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ORIGINAL PAPER

# An in vitro analysis of intestinal ammonia handling in fasted and fed freshwater rainbow trout (*Oncorhynchus mykiss*)

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**Abstract** Ammonia transport and metabolism were investigated in the intestinal tract of freshwater rainbow trout which had been either fasted for 7 days, or fasted then fed a satiating meal of commercial trout pellets. In vivo, total ammonia concentrations ($T_{amm}$) in the chyme were approximately 1 mmol $L^{-1}$ across the entire intestine at 24 h after the meal. Highest chyme pH and $P_{NH3}$ values occurred in the posterior intestine. In vitro gut sac experiments examined ammonia handling with mucosal ($Jm_{amm}$) and serosal ($Js_{amm}$) fluxes under conditions of fasting and feeding, with either background (control $\leq$0.013 mmol $L^{-1}$) or high luminal ammonia concentrations (HLA = 1 mmol $L^{-1}$), the latter mimicking those seen in chyme in vivo. Feeding status (fasted or fed) appeared to influence ammonia handling by each individual section. The anterior intestine exhibited the greatest $Jm_{amm}$ and $Js_{amm}$ values under fasted control conditions, but these differences tended to disappear under typical post-feeding conditions when total endogenous ammonia production ($Jt_{amm}$ = $Js_{amm}$ − $Jm_{amm}$, signs considered) was greatly elevated in all intestinal sections. Under fasted conditions, glutamate dehydrogenase (GDH) and glutaminase (GLN) activities were equal across all sections, but the ammonia-trapping enzyme glutamine synthetase (GS) exhibited highest activity in the posterior intestine, in contradiction to previous literature. Feeding clearly stimulated the total rate of endogenous ammonia production ($Jt_{amm}$), even in the absence of a high luminal ammonia load. This was accompanied by an increase in GDH activity of the anterior intestine, which was also the site of the largest $Jt_{amm}$. In all sections, during HLA exposure, either alone or in combination with feeding, there were much larger increases in endogenous $Jt_{amm}$, most of which was effluxed to the serosal solution. This is interpreted as a response to avoid potential cytotoxicity due to overburdened detoxification mechanisms in the face of elevated mucosal ammonia. Thus $T_{amm}$ of the intestinal tissue remained relatively constant regardless of feeding status and exposure to HLA. Ammonia production by the gut may explain up to 18 % of whole-body ammonia excretion in vivo under fasting conditions, and 47 % after feeding, of which more than half originates from endogenous production rather than from absorption from the lumen.

**Keywords** Intestine · Ammonia · Chyme · Feeding · Fasting · Rainbow trout

## Introduction

Ammoniotelic fish generate ammonia mainly through the deamination of amino groups during protein catalysis, and the major site of production appears to be the liver (Walton and Cowley 1977). One of the best-studied natural factors leading to an alteration of ammonia excretion is feeding. Following feeding, fish generally demonstrate an increase in both plasma ammonia concentration and whole-body ammonia excretion (e.g., Brett and Zala 1975; Bucking and Wood 2008, 2012; Zimmer et al. 2010). At least part of this response is due to the fact that fish lack an ability to store

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protein, and must metabolize any excess protein in the diet, which contributes to the overall ammonia load (Ballantyne 2001; Stone et al. 2003). Indeed Murai et al. (1987) have shown that trout fed high protein diets demonstrated elevated plasma ammonia levels in comparison to trout fed a normal diet.

Two other, generally overlooked sources of systemic ammonia loading may be ammonia produced in the lumen by digestive processes acting on the chyme, as well as ammonia produced by the metabolism of the gut cells themselves. In the intestine of rainbow trout, Bucking and Wood (2012) reported chyme ammonia concentrations as high as 1.8 mmol $L^{-1}$, more than sixfold greater than the concentration in plasma. Furthermore, after feeding, the anterior and posterior intestine exhibited time-dependent increases in the mRNA expression of Rhbg1 (Bucking and Wood 2012), a Rhesus (Rh) glycoprotein. Rh glycoproteins are ammonia channels which have recently been implicated in ammonia excretion in the gills (Nakada et al. 2007; Nawata et al. 2007; Wood and Nawata 2011; Wright and Wood 2009, 2012) and potentially they could increase the permeability of the intestinal wall to ammonia. Curiously, ureotelic elasmobranch fish have also been shown to express Rhbg in their intestine, to a much higher extent than urea transporters (Anderson et al. 2010). In these fish, Rhbg is proposed to function in the reabsorption of ammonia produced via ureolytic bacteria in the gut. Ultimately, Rh proteins seem to play an important role in aquatic organisms (Wright and Wood 2009). Various isoforms of these proteins exist, with different patterns of cellular localization. Typically, Rhbg tends to localize to the basolateral membrane, whereas the Rhcg localizes to the apical membrane (Wright and Wood 2009). Although the body of evidence surrounding intestinal Rh functionality is relatively low, both isoforms of Rh proteins have been identified by immunohistochemistry in the intestines of two Batrachoidid fishes, the gulf toadfish and the plainfin midshipman (Bucking et al. 2013a). In addition, using an in vitro approach, Bucking et al. (2013b) found that the intestine of the midshipman is indeed permeable to ammonia. These authors proposed an intimate link between intestinal ammonia absorption and urea synthesis, though it is unknown if the ammoniotelic rainbow trout employs this same mechanism. Karlsson et al. (2006) has proposed that the gastrointestinal tract may contribute to plasma ammonia increases following feeding in trout, given the observed post-prandial elevations of ammonia concentrations in the hepatic portal vein prior to blood perfusion through the liver.

Though feeding naturally increases plasma ammonia levels, ammonia is a neurotoxin (Randall and Tsui 2002). Thus, mechanisms must exist for fish to cope with and/or minimize these ammonia loads following feeding. One important mechanism is the recycling of ammonia to synthesize amino acids. Glutamate dehydrogenase (GDH) may react ammonia with alpha-ketoglutarate to form glutamate, and then transamination reactions may transfer the amino groups to form other amino acids, or an additional ammonia may be fixed by glutamine synthetase (GS) to form glutamine. GS and GDH enzyme activity and/or mRNA expression occur in the intestine of salmonids and the midshipman (Chamberlin et al. 1991; Mommsen et al. 2003a, b; Murray et al. 2003; Bucking and Wood 2012; Bucking et al. 2013b). GS in particular has been shown to perform detoxification functions in various tissues under high ammonia stress (Wicks and Randall 2002; Wright et al. 2007). In turn, synthesized glutamine may be deaminated by glutaminase (GLN) to serve as an oxidative substrate fuel for the intestinal cells, as in mammals, thereby liberating ammonia again (Windmueller and Spaeth 1977). In other species, such as insects, cellular metabolic enzymes appear to play a large role in the detoxification of ammonia (Weihrauch et al. 2012). Particularly, mosquitos that digest blood meals rely heavily upon GS to detoxify the large nitrogen loads associated with degradation of blood proteins (Scaraffia et al. 2005). In addition, digestion in these insects triggers the transcription of GS mRNA to aid in the fixation of ammonia onto glutamate (Scaraffia et al. 2005). Insects also use proline as a fuel source in rectal cells, and its metabolism results in the formation of ammonia, which is transported directly into the rectal lumen to be subsequently excreted (Chamberlin and Phillips 1983). Insects, however, are flexible in their capabilities to excrete their nitrogenous wastes, unlike trout, and processes involved in the formation of uric acid and urea may be activated (Weihrauch et al. 2012). In general, the potential for altered enzyme activity dependent on feeding status has been studied only sparsely in fish. Mommsen et al. (2003b) reported negligible change in GDH activities in the intestine of tilapia with 5 days of fasting, though Bucking and Wood (2012) did observe increased GS activity in the posterior intestine 24 h following feeding in trout, and this was also observed in the midshipman gut (Bucking et al. 2013b).

Our objectives for this study were to use the in vitro gut sac technique (Nadella et al. 2006) to assess the production and transport of ammonia in the anterior, mid, and posterior intestine of rainbow trout, to determine if there are differences in ammonia metabolism between fed and fasted fish, and to assess the site-specific activities of GS, GDH, and GLN and their responses to feeding. Our overarching hypothesis was that feeding status will influence intestinal ammonia handling so as to minimize the potential for cytotoxity to gut tissue. Our first prediction was that there would be site-specific differences in ammonia handling along the tract, with the most

pronounced effects in the sections that exhibit the highest Rhbg1 mRNA expression outlined by Bucking and Wood (2012). Secondly, based on previous studies, which have shown activity of ammonia-producing enzymes in the teleost intestine (Mommsen et al. 2003a, b), we postulated that the gut tissue itself would endogenously produce ammonia, some of which would appear in the serosal solution, indicative of absorption into the bloodstream. Our third prediction was that endogenous ammonia production of the intestinal tissue would increase following feeding, in response to the increased metabolic activity of digestion and absorption. Fourthly, we predicted that when luminal concentrations of ammonia were experimentally elevated to duplicate those measured in chyme after feeding (high luminal ammonia = HLA), ammonia transport into the serosal solution would increase greatly, which would indicate the gut is permeable to chyme ammonia, as previously suggested by Karlsson et al. (2006). Our fifth postulate was that ammonia permeability would reflect the changes in Rhbg expression following feeding seen by Bucking and Wood (2012). Finally, our last prediction was that the activities of GS, GDH, and GLN would vary regionally and would change depending on feeding status, as the activity of some of these enzymes has been shown previously to be influenced by feeding status (Mommsen et al. 2003a, b; Bucking and Wood 2012; Bucking et al. 2013b). With these studies, we hope to obtain a first glance at the ammonia-producing and handling properties of the intestine in rainbow trout.

## Methods and materials

### Experimental animals

Rainbow trout, *Oncorhynchus mykiss,* weighing 110–240 g, were obtained from Humber Springs Trout Hatchery, Ontario, Canada, and kept in aerated 500-L tanks (30 fish per tank) with a flow-through system of dechlorinated Hamilton tapwater (moderately hard: $[Na^+] = 0.6$ mequiv $L^{-1}$, $[Cl^-] = 1.8$ mequiv $L^{-1}$, $[Ca^{2+}] = 0.8$ mequiv $L^{-1}$, $[Mg^{2+}] = 0.3$ mequiv $L^{-1}$, $[K^+] = 0.05$ 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/sec, background ammonia concentration ≤10 μmol $L^{-1}$). Fish were fed a light meal (approximately 1 % body mass) three times a week (Martin Profishent Aquaculture Nutrition, Tavistock, ON, Canada; crude protein 45 %, crude fat 9 %, crude fiber 3.5 %) and allowed to acclimate for 4 weeks prior to any experimentation. Animal handling was in compliance with the regulations of the Canada Council for Animal Care under McMaster University Animal Utilization Protocol 09-04-10.

### Chyme analysis

Initial experiments were performed to assess chyme total ammonia ($T_{amm}$) concentrations, $P_{NH3}$ and pH in vivo. Fish were fasted for 1 week prior to experimentation. Fish were then fed to satiation (~3 to 5 % of body mass) and following 24 h, randomly selected fish were anesthetized (0.07 g $L^{-1}$ neutralized MS222) and the body cavity was revealed using an anterior to posterior incision on the ventral side of the fish from the pectoral fins to the anus. Each individual gut section (anterior, mid, and posterior intestines) was freed from connective tissue, then tied off using 2–0 silk thread prior to excision of the tissue. The bile duct into the anterior intestine was also tied off to prevent bile spillover. Following excision, the sections were then individually opened and chyme from each section was transferred to bullet tubes, centrifuged (13,000*g*, 60 s), and the resultant supernatant was assayed for pH using a microelectrode (PerpHecT ROSS pH microelectrode, Thermo Scientific; Beverly, MA). Samples were then flash-frozen in liquid nitrogen and stored at −80 °C for later analysis of $T_{amm}$.

### In vitro gut sac experiments

Gut sac experiments were performed to quantify ammonia fluxes with mucosal and serosal solutions and tissue total ammonia levels ($T_{amm}$) of each individual gut section under various conditions. A common protocol was used across all treatments. Trout were anesthetized as above, and the gut sections were excised, cleaned of excess connective tissue, and placed in an ice-cold Cortland saline bath (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). The bile duct connecting the liver to the anterior intestine was tied off using 2–0 silk thread to prevent leakage, and the internal surface of each section was rinsed with Cortland saline and cleaned to remove chyme and feces. This was done to ensure that starting mucosal saline ammonia concentrations of all preparations for both fasted and fed fish were similar, and ammonia levels were measured (see below) to confirm this. Each section was then tied off at one end using 2–0 silk thread. Through the open end, a flared polyethylene (Intramedic Clay-Adams PE-60; Becton–Dickinson and Company, Sparks, MD) tube was inserted. The tubes were then secured in place using silk thread. The desired saline (depending on treatment) was then infused into each individual gut section using a syringe. This saline was then withdrawn and re-infused multiple times to allow mixing of the saline and the mucosal contents and a sample of this mixture was taken as the initial mucosal sample. The section itself was filled with experimental saline (see below) until taut and the PE tube

was sealed. The section was then gently dried by blotting and immersed into 15-mL (mid and posterior intestines; length approximately 3 and 4 cm, respectively) or 50-mL (anterior intestine; length approximately 8 cm) plastic centrifuge tubes (Falcon™; Corning Incorporated, Corning, NY) containing Cortland saline. The saline in these tubes was bubbled with a 99.7 % $O_2$: 0.3 % $CO_2$ mix to mimic physiological $PCO_2$, and to maximize $O_2$ supply in the preparation. Following the 2-h flux period, a 5-mL sample was taken from the bathing Cortland saline for each gut section, as well as a sample from a control tube which contained only saline which was gassed for a 2-h period to control for background ammonia concentrations in the bathing saline. Gut sections were then removed, blotted dry, and weighed. Mucosal contents were then taken and stored for ammonia quantification. Surface area of the tissue was measured using a standard technique for gut sacs first outlined by Grosell and Jensen (1999) wherein individual gut sections were cut in half and the area was traced onto 0.5-mm graph paper. The gut tissue was then flash-frozen in liquid nitrogen and stored at −80 °C for further analysis. As a control, in parallel experiments gut tissue that had not undergone the above post-flux processing was taken immediately and flash-frozen to test for potential increases in tissue $T_{amm}$ due to the brief air exposure period involved in surface area measurements. It was determined that there were negligible differences between the two processing treatments.

All sets of gut sac experiments were performed on both fed and fasted fish. Unfed fish were fasted for 1 week prior to experimentation, and fed fish were allowed 24 h to digest a single satiating meal (~3 to 5 % of body mass). The first set of experiments was performed to determine baseline ammonia production by each gut section (control). For these experiments, an unmodified Cortland saline was used on the mucosal (luminal) surface and the sections were bathed in the same serosal saline. The measured starting mucosal $T_{amm}$ saline was always $<0.013$ mmol $L^{-1}$. The second set of experiments were performed to observe permeability of the intestinal sections to ammonia, as well as ammonia production rates, when the lumen was filled with a physiologically relevant concentration of ammonia (HLA) in the mucosal saline, as determined from the in vivo experiments. In these experiments, a modified Cortland saline containing 1 mmol $L^{-1}$ $NH_4Cl$ was used on the mucosal surface, and these sections were bathed in an unmodified Cortland saline on the serosal surface.

## Analytical procedures

Previously collected chyme and mucosal saline samples were deproteinized using ice-cold 20 % perchloric acid (PCA). Samples were then pH neutralized using 1 mol $L^{-1}$ KOH prior to further analysis. Gut tissue samples which had been flash-frozen in liquid $N_2$ were ground to a fine powder using a liquid $N_2$-cooled mortar and pestle and were deproteinized using a solution containing 8 % PCA and 1 mmol $L^{-1}$ EDTA. Samples were then pH neutralized using 1 mol $L^{-1}$ KOH prior to further analysis. Tests showed that serosal saline samples could be analyzed without deproteinization.

Quantification of ammonia in serosal saline and deproteinized mucosal saline, chyme, and tissue samples was performed using a commercial assay (Raichem Cliniqa® ammonia assay; glutamate dehydrogenase method).

## Enzyme activity

Fresh gut tissue was taken from randomly selected trout exposed to the same fasted and fed conditions. Gut tissue was sectioned into the anterior, mid, and posterior intestine and immediately flash-frozen in liquid $N_2$ following excision. Additional tissues were taken from a separate batch of fish following the same feeding conditions, however, in these fish the epithelial layer of each intestinal section was scraped from the muscle layer using a glass slide. This was performed to determine enzymatic activity within these individual components. Whole tissues, as well as separated muscle layer and epithelial scrapings were then homogenized using a sonicator (Misonix™ Microson Ultrasonic Cell Disruptor) in approximately 4.9 times the tissue mass in volumes of ice-cold homogenization buffer (in mmol $L^{-1}$: 20 $K_2HPO_4$, 10 HEPES, 0.5 EDTA, 1 dithiothreitol, 50 % glycerol by volume; pH 7.5). All assays were conducted at 23 °C using absorbance spectrophotometry.

The assays used to determine glutamine synthetase (GS, EC 6.3.1.2), glutaminase (GLN, EC 3.5.1.2), and glutamate dehydrogenase (GDH, EC 1.4.1.2) activity were similar to those outlined by Chamberlin et al. (1991). All reactions performed were zero-order reactions and all kinetic assays were linear with time using 50, 10, and 25 μL of homogenate for each enzyme assay, respectively. GS activity was determined through the disappearance of γ-glutamylmonohydroxamate, measured at 540 nm, using the γ-glutamyl transfer reaction. GLN activity was determined via the appearance of NADH in solution in the presence of GDH, measured at 340 nm. GDH activity was determined by the disappearance of NADH, measured at 340 nm, using α-ketoglutarate as a substrate in the presence of ADP, which was required for enzyme activation. Activity units of all enzymes were quantified as the micromolar appearance of reaction product in the measured solution per min (U/g tissue). GDH and GS activity analysis was then performed on separated epithelial scrapings and muscle tissue for the anterior and posterior intestine, respectively, as these

enzymes showed the largest changes in these tissues in response to feeding (see "Results").

## Calculations

### *Chyme analysis*

$P_{NH3}$ for chyme was determined using pK′ and $\alpha_{NH3}$ values reported by Cameron and Heisler (1983). The solubility of ammonia ($\alpha_{NH3} = 51.271$ mmol Torr$^{-1}$ L$^{-1}$) and pK′ (9.64) values in chyme at 13 °C were assumed to be similar to those in 125 mmol L$^{-1}$ NaCl, based on the analysis of chyme performed by Bucking and Wood (2006a) on trout fed the same diet. $P_{NH3}$ values were calculated using the following formulae:

$$NH_3 = \frac{T_{amm} \times \text{anti log}\,(pH - pK')}{1 + \text{anti log}\,(pH - pK')} \quad (1)$$

$$P_{NH3} = [NH_3]/\alpha_{NH_3} \quad (2)$$

### *Serosal and mucosal ammonia flux rates*

To obtain serosal ammonia flux rates ($Js_{amm}$; μmol cm$^{-2}$ h$^{-1}$) for the intestinal sac experiments, the following formula was used:

$$Js_{amm} = \frac{[(Ts_{ammf} - Ts_{ammi}) \times V_s]}{SA \times t} \quad (3)$$

where $Ts_{ammf}$ and $Ts_{ammi}$ are the final and initial ammonia concentrations (μmol L$^{-1}$) in the serosal saline, $V_s$ is volume of the serosal solution (L), SA is intestinal surface area (cm$^2$), and $t$ is time (h). All $Js_{amm}$ fluxes were positive, into the serosal saline.

Mucosal ammonia flux rates ($Jm_{amm}$) for the intestinal sacs were calculated via the following formula:

$$Jm_{amm} = \frac{[(Tm_{ammi} \times Vm_i) - (Tm_{ammf} \times Vm_f)]}{SA \times t} \quad (4)$$

where $Tm_{ammf}$ and $Tm_{ammi}$ are the final and initial ammonia concentrations (μmol L$^{-1}$) in the mucosal saline, and $Vm_f$ and $Vm_i$ are the final and initial volumes of mucosal saline (L). Positive $Jm_{amm}$ fluxes were out of the mucosal saline; negative $Jm_{amm}$ fluxes were into the mucosal saline.

### *Total tissue ammonia production rates*

Total tissue ammonia production rates ($Jt_{amm}$), representing the net rate of endogenous production by the gut tissue itself, were determined using the following formula:

$$Jt_{amm} = (Js_{amm} - Jm_{amm}) \quad (5)$$

The calculation assumes that there is no net change in the $T_{amm}$ content of the tissue itself, an assumption that was generally supported by the data (see "Results").

## Statistical analyses

Data are expressed as mean ± SEM ($N$ number of fish). All comparisons made between fasted and fed data within each treatment (significant differences represented by asterisks) were conducted via Student's unpaired $t$ test. Comparisons between controls and HLA treatments of an individual intestinal section of the same feeding status were conducted using a Student's unpaired $t$ test (significant differences marked by daggers). When making comparisons amongst intestinal sections of a single experimental group (significant differences represented by letters), a one-way ANOVA followed by a Bonferroni's correction post hoc test was conducted. In the case of a failed normality test, an ANOVA on ranks was conducted followed by a Tukey's post hoc test. Comparisons made between intestinal sections of a single treatment for enzyme activities (significant differences represented by letters) were determined using a one-way ANOVA followed by a Bonferroni's correction post hoc test. When making comparisons for enzyme activity amongst all experimental groups (significant differences represented by daggers) analyses were conducted using a two-way ANOVA, comparing the variables of feeding status and intestinal section followed by a Bonferroni's correction post hoc test. In the case of a failed normality test, a two-way ANOVA on ranks was conducted followed by a Tukey's post hoc test. In all cases, statistical significance was accepted at the $p < 0.05$ level.

# Results

## Chyme analysis

Table 1 presents mean values for chyme pH, $T_{amm}$, and $P_{NH3}$ at 24 h after feeding. Chyme pH in the posterior intestine was substantially higher than in the anterior intestine, indicating pH gradually increases along the tract. $P_{NH3}$ in the posterior intestine was also elevated compared to the anterior intestine. No differences in chyme total ammonia concentration were noted amongst sections, which provided an ammonia concentration of 1 mmol L$^{-1}$ to be used in HLA experiments.

## Serosal and mucosal ammonia fluxes

Electronic Supplementary Table S1 reports the mean serosal and mucosal total ammonia concentrations measured during these flux experiments.

Control $Js_{amm}$ (i.e., flux into the serosal solution) for the anterior intestine in fasted fish was about 0.1 μmol cm$^2$ h$^{-1}$, which was greater than in the mid and posterior intestine by four- to fivefold (Fig. 1). In all three

**Table 1** pH, $T_{amm}$ (mmol $L^{-1}$), and $P_{NH3}$ (mTorr) measurements in chyme extracted 24 h following a single satiating meal

| Section | pH | $T_{amm}$ | $P_{NH3}$ |
|---|---|---|---|
| Anterior ($N = 6$) | $7.87 \pm 0.16^{a}$ | $1.068 \pm 0.14^{a}$ | $0.390 \pm 0.077^{a}$ |
| Mid ($N = 6$) | $8.28 \pm 0.12^{ab}$ | $0.893 \pm 0.10^{a}$ | $0.839 \pm 0.20^{ab}$ |
| Posterior ($N = 5$) | $8.57 \pm 0.12^{b}$ | $0.981 \pm 0.16^{a}$ | $1.76 \pm 0.54^{b}$ |

Data are expressed as mean ± SEM. Statistical analysis ($P < 0.05$) was conducted using a one-way ANOVA followed by a Bonferroni's correction post hoc test. Means within a column sharing the same letter are not significantly different

sections, $Js_{amm}$ increased 24 h post-feeding even though the mucosal solution was still control saline, and the flux rates in the three sections all differed from one another (anterior > mid > posterior).

In response to HLA in the mucosal saline, both fasted and fed fish exhibited large increases in $Js_{amm}$ in all three sections (Fig. 1). In fasted fish, the HLA flux rates became similar in all three sections, representing increases ranging from 2.5-fold (anterior) to ninefold (posterior). In the presence of HLA, the stimulatory effect of feeding was no longer seen: $Js_{amm}$ was not different between fasted and fed fish in the anterior or mid intestine (Fig. 1a, b), and in the posterior intestine, feeding resulted in a 30 % depression in $Js_{amm}$ in the HLA treatments (Fig. 1c). In consequence, $Js_{amm}$ of the posterior intestine was lower than of the other two sections.

Control $Jm_{amm}$ values were negative (i.e., flux into the mucosal solution) and much lower than $Js_{amm}$ values, and not different among sections in control fasted fish (Fig. 2). However, unlike $Js_{amm}$ values, an effect of feeding on $Jm_{amm}$ was only observed in the mid intestine (Fig. 2B).

The HLA treatment caused all $Jm_{amm}$ fluxes to change into positive values (i.e., flux out of the mucosal solution). In all cases, the positive fluxes were very large relative to the negative fluxes in control preparations. In the presence of HLA, no significant effect of feeding on $Jm_{amm}$ was observed, and the only difference among segments was a lower $Jm_{amm}$ in the anterior intestine in the unfed HLA treatment (Fig. 2a). Note that these positive $Jm_{amm}$ fluxes induced by mucosal HLA were less than half the positive $Js_{amm}$ fluxes induced by the same treatment. Thus under HLA treatments, much more ammonia was leaving the gut tissue into the serosal solution than was entering from the mucosal solution.

## Tissue ammonia content

Tissue total ammonia concentrations ($T_{amm}$) in intestinal tissue are displayed in Table 2. $T_{amm}$ did not vary among

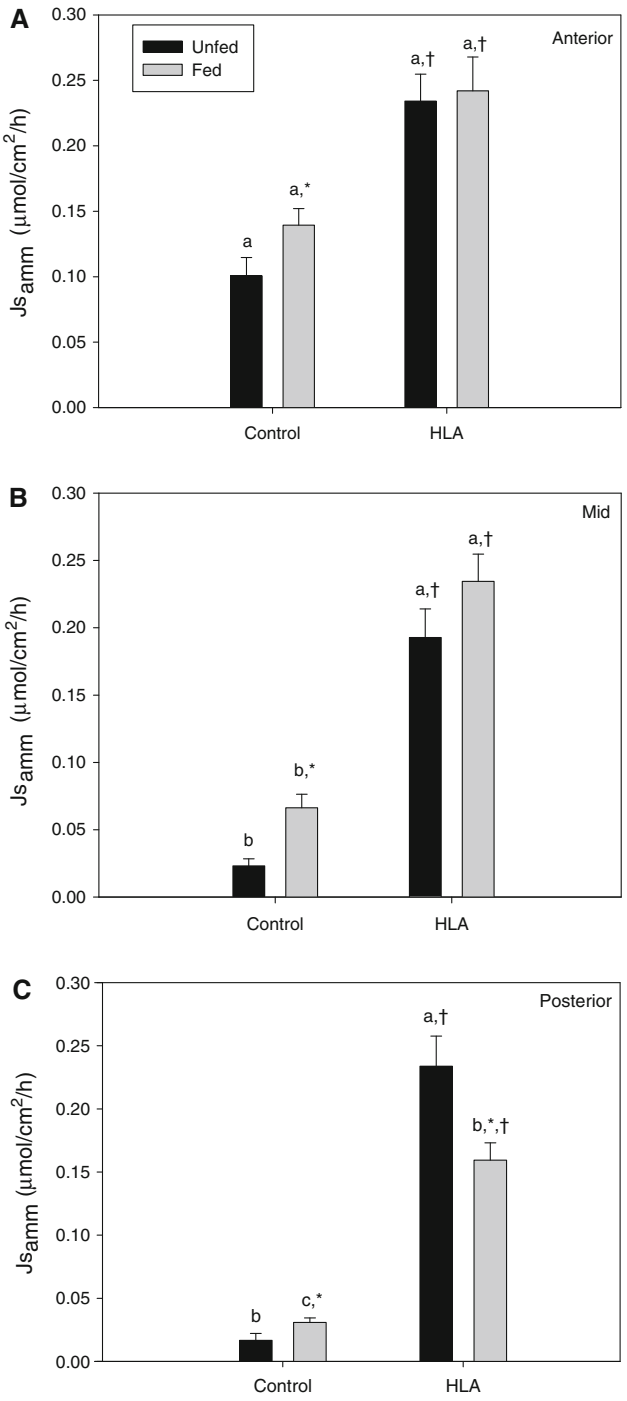


**Fig. 1** Ammonia flux rates ($Js_{amm}$, µmol $cm^{-2}$ $h^{-1}$) of the anterior (**a**), mid (**b**), and posterior intestine (**c**) into the serosal saline of unfed (*black bars*) and fed (*gray bars*) fish under control and HLA treatments. Significant differences ($P < 0.05$) between unfed and fed treatments (*asterisks*) and between control and HLA treatments (*daggers*) were determined using a Student's unpaired *t* test. Significant differences within a treatment (*letters*) among all intestinal sections were determined using a one-way ANOVA followed by a Bonferroni's post hoc test ($N = 5$ for each treatment)

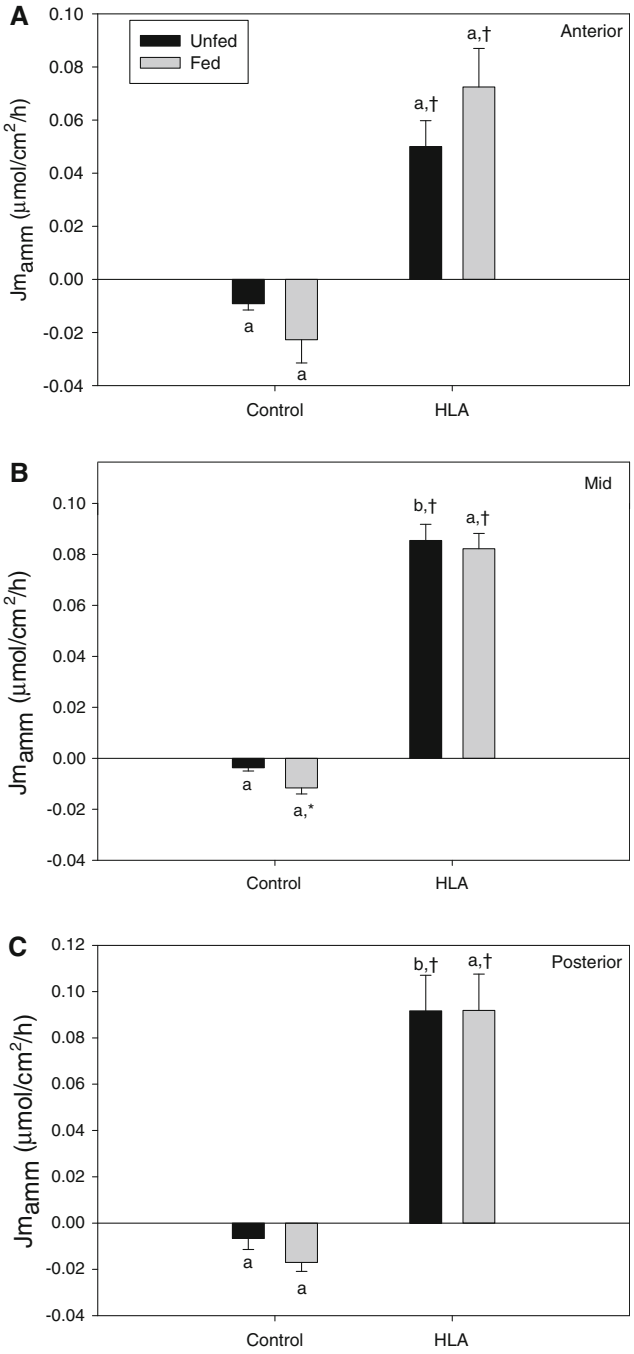


**Fig. 2** Ammonia flux rates ($Jm_{amm}$, µmol $cm^{-2}$ $h^{-1}$) of the anterior (**a**), mid (**b**), and posterior intestine (**c**) into or out of the mucosal saline of unfed (*black bars*) and fed (*gray bars*) fish under control and HLA treatments. *Positive values* indicate net efflux of ammonia from the mucosal solution over the 2-h flux. *Negative values* indicate net influx of ammonia into the mucosal solution. Significant differences ($P < 0.05$) between unfed and fed treatments (*asterisks*) and between control and HLA treatments (*daggers*) were determined using a Student's unpaired *t* test. Significant differences within a treatment (*letters*) among all intestinal sections were determined using a one-way ANOVA followed by a Bonferroni's post hoc test ($N = 5$ for each treatment)

sections in any treatment. Elevations in $T_{amm}$ with feeding were significant only in the mid intestine in control fish, and in the anterior intestine for HLA fish. There were no effects of the HLA treatment on $T_{amm}$ within individual sections in either fed or fasted preparations.

## Endogenous tissue ammonia production

The net rate of endogenous ammonia production by the gut tissue itself ($Jt_{amm}$) was estimated as the difference between $Js_{amm}$ and $Jm_{amm}$ [see "Methods and materials", Eq. (5)], a calculation which assumes that there is no net change in the $T_{amm}$ content of the tissue itself. This assumption was supported by the data of Table 2, except for the two responses to feeding noted above.

Control $Jt_{amm}$ for unfed preparations was about 0.11 µmol $cm^2$ $h^{-1}$ in the anterior intestine, a rate which was about four- to sixfold higher than the much lower values in the mid and posterior intestine (Fig. 3). Feeding stimulated an increase in $Jt_{amm}$ in all sections under control conditions (Fig. 3). In addition, the threefold increase in the mid intestine was probably an underestimate in light of the fact that tissue $T_{amm}$ also increased in this section in response to feeding (Table 2). Despite this increase in the mid intestine, the anterior intestine still exhibited a higher endogenous ammonia production rate under the feeding treatment than the other two sections. The $Jt_{amm}$ values in the three sections all became different from one another (anterior > mid > posterior; Fig. 3).

The HLA treatment caused much more marked stimulations of $Jt_{amm}$ than did feeding, with increases in all three sections for fasted fish (Fig. 3). On a relative basis, the increases in $Jt_{amm}$ caused by HLA were greatest in the

**Table 2** Total tissue ammonia (µmol g $tissue^{-1}$) concentration of the anterior, mid, and posterior intestine in unfed and fed fish under control and HLA treatments

| Section | Control | HLA |
|---|---|---|
| Unfed | | |
| Anterior ($N = 5$) | 1.44 ± 0.14 | 1.76 ± 0.13 |
| Mid ($N = 5$) | 2.01 ± 0.26 | 2.07 ± 0.29 |
| Posterior ($N = 5$) | 2.11 ± 0.22 | 2.31 ± 0.21 |
| Fed | | |
| Anterior ($N = 5$) | 2.06 ± 0.38 | 2.50 ± 0.20* |
| Mid ($N = 5$) | 2.87 ± 0.24* | 2.48 ± 0.35 |
| Posterior ($N = 5$) | 2.32 ± 0.21 | 2.42 ± 0.18 |

Data are expressed as mean ± SEM. Significant differences ($P < 0.05$) between unfed and fed fish (asterisks) were evaluated using a Student's unpaired *t* test. There were no significant differences among sections within treatments, and no significant effects of the HLA treatment within a section

posterior intestine (sixfold), intermediate in the mid intestine (threefold) and least in the anterior intestine (1.7-fold). In fed fish, responses to HLA exhibited a somewhat different pattern with smaller (1.6–1.9 fold) increases only in the mid and posterior sections (Fig. 3b, c). While there was no increase with HLA in anterior preparations from fed fish (Fig. 4a), there was an increase in tissue $T_{amm}$ content in this section (Table 2), so the effect appears to be universal. For preparations from unfed fish treated with HLA, the $Jt_{amm}$ values were greater in the anterior section than in the midsection, and intermediate in the posterior section. Feeding did not greatly alter the response to HLA, except in the posterior intestine, where feeding lowered the endogenous production rate by about 50 %, so that it became lower than in the other two sections, although still greater than the fed control rate (Fig. 3c).

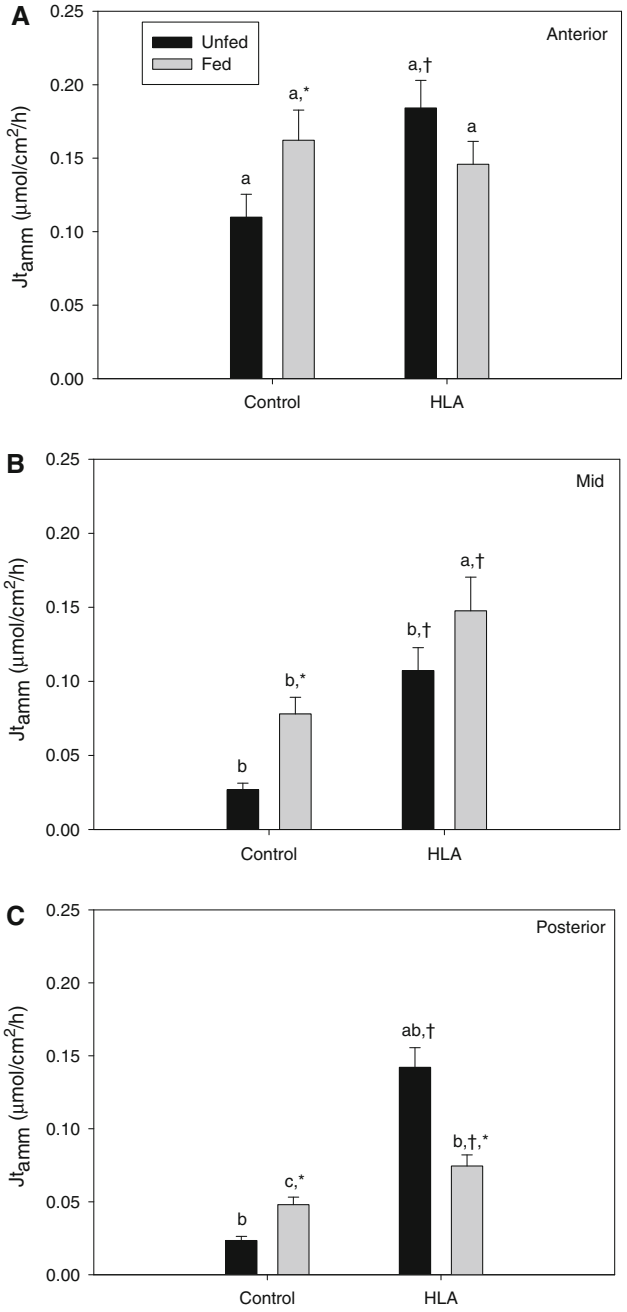


**Fig. 3** Tissue ammonia production rate ($Jt_{amm}$, µmol $cm^{-2}$ $h^{-1}$) of the anterior (**a**), mid (**b**), and posterior (**c**) intestine of unfed (*black bars*) and fed (*gray bars*) fish under control and HLA treatments. Significant differences ($P < 0.05$) between unfed and fed treatments (*asterisks*) and between control and HLA treatments (*daggers*) were determined using a Student's unpaired *t* test. Significant differences within a treatment (*letters*) among all intestinal sections was determined using a one-way ANOVA followed by a Bonferroni's post hoc test ($N = 5$ for each treatment)

## Enzyme activity

Enzyme activities were measured only in tissues taken from freshly sacrificed trout, either fasted or fed, so there were no HLA treatments. Glutamine synthetase (GS) activity increased from the anterior to the posterior intestine in unfed fish, with the latter having significantly higher activity than the other two sections (Fig. 4a). This pattern was abolished by feeding, with all three segments exhibiting similar activities. No changes in GS activity in the anterior and mid intestine occurred in response to feeding, however, a substantial 70 % decrease in the posterior intestine relative to the unfed treatment was observed. As this pattern was unexpected relative to previous literature (see "Discussion"), the experiment was repeated with a different batch of trout, with the same result, a decrease in GS activity in the posterior intestine after feeding (data not shown). Glutaminase (GLN) activity did not vary across the intestine, and did not change in response to feeding (Fig. 4b). Glutamate dehydrogenase (GDH) also did not vary across the intestine in preparations from unfed fish (Fig. 4c). However, feeding caused a 60 % increase in GDH activity in the anterior intestine, so that it also became higher than in the posterior intestine, with intermediate levels in the mid intestine. Feeding did not alter GDH activities in these latter two sections.

As the anterior and posterior intestine were the only sections to exhibit large changes in enzyme activity (GDH in anterior intestine and GS in posterior intestine) in response to feeding (Fig. 4a, c), activities of these enzymes in the epithelial scrapings and the muscle tissue were measured separately in an additional experiment (Table 3). GDH activity was broadly distributed in the anterior intestine, with similar activity in both the muscle layer and epithelial scrapings (~5 % whole-tissue mass). Furthermore, GDH activity increased in both the epithelial scrapings and the muscle tissue in response to feeding. In the posterior intestine, GS activity was localized to the epithelial scrapings (~30 % whole tissue mass), with

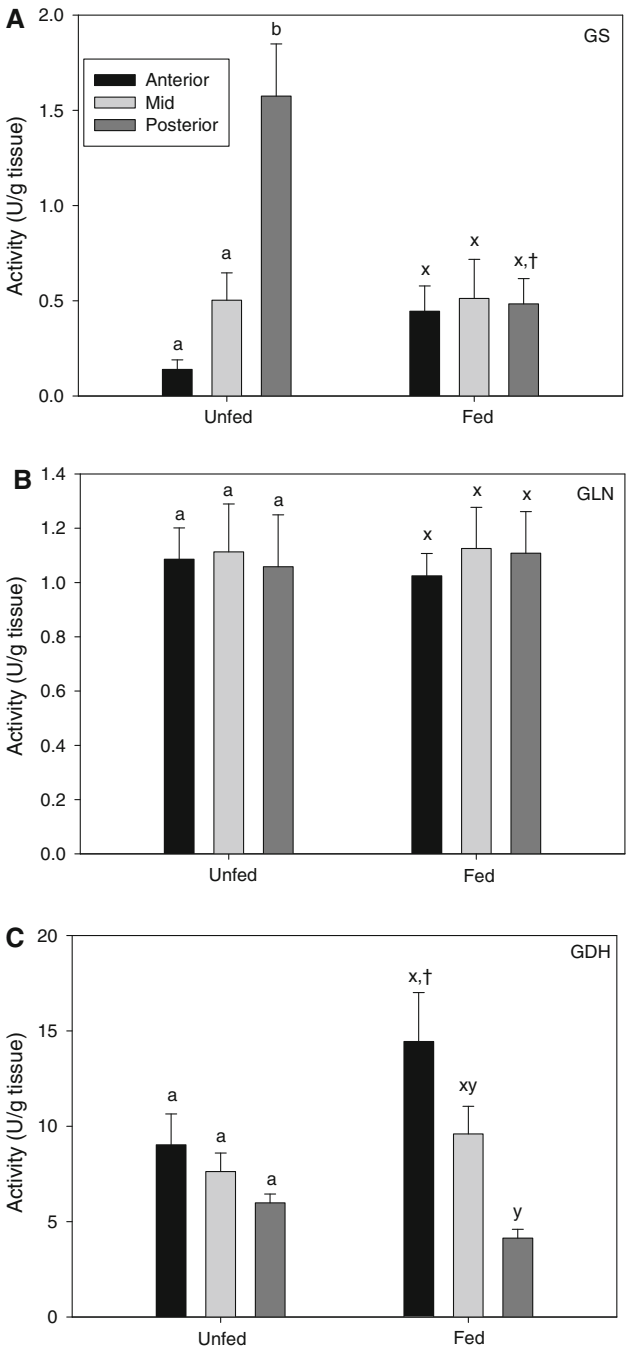


**Fig. 4** Glutamine synthetase (**a**, GS), glutaminase (**b**, GLN) and glutamate dehydrogenase (**c**, GDH) activity (units g tissue$^{-1}$) for the anterior, mid, and posterior intestine under unfed and fed conditions. Significant differences ($P < 0.05$) among all sections within an experimental group (*letters*) were determined using a one-way ANOVA followed by a Bonferroni's post hoc test. Significance comparing unfed and fed fish (*daggers*) was determined using a two-way ANOVA followed by a Bonferroni's correction post hoc test. In the case of failed normality test, a one- or two-way ANOVA followed by a Tukey's post hoc test was conducted (GS, $N = 10$; GLN, $N = 5$; GDH, $N = 5$)

**Table 3** Comparison of enzyme activity values (U g$^{-1}$) for glutamate dehydrogenase (GDH) in the anterior intestine, and glutamine synthetase (GS) in the posterior intestine between epithelial scrapings and muscle tissue

| Enzyme | Section/feeding status | Epithelial scrapings | Muscle tissue |
|---|---|---|---|
| GDH | Unfed anterior ($N = 5$) | 18.78 ± 2.67 | 15.85 ± 3.73 |
| | Fed anterior ($N = 5$) | 43.28 ± 10.97* | 38.98 ± 11.52* |
| GS | Unfed posterior ($N = 5$) | 8.73 ± 1.33 | 1.17 ± 0.67$^{\dagger}$ |
| | Fed posterior ($N = 5$) | 2.66 ± 0.40* | 0.27 ± 0.17$^{\dagger}$ |

Data are expressed as mean ± SEM. Statistical analysis ($P < 0.05$) comparing enzyme activity between epithelial scrapings and muscle tissue (daggers) was conducted using a Student's paired *t* test. Comparisons made for the effect of feeding on enzyme activity within either epithelial scrapings or muscle tissue (asterisks) were conducted using a Student's unpaired *t* test

minimal activity in the muscle tissue. In addition, feeding caused a marked decrease in GS activity of the epithelial scrapings, similar to the observed decrease using whole-tissue homogenates (Fig. 4a), but had no effect on GS activity in the muscle layer.

## Discussion

### Overview

To our knowledge, this study is the first in vitro analysis of ammonia handling in the intestinal tract of the rainbow trout. Prior to this, the majority of research had been centered upon the effects of feeding on plasma ammonia concentration and ammonia excretion rates and mechanisms at the level of the gill. The chyme ammonia concentration found 24 h post-feeding was used to provide a physiologically relevant mucosal ammonia concentration (1.0 mmol L$^{-1}$) for HLA gut sac incubations, which was similar to the values reported by Bucking and Wood (2012). In addition, we found an increase in $P_{NH3}$ from anterior to posterior intestine which is attributed to the increasing chyme pH along the tract (Table 1). In all three segments, these chyme pH and $P_{NH3}$ values were much higher than levels previously reported in trout blood plasma in vivo (e.g., Bucking and Wood 2008; Wood and Nawata 2011) suggesting an inward diffusion gradient for ammonia. The time points at which gut sac experiments were conducted (fasted and 24 h post-feeding) were chosen based on two previous studies. Firstly, Bucking and Wood (2012) reported that mRNA expression of Rhbg1 increased 24 h following feeding, and thus we had predicted there

would be a concomitant physiological difference at this time. In addition, Bucking and Wood (2006b) tracked the passage of chyme along the intestine of trout following a similar feeding protocol to the present study. Although chyme began to appear in the posterior intestine 12 h following ingestion, at 24 h chyme was present throughout the whole intestine, ensuring that all tissues had been pre-exposed to elevated luminal ammonia at the time of our experiments.

The gut sac technique was a useful tool to evaluate our predictions. The various in vitro procedures for making physiological measurements using intestinal tissues have been reviewed by Wood and Bucking (2011). When compared against Ussing chamber and perfused gut approaches, the advantages of the gut sac technique are simplicity and scale, allowing the efficient testing of a number of different treatments. In addition, this technique allows for the maintenance of gut morphology, which is crucial for gut function. Upon dissection, the gut was relatively taut in fed fish due to the presence of chyme, but less so in fasted fish. Therefore, one caveat is that we used a relatively constant filling regime for gut sacs from both fed and fasted trout, whereas under physiological conditions, a fasted fish would probably not experience the same degree of gut stretching as a fed fish. Gut stretching may influence ion permeability due to activation of mechanoreceptors, with potential effects on voltage-gated ion channels (Larsson et al. 1998). An additional caveat relates to limitations in truly duplicating in vivo total ammonia conditions on the blood side during flux experiments. In vivo, arterial and hepatic vein total ammonia concentrations are reported to rise from approximately 50–100 μmol $L^{-1}$ in fasted trout, up to 250–350 μmol $L^{-1}$ after feeding (Karlsson et al. 2006; Bucking and Wood 2008), whereas we used a constant starting concentration close to 0 μmol $L^{-1}$ in the serosal saline. Nevertheless, as illustrated in Electronic Supplementary Table S1, the mean serosal concentrations averaged over the 2-h flux periods were very representative of these in vivo plasma concentration ranges, so the overall discrepancy was not large.

With respect to our original predictions, most but not all were confirmed. Firstly, clear differences in ammonia handling appear to exist along the tract, with the largest area-specific flux in the anterior intestine and the lowest in the posterior intestine. However, these differences were attenuated or disappeared with HLA (Figs. 1, 2, 3). Secondly, there was substantial endogenous ammonia production by the gut tissue itself (Fig. 3), with most of this appearing in the serosal solution (Fig. 1), indicative of absorption into the bloodstream. Again, under control conditions, these endogenous rates were highest in the anterior intestine and lowest in the posterior intestine (Fig. 3). Our third postulate, that endogenous ammonia production of the intestine would increase following feeding was clearly supported in all three segments in control preparations, but this pattern was lost with HLA treatment (Fig. 3). Our fourth prediction, that a mucosal HLA treatment (representative of postprandial chyme ammonia concentrations) would greatly increase ammonia transport into the serosal solution was unequivocally confirmed (Figs. 1, 2). However our fifth prediction, that ammonia permeability would reflect the changes in Rhbg glycoprotein expression following feeding seen by Bucking and Wood (2012) was not supported. The serosal flux of ammonia actually decreased in the posterior intestine of HLA-treated preparations from fed fish relative to unfed fish, in contrast to the predicted increase (Fig. 1c). Thus, our physiological data do not correlate with the increased Rhbg1 mRNA expression in the posterior intestine reported by Bucking and Wood (2012); mRNA expression does not necessarily correlate with functional protein expression. Finally, our prediction that the activities of GS, GDH, and GLN would vary regionally and would change depending on feeding status was supported for GS (Fig. 4a) and GDH (Fig. 4c), but not for GLN (Fig. 4b). It was also seen that GS activity in the posterior intestine differed between epithelial scrapings and muscle tissue, while GDH activity was broadly distributed in the anterior intestine (Table 3). These results generally support the notion of metabolic zonation across the teleost intestine (Mommsen et al. 2003a, b).

However there were several surprising findings. In addition to the unexpected decrease in posterior intestinal ammonia flux in HLA fed preparations (Fig. 1c), there was also an unexpected decrease in GS activity with feeding in this segment (Fig. 4c), contrary to the report of Bucking and Wood (2012). However, most surprising of all was the very marked stimulation of endogenous ammonia production in all segments caused by the mucosal HLA treatment under unfed conditions (Fig. 3). These responses are discussed subsequently.

## Gut ammonia handling in fasted fish under control conditions

As predicted, we observed that the gut is capable of endogenous ammonia production (Fig. 3). Under control conditions, this was evident through the observed bi-directional ammonia flux into both the serosal and mucosal salines (Figs. 1, 2). Endogenous intestinal production of ammonia under fasted conditions using similar gut sac techniques has also been observed in the plainfin midshipman (Bucking et al. 2013b). Presumably, ammonia is being produced through cellular metabolic processes, as chyme is absent under fasted conditions, and the rate is clearly greatest in the anterior segment on an area-specific basis. Notably, in all sections, $Js_{amm}$,

representative of flux into the blood, was much greater than $Jm_{amm}$, representative of flux into the lumen of the intestine (Figs. 1, 2). Given that ammonia is potentially toxic (Randall and Tsui 2002), one might expect that the endogenously produced N-waste of the gut tissue itself would be simply dumped into the lumen to be eventually cleared through the rectum. However, in fasted fish, $Js_{amm}$ by the entire intestinal tract was estimated to be about 70 μmol/kg/h, based on the $Js_{amm}$ of each individual section in unfed controls (Fig. 1) multiplied by the relevant gut surface area, and divided by the average weight of the trout. This would account for approximately 18 % of the whole-body $J_{amm}$ values previously reported for fasted trout (Bucking and Wood 2008).

Under fasted conditions, the activities of GDH and GLN were equal across all sections (Fig. 4b, c). GLN, and potentially GDH, could contribute to intestinal $Jt_{amm}$, but they do not explain the regional differences in $Jt_{amm}$. A likely explanation is the activity of GS, the ammonia-trapping enzyme, was lowest in the anterior segment (Fig. 4a), so that more of the endogenously produced ammonia was released rather than recycled. Other possible explanations include alternate ammonia-producing pathways and/or the fact that enzymatic activities in situ may differ from the activities measured in vitro (under saturating conditions and in the reverse direction). Notably, in contrast to findings of Bucking and Wood (2012) of no regional differences in GS activity across the intestine in unfed trout, ammonia-fixing GS exhibited the highest activity in the posterior intestine under fasted conditions (Fig. 4a), with almost all activity localized to the epithelial scrapings (Table 3). Possibly, the epithelial location may reflect intestinal bacteria which are known to use glutamine synthetase to fix ammonia, which is their preferred nitrogen source (Magasanik 1977). Teleost intestinal flora has not been well studied, though treatment of the plainfin midshipman gut with antibiotics has been shown to greatly reduce ammonia flux (Bucking et al. 2013b) suggesting a strong influence of intestinal bacterial flora on ammonia handling in fish. Under fasting conditions, GS activity may also be important to supply the intestinal cells with glutamine for a variety of downstream processes (Taylor et al. 2011), including use as an oxidative substrate by the intestinal cells, which has been observed in mammals (Hanson and Parsons 1977; Windmueller and Spaeth 1977). Interestingly, despite the regional differences observed in $Jt_{amm}$ and enzyme activity across the intestine, intestinal tissue $T_{amm}$ was unchanged across all sections (Table 2), suggesting tight homeostatic control of tissue $T_{amm}$ in the intestine.

## Gut ammonia handling in fasted fish under HLA conditions

While this scenario is unlikely to occur in vivo, the purpose of the HLA experiments in fasted fish was to establish baseline ammonia permeability of this tissue to make comparisons against fed fish. Based on Rhbg mRNA expression patterns reported by Bucking and Wood (2012), we expected to observe lower ammonia uptake fluxes in fasted conditions compared to those in fed fish, and we did not expect to see differences, given the relatively low and equal expression of this transcript across all sections. However, the results only partially support these predictions, at least in part because the large stimulatory effects of HLA treatment on endogenous net ammonia production (i.e., $Jt_{amm}$) had not been foreseen. As discussed subsequently, HLA treatment under fed conditions did not result in higher $Js_{amm}$ than under unfed conditions (Fig. 1). However, during HLA treatment under unfed conditions, $Js_{amm}$ was greater in all sections relative to control unfed conditions, and the absolute values were similar amongst sections (Fig. 1). This is in accord with similar Rhbg expression levels under fasting conditions in the three sections (Bucking and Wood 2012), suggesting the presence of a constitutively expressed mechanism through which ammonia transport can occur. However, less than half of the ammonia appearing in the serosal solution (i.e., $Js_{amm}$; Fig. 1) was ammonia that had been removed from the mucosal solution (i.e., $Jm_{amm}$; Fig. 2). Instead, the majority of the ammonia that appeared in the serosal saline originated from greatly elevated endogenous ammonia production (i.e., $Jt_{amm}$; Fig. 3). Indeed, $Jm_{amm}$ of the anterior intestine was significantly lower than in the mid and posterior intestines within the unfed HLA treatment (Fig. 2), yet $Js_{amm}$ fluxes were the same (Fig. 1), because of the higher endogenous production rate ($Jt_{amm}$) in this section, which again fits with the lower GS activity here (Fig. 4a).

Why does HLA treatment cause such large increases in endogenous ammonia production rates (i.e., $Jt_{amm}$)? We hypothesize this occurs because the normal detoxification mechanisms (e.g., GS, GDH, associated transamination pathways, perhaps even adenylate synthases) become overwhelmed under ammonia loading conditions. Therefore to prevent cytotoxicity, the excess ammonia that cannot be "mopped up" metabolically is effluxed to the bloodstream (or in this case to the serosal solution) for disposal by excretion through the gills. Homeostasis of intracellular $T_{amm}$ concentrations appears to be very precise in the intestinal tissues (Table 2), so these appear to be regulated at the expense of the whole animal. It is tempting to speculate that the progressively higher levels of GS activity from anterior to mid to posterior intestine (Fig. 4a) may correlate with the greater chyme $P_{NH3}$ levels (Table 1), and therefore the greater requirement for detoxification. On the other hand, the reduction in GS activity with feeding in the posterior intestine is not in agreement with this greater requirement for detoxification.

### Gut ammonia handling in fed fish under control conditions

While the absence of luminal ammonia upon feeding is unlikely to occur in vivo, these conditions were helpful in unraveling the complexities of the system. Most importantly, this treatment demonstrated that feeding itself, in the absence of increased mucosal ammonia, results in increased net endogenous production of ammonia by the gut tissue itself (Fig. 3), some of which is effluxed to the bloodstream (i.e., serosal solution) as $Js_{amm}$ (Fig. 1). Bucking et al. (2013b) also observed increases in endogenously produced ammonia in the gut of plainfin midshipman in response to feeding. Presumably, the metabolic activity of the gut for mechanical, secretory, and absorptive work is increased, and therefore more ammonia is produced by deamination reactions and/or adenylate breakdown. The ammonia scavenging mechanisms become challenged, so some of this ammonia is effluxed, although this is not entirely successful at maintaining homeostasis of intracellular $T_{amm}$ (Table 2). However, in contrast to the HLA treatments, the detoxification systems are not saturated due to the absence of HLA, so regional differences in $Js_{amm}$ apparent under fasted control conditions can still be seen in these fed preparations (Fig. 1). Such site-specificity does not come as a surprise, as zonation of gut transporters for other moieties has been observed in the teleost gut (e.g., $Na^+$:glucose cotransporter, $H^+$:peptide cotransporter, Gonçalves et al. 2007; $Na^+/H^+$ exchanger, carbonic anhydrase, $Na^+/HCO_3^-$ cotransporter, Grosell et al. 2007).

Under fed conditions, $Js_{amm}$ increased significantly in control treatments relative to unfed controls in all sections (Fig. 1) but such an increase in $Jm_{amm}$ (i.e., to more negative values) occurred only in the mid intestine (Fig. 2). In the anterior intestine, the observed increase in $Js_{amm}$ may be explained, at least in part, by the observed increase in GDH activity in this tissue following feeding (Fig. 4c), which was broadly distributed across the epithelial and muscle layers (Table 3). This is different from GS, which showed highly localized activity in the mucosal epithelium of the posterior intestine (Table 3). Future studies should examine the precise localization of these and other enzymes in all gut sections. Site-specific activity of GDH (anterior > posterior; Fig. 4c) is in agreement with previous findings by Mommsen et al. (2003b), who observed high activity in the anterior intestine of actively fed tilapia. Feeding did not result in any significant changes in tissue $T_{amm}$ in the anterior intestine (Table 2). The mid intestine, however, was the only section in which tissue $T_{amm}$ increased significantly in response to feeding alone (Table 2) and was also the only tissue where $Jm_{amm}$ increased in response to feeding (Fig. 2). This was a surprising finding, given the lack of evidence in the literature suggesting the mid intestine as an ammonia-producing site of the gut during feeding (Mommsen et al. 2003a, b; Bucking and Wood 2012). The significance of this increase in $Jm_{amm}$ is unclear but may reflect the recruitment of apical excretion mechanisms, in addition to basolateral mechanisms, to handle the increased tissue $T_{amm}$ produced by feeding. Based on our analyses, there were no increases in the activity of the ammonia-producing enzymes in the mid intestine (Fig. 4b, c).

### Gut ammonia handling in fed fish under HLA conditions

This treatment was representative of in vivo luminal conditions at 24 h after feeding. The results, showing greatly elevated $Js_{amm}$ fluxes relative to both control fasted and control fed preparations, provide strong support for the conclusions of Karlsson et al. (2006), who suggested that the gut may be partially responsible for the increases in plasma $T_{amm}$ observed post-feeding based on in vivo evidence of elevated plasma $T_{amm}$ levels in the hepatic portal vein (HPV), prior to drainage into the liver. The liver, not the gut, is classically considered to be the major site of ammonia production (Walton and Cowley 1977). However, by using gut sac data from fed HLA preparations, and performing an analogous calculation to that used earlier for fasting control fish, we calculate that total intestinal $Js_{amm}$ would amount to about 350 μmol/kg/h, accounting for approximately 47 % of the whole-body $J_{amm}$ values for fed trout reported by Bucking and Wood (2008). Of this total, slightly more than half would be due to increased endogenous production (i.e., $Jt_{amm}$; Fig. 3), and the remainder due to increased absorption from the lumen (i.e., $Jm_{amm}$; Fig. 2). If some of the ammonia remaining in the lumen were eventually excreted through the rectum, then even more of the whole-body $J_{amm}$ would be of intestinal origin. Though we cannot be sure of any potential in vivo effects on gut ammonia production, such as neural or hormonal inputs which have a large role in regulating digestion in mammals (Rogers et al. 1996), it appears evident from the present study that following feeding, the gut is a major site of ammonia production in the rainbow trout.

Clearly as with feeding alone, there was regional heterogeneity in the combined HLA-feeding responses. The most marked difference was the lower $Js_{amm}$ in the posterior intestine of the combined HLA-feeding treatment relative to HLA alone (Fig. 1c). In turn, this was explained by the lower endogenous ammonia production rate ($Jt_{amm}$; Fig. 3c) in this treatment. Overall, this observation conflicts with Bucking and Wood (2012), who reported increased Rhbg1 mRNA expression in the anterior and posterior intestine post-feeding, which we had predicted

would lead to an increase in ammonia permeability. However, the potential for modifications of endogenous ammonia production rates had not been taken into account in these predictions. In addition, our findings suggest that the physiological impacts of increased Rhbg1 mRNA expression are not observed at the same time points of our experiments, thus further investigations using gut sacs at multiple time points post-feeding would be beneficial to more clearly understand this relationship.

Interestingly, in the posterior intestine, GS activity was significantly lower in response to feeding, again contrary to the results presented by Bucking and Wood (2012) where feeding induced an increase in the activity of this enzyme in this gut section. Likely there were unknown differences in stocks, or feeding/fasting protocols between the present study and that of Bucking and Wood (2012), emphasizing the importance of making all measurements on the same batch of animals at the same time. Perhaps there are alternative detoxification mechanisms which are up-regulated in the posterior intestine so as to reduce $Jt_{amm}$, a possibility that deserves future study. Consistent with a decrease in GS activity in the present study, however, was an increase in $Jt_{amm}$ by this section in fed control conditions relative to fasted control conditions (Fig. 4). The decrease in GS following feeding may be reflective of the availability of luminal dietary glutamine, though the concentration of this amino acid in the chyme of trout on the present diet has, to our knowledge, yet to be measured. Furthermore, as GS carries energetic costs to function (Webb and Brown 1980), fish may be sparing ATP for other digestive processes. Thus, GS activity may be dependent upon its substrate concentration, and could undergo post-translational modifications following feeding to alter its activity. Based on this hypothesis, GS would have a limited role for fixing ammonia in the intestine during feeding. This is in contrast to other species, such as insects, which place large dependence upon GS for detoxification during digestion (Weihrauch et al. 2012). In addition, fed rats experience an increase in intestinal GS activity (Arola et al. 1981), which could be due to the enhanced demand for this oxidative substrate (Windmueller and Spaeth 1977). GS is also known to show altered gene expression or activity in response to ammonia stress in a variety of organisms (fish Wright et al. 2007; insects Scaraffia et al. 2005; bacteria Wax et al. 1982). Future gut sac experiments, using modified serosal and mucosal salines containing amino acids, would aid in understanding the influence of the availability of amino acids, such as glutamine, on intestinal ammonia handling. Specifically, they should assess the roles of GS in the intestine of trout in preventing ammonia toxicity and in providing fuel for trout intestinal cells.

## Conclusions

Ammonia handling in the intestinal tract of freshwater teleosts is an area of research which is evidently void of information. Our study provides some of the first insights into this topic, showing clear site-specific patterns of ammonia handling across the tract. The anterior intestine exhibits the greatest fluxes under fasted control conditions, but these differences tend to disappear under typical post-feeding conditions, and indeed the posterior section may exhibit the greatest detoxification capacity. Feeding clearly stimulated endogenous ammonia production. However, of all our findings, one of the most interesting was the large increase in endogenous $Jt_{amm}$ during HLA exposure, either alone or in combination with feeding. We propose that this is a response to avoid potential cytotoxicity due to overburdened ammonia detoxification mechanisms in the face of elevated mucosal ammonia. This may explain why $T_{amm}$ of each intestinal section remained relatively constant regardless of feeding status and exposure to HLA. For such a response, the recruitment of apical and basolateral transport mechanisms would need to be elevated to avoid lethal increases in intestinal $T_{amm}$; this will provide a rich area for future investigation. Indeed, Bucking et al. (2013a) have recently immunolocalized several apical and basolateral Rh proteins in the plainfin midshipman gut, though their exact functions are still unclear. In vivo, subsequent gill ammonia excretion could provide a less energetically costly means of excretion compared to excretion of ammonia against an unfavorable concentration gradient into the intestinal lumen. Indeed, our flux data shows that approximately half of whole-body $J_{amm}$ observed following feeding (Bucking and Wood 2008) might be of intestinal origin. Further analysis into alternative detoxification mechanisms should be explored, including the possibility of a potential relationship between ammonia handling and intestinal $Na^+$ uptake. Such a relationship has been extensively studied in the gills (Wright and Wood 2012), and may provide directions for pharmacological analysis that can be conducted in the gut. In addition, mammalian models suggest the colon prevents large accumulations in blood ammonia through $Na^+$-dependent intestinal scavenging mechanisms (Worrell et al. 2008; Ramirez et al. 1999). Future studies should search for comparable mechanisms in fish. Overall, the findings presented in this study lay a foundation for further explorations in this relatively new field of research.

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conform to the animal care guidelines implemented by the institutional animal care committee at McMaster University.

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