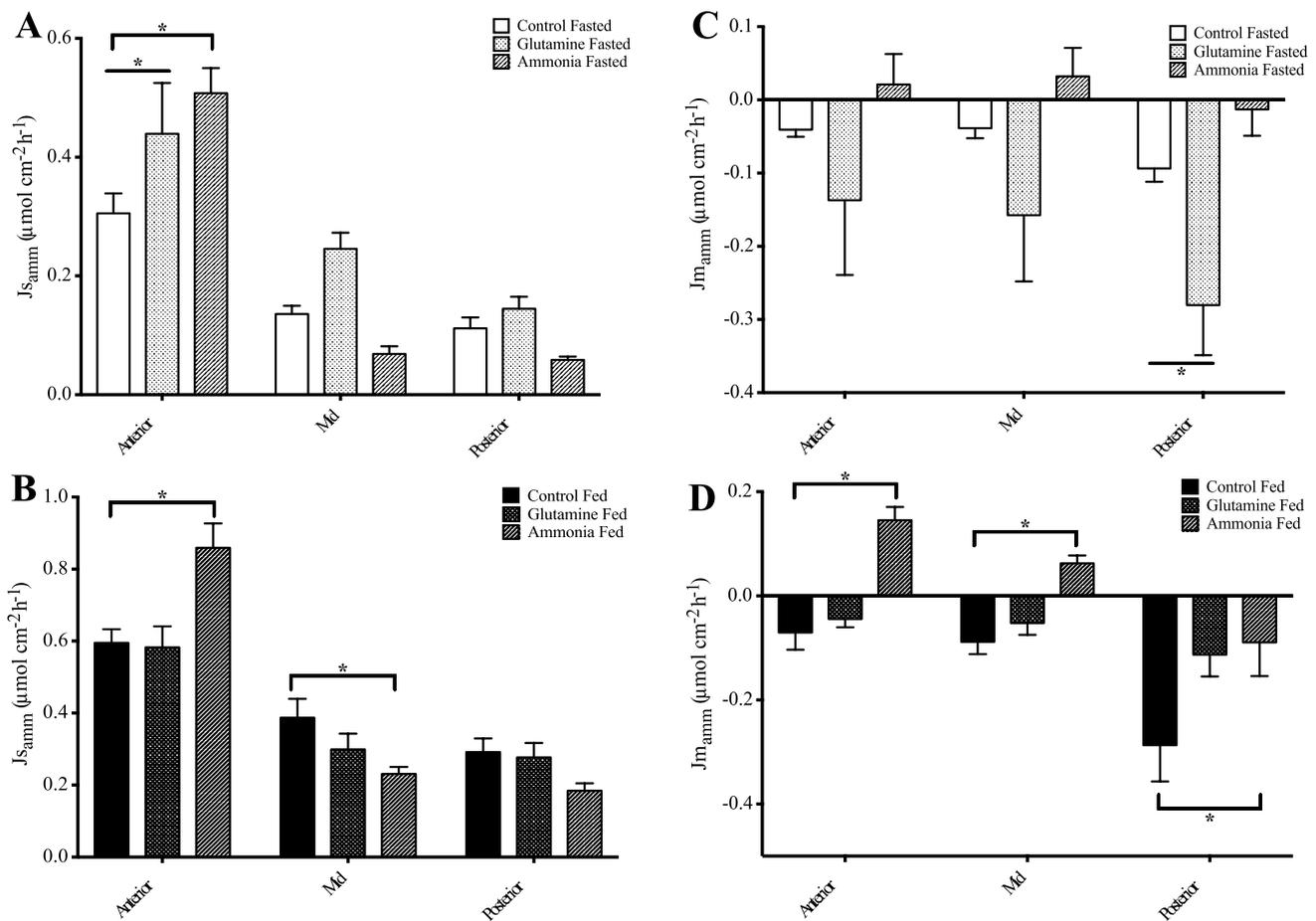


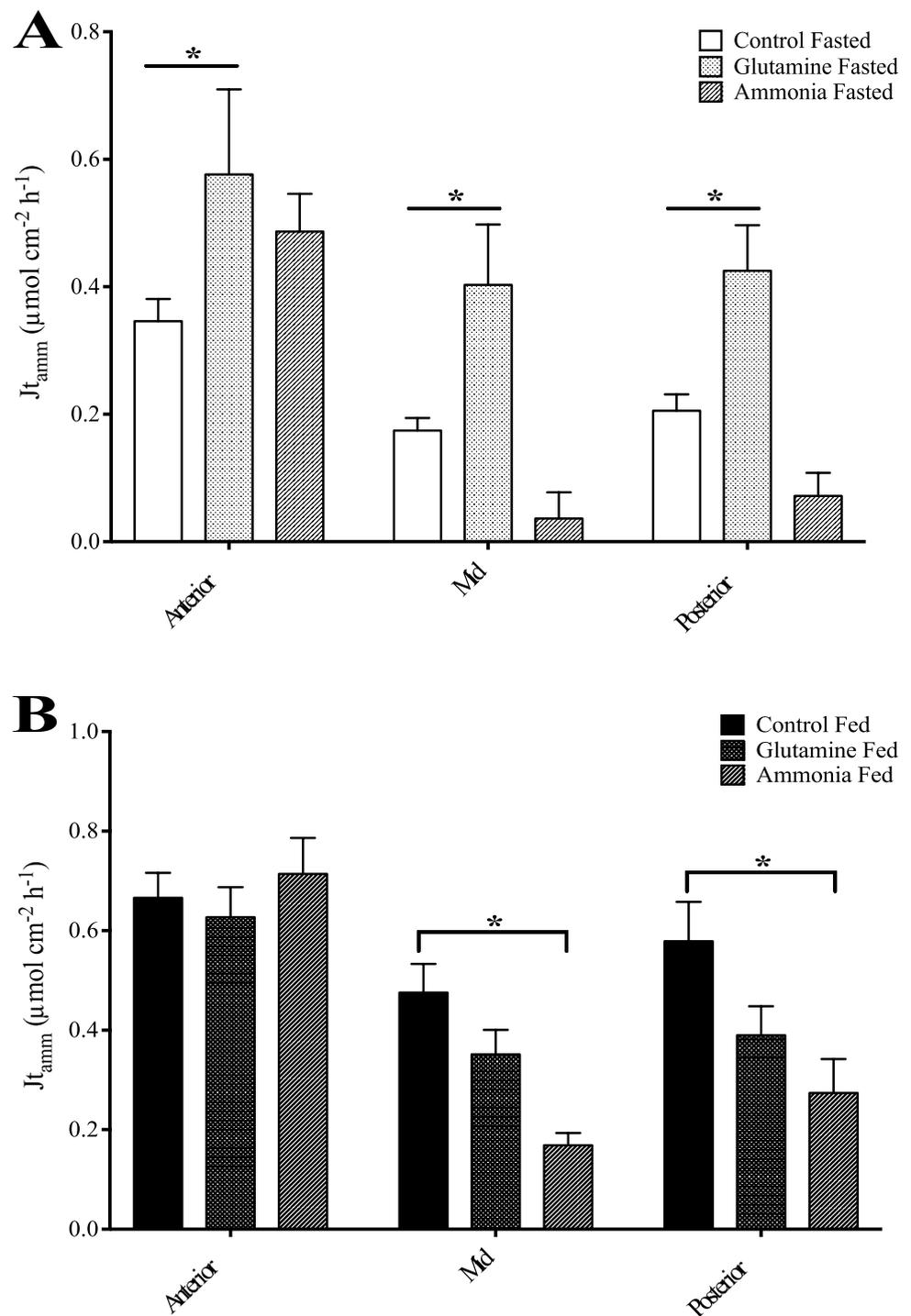
Fig. 4 Serosal (a, b; J_{s_amm}) and mucosal (c, d; J_{m_amm}) ammonia flux rates ($\mu\text{mol cm}^{-2} \text{h}^{-1}$) of the anterior, mid, and posterior intestines of fasted (a, c; white bars) and fed (b, d; filled bars) fish in the presence of either control saline (no pattern), high glutamine (dotted

pattern), or high ammonia (diagonal pattern) in the lumen. Asterisk (*) represents significant difference between high glutamine or high ammonia treated group relative to control group ($p < 0.05$). Values are means \pm SEM ($N=5$)

Rubino et al. 2014, 2019), explaining the greatly elevated blood ammonia levels measured in the HPV of trout after feeding (Karlsson et al. 2006). A novel finding of our study was that there were also overall significant increases in J_{t_urea-N} and J_{s_urea-N} in the intestine of fed fish (Table 1). Again, as the original chyme was not present, this supports the idea that urea can be synthesized in some way from dietary amino acids by the intestinal tissues after feeding, then subsequently transferred to the blood, draining to the HPV, as also evidenced by Karlsson et al. (2006). Although Kajimura et al. (2004) provided indirect evidence (by anal suturing) that rectal excretion of both ammonia (especially) and urea increased after feeding in trout, only the increased ammonia flux to the lumen (J_{m_amm}) was significant in the present study, occurring mainly in the posterior intestine (Table 1). Nevertheless, the intestinal cells of ammoniotelic freshwater rainbow trout clearly have the ability to not only transport urea in

a directional fashion, but also likely produce it after feeding. This agrees with previous whole animal studies on rainbow trout that showed an increase in urea excretion rate depending on the diet (Kaushik et al. 1983; Alsop and Wood 1997; Kajimura et al. 2004). Similarly, isolated GIT tissues of plainfin midshipman significantly increased production and transport of urea across both mucosal and serosal surfaces following a meal (Bucking et al. 2013b). In this particular species, the urea secreted into the GIT lumen appeared to be both excreted out in the feces and used by ureolytic GIT bacteria, with possible recycling of ammonia (Bucking et al. 2013b). Both ammonia (Rh glycoproteins) and urea transporters (UT) have been identified in the GIT of the plainfin midshipman, and the UT is upregulated after feeding (Bucking et al. 2013a). In trout intestine, Rhbg expression increases after feeding (Bucking and Wood 2012), but as yet there is no information on the presence of UT.

Fig. 5 Total tissue ammonia production rates ($J_{t_{\text{amm}}}$; $\mu\text{mol cm}^{-2} \text{h}^{-1}$) of the anterior, mid, and posterior intestine of **a** fasted and **b** fed fish in the presence of either control saline (no pattern), high glutamine (dotted pattern), or high ammonia (diagonal pattern) in the lumen. Values are calculated from Fig. 4 data. Asterisk (*) represents significant difference between high glutamine or high ammonia treated group relative to control group ($p < 0.05$). Values are means \pm SEM ($N = 5$)

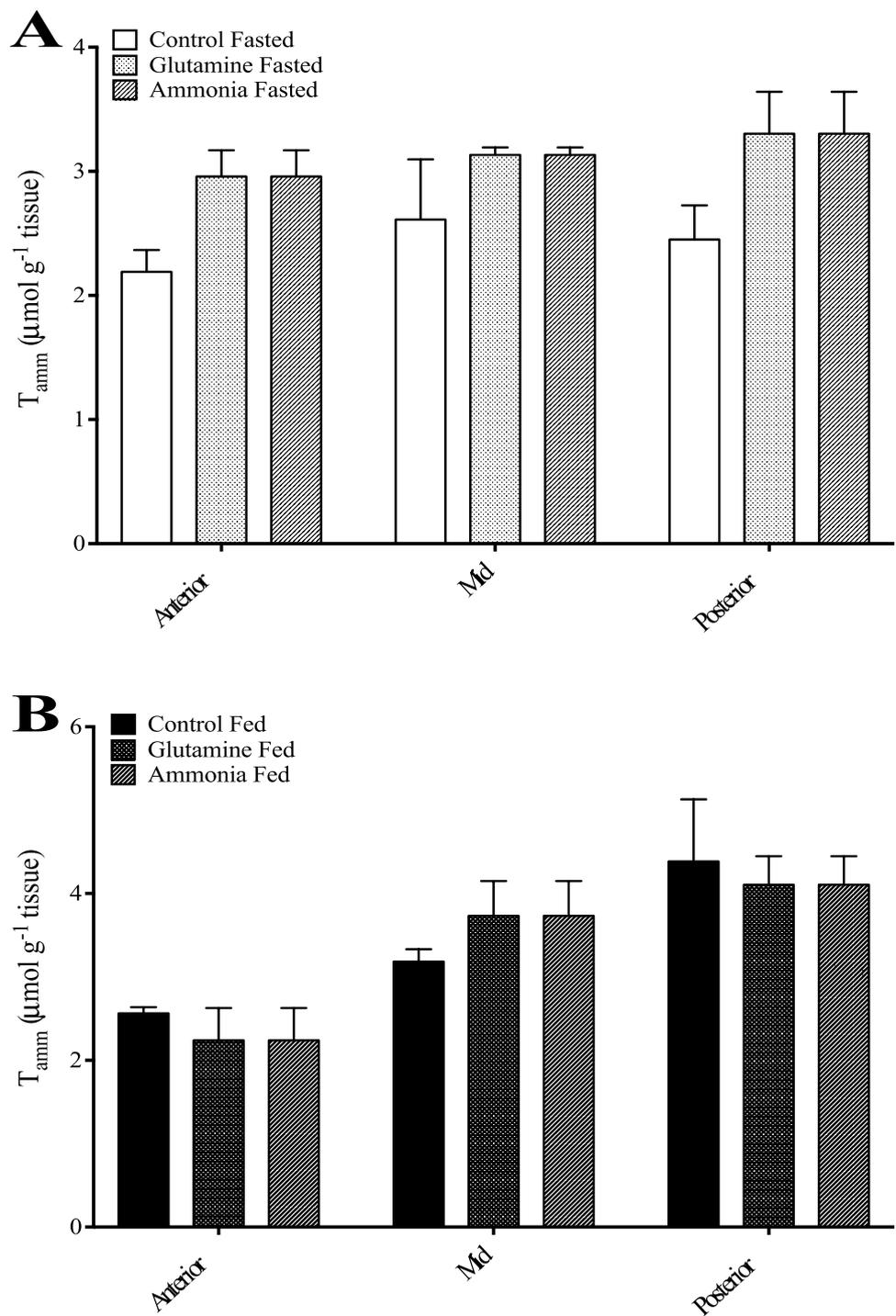


CPS III and arginase enzyme activity levels after feeding

In association with the increased $J_{t_{\text{urea-N}}}$ and $J_{s_{\text{urea-N}}}$ discussed above, we hypothesized that the intestinal cells would increase CPS III and arginase activity after feeding. However, feeding had minimal or negative overall effect on CPS III activity in either epithelial scrapings or muscle

(Table 2). Previous studies also found below detectable or low levels of CPS III activity in adult rainbow trout intestine (Wright et al. 1995; Korte et al. 1997; Bucking et al. 2013b) and in the intestine of other adult teleosts (Felskie et al. 1998; Kong et al. 1998). This contrasts with the plainfin midshipman where intestinal CPS III activity increased greatly after feeding (Bucking et al. 2013b). Rainbow trout, similar to other teleosts, have relatively

Fig. 6 Total tissue ammonia concentration (T_{amm} , $\mu\text{mol g}^{-1}$ tissue) of the anterior, mid and posterior intestines of **a** fasted and **b** fed fish in the presence of either control saline (no pattern), high glutamine (dotted pattern), or high ammonia (diagonal pattern) in the lumen. Asterisk (*) represents significant difference between high glutamine or high ammonia treated group relative to control group ($p < 0.05$). Values are means \pm SEM ($N = 5$)



higher CPS and other OUC enzyme activities during embryogenesis to prevent possible ammonia toxicity, that is then reduced at about 70 days post fertilization (Korte et al. 1997; Wright et al. 1995); reviewed by Zimmer et al. (2017). Therefore, the low enzymatic activities we found may not have physiological importance for urea production, but rather simply represent the products of low-level gene expression left over from embryonic life.

On the other hand, arginase activity increased significantly after feeding, specifically in the mid intestine epithelial scraping (Table 3). In contrast to CPS III, arginase activity increases slowly during post-fertilization development of rainbow trout (Wright et al. 1995). This is similar to a previous finding on adult Atlantic cod (*Gadus morhua*) with undetectable level of CPS III but the presence of arginase in its intestine (Chadwick and Wright 1999). Unlike

CPS III that is exclusively involved with the OUC pathway, arginase is also involved in arginolysis, metabolizing dietary arginine directly to urea. This may explain the increase in urea production and transport after feeding. Another mechanism that may have contributed to intestinal urea production is uricolysis, by the conversion of uric acid to urea (reviewed by Anderson 2001; Cvancara 1969; Goldstein and Forster 1965). This pathway should be investigated in future studies.

High luminal glutamine treatment

Glutamine can be metabolized to α -ketoglutarate as an energy source for immediate energy production by the citric acid cycle (or for carbon storage), releasing ammonia, or the amino group can be transferred by transamination to form other amino acids. It can also feed into the OUC pathway by CPS III, producing urea to be excreted. We predicted that the surplus supply of glutamine in the lumen would increase both ammonia and urea-N production and flux rates as the intestinal cells break down the amino acid. We found an increase in ammonia flux rates and production in intestinal preparations from fasted trout, but not fed trout (Figs. 4, 5, 6), suggesting that glutamine is deaminated and metabolized as an energy source in fasted fish, whereas in fed fish there may be alternate fuels available from the chyme. This response pattern differs from that of the plainfin midshipman where high mucosal glutamine stimulated ammonia production in intestinal preparations from both fasted and fed fish (Bucking et al. 2013b).

There were minimal changes in urea-N handling in response to elevated mucosal glutamine (Figs. 1, 2, 3). This is in accord with the low and generally unresponsive CPS III activity we found (Table 2), further suggesting that the intestine does not use the OUC to make urea. However, at present we should not dismiss the significant stimulations of $J_{\text{S}_{\text{urea-N}}}$ and $J_{\text{t}_{\text{urea-N}}}$ in the anterior intestine of fed trout only (Figs. 1b, 2b) which occurred in parallel to significantly decreased CPS III activity in this tissue (Table 2), a puzzling result. Another puzzling result was reported by Bucking et al. (2013b) in the plainfin midshipman, which is thought to have an intestinal OUC. Plainfin midshipman intestinal preparations exhibited decreased urea production in response to high glutamine in the lumen (Bucking et al. 2013b). The authors speculated that ammonia was the substrate for the OUC in this species, and that the excess glutamine was interfering with the ammonia transport across mitochondrial matrix, thereby reducing the substrate available for the OUC.

High luminal ammonia treatment

The suggestion of Bucking et al. (2013b) that ammonia rather than glutamine could be the preferred substrate for

the intestinal OUC of the plainfin midshipman is a rare but not unprecedented phenomenon in fish, having been documented in the ureotelic Magadi tilapia (Lindley et al. 1999). Magadi tilapia increases urea excretion rate when exposed to high external ammonia (Wood et al. 1989, 2013). Ureogenic largemouth bass (*Micropterus salmoides*) also increase urea excretion in high ammonia water (Kong et al. 1998). To evaluate this suggestion in the rainbow trout intestine, we loaded high ammonia into the lumen to investigate whether the rainbow trout has the ability to detoxify ammonia to less toxic urea. The treatment had significant effects on ammonia handling and production (Figs. 4, 5, 6), somewhat parallel to earlier studies with lower levels (1 mmol L^{-1}) of ammonia loading (Rubino et al. 2014, 2019). At this higher level (2 mmol L^{-1}), there were no significant overall effects of high ammonia on $J_{\text{t}_{\text{amm}}}$ of fasted fish (Fig. 5a) but an overall reduction of $J_{\text{t}_{\text{amm}}}$ occurred in fed fish, prominent in the mid and posterior intestine (Fig. 5b). This suggests that ammonia detoxification was induced. However, there were minimal effects on urea-N handling in both fasted and fed fish (Figs. 1, 2, 3). Since the adult rainbow trout does not appear to use the OUC, the formation of urea may not be the primary mechanism for the detoxification of ammonia. There were some inhibitory effects on both the flux rates and production of urea. Presumably, this is because the primary mechanism for detoxification of ammonia in the intestine of the rainbow trout is by synthesis of glutamine, via the enzyme glutamine synthetase (GS), with potential transamination to other amino acids. We found the most marked changes in the posterior intestine and correspondingly, the GS enzyme activity is reported to be highest in this section in the trout (Mommsen et al. 2003; Rubino et al. 2014). Although we have not demonstrated that the trout intestine was making glutamine specifically, glutamine in general acts as an ammonia storage molecule which can be used as energy immediately or later on (reviewed by Anderson 2001). Interestingly, in vitro preparations of the intestine of the plainfin midshipman also did not exhibit changes in urea production with high mucosal ammonia, possibly because an in vivo signal is needed to regulate the OUC enzymes (Bucking et al. 2013b). There are a few pieces of evidence suggesting that the intestinal bacteria community (microbiome) influences ammonia handling in some fish species. Ammonia appearance in the gut of plainfin midshipman was greatly reduced with antibiotic treatment (Bucking et al. 2013b). Similarly, in the ureotelic dogfish shark, inhibition of bacterial urease decreased ammonia appearance in the mucosal chyme of the intestine (Wood et al. 2019). Intestinal bacteria and the activity of ammonia detoxifying enzymes were correlated in the herbivorous fish (*Camptostoma anomalum*), but not in the carnivorous fish (*Etheostoma caeruleum*) (Turner and Bucking 2019). It is still unclear whether the rainbow trout also carries an intestinal bacteria

community that is capable of detoxifying ammonia or glutamine, or converting urea to ammonia, but we assume that a lot of the flora was flushed out during our gut sac preparations. We used relatively high concentrations of ammonia and glutamine in short flux periods, which likely would have minimized the influence of any residual microbial activity. Clearly, there is a need for better mechanistic understanding of N-handling by the GIT in fish, including the contributions of the microbiome.

Author contributions JS, JGR, and CMW: conceptualization, methodology, investigation. EHJ: formal analysis, writing—original draft preparation. EHJ, JS, JGR, and CMW: writing. All authors—review and editing.

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Compliance with ethical standards

Conflict of interest No conflict of interest, financial or otherwise, are declared by the authors.

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