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RESEARCH ARTICLE

Nitrogen metabolism of the intestine during digestion in a teleost fish, the plainfin midshipman (*Porichthys notatus*)

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SUMMARY

Digestion affects nitrogen metabolism in fish, as both exogenous and endogenous proteins and amino acids are catabolized, liberating ammonia in the process. Here we present a model of local detoxification of ammonia by the intestinal tissue of the plainfin midshipman (*Porichthys notatus*) during digestion, resulting in an increase in urea excretion of gastrointestinal origin. Corroborating evidence indicated whole-animal ammonia and urea excretion increased following feeding, and ammonia levels within the lumen of the midshipman intestine increased to high levels (1.8±0.4 μmol N g^{-1}). We propose that this ammonia entered the enterocytes and was detoxified to urea *via* the ornithine-urea cycle (O-UC) enzymes, as evidenced by a 1.5- to 2.9-fold post-prandial increase in glutamine synthetase activity (0.14±0.05 and 0.28±0.02 μmol min^{-1} g^{-1} *versus* 0.41±0.03 μmol min^{-1} g^{-1}) and an 8.7-fold increase in carbamoyl phosphate synthetase III activity (0.3±1.2 *versus* 2.6±0.4 nmol min^{-1} g^{-1}). Furthermore, digestion increased urea production by isolated gastrointestinal tissue 1.7-fold, supporting our hypothesis that intestinal tissue synthesizes urea in response to feeding. We further propose that the intestinal urea may have been excreted into the intestinal lumen *via* an apical urea transporter as visualized using immunohistochemistry. A portion of the urea was then excreted to the environment along with the feces, resulting in the observed increase in urea excretion, while another portion may have been used by intestinal ureolytic bacteria. Overall, we propose that *P. notatus* produces urea within the enterocytes *via* a functional O-UC, which is then excreted into the intestinal lumen. Our model of intestinal nitrogen metabolism does not appear to be universal as we were unable to activate the O-UC in the intestine of fed rainbow trout. However, literature values suggest that multiple fish species could follow this model.

Key words: ammonia, urea, prandial, ureolytic bacteria, *Porichthys notatus*, *Oncorhynchus mykiss*.

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INTRODUCTION

There are two distinct strategies that fish adopt concerning nitrogen metabolism and waste regulation. The majority of fish directly excrete ammonia (known as ammoniotely) to the surrounding water, using the vast aquatic environment to dilute their toxic waste (e.g. Randall and Wright, 1987; Campbell, 1991; Mommsen and Walsh, 1992; Wood, 1993; Anderson, 2001; Wright and Wood, 2009). Alternatively, some fish can detoxify ammonia into urea and excrete it (known as ureotely) (e.g. Anderson, 1995; Walsh, 1997; Anderson, 2001) *via* three different (energetically costly) enzymatic pathways – uricolysis, arginine catabolism and the ornithine-urea cycle (O-UC) (Goldstein and Forster, 1965; Brown et al., 1966; Cvancara, 1969; Hayashi et al., 1989; Mommsen and Walsh, 1992; Wood, 1993; Korsgaard et al., 1995; Anderson, 1995), which is the most prevalent pathway in mammals. In fact, one of the criteria to identify ureotelic fish is the presence/absence of O-UC enzyme activities [glutamine synthetase (GS), carbamoyl phosphate synthase III (CPS III) and ornithine carbamoyltransferase (OCT)] in tissues such as the liver, as it is the organ primarily used for the O-UC in mammals, as well as muscle, a sometimes equally important tissue in ureotelic fish (e.g. Julsrud et al., 1998; Lindley et al., 1999; Anderson et al., 2002; Kajimura et al., 2006). Interestingly, the genes responsible for generating the O-UC enzymes have been conserved in fish that are ammoniotelic and lack expression of an active O-UC in most tissues [e.g. see Anderson (Anderson, 2001) for a species comparison of CPS III sequences, and Murray et al. (Murray et al., 2003) for a comparison of GS sequences]. An explanation for this conservation of ostensibly idle genes rests in recent findings that embryonic stages of fish that are ammoniotelic as adults are ureotelic during development (Dépêche et al., 1979; Chadwick and Wright, 1999; Terjesen et al., 2002; Braun et al., 2009), a trait thought to prevent ammonia toxicity to the embryo during critical developmental stages.

Regardless, excretion of either ammonia or urea in fish is currently thought to proceed *via* dedicated transporters, belonging to the Rhesus glycoprotein (Rh) family (reviewed by Wright and Wood, 2009; Weihrauch et al., 2009) and the urea transporter (UT) family (reviewed by Walsh, 1997; McDonald et al., 2006; McDonald et al., 2012; Weihrauch et al., 2009), respectively. The bulk of nitrogen excretion in freshwater fish occurs *via* the gill – accounting for over 80% of total nitrogen excretion regardless of the form – while a smaller fraction occurs *via* the kidney and other unidentified routes [e.g. the skin and the gastrointestinal (GI) tract] (e.g. Wood et al., 1995a; Wood et al., 1995b; Wright et al., 1995b; McDonald et al., 2002; Bucking et al., 2010; Smith et al., 2012), although the contribution of these alternative routes may become greater (~40%) in marine fish (e.g. Read, 1968; Morii et al., 1978; Sayer and Davenport, 1987; for a review, see Wood 1993). The majority of studies examining piscine nitrogen metabolism, including many cited above, have been conducted on fasting fish; however, digestion

has large implications for nitrogen metabolism. For example, digestion can increase total nitrogen excretion many fold, sometimes even by an order of magnitude (e.g. Bucking and Wood, 2008; Lam et al., 2008; Tng et al., 2008; Bucking et al., 2009; Bucking et al., 2010; reviewed by Handy and Poxton, 1993; Wood, 1995; Leung et al., 1999; Wood, 2001), primarily through increased branchial nitrogen excretion (Bucking et al., 2010).

However, fecal nitrogen excretion can account for a significant proportion of total nitrogen excretion, greater than renal contributions in fact (Beamish and Thomas, 1984; Kajimura et al., 2004). Additionally, we now know that the concentration of ammonia along the GI tract during digestion is very high in species examined to date [~2 mmol l^{-1} (Wood et al., 2009; Bucking and Wood, 2012)], presenting potentially toxic circumstances on the local scale of the gut. One potential way that the GI tissue could limit the toxicological effects of this extreme ammonia concentration is by blocking uptake of intestinal ammonia through a downregulation or an entire lack of enterocyte ammonia transporter expression. Indeed, detection of intestinal Rh transcripts has shown a variable and inconclusive pattern to date (Hung et al., 2007; Nakada et al., 2007a; Nakada et al., 2007b; Nawata et al., 2007; Nawata and Wood, 2008; Braun et al., 2009; Rodela et al., 2012), suggesting a lack of dedicated ammonia transporters in the intestine. However, at ~2 mmol l^{-1} lumenal concentrations, some diffusion of ammonia down its gradient is likely even without the aid of Rhs (typical teleost plasma ammonia concentrations are ~100–200 μmol l^{-1}), and entry into the enterocyte is probable. Interestingly, intestinal activities of O-UC enzymes have been observed not only in the ureotelic toadfish *Opsanus beta* (Wood et al., 1995b; Julsrud et al., 1998), but also the ammoniotelic largemouth bass (*Micropterus salmoides*) (Kong et al., 1998) and bowfin (*Amia calva*) (Felskie et al., 1998). While the physiological significance of these findings is unknown, urea-N excretion rates of ammoniotelic fish were responsive to alterations in ration, dietary composition and starvation (Beamish and Thomas, 1984; Wright, 1993; Alsop and Wood, 1997; Kajimura et al., 2004; Lam et al., 2008), suggesting a role for the GI tract in urea synthesis.

With the above background in mind, using plainfin midshipman (*Porichthys notatus*), we hypothesized a model of urea excretion wherein this species could locally detoxify lumen ammonia to urea in the gut tissue and then transport all or a portion of this urea back to the lumen for excretion with rectal fluids. *Porichthys notatus* was chosen due to its ammoniotelic nature but close phylogenetic relationship with the facultatively ureotelic *O. beta*. To examine facets of this model, we tested several predictions. First, that digestion would increase whole-animal nitrogen excretion and generate high ammonia concentrations in the intestine. A second prediction of this model is that midshipman gut tissue should contain relatively high activities and transcripts of O-UC enzymes, and should be able to convert ammonia into urea in *in vitro* preparations. To test a third prediction of the hypothesis, we examined through immunohistochemical methods and qPCR the presence/absence and localization of urea transporters in midshipman gut tissue. As the above predictions were supported by our results, we wished to determine whether this model was specific to midshipman, or more broadly applicable to other teleosts. So, finally, we measured the activities of O-UC enzymes in the intestines of fed rainbow trout and compared all data with literature values.

MATERIALS AND METHODS

Animals

Adult midshipman fish (*Porichthys notatus* Girard; 67–218 g) were collected from the wild (Nanoose Bay, BC, Canada; 49°15 N, 124°10 W) from the intertidal zone and transported to the Bamfield Marine Sciences Centre (Bamfield, BC, Canada) where they were acclimated to laboratory conditions with running seawater (~32 ppt, 9–12°C) and constant aeration before experimentation began. The studies were conducted over two summer seasons, with animals collected in June 2011 and June 2012. The animals were not fed while in captivity except where noted within each series. Animal husbandry and all experiments were conducted according to approved animal care protocols from both the University of Ottawa and Bamfield Marine Sciences Centre (protocol BL-255).

Series 1 – whole-animal nitrogen fluxes

Midshipman fish (*N*=5) were weighed and placed in individual containers (2 litres) that were supplied with their own running seawater and aeration lines. Water flow was ceased (but aeration continued) and water samples (1 ml) were taken at various time points (0, 3 and 6 h) following placement in the chambers. Following the 6 h acclimation to the chambers, the fish were individually removed and hand-fed pieces of thawed shrimp rinsed with seawater until ~2.5% of their body mass had been consumed. They were then placed back into their individual chambers and the water was changed. An initial water sample was then taken followed by subsequent samples at several time points following feeding. The water within the chambers was thoroughly changed 3 h following feeding and then every subsequent 12 h after the feeding interval. Following the water changes, an initial water sample was taken. At the end of the experiment, the animals were returned to the holding tanks for recovery.

The water samples were examined for ammonia nitrogen (ammonia-N) and urea nitrogen (urea-N) content according to Ivancic and Deggobis (Ivancic and Deggobis, 1984) and Rahmatullah and Boyde (Rahmatullah and Boyde, 1980), respectively. Ammonia fluxes to the water (J_{NH_3/NH_4^+}; μmol N kg^{-1} h^{-1}) and urea fluxes to the water (J_{Urea}; μmol N kg^{-1} h^{-1}) were calculated as the appearance of ammonia or urea in the water after accounting for the time of the flux period, the mass of the animal, and the concentration of ammonia or urea at the preceding time point or water change. Measured urea concentrations were multiplied by a factor of two in order to account for the two nitrogen atoms in urea. The percentage of total nitrogenous waste excretion (ammonia-N + urea-N) accounted for by urea-N was also calculated.

Series 2 – *in vitro* ammonia and urea production

Midshipman fish (*N*=6) were killed with an overdose of anaesthetic [1 g l^{-1} tricaine methanesulfonate (MS-222); Syndel Laboratories, BC, Canada] following 7–12 days of fasting in the laboratory. Another group of midshipman (*N*=6) was hand-fed ~2.5% of their body mass with shrimp (as above) for 5 days and killed in the same manner 10 h following the last meal. Finally, in order to test the role of gut microbes, a group of midshipman (*N*=5) was held in a static tank (10 litres) that was spiked with antibiotics (75 p.p.m. each of sisomycin, ampicillin, amphomycin and penicillin), a regimen that substantially reduced gut microbe populations in the related gulf toadfish (Walsh et al., 1990; Walsh et al., 1991). The water was changed every 12 h, and fresh antibiotics were added. The antibiotic exposure lasted 48 h before the fish were killed.

The GI tract (without the stomach/esophagus) was removed in its entirety, the contents were collected (and placed at −20°C for future analysis), and four equal sections were cut from the anterior portion of the intestine. The nitrogen metabolism of the intestinal tissue was examined using an *in vitro* gut sac technique (e.g. Bury et al., 2001, Nadella et al., 2006; Bucking and Schulte, 2012). The

intestinal sections were cleared of digesta and thoroughly rinsed with Cortland saline (see below). The proximal end of each intestinal section was tied off using a silk ligature while a small piece of polyethylene tubing (PE 160) was inserted into the distal end and secured in place with another ligature. The tubing was used to fill the sac with one of four mucosal salines (see below) until the intestine was taut. The end of the tubing was then heat-sealed and the intestinal sac was weighed and then incubated in 3 ml of aerated serosal saline (see below) for 3 h. The temperature was maintained at 12°C using a water bath. Following incubation, the sacs were removed from their serosal baths, blotted dry and re-weighed. Samples were then taken of the serosal saline (1 ml) and the mucosal saline (500 μl). The surface area of the intestinal sac was measured by cutting open the sac and tracing the outline on a piece of graph paper (Grosell and Jensen, 1999).

A modified Cortland saline (Wolf, 1963) was used for the serosal solutions for all gut sac preparations (144 mmol l^{-1} NaCl, 5.1 mmol l^{-1} KCl, 1.4 mmol l^{-1} $CaCl_2.2H_2O$, 1.9 mmol l^{-1} $MgSO_4.7H_2O$, 11.9 mmol l^{-1} $NaHCO_3$, 2.9 mmol l^{-1} Na_2HPO_4, 5.5 mmol l^{-1} glucose, pH 7.8). Four asymmetrical mucosal solutions were also prepared and adjusted to pH 8 (Bucking et al., 2009; Wilson et al., 1996): a control saline (100 mmol l^{-1} NaCl, 50 mmol l^{-1} $MgSO_4$, 35 mmol l^{-1} $MgCl_2$, 5 mmol l^{-1} $CaCl_2$, 5 mmol l^{-1} KCl, 1 mmol l^{-1} $KHCO_3$, 0.1 mmol l^{-1} NH_4Cl), a high ammonia saline (control saline with 2 mmol l^{-1} NH_4Cl), a high glutamine saline (control saline with 2 mmol l^{-1} glutamine), and a high ammonia and high glutamine saline (control saline with 2 mmol l^{-1} NH_4Cl and 2 mmol l^{-1} glutamine). Final mucosal and serosal samples, along with samples of the starting salines, were analyzed for ammonia and urea concentrations as in Series 1. Urea and ammonia appearance in the serosal and mucosal solutions was calculated according to: [(final concentration (nmol) – initial concentration (nmol))/surface area (cm^2) × time (h)].

Series 3 – effects of feeding on enzyme activity

Midshipman fish were killed using MS-222 (see Series 2) after fasting for 4–10 days (N=7) or 16–21 days (N=7) in the laboratory, and the intestinal tract was removed in its entirety. A subsample of intestinal tissue was excised from the middle of the tract and was freeze-clamped and stored at –80°C. An additional group of midshipman (N=7) was placed on a feeding schedule for 7 days, where they were hand-fed thawed, cleaned shrimp until satiation at a synchronized time. On the last day, the fish were fed to satiation and killed 10 h following the consumption of the meal. The intestinal tracts were removed, cleaned of chyme and frozen as above. The tissues were then ground with a mortar and pestle under liquid nitrogen and stored at –80°C until the following enzymes (detailed below) were analyzed. Care was taken to ensure that the tissue did not thaw until placed in extraction buffer as described below.

All enzyme assays were carried out at 26°C. Na^+-K^+-ATPase (NKA) activity was measured according to previously published methods (e.g. McCormick, 1993; Craig et al., 2013). The homogenization buffers [SEI buffer (150 mmol l^{-1} sucrose, 10 mmol l^{-1} EDTA, 50 mmol l^{-1} imidazole, pH 7.5) and SEID buffer (0.5 g sodium deoxycholate in 100 ml SEI buffer)] and reaction buffers [salt solution (50 mmol l^{-1} NaCl, 10.5 mmol l^{-1} $MgCl_2$, 42 mmol l^{-1}, KCl, 50 mmol l^{-1} imidazol), Solution A (50 mmol l^{-1} imidazole, 2.8 mmol l^{-1} PEP, 0.22 mmol l^{-1}, NADH, 0.7 mmol l^{-1}, ATP, 4 units ml^{-1} LDH, 5 units ml^{-1} PK) and Solution B (0.5 mmol l^{-1} ouabain in 10 ml Solution A)] were made fresh daily and immediately before use.

An additional aliquot of the ground frozen tissue was added to four volumes of extract buffer (50 mmol l^{-1} Hepes, pH 7.5, 50 mmol l^{-1} KCl, 1 mmol l^{-1} dithiothreitol and 0.5 mmol l^{-1} EDTA) and homogenized with a Wheaton Overhead Stirrer (with a modified probe fitted for 1.5 ml bullet tubes; Rochdale, UK) for 30 s, and briefly sonicated (10 s). The homogenate was then centrifuged at 14,500 $\boldsymbol{g}$ for 5 min at 4°C. Briefly, GS was measured by the glutamyl transferase assay [reaction mixture contained glutamine (6 mmol l^{-1}), hydroxylamine (15 mmol l^{-1}), ADP (0.4 mmol l^{-1}), KH_2AsO4 (20 mmol l^{-1}), $MnCl_2$ (3 mmol l^{-1}) and Hepes (50 mmol l^{-1}); pH 6.7)]; glutamate dehydrogenase (GDH) was measured in the forward direction only [the reaction mixture contained NH_4Cl (0.2 mol l^{-1}), NADH (0.1 mmol l^{-1}), α-ketoglutarate (10 mmol l^{-1}), Hepes (35 mmol l^{-1}; pH 7.4), KCl (35 mmol l^{-1}), EDTA (0.35 mmol l^{-1}) and dithiothreitol (DTT) (0.75 mmol l^{-1})]; and OCT [the reaction mixture contained ornithine (10 mmol l^{-1}), carbamoyl phosphate (5 mmol l^{-1}), KCl (50 mmol l^{-1}), Hepes (50 mmol l^{-1}, pH 7.4), DTT (0.5 mmol l^{-1}), EDTA (0.25 mmol l^{-1})] activity was measured as previously described (Xiong and Anderson, 1989; Anderson, 1989; Shankar and Anderson, 1985; Casey and Anderson, 1982; Walsh, 1995; Walsh, 1996).

Carbamoyl phosphate synthetase activity was determined by measuring the [^{14}C]carbamoyl phosphate formed from [^{14}C]bicarbonate after reactions lasting for 60 min, as previously described (Anderson et al., 1970; Anderson, 1980; Korte et al., 1997; LeMoine and Walsh, 2013). The reaction mixture contained 20 mmol l^{-1} ATP, 25 mmol l^{-1} $MgCl_2$, 25 mmol l^{-1} phosphoenolpyruvate, 2 units of pyruvate kinase, 5 mmol l^{-1} [^{14}C]bicarbonate (3×10^6 c.p.m.), 0.04 mol l^{-1} Hepes, pH 7.6, 0.04 mol l^{-1} KCl, 0.5 mmol l^{-1} DTT and 0.5 mmol l^{-1} EDTA. Glutamine (20 mmol l^{-1}) was used to determine the activity level of CPS II and 1.7 mmol l^{-1} N-acetylglutamate was used to activate CPS III activity while 1.7 mmol l^{-1} UTP was used to inhibit CPS II activity.

Additionally, rainbow trout (N=3, 318–337 g) were fed to satiation with 5 pt floating pellet food and killed (1 g l^{-1} MS-222) 24 h following ingestion of the meal [based on Bucking and Wood (Bucking and Wood, 2012)]. The three intestinal sections (anterior, mid and posterior) were visually identified based on anatomical structures and individually removed, cleaned of chyme, freeze-clamped and stored at −80°C as with the midshipman tissues. The samples were then ground under liquid nitrogen and GS, OCT, CPS II and CPS III activities were measured as described above.

Series 4 – effects of feeding on gene expression

Midshipman fish were killed using MS-222 (1 g l^{-1}) after fasting for 4–10 days (N=7) or 16–21 days (N=7) in the laboratory, or 10 h following a feeding to satiation (N=7), and the intestinal tract was removed, sectioned, freeze-clamped and stored at −80°C as in Series 3. Total RNA was isolated from the intestinal tissue samples (~5–20 mg) using Trizol (Invitrogen, Carlsbad, CA, USA), following the manufacturer's instructions. RNA concentrations were determined spectrophotometrically (NanoDrop, Thermo Scientific, Ottawa, ON, Canada), and RNA integrity was verified by agarose gel electrophoresis [1.5% agarose-TAE (40 mmol l^{-1} Tris-acetate, 2 mmol l^{-1} EDTA)]. First-strand cDNA was synthesized by reverse transcribing 5 μg of total RNA using 10 pmol of random hexamers and 20 units of SuperScript II reverse transcriptase (Invitrogen), again following the manufacturer's instructions.

mRNA levels were assessed in all tissue collected from the feeding and fasting protocol using quantitative real-time PCR (qRT-PCR) on an Qiagen sequence analysis system (Qiagen Rotor-Gene Q, Toronto,

ON, Canada) with Rotor-Gene SYBR Green. Primers for GS, CPS III and pnUT (Rodela et al., 2011; Rodela et al., 2012) were previously designed for toadfish gene sequences, but due to the closely related nature of the midshipman and toadfish, we hypothesized that they would amplify midshipman sequences as well. To test this hypothesis, the primers were used to amplify midshipman cDNA in a standard PCR reaction and the product was sent for sequencing. Once the identity of the product was confirmed, the primers were then used in qRT-PCR. qRT-PCR reactions contained 1 μl of cDNA, 5 pmol of each primer and Rotor-Gene SYBR green master mix (Qiagen) in a total volume of 12.5 μl. All qRT-PCR reactions were performed as follows: 1 cycle of 95°C for 5 min, followed by 40 cycles of 95°C for 5 s and 60°C for 10 s (set annealing temperature of all primers). Curve analyses were performed for all reactions to confirm the presence of a single band, and representative samples (five for each product) were selected to confirm the identity of the product through sequencing, following standard sequencing protocols. The efficiency of the primer sets was confirmed using standard curves to determine the best linear amplification of the genes of interest. Control reactions were conducted with no cDNA template or with non-reverse-transcribed RNA to determine the level of background or genomic DNA contamination, respectively. Genes were quantified according to standard curve generation (Scott et al., 2004; Bucking and Schulte, 2012). A standard curve was run with each set of qRT-PCR reactions to control for run-to-run variation in reaction efficiency. Results were then normalized to the control gene 18S rRNA, the expression of which did not change with treatment.

Series 5 – immunohistochemistry

Midshipman fish were killed using MS-222 after fasting for 16–21 days, or 10 h following a feeding to satiation as in Series 3 and 4, and the intestines were removed and incubated for 24 h at 4°C in a solution of 4% paraformaldehyde [prepared in phosphate buffered saline (PBS), pH 7.4]. Overnight incubations in 15 and 30% sucrose solutions (in PBS) were then used to cryoprotect the tissue samples. The tissues were subsequently embedded in OCT cryo-sectioning medium (VWR, Mississauga, ON, Canada) and stored at −80°C until use. Each sample was then sectioned at 8 μm thickness using a cryostat (Leica CM 1850, Leica Biosystems, Nussloch, Germany) and sections were collected on SuperFrost Plus glass slides (Fisher Scientific, Ottawa, ON, Canada) before being stored at −20°C. The sections were then used for immunohistochemistry according to standard procedures. Briefly, the slides were rehydrated with PBS (3×5 min) and subsequently blocked with 5% goat serum albumin (GSA; Sigma-Aldrich, St Louis, MO, USA) in PBST (PBS with 0.5% Triton X-100) for 1 h. Following blocking, the slides were washed (3×5 min) with PBS before they were incubated overnight at 4°C with PBS-diluted primary antibodies. A previously described toadfish UT primary antibody (tUT) (Bucking et al., 2013) was used to detect the midshipman UT (pnUT) at a concentration of 1:500 and the α5, a mouse anti-chicken antibody for the NKA (University of Iowa, Hybridoma Bank), was used at a concentration of 1:50 (Braun et al., 2009; Bucking et al., 2013). As a negative control for background staining, slides were also incubated with PBS (with 5% GSA) lacking primary antibody. Sections were then washed (3×5 min) with PBS before incubation for 1 h with 1:500 Alexa-546 anti-rabbit (Invitrogen) and 1:500 Alexa-488 goat anti-mouse (Molecular Probes, Burlington, ON, Canada). The sections were then washed (3×5 min) in PBS and coverslipped with mounting medium (Prolong Gold antifade reagent, Molecular Probes). Sections were then examined with a confocal scanning system (Zeiss LSM 510, AxioImager.M1, Carl Zeiss Microscopy, Toronto, Canada) equipped with an argon laser. Images were collected using ZEN imaging software (Carl Zeiss Microscopy) at 40× magnification, under oil immersion unless otherwise stated.

Statistics

Whole-animal ammonia ($J_{NH3/NH4^+}$) and urea (J_{Urea}) fluxes to the water were examined using a repeated-measures one-way ANOVA followed by a Tukey's *post hoc* test. *In vitro* tissue urea transport/production was examined using a two-way ANOVA with condition (fasted, fed, fasted + antibiotics) and saline treatment (see Table 1) as factors, followed by appropriate *post hoc* testing (Tukey's test). Tissue enzyme activity levels and mRNA expression levels were examined using a one-way ANOVA followed by a Tukey's *post hoc* test.

RESULTS

Series 1 – whole-animal nitrogen fluxes

During the 6 h control period before the consumption of a meal, $J_{NH3/NH4^+}$ and J_{Urea} rates were not significantly different and were collapsed into a single control value of 83.6±19.9 and 4.1±1.7 μmol N kg^{-1} h^{-1}, respectively, resulting in a pre-prandial percent urea-N excretion of 4.8±2.0% of total N (Fig. 1). A spike in $J_{NH3/NH4^+}$ immediately after feeding was most likely due to the stress of the feeding protocol. Thereafter, $J_{NH3/NH4^+}$ increased from control values until 36 h following feeding, increasing threefold from 182.4±30.7 μmol N kg^{-1} h^{-1} at 12 h to 549.3±49.9 μmol N kg^{-1} h^{-1}, almost sixfold higher than control values, before falling back to control levels by 60 h (Fig. 1A). In contrast, post-prandial J_{Urea} remained stable until 30 h following feeding, when it rapidly increased over fivefold to 21.0±9.1 μmol N kg^{-1} h^{-1} (Fig. 1B). This spike in J_{Urea} coincided with the appearance of feces and carbonate pellets (Walsh et al., 1991) in the experimental chambers. J_{Urea} continued to increase until 60 h, when it peaked at 43.3±4.8 μmol N kg^{-1} h^{-1} before falling slightly at 96 h (Fig. 1B). As a consequence, percent urea-N initially fell 50% over the first 24 h following feeding, decreasing to ~2% of total N due to the increasing $J_{NH3/NH4^+}$ rate but stable J_{Urea}. Percent urea-N then proceeded to increase back to control levels at 30 h, due to the rising post-prandial J_{Urea} rate (Fig. 1C). At 60 h following feeding, percent urea-N excretion was fivefold higher than control levels due to a combination of rising J_{Urea} rates and falling $J_{NH3/NH4^+}$ rates (Fig. 1C). Notably, percent urea-N did not exceed 25% over the course of the experiment.

Table 1. Effects of fasting and feeding on intestinal enzyme activities and relative mRNA expression levels in the plainfin midshipman (*Porichthys notatus*)

	Fasted		
	16–21 days	4–10 days	Fed
Enzyme activity			
NKA	4.55±0.61^a	3.48±0.69^a	6.10±0.77^b
OTC	3.91±0.27^a	4.79±0.43^a	3.48±0.74^a
GDH	1.88±0.08^a	1.77±0.26^a	2.13±0.23^a
CPS II	0.17±0.08^a	0.40±0.21^a	0.19±0.05^a
mRNA expression			
GS	0.37±0.09^a	1.00±0.10^b	0.67±0.07^b
CPS III	0.55±0.07^a	1.00±0.08^b	0.83±0.10^{a,b}
pnUT	0.73±0.10^b	1.00±0.11^{a,b}	1.21±0.09^a

Enzyme activities are in μmol min^{-1} g^{-1} except CPS II, which was in nmol min^{-1} g^{-1}. mRNA expression is relative to 18S mRNA expression and values at 4–10 days fasting have been set to 1. Values sharing letters are not significantly (P>0.05) different. N=7 for each condition.

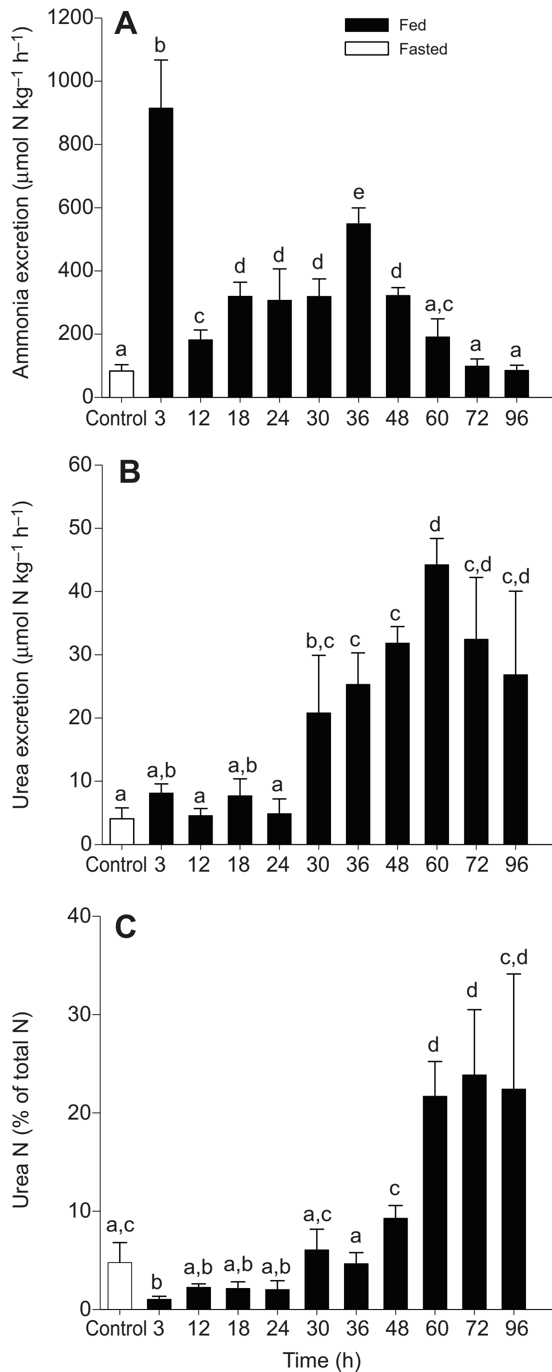

Fig. 1. Whole-animal nitrogen excretion rates during digestion in the plainfin midshipman (*Porichthys notatus*). (A) Ammonia excretion rates (J_{NH_3/NH_4^+}; µmol N kg^{-1} h^{-1}). (B) Urea excretion rates (J_{Urea}; µmol N kg^{-1} h^{-1}). (C) Percent of nitrogen excreted as urea. Bars sharing letters are not significantly different (P>0.05). Animals were fed at 0 h. Control represents fasted animals before feeding. N=5.

Series 2 – *in vitro* ammonia and urea production

Chyme ammonia (1.8±0.4 µmol N g^{-1}) and urea (0.11±0.05 µmol N g^{-1}) content 10 h following the ingestion of a meal was measured to determine realistic ammonia concentrations for use in the mucosal salines.

The appearance rate of ammonia in the mucosal solution of fasted fish exposed to the control saline was twofold lower than the appearance of urea under the same conditions (16.8±1.0 nmol N h^{-1} cm^{-2}; Fig. 2A,C). Exposure to high ammonia, high glutamine, and high ammonia and high glutamine mucosal salines increased the appearance rate of mucosal ammonia by approximately threefold (43.3±8.6 nmol N h^{-1} cm^{-2}; averaged across treatments) regardless of saline composition (Fig. 2A). The appearance rate of ammonia in the mucosal solution of fed fish was higher than that of fasted fish, and the control saline produced rates similar to values seen with urea (Fig. 2A,C). Similar to fasted fish, exposure to each of the three other salines increased ammonia appearance rates in the lumen by approximately twofold (141.2±18.6 nmol N h^{-1} cm^{-2}; averaged across treatments), threefold higher than the respective values in fasted tissues and 1.5-fold higher than urea values (Fig. 2A,C). Exposure of fasted fish to antibiotics reduced the appearance rate of ammonia in the mucosa compared with both fed and fasted fish (3.1±0.7 nmol N h^{-1} cm^{-2}), while as before, exposure to each of the three salines increased ammonia in the mucosa fivefold regardless of saline composition (16.2±4.3 nmol N h^{-1} cm^{-2}; averaged across treatments; Fig. 2A).

The appearance rate of ammonia in the serosal solution of fasted fish exposed to the control saline was similar to the appearance rate of both serosal urea and mucosal ammonia (Fig. 2B). Exposure to high ammonia, high glutamine, and high ammonia and high glutamine mucosal salines increased the appearance rate of serosal ammonia by approximately threefold (27.4±5.7 nmol N h^{-1} cm^{-2}; averaged across treatments) regardless of saline composition (Fig. 2B). The appearance rate of ammonia in the serosal solution in fed tissues was variable, lower with control (12.5±1.8 nmol N h^{-1} cm^{-2}) and high glutamine (16.1±1.4 nmol N h^{-1} cm^{-2}) salines, and was higher in high ammonia (20.3±3.2 nmol N h^{-1} cm^{-2}) and high ammonia and high glutamine salines (19.7±2.4 nmol N h^{-1} cm^{-2}). Overall, fed ammonia sersosal appearance rates were very similar to values seen with fasted tissues, and greatly lower than mucosal values (Fig. 2B). The appearance rate of ammonia in the serosal solution in fasted tissues exposed to antibiotics was also variable, lower with control (3.1±0.6 nmol N h^{-1} cm^{-2}) and high glutamine (6.6±1.7 nmol N h^{-1} cm^{-2}) salines and higher in high ammonia (11.5±2.7 nmol N h^{-1} cm^{-2}) and high ammonia and high glutamine salines (11.7±1.7 nmol N h^{-1} cm^{-2}), as seen with fed tissues. Generally, fasted fish that were exposed to antibiotics had lower serosal ammonia appearance rates compared with fasted and fed tissues, but similar to mucosal appearance rates (Fig. 2B).

The mucosal appearance rate of urea in control fasted tissues was 43.8±8.1 nmol N h^{-1} cm^{-2} (Fig. 2C). Treatment with the high ammonia mucosal saline did not affect the appearance rate of urea; however, treatment with high glutamine mucosal saline significantly reduced the rate of urea appearance twofold to 21.3±5.2 nmol N h^{-1} cm^{-2} compared with the rate seen with control saline (Fig. 2C). Treatment with a high ammonia and high glutamine mucosal saline recovered urea appearance rates to high ammonia saline levels and to control saline values (Fig. 2C). Generally speaking, urea appearance rates in the mucosal saline were higher in fed tissues compared with the respective fasted tissues. Specifically, mucosal urea appearance rates were 1.5-fold higher in fed tissues exposed to control mucosal saline (70.8±9.9 nmol N h^{-1} cm^{-2}), rates similar to fed tissues exposed to high ammonia saline (58.5±7.2 nmol N h^{-1} cm^{-2}; Fig. 2C). As seen with fasted tissue, exposure to a high glutamine mucosal saline reduced urea mucosal appearance rates threefold to 23.5±7.0 nmol N h^{-1} cm^{-2}, and exposure to a high ammonia and high glutamine mucosal saline recovered urea appearance rates to control saline values (Fig. 2C). The appearance rate of urea in the mucosal saline was higher in fasted

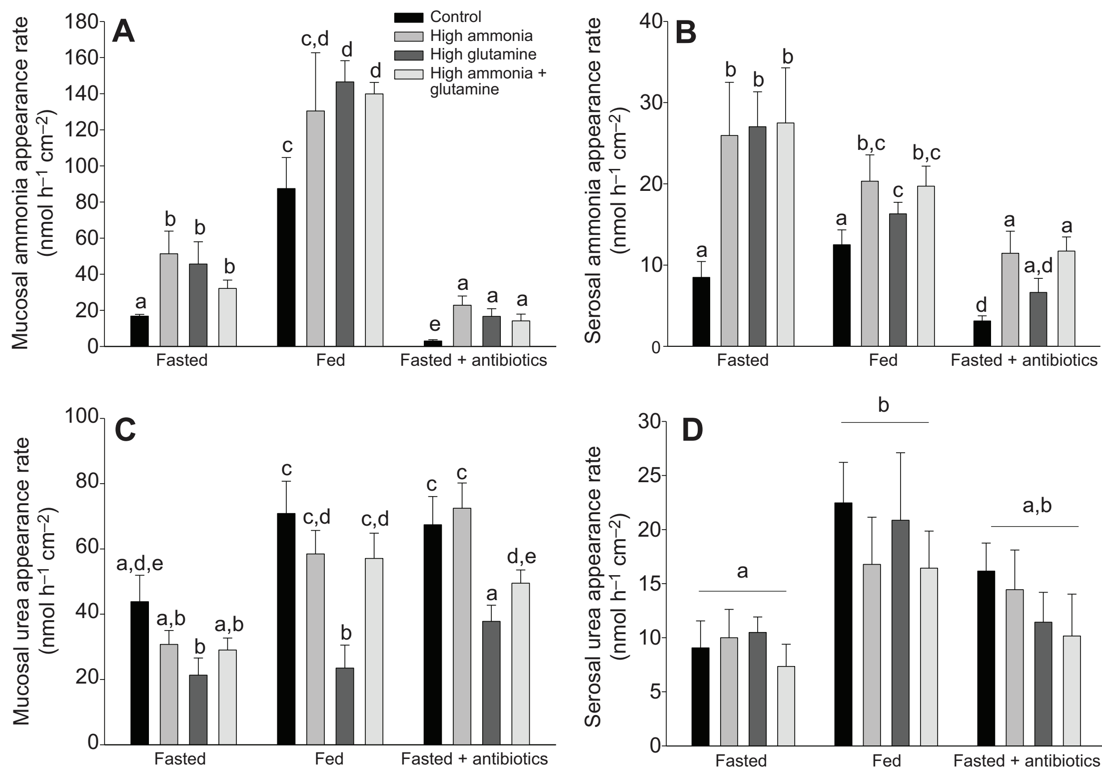

Fig. 2. Intestinal tissue urea and ammonia production in the plainfin midshipman (*Porichthys notatus*) by tissues obtained from fasted (7–12 days; N=6) or fed (N=6) animals and fasted (7–12 days; N=5) animals incubated with antibiotics that have been exposed to four different mucosal salines (control, high ammonia, high glutamine, and high ammonia + high glutamine). (A) Urea appearance rate in the mucosal saline (nmol N h^{-1} cm^{-2}). (B) Urea appearance rate in the serosal saline (nmol N h^{-1} cm^{-2}). (C) Ammonia appearance rate in the mucosal saline (nmol N h^{-1} cm^{-2}). (D) Ammonia appearance rate in the serosal saline (nmol N h^{-1} cm^{-2}). Bars that share letters are not significantly different (P>0.05).

fish exposed to antibiotics compared with fasted fish and similar to concentrations found in fed fish with each respective mucosal treatment. Once again, exposure to high glutamine significantly reduced the appearance rate of urea in the mucosal solution; however, values remained above those seen in both fasted and fed fish (38.9±4.9 nmol N h^{-1} cm^{-2}; Fig. 2C). Treatment with high ammonia and high glutamine partially recovered the appearance rate of urea in the lumen (49.4±4.1 nmol N h^{-1} cm^{-2}), but not back to control saline values as seen without antibiotic treatment (Fig. 2C). The serosal appearance rate of urea was influenced only by condition, and not mucosal saline treatment. The serosal appearance rate of urea in fasted fish was significantly lower than the serosal appearance rate in fed fish, while treatment of fasted fish with antibiotics produced an intermediate appearance rate that was similar to both fasted and fed tissue values (Fig. 2D).

Finally, the sum of mucosal and serosal ammonia production rates by tissue exposed to control salines were ~26 and 98 nmol N h^{-1} cm^{-2} for tissues obtained from fasted and fed animals, respectively. In comparison, the sum of mucosal and serosal urea production rates were ~53 and 94 nmol N h^{-1} cm^{-2} for tissues obtained from fasted and fed animals, respectively.

Series 3 – effects of feeding on enzyme activity

Intestinal NKA activity was not affected by fasting for 16–21 days compared with 4–10 days; however, NKA activity did increase during digestion (Table 1). In contrast, GS activity was lowest following 16–21 days fasting (0.14±0.05 μmol min^{-1} g^{-1}), doubling to 0.28±0.02 μmol min^{-1} g^{-1} with 4–10 days fasting, and increasing a further 1.5-fold to 0.41±0.03 μmol min^{-1} g^{-1} following the consumption of a meal (Fig. 3A). Intestinal GDH, OCT and CPS II activity was not affected by fasting or digestion (Table 1). CPS III activity was maintained similar to NKA during fasting (0.30±0.12 nmol min^{-1} g^{-1}; averaged over fasting) but increased by an order of magnitude to 2.62±0.35 nmol min^{-1} g^{-1} during digestion (Fig. 3B).

Series 4 – effects of feeding on gene expression

The relative expression levels of GS and CPS III mRNA were unchanged 10 h following feeding compared with 4–10 day fasted values, unlike the enzyme activity levels of GS and CPS III (Table 1). There was an increase in relative GS transcript levels when examining 16–21 days fasting and 4–10 days fasting, which may account for the coinciding increase in enzyme activity; however, the increase in relative levels of CPS III mRNA with a shorter, fast duration was not associated with increased CPS III activity levels (Table 1). There was a trend (P=0.057) towards a significant increase in post-prandial pnUT mRNA expression levels when compared with 4–10 days fasting, and a significant increase in pnUT expression levels when compared with 16–21 days fasting (Table 1).

Series 5 – immunohistochemistry

NKA reactivity occurred along the basolateral membranes of the enterocyte located along the villi of the mucosa of the midshipman intestine (Fig. 4A). In contrast, the UT antibody reacted along the apical membrane of the enterocytes and was associated with vesicles located along the villi (Fig. 4A). In comparison, the villi of the

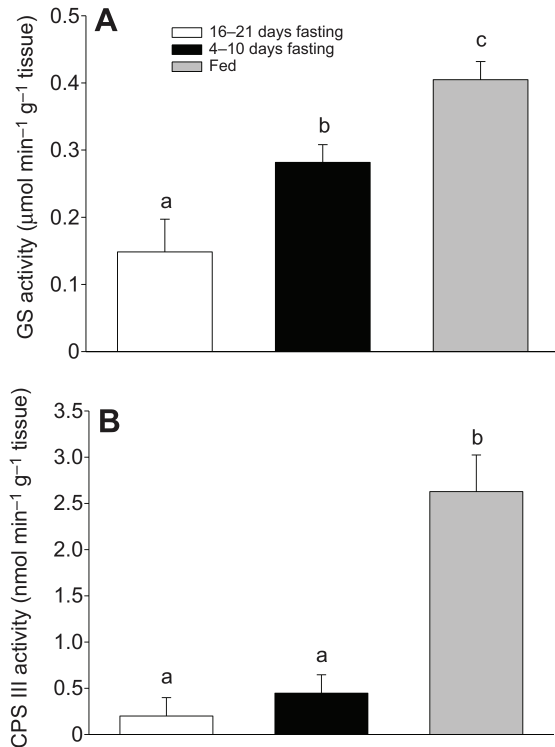

Fig. 3. Intestinal enzyme activities in the plainfin midshipman (*Porichthys notatus*) in tissues obtained from animals that were fasted 16–21 days, fasted 4–10 days or 10 h following the ingestion of a meal. (A) Glutamine synthetase activity (μmol min^{-1} g^{-1}). (B) Carbamoyl phosphate synthetase III activity (nmol min^{-1} g^{-1}). Bars that share letters are not significantly different (P>0.05). N=7 for each condition.

mucosa of fed tissue likewise reacted with NKA along the basolateral membranes of the enterocytes, while the UT antibody again reacted along the apical membrane and amongst numerous vesicles (Fig. 4B). Magnification of the tissue and vesicles (arrows) revealed granular staining of the vesicles with evidence of the vesicles tracking out to the apical membrane (trails following the vesicles; Fig. 4C,D). Overall, positive pnUT staining was similar between unfed and fed tissues; however, the number of vesicles was greater in fed tissue sections, as was evidence of vesicle tracking to the apical membrane.

DISCUSSION

In this study, we sought to address the hypothesis that midshipmen could use enterocytes to locally detoxify gut lumen ammonia to urea and then transport all, or a portion, of this urea back to the lumen for excretion. Several predictions of this hypothesis have been confirmed in this study. First, we have verified that digestion has a large impact on gut luminal ammonia concentrations and whole-animal nitrogen excretion – maximally increasing ammonia and urea excretion in the midshipman by sixfold and fourfold, respectively, in accord with previous observations (see Introduction). Secondly, we have shown that the intestinal tissue is capable of producing urea in an *in vitro* environment. Furthermore, we have shown that the activity of key enzymes responsible for urea production transiently increases in enterocytes during digestion. And finally, we have visualized a potential transport pathway to export urea across the apical membrane of enterocytes into the lumen.

This study has confirmed a temporal disconnect between the peak ammonia and urea excretion rates previously observed in the marble goby (Tng et al., 2008; Lam et al., 2008), which suggests that the routes of excretion may differ, with the bulk of ammonia excretion occurring across the gills while urea excretion may occur rectally. The delay in urea excretion compared with ammonia excretion appears variable – 3–6 h in the marble goby (Tng et al., 2008; Lam et al., 2008) *versus* 24 h in the midshipman (Fig. 1B). This difference may relate to differences in metabolic rates and rearing conditions (25°C water *versus* 12°C water), and their subsequent effects on meal processing rates, digestion and fecal excretion (Jobling, 1981; Johnston and Battram, 1993). Interestingly, previous studies with rainbow trout and tilapia (Brett and Zala, 1975; Beamish and Thomas, 1984; Wright, 1993; Alsop and Wood, 1997; Kajimura et al., 2004) failed to detect a temporal delay in increased urea excretion compared with ammonia excretion during digestion. Methodological differences (i.e. a fine time scale *versus* a course time scale) could explain this incongruity. However, as we were not able to stimulate detectable levels of intestinal CPS III activity in the rainbow trout, these differences may highlight species-specific responses to digestion where alternative strategies are used to generate urea and prevent ammonia toxicity. Indeed, previous studies examining the activities of O-UC enzymes in the GI tract of ammoniotelic fish have been somewhat variable. Intestinal CPS III activity was minimal in the common carp, the channel catfish, the goldfish and the trout, in contrast to detectable activity in the walking catfish, the largemouth bass, the toadfish and arguably the bowfin (Table 2). It is important to note that the majority of the studies reported in Table 2 were conducted on fasted fish and the low level of CPS III activity in fasted midshipman (Fig. 3B) is in line with these previous studies. While we were not able to induce CPS III activity in the intestine of the rainbow trout (although GS activity did increase; Table 2), it remains to be seen how other fish species will respond to feeding and further investigation is required. Certainly the high level of CPS III activity in the intestine of the largemouth bass and bowfin suggests that while an active intestinal O-UC during digestion is not universal in fish species, it may be present in multiple distantly related species. Furthermore, perhaps the conclusion that adult ammoniotelic teleost fish lack an active O-UC and that preservation of the O-UC genes is a result of embryonic urea production (Griffith, 1991; Wright et al., 1995a) has been based on studies focusing only on the liver, although O-UC enzymes in the skeletal muscle of rainbow trout were detectable but not inducible under several conditions (Todgham et al., 2001). Studies examining the intestine during digestion may reveal a conditionally active O-UC and disclose an additional evolutionary rationale for the preservation of O-UC enzyme expression in adult ammoniotelic fish.

Although the overall contribution of urea excretion to the total of ammonia plus urea remains relatively low even in feeding [consistent with fasted observations in the present study and a previous study (Walsh et al., 2001)], the delayed and discontinuous appearance of urea and its apparent correspondence with the first appearance of fecal matter and carbonate pellets (30 h following the consumption of a meal) suggests an important role for the intestine in this process. Our measurements of urea production rates in isolated intestinal tissues confirmed that this tissue has the ability to produce urea from ammonia and the rates of production of urea *in vitro* appear to match reasonably well with excretion rates observed *in vivo*. [The increase in mucosal urea production comparing fasted and fed tissue was ~30 nmol N h^{-1} cm^{-2} along with an average increase in serosal urea appearance of ~10 nmol N h^{-1} cm^{-2} (Fig. 2C,D).] With an average total intestinal area of 150 cm^{2} in a 100 g fish, the total urea production capabilities of the fed intestinal

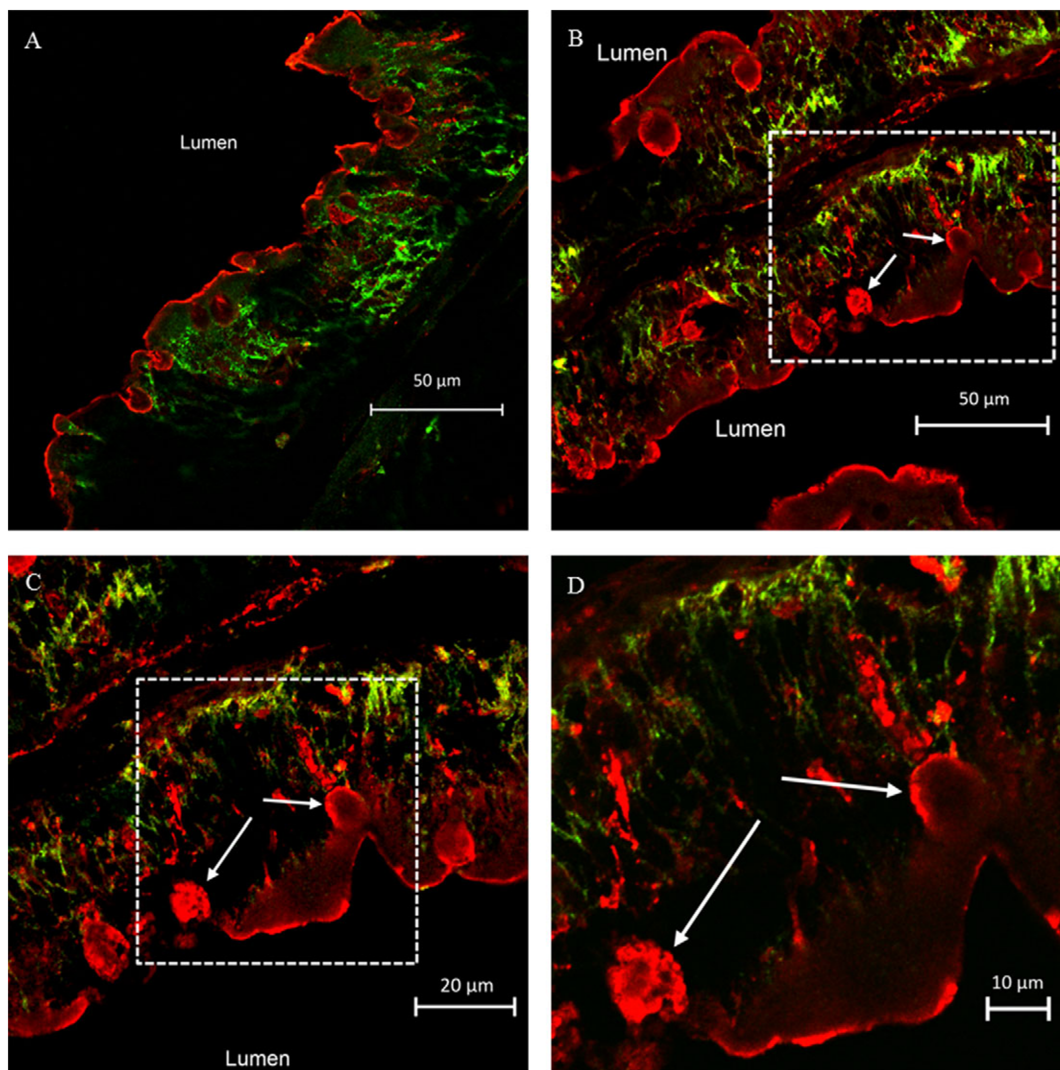

Fig. 4. Immunohistochemistry of intestinal villi in the plainfin midshipman (*Porichthys notatus*) from fasted and fed animals showing reactivity of enterocytes with pnUT (red) and NKA (green). (A) Fasted animal. (B) Fed animal at 40× magnification. (C) Fed animal at 60× magnification. (D) Fed animal at 120× magnification. NKA positively reacted with the basolateral membrane of enterocytes while pnUT was located along the apical membrane of the enterocytes in contact with the lumen of the gastrointestinal tract. pnUT was also located within vesicles (arrows, verified using a vesicle-specific antibody; data not shown). White box highlights region of interest that is magnified in subsequent image.

tissue over fasted controls was ~60 μmol N kg^{-1} h^{-1}, in accord with the overall increase in urea excretion by the animal *in vivo* (Fig. 1B). Data from prior studies in this regard are limited. Kajimura et al. (Kajimura et al., 2004) showed that by suturing the anus of trout, urea-N excretion to the water was decreased by 30% during digestion, accounting for roughly 34 μmol N kg^{-1} h^{-1}, which is similar to the increase in urea excretion seen in the present study (39 μmol N kg^{-1} h^{-1}; Fig. 1B), and the marble goby [~50 μmol N kg^{-1} h^{-1} (Lam et al., 2008)]. One future direction for studies with midshipmen could be to conduct similar suturing experiments and/or divided chamber and urinary/rectal catheter experiments that separate the various components of excretion, as has been done for the related toadfish (Wood et al., 1995b).

Urea production in the intestine could potentially occur by one of three pathways (the O-UC, uricolysis, or direct hydrolysis of arginine). We were able to confirm that the intestinal tissue of the midshipman has key enzymes necessary to synthesize urea *via* the O-UC. Furthermore, the enzyme activities of both CPS III and GS increase with feeding, suggesting that urea is being generated by the intestinal tissue (Fig. 3A,B). Interestingly, the activation of these two enzymes does not appear to involve an overwhelming response at the transcriptional level as there were no increases upon feeding compared with animals not fed for less than 2 weeks (Table 1). Possibly, increases in the activities of these enzymes may involve mechanisms at the translational or post-translational levels. Additionally, the urea production of the midshipman intestinal tissue was stimulated by treatment (i.e. feeding *versus* fasting) and not by exposure to high mucosal ammonia concentrations (Fig. 2C). Feasibly, the regulation of the enzyme activity level of the enterocytes proceeds *via* a signal (hormonal, neuronal, metabolic, etc.) *in vivo* that triggers initiation. For example, the alkaline tide is suspected of acting as a trigger for the post-prandial activation of several enzymes in the dogfish (Wood et al., 2008).

If we assume that the maximal rate of post-prandial intestinal CPS III activity (~3 nmol min^{-1} g^{-1} tissue) translates into maximal urea production, the midshipman intestine would be capable of producing approximately 18 μmol N h^{-1} kg^{-1} (assuming an approximate intestinal mass of 10 g in a 100 g fish), which corresponds to ~46% of the urea production recorded *in vivo*. However, this calculation suggests that in addition to a functional O-UC during digestion, urea is also being produced *via* other enzymatic pathways such as the degradation of dietary arginine by arginase (Mommsen and Walsh, 1992; Korsgaard et al., 1995). While data are scarce on the contribution of this pathway to overall urea synthesis in teleost fish, intestinal arginase activity in feeding lamprey was suspected of providing a small but significant fraction of total post-prandial urea production (Wilkie et al.,

Table 2. Activities of intestinal ornithine-urea cycle enzymes in fed and fasted ammoniotelic and ureotelic fish

	Intestinal enzyme activities			
	GS	OTC	CPS II	CPS III
Fed ammoniotelic fish				
Rainbow trout				
Anterior intestine	0.16±0.03[a]; 0.51±0.02[b]	0.07±0.02[a]	BDL[a]	BDL[a]
Mid intestine	0.75±0.13[a]; 0.98±0.08[b]	0.11±0.05[a]	0.85± 0.08[a]	BDL[a]
Posterior intestine	2.03±0.31[a]; 4.20±1.20[b]	0.10±0.04[a]	1.45±0.15[a]	0.08±0.04[a]
Fasted ammoniotelic fish				
Rainbow trout	0.06±0.02[c]	0.03±0.03[c]	BDL[c]	BDL[c]
Common carp	0.99±0.37[d]	0.002±0.001[d]	0.42±0.20[d]	0.05±0.04[d]
Channel catfish	0.33±0.18[d]	0.15±0.02[d]	0.84±0.11[d]	0.11±0.01[d]
Goldfish	1.02±0.51[d]	0.10±0.05[d]	0.33±0.12[d]	0.10±0.05[d]
Bowfin	0.50±0.11[d]	0.17±0.02[d]	0.41±0.18[d]	0.26±0.03[d]
Largemouth bass	0.36±0.20[e]	0.66±0.18[e]	3.40±1.74[e]	5.80±3.21[e]
Fasted ureogenic/ureotelic fish				
Walking catfish	0.45±0.04[f]	0.20±0.01[f]	10.83±1.33[f]	16.33±1.67[f]
Toadfish	7.36±1.37[h]	2.41±0.55[g]; 3.41±1.39[h]		3.6±1.2[g]
Fed elasmobranch				
Dogfish	19.57±0.57[i]	2.23±0.72[i]		1.21±0.37[i]
Fasted elasmobranch				
Dogfish	16.61±1.73[i]	1.27±0.60[i]		1.50±0.3[i]

All values were from unknown portions of the intestine unless otherwise noted. Activity of GS and OTC is in μmol min^{-1} g^{-1} and activity of CPS II and CPS III is in nmol min^{-1} g^{-1}.

BDL, below detection limit.

[a]Present study (*N*=3).

[b]Bucking and Wood, 2012 (*N*=7).

[c]Korte et al., 1997 (*N*=4).

[d]Felskie et al., 1998 (*N*=4–5).

[e]Kong et al., 1998 (values are an average of 3 presented in the paper).

[f]Saha et al., 1999 (*N*=3).

[g]Julsrud et al., 1998 (*N*=4).

[h]Wood et al., 1995b (*N*=3).

[i]Kajimura et al., 2006 (fed values are peak post-prandial values; *N*=4–8).

2004). Finally, uricolysis, in conjunction with purine nucleotide metabolism, most likely provides a portion of the urea synthesis (reviewed by Anderson, 2001). However, while urea excretion rates increased in fed tilapia, uricolytic enzymes activities in the liver were unchanged or even decreased with feeding (Wright, 1993). Unfortunately, enzyme activities in the intestine were not measured, highlighting the need to investigate the intestine directly in addition to the liver.

The observation of the ability of ammonia and not glutamine to serve as a substrate for urea production in the intestine, despite the fact that piscine CPS III preferentially utilizes glutamine as a substrate over ammonia (Anderson and Casey, 1984), is intriguing. Essentially, during urea production in fish, ammonia is first converted to glutamine by a cytosolic GS and then transported to the mitochondria, or passed to the mitochondrial matrix to a mitochondrial GS (Casey and Anderson, 1982; Anderson, 2001). The former is consistent with enzyme compartmentalization seen in elasmobranchs, while the latter is consistent with other teleost fish (Casey and Anderson, 1982; Anderson, 2001). With excess glutamine available as a substrate, one could predict that it would be transported to the mitochondrial matrix where it could directly feed into CPS III and urea production. However, urea synthetic rates with high glutamine salines were lower than with control salines in tissues from both fed and fasted animals (Fig. 2C). In order to explain the lower urea production rate with glutamine, one could postulate that the excess glutamine interfered with the trace ammonia transport and reduced the substrate available for the O-UC pathway. The recovery of urea production rates with combined excess glutamine and ammonia supports this hypothesis, although the full recovery to control urea production values was not seen with tissue from antibiotic-treated animals.

Regardless of the exact enzymatic pathway utilized, the intestinal tissue appears to be generating urea, a phenomenon that increased dramatically in response to feeding. A final prediction of the hypothesis is that urea generated in this fashion would need to be transported to the lumen for excretion. We were able to detect mRNA for a urea transporter for this species, and this mRNA was upregulated by feeding and downregulated by long-term fasting. Furthermore, the fact that pnUT was immunolocalized to the apical surface of the enterocytes is in accord with a higher mucosal appearance rate of urea compared with the serosal appearance rates (Fig. 2C,D). The additional localization of pnUTs within vesicles was surprising. There is a possibility that the antibody is cross reacting with mucus within the vesicles; however, western blots indicated a high fidelity of the antibody to a protein with the expected size of the UT [along with a dimer (Bucking et al., 2013)]. Additionally, our model requires ammonia entry into the enterocyte to serve as a substrate for urea production. In the first study to immunologically visualize the proteins thought to be responsible for a majority of ammonia transport (the Rh glycoproteins; see the Introduction) in the intestine of a teleost fish, it was previously shown that midshipman express a basolateral Rh transporter (Rhbg) and two apical Rh transporters (Rhcg1, Rhcg2) in the enterocytes (Bucking et al., 2013). This would allow both exogenous ammonia from the lumen and endogenous ammonia from the plasma to enter the enterocyte.

The removal of bacteria from the intestine (*via* incubation with water dissolved antibiotics) greatly increased the appearance rate

of urea in the intestinal lumen (Fig. 2C) as well as decreased the coinciding appearance rate of ammonia (Fig. 2A), suggesting that ureolytic bacteria play a role in the nitrogen metabolism of the teleost intestine. These bacteria can use urea for protein generation – breaking urea down into ammonia *via* urease [for reviews on microbial ureases, see Mobley and Hausinger (Mobley and Hausinger, 1989), Stewart and Smith (Stewart and Smith, 2005) and Solomon et al. (Solomon et al., 2010)] and using the liberated nitrogen groups to build proteins. The bacteria most likely use the waste nitrogen product for growth and survival while providing the host with salvaged energy from non-digestible compounds (reviewed by Hooper et al., 2002). Several attempts were made to feed animals incubated with antibiotics; however, only two fish were able to digest their meal. While no firm conclusions can be drawn, mucosal urea appearance rates from intestinal tissue obtained from fed fish incubated with antibiotics ($102.8\pm8.1\,\mathrm{nmol\,N\,h^{-1}\,cm^{-2}}$) were $\sim35\,\mathrm{nmol\,N\,h^{-1}\,cm^{-2}}$ higher than unfed fish incubated with antibiotics. This is similar to the difference in urea appearance rates between fed and unfed tissue from animals not exposed to antibiotics, suggesting that the urease activity of the bacteria was constant in fed and unfed fish and consumed a similar proportion of urea produced.

As a final note, when the responses of midshipmen to the nitrogen load during digestion are compared with the responses of ureotelic and elasmobranch fish, which have a hepatic O-UC that may be able to compensate for the high ammonia exposure, interesting differences appear. For example, the dogfish shark (*Squalus acanthias*) responds to feeding with a dramatic activation of the O-UC in both the liver and muscle but not the intestine (Kajimura et al., 2006), while the toadfish exhibited no effect of feeding on hepatic O-UC enzyme activities (Walsh and Milligan, 1995). Despite this, urea excretion increased following feeding. Unfortunately the toadfish intestine was not examined in this case.

Conclusions

Overall, we suggest a model where excess ammonia generated in the intestine of the midshipman during digestion is detoxified to urea by enterocytes, at least partially through the induction of the O-UC. The urea is then excreted back into the intestinal lumen *via* apical or vesicular UTs and in turn is partially used by ureolytic bacteria in the gut and the rest excreted with the feces. Quantitatively, the urea production observed was not enough to transition the midshipman from ammoniotelism to ureotelism. However, we hypothesize that qualitatively the urea production is instrumental in maintaining enterocyte viability and intestinal epithelium integrity, along with a mutually beneficial relationship with GI tract bacteria. This phenomenon is not universal amongst teleost fish, as rainbow trout lacked a fed-inducible O-UC in the intestine. However, enzyme activities suggest that an active O-UC is present in at least three other fish species and further research is clearly required to determine the exact function of this post-prandial upregulation of O-UC enzymes in the intestine.

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AUTHOR CONTRIBUTIONS

All authors contributed substantially to the study and manuscript.

COMPETING INTERESTS

No competing interests declared.

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