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RESEARCH ARTICLE

SPECIAL ISSUE
THE INTEGRATIVE BIOLOGY OF THE GUT

Accounting for the role of the gastro-intestinal tract in the ammonia and urea nitrogen dynamics of freshwater rainbow trout on long-term satiation feeding

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ABSTRACT

The contribution of the gut to the ingestion, production, absorption and excretion of the extra ammonia and urea nitrogen (urea-N) associated with feeding ('exogenous' fraction) has received limited attention. Analysis of commercial pellet food revealed appreciable concentrations of ammonia and urea-N. Long-term satiation feeding increased whole-trout ammonia and urea-N excretion rates by 2.5-fold above fasting levels. Blood was sampled from the dorsal aorta, posterior, mid- and anterior sub-intestinal veins, as well as the hepatic portal vein *in situ*. Ammonia, urea-N and fluid flux rates were measured *in vitro* using novel gut sac preparations filled with native chyme. The sacs maintained the extreme physico-chemical conditions of the lumen seen *in vivo*. Overall, these results confirmed our hypothesis that the stomach, and anterior intestine and pyloric caecae regions play important roles in ammonia and urea-N production and/or absorption. There was a very high rate of urea-N production in the anterior intestine and pyloric caecae, whereas the posterior intestine dominated for ammonia synthesis. The stomach was the major site of ammonia absorption, and the anterior intestine and pyloric caecae region dominated for urea-N absorption. Model calculations indicated that over 50% of the exogenous ammonia and urea-N excretion associated with satiation feeding was produced in the anaerobic gut. This challenges standard metabolic theory used in fuel-use calculations. The novel gut sac preparations gained fluid during incubation, especially in the anterior intestine and pyloric caecae, owing to marked hyperosmolality in the chyme. Thus, satiation feeding with commercial pellets is beneficial to the water balance of freshwater trout.

KEY WORDS: Voluntary feeding, Sub-intestinal vein, Hepatic portal vein, Pyloric caeca, Chyme-filled gut sacs, Whole-body ammonia and urea-N excretion, Gut fluid fluxes

INTRODUCTION

It has been known for a century that bony fish produce ammonia and urea nitrogen (urea-N) as, respectively, major and minor products of nitrogen metabolism, and that these are largely excreted by the gills (Smith, 1929). Essentially, all of this nitrogen enters in the form of food via the gastro-intestinal tract, but only in the past 15 years has the role of the gut in producing and transporting ammonia and urea-N been studied. Key studies include that of Karlsson et al. (2006), who monitored elevated levels of ammonia and urea-N, as well as amino acids, in the blood of the hepatic portal vein (HPV) and dorsal aorta of rainbow trout after force-feeding of an involuntary meal. The HPV transports venous blood draining from the entire gastro-intestinal tract into the liver (Thorarensen et al., 1991). Several subsequent studies have demonstrated that the level of total ammonia in the chyme of salmonids can be very high (several mmol l^{-1}) after feeding (Bucking and Wood, 2012; Rubino et al., 2014; Jung et al., 2022; 2023a). Rubino et al. (2014, 2019) used *in vitro* gut sac preparations of voluntarily fed rainbow trout to demonstrate that both the absorptive transport capacity of the intestine for ammonia and the rate of endogenous ammonia production by the gut tissue itself increase after feeding. On an area-specific basis, both transport rates and endogenous production rates were highest in the anterior intestine. Indeed, Rubino et al. (2014) suggested that the combination of ammonia production in the chyme plus endogenous ammonia production by the intestinal tissue could account for almost half of the post-prandial ammonia excretion by the whole animal. However, none of the above-cited studies considered the possible role of the stomach or the pyloric caecae in ammonia production and transport. The recent report of Jung et al. (2023b), that stomach gut sacs have much higher ammonia removal rates from mucosal saline than any of the intestinal segments on an area-specific basis, pointed to an important role for the stomach in the absorptive transport of ammonia, but was not confirmed by appearance rates in the serosal saline. Overall, these previous investigations raise questions as to where most of the ammonia is actually absorbed.

Much less is known about urea-N dynamics in the gastro-intestinal tract of teleost fish. Urea-N levels in chyme have not been reported in salmonids, but in plainfin midshipman (*Porichthys notatus*) (Bucking et al., 2013b) and other marine teleosts (Wood et al., 2019), they are very low relative to chyme ammonia concentrations. However, Bucking et al. (2013b) and Jung et al. (2021), using *in vitro* gut sac preparations of the midshipman and rainbow trout, respectively, demonstrated that both urea-N transport rates and endogenous urea-N production rates increased after feeding. In the trout, the anterior intestine again dominated on an area-specific basis.

Neither the pyloric caecae nor stomach (with the exception of Jung et al., 2023b) were examined in any of the above-cited studies, despite the long-known role of the caecae as a key digestive and absorptive region (Buddington and Diamond, 1986, 1987), and accelerating evidence of the importance of the stomach as an absorptive organ in fish (reviewed by Wood and Bucking, 2011; Bucking et al., 2024). Where the effects of feeding were examined, the protocols were single meals after fasting, so that different parts of the tract would have been in different stages of meal processing.

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In addition to these limitations, none of these investigations considered the possibility that, in the intact animal, significant amounts of ammonia and urea-N could have been ingested in the food rather than produced in the tract. Comparing reviews by Bucking (2017) and Wood (2001), it is obvious that interest in the gut as a site of ammonia and urea metabolism is increasing, but our knowledge remains fragmentary relative to that on gills or liver.

Against this background, the primary objective of the present study was to quantitatively assess the sites and rates of ammonia and urea-N production and absorption in all parts of the tract, including the stomach and pyloric caecae, using rainbow trout that had been on a daily satiation-feeding regime for a month. This was accomplished by measuring ammonia and urea-N concentrations in the food (commercial trout pellets), in blood sampled *in situ* from sites along the sub-intestinal vein (draining various parts of the intestine), as well as from the HPV (draining the entire gut, including the stomach) and dorsal aorta in anaesthetized ventilating trout. We also quantified ammonia and urea-N concentrations in the chyme of each section, and the rates at which they were absorbed. These were compared with total ammonia and urea-N excretion rates measured in intact satiation-fed and fasted trout. To this end, we used gut sac preparations incubated *in vitro*. A recent Commentary article in this journal (Bucking et al., 2024) criticized gut sacs and other *in vitro* techniques, because the mucosal saline failed to duplicate *in vivo* conditions in terms of unrealistic P_{O_2}, P_{CO_2}, P_{NH_3}, pH, HCO_3^- and osmolality levels, as well as the absence of the microbiome normally present in chyme. Therefore, we adopted a novel approach, making gut sacs that were filled not with saline, but rather with their endogenous chyme. These sacs were taken from the same trout from which blood ammonia and urea-N concentrations had been measured.

Our overall working hypothesis was that the previously overlooked stomach and pyloric caecae regions would play quantitively important roles in ammonia and urea-N absorption and/or production in light of their high volume and surface areas, and their known importance in the general digestive physiology of fish. We also posited that the gut sacs filled with their own chyme rather than saline would better duplicate *in vivo* conditions and would provide more realistic estimates of gastro-intestinal ammonia and urea-N transport rates in intact trout. An unexpected outcome of these trials is that they shed new light on fluid balance in the tract of trout fed to satiation with commercial pellets.

MATERIALS AND METHODS

Experimental animals

Experiments in were performed in January and February 2024 on a single batch of 24 adult diploid rainbow trout [*Oncorhynchus mykiss* (Walbaum 1792) (1.356±0.046 kg)] of mixed sex. They had been held for approximately 3 years under seasonal temperatures in flowing dechlorinated Vancouver tap water (in mmol l^{-1}: Na^+~0.09, Cl^-~0.10, Ca^{2+}~0.10, Mg^{2+}~0.011, K^+~0.004; hardness ~3.3 mg $CaCO_3$ l^{-1}, pH~7.0) in the aquatic facility of the Department of Zoology, University of British Columbia, Vancouver, Canada. The fish had been purchased as juvenile diploids from the Miracle Springs Trout Hatchery (Fraser Valley, British Columbia, Canada), and diploidy was confirmed by the fact that the fish had large identifiable gonads, most with eggs and sperm present at the time of the present study. Although we kept track of sex in series 1 and 2, we could see no clear effects of sex differences in any of the results.

Nine of these trout were used for the *in situ* measurements of series 1 (four females, five males) and then the gastro-intestinal tracts of these same fish were immediately used in the *in vitro* gut sac experiments of series 2. The other 15 trout were used in the *in vivo* experiments of series 3. For 1 month prior to the present experiments, the fish were acclimated to the experimental temperature (9.5°C) and fed daily to satiation at 18:00 with commercial trout pellet feed (6.0 mm Oregon Biotrout™, Bio-Oregon, Longview, WA, USA). In practice, the ration amounted to 2% of the body mass per day, and the last feeding was 16–22 h before the experiments reported here, except for the fasted treatment of series 3. All experiments were approved by the University of British Columbia Animal Care Committee (AUP A22-0195) under the guidelines of the Canadian Council on Animal Care.

Ammonia and urea-N concentrations in food

The certified composition of 6.0 mm Oregon Biotrout™ pellets is 43% protein, 24% lipid 8.5% moisture, 12.0% ash and 2.0% fibre, but the manufacturer does not provide information on the possible ammonia and urea-N content of the food. This could potentially make up an important part of the nitrogen-budget of the animal. To address this, pellets (approximately 0.90 g) were incubated with exactly 0.57 ml g^{-1} of either tap-water (N=6, to mimic water ingested with food) or 0.57 ml g^{-1} of 100 mmol l^{-1} HCl (N=6, to approximate gastric acid) in sealed 1.5 ml tubes at 9.5°C. This fluid to pellet ratio was the same as used by Wood and Eom (2019) and was chosen to represent the overall percentage of water measured in chyme sampled from the stomach at 2–24 h post-feeding by Bucking and Wood (2006) in trout fed a similar pellet diet. In the present study, the tubes were sampled at 1 h and again after 18 h of incubation. At time 0 h, as well as at each sampling time, the tubes were thoroughly vortexed. They were then centrifuged at 5000 ***g*** for 2 min, and samples (30 µl) of the supernatant were taken for ammonia and urea-N analysis, to represent the concentrations that would occur in chyme by dissolution of the pellets. After the 1 h sampling, the tubes were vortexed again and incubated until 18 h. For each of the two fluids, half of incubations were performed with whole pellets, and half with pellets that had been ground to a fine powder with a mortar and pestle. There were no detectable differences among the four methods, so the results were averaged.

Series 1: *in situ* blood measurements

The primary purpose of these experiments was to localize the areas of the gastro-intestinal tract where ammonia and urea-N are absorbed by sampling venous blood from selected sites along the tract. An approach similar to that of Wood and Eom (2019) was used in which the anaesthetized trout was maintained alive and ventilating on a operating table. Each fish was carefully netted from the holding tank with minimal disturbance, and immediately placed in a 20 l volume of high dose anaesthetic (0.2 g l^{-1} MS-222, NaOH neutralized, Syndel Laboratories, Parksville, British Columbia, Canada) at the acclimation temperature (9.5°C) for an initial knockdown. As soon as persistent overturn occurred, but ventilation continued, the fish was quickly weighed, and then the fork length was measured. The fish was then transferred to an operating table, where it was continuously irrigated via the gills with a more dilute anaesthetic solution (0.05 g l^{-1} neutralized MS-222 at the acclimation temperature of 9.5°C). This maintained stage 5 anaesthesia – 'loss of reflex activity, total loss of reactivity, shallow opercular movements' (McFarland, 1959) – over the next 2–2.5 h during which the *in situ* measurements were made.

The dorsal aorta was cannulated (Soivio et al., 1972) with a polyethylene PE50 catheter (Clay Adams™, Becton Dickinson, Franklin Lakes, NJ, USA) filled with Cortland salmonid saline (Wolf, 1963) to facilitate later sampling of arterial blood. The saline was heparinized with 50 i.u. ml^{-1} lithium heparin (Sigma-Aldrich,

Journal of Experimental Biology (2025) 228, jeb249654. doi:10.1242/jeb.249654

St Louis, MO, USA). The peritoneal cavity was then surgically opened from close to the anus to close to the pectoral girdle to expose the gastro-intestinal tract. The tract was gently raised on forceps to allow cannulation of the sub-intestinal vein close to the caudad end of the posterior intestine. This catheter was also filled with heparinized Cortland saline. An approach modified from that of Eliason et al. (2007) was used. A 15 cm length of soft, highly flexible silastic tubing (0.5 mm ID, 0.94 mm OD; Dow Corning, Midland, MI, USA) was attached to a 50 cm length of PE50, which is much stiffer. We found this allowed us to feed the catheter along the sub-intestinal vein without puncture of the delicate vein wall. However, to allow initial insertion, we found it helpful to cut the tip at a 45 deg angle and to stiffen it by immersion in liquid N_2. A small hole was made in the vein using a #22 needle, and the silastic catheter tip was inserted in the cephalad direction. Prior to cannulation, two 2-0 silk sutures had been placed into the intestinal wall bracketing the intended insertion site; the anterior suture was subsequently tied around the catheterized vessel to prevent leakage, and the posterior suture was tied to occlude the vessel and anchor the catheter. Once the cannulation was completed, the forceps were removed and the intestine was returned to the coelomic cavity.

For blood sampling, the sub-intestinal vein was occluded externally with forceps at the cephalad end of the posterior intestine, such that venous blood would be collected only from this section. The blood was allowed to slowly siphon into the bottom of a 0.5 ml plastic centrifuge tube. The initial 0.2 ml of blood was put aside as it was possibly contaminated with saline. Then approximately 0.3 ml of blood was collected for analysis, and the remaining blood was re-injected. The silastic catheter tip was then advanced to the caudad end of the mid-intestine, where the procedure was repeated. As the catheter occluded the vein behind it, and forceps were used to occlude the vessel at the cephalad end of the mid-intestine, blood was collected only from this section. Finally, the catheter was advanced as far as possible in the cephalad direction; post mortem examination showed that the tip was generally immediately caudad of the region where the pyloric caecae enter the anterior intestine. It was not possible to occlude the vessel cephalad to this point. A 0.3 ml blood sample was taken. In addition, an arterial blood sample (again 0.3 ml) was taken in random order relative to the three sub-intestinal vein samples, by drawing it from the dorsal aortic catheter into a gas-tight glass syringe equipped with a #22 needle (Hamilton, Reno, NV, USA). Finally, a 0.3 ml blood sample was taken by direct puncture of the HPV with the same Hamilton syringe. Sampling and initial analysis of blood at each site took about 15 min. The external surface of the intestine was kept moist throughout with physiological saline. Blood samples were obtained successfully from all sites in only seven of the nine fish, and these are the data reported in the figures and tables to preserve the repeated measures design.

The blood samples were suspended in a water bath at 9.5°C and their pH was immediately measured. They were then centrifuged at 5000 ***g*** for 1 min. The plasma was decanted and frozen in liquid N_2 for later measurements of total ammonia, urea-N and osmolality.

After sampling was completed, the fish was then euthanized by raising the MS-222 concentration to 0.5 mg l^{-1} followed by cervical section. The gastro-intestinal tract was then immediately prepared for the gut sac experiments of series 2.

Series 2: gut sac experiments

The primary objective of these experiments was to incubate gut sacs that were filled with the native chyme of the fish, so as to quantify ammonia and urea-N absorption rates in each section of the tract *in vitro*. Terminal sampling of the sac contents would yield a profile of ammonia and urea-N concentrations in the chyme, as well as the chemical conditions inside the gut sac preparations. The experiments also provided information on osmotic conditions and fluid fluxes in the preparations.

After euthanasia of the fish at the end of series 1, the gastro-intestinal tract was tied off at the cephalad end of the stomach, and at the caudad end of the posterior intestine, then excised. The total gut length was measured, and the tract was placed on ice for further processing. Ties with silk suture (2-0) were placed around the tract to separate the contents of the stomach, the anterior intestine, the mid-intestine and the posterior intestine. Chyme was abundant in all sections in all fish. The anterior section included the many pyloric caecae that were attached to it in these mature trout. These caecae populate almost the entire length of the anterior intestine, so it was not possible to make a separate sac for the anterior intestine alone, which is often carried out in smaller trout. To our knowledge, no previous studies have incorporated the pyloric caecae in gut sac experiments. The bile duct was tied off and external fat and fascia adhering to the serosal surface of the tract were carefully removed. Additional sutures were placed next to the original ones, such that each section of the tract could be made into a separate gut sac. For the stomach, it was necessary to reinforce the silk ties with ties of strong cotton string. Gut sacs were then surgically separated from one another, rinsed externally with Cortland saline, carefully blotted dry in a standardized fashion, and weighed to 0.1 mg accuracy on a model CP2245 microbalance (Sartorius AG, Göttingen, Germany).

Each gut sac was placed in a glass beaker of Cortland saline of known volume. Volumes varied based on the sizes of the preparations but were typically 40–60 ml for the stomach, and the anterior intestine and pyloric caecae, and 20–30 ml for the mid and posterior intestine. These volumes and the shapes of the beakers were chosen to allow full immersion of the preparations without compression, while ensuring good mixing by bubbling. The saline had been pre-equilibrated with a 1% CO_2/99% air gas mixture, so as to provide a P_{CO_2} of 7.5 Torr (1 Torr=1.33322 kPa) on the serosal side of the preparation. The beakers were partially immersed in a water bath to maintain a temperature of 9.5°C. Gassing continued during the incubations, to provide mixing. Samples of the external saline (5 ml) were taken at the start and end of the incubations, and measured immediately for pH and P_{O_2}, then frozen in liquid N_2 for later measurements of total ammonia, urea-N and osmolality. After approximately 2 h (exact time noted), the gut sacs were removed, blotted in a standardized fashion and weighed as before. The sacs were then opened at the caudad end, and samples of chyme were extruded into 1.5 ml centrifuge tubes. P_{O_2} and pH of the chyme were measured immediately. The tubes were then centrifuged as for blood samples, to yield supernatant, and frozen in liquid N_2 for later analyses of total ammonia, urea-N, osmolality and total CO_2. After all the chyme had been extruded from the sacs, they were then opened longitudinally, blotted again both internally and externally, and weighed to determine the mass of the tissue in each section. Each sac was then spread out on 0.5 mm graph paper to determine its area and length, as described by Grosell and Jensen (1999). As these mature fish had up to 50 pyloric caecae attached to the anterior intestine, the mean area per caeca was determined by measurements on five representative caecae, then multiplied by the total number of caecae. This was then added to the surface area of the main tract of the anterior intestine. The length reported for the anterior intestine is that of the main tract.

The mass of the chyme in each section was determined by subtracting the mass of the gut tissue from the initial mass of the gut sac. The fluid flux rate into or out of the chyme over the incubation

Journal of Experimental Biology (2025) 228, jeb249654. doi:10.1242/jeb.249654

period was calculated as the difference between initial and final masses of the gut sac, factored by time. The ammonia and urea-N flux rates were calculated from the increases in concentration in the serosal saline, factored by the volume of saline and time. Fluxes out of the gut sacs (net absorption) are therefore positive, and into the gut sacs (net secretion) are negative. The flux rates for each section have been reported per kilogram of body mass in figures, as well as per unit surface area of gut sac, per unit gut tissue mass of gut sac and per unit length of gut sac in Tables S1 and S2. When making the gut sacs, there was some unavoidable loss of tissue and chyme at the ends outside the suture ties. A comparison of the total length of the whole gastro-intestinal tract when it was originally excised with the total functional length of the four gut sacs added together from each fish revealed a ratio of 1.20±0.04 (N=9). This was used as a correction factor in calculating, for each section, the flux rates, mass of chyme, total length, total tissue mass and total surface area.

Series 3: whole-animal experiments

These experiments quantified the elevations in whole-animal ammonia and urea-N excretion rates in satiation-fed trout relative to fasted trout for comparison with the gastro-intestinal ammonia and urea-N absorption measurements in series 1 and 2. The fed trout were on their regular daily feeding schedule, whereas the fasted trout had not been fed for 7 days. Approximately 1 h after the regular feeding time, the fish were transferred to black plexiglass chambers (12×20×60 cm) served with aeration and flowing water at 9.5°C, and allowed to settle overnight. The next day, aeration and flow were stopped and the volume was set to 9.5 l. Water samples were taken at 1 h intervals for 4 h, after which the fish were weighed. Ammonia and urea-N flux rates were calculated from the increments in water concentrations over each hour, factored by volume and mass.

Analytical techniques

Total ammonia and urea-N concentrations in water, and urea-N concentrations in plasma and saline were determined using the colorimetric assays of Verdouw et al. (1978) and Rahmatullah and Boyde (1980), respectively. A commercial enzymatic assay kit designed specifically for plasma (Raichem ammonia kit based on glutamate dehydrogenase, Cliniqa Corporation, San Marcos, CA, USA) was used to measure ammonia concentrations in the plasma as well as in the saline, because even a small amount of protein in the saline from the gut sac incubations interfered with the Verdouw et al. (1978) assay. In the supernatant of centrifuged trout pellets and chyme, unknown substances interfered with all assays, so the procedure developed by Wood et al. (2023) was used. In brief, an equal volume of ice-cold 8% $HClO_3$ was added to the sample. After a 10 min incubation on ice, followed by centrifugation at 10,000 ***g*** for 15 min, the supernatant was removed. For urea-N, an aliquot was appropriately diluted with 4% $HClO_3$ and assayed by the method of Rahmatullah and Boyde (1980) against urea-N standards that had been put through the same procedure. For ammonia, a second aliquot was neutralized with 1.5 volumes of saturated Tris buffer (pH 7.5), then analysed with the Raichem kit against ammonia standards that had been put through the same procedure. Both assays were cross-validated among the different media using common standards.

The pH of blood, saline and chyme supernatant samples was measured at 9.5°C with a 238140 Biotrode™ (Hamilton) glass combination micro-electrode coupled to an Accumet™ meter (Fisher Scientific, Toronto, ON, Canada) and calibrated with precision buffers (Radiometer, Copenhagen, Denmark and Fisher Scientific). Total CO_2 in chyme supernatant and saline samples was measured with a Corning 965 analyser (Corning Instruments, Corning, NY, USA), calibrated with $NaHCO_3$ standards. P_{O_2} in chyme and saline samples was determined at 9.5°C using an O_2 micro-optode (PreSens Precision Sensing GmbH, Regensburg, Germany) calibrated with air (100% air saturation) and 1% sodium sulphite (0%). The osmolality of plasma, saline and chyme supernatant was measured with a Model 5100B vapor pressure osmometer (Wescor, Salt Lake City, UT, USA) calibrated with Wescor standards.

P_{CO_2}, P_{NH_3}, HCO_3^- and NH_4^+ concentrations were calculated using rearrangements of the Henderson-Hasselbalch equation. CO_2 solubility and pK′ values, and NH_3 solubility and ammonia pK values for teleosts were taken from Boutilier et al. (1984) and Cameron and Heisler (1983), respectively.

Statistics

Data are expressed as means±s.e.m. (N), where N equals the number of trout or preparations from different fish. As a repeated-measures approach was used throughout, data points from each fish are also shown in the figures to illustrate variation and trends within individual animals. Data were analysed by one-way repeated measures ANOVA followed by the Holm-Šídák *post hoc* test to identify significant differences. Prior to testing, all data were evaluated for normality of distribution and homogeneity of variance. If data failed, they were then subjected to standard transformations (log, square root). If data still failed, the non-parametric Friedman repeated-measures test on ranks was employed, followed by Dunn's *post hoc* test. Student's *t*-test (paired, unpaired or one-sample, as appropriate) was used for simple pair-wise comparisons. $P \leq 0.05$ was considered significant.

RESULTS

Ammonia and urea-N concentrations in food

Substantial concentrations of both ammonia and urea-N were found in the trout pellets and the associated fluid phase in the incubations, with greater variation amongst replicates for ammonia than urea-N (Table 1). Interestingly, there were differences between samples incubated for 1 h versus 18 h, with urea-N concentrations increasing significantly (P=0.043) and ammonia concentrations dropping non-significantly (P=0.104) by a similar amount at 18 h. Using the 1 h data, a trout eating a 2% ration (20 g food kg^{-1} body mass) would directly ingest 147 µmol kg^{-1} of ammonia and 63 µmol kg^{-1} of urea-N each day.

Series 1: *in situ* blood measurements

Plasma total ammonia concentrations increased significantly by approximately 2-fold from the arterial blood (mean=522 µmol l^{-1}) to the posterior sub-intestinal vein blood (~1050 µmol l^{-1}), and remained more or less constant at this level in the mid and anterior sub-intestinal vein, before again increasing significantly to 2258 µmol l^{-1} in the HPV (Fig. 1A). A similar pattern was seen in PNH_3 reflecting the fact that there was minimal variation in blood pH among the five sampling sites (Table S1).

Table 1. Concentrations of ammonia and urea-nitrogen in pellet food incubated for 1 or 18 h at 9.5°C with 0.57 ml of fluid per gram of pellet

	1 h incubation	18 h incubation
Ammonia in pellets (µmol kg^{-1})	7335±1689	4292±858
Ammonia in fluid phase (µmol l^{-1})	4672±1044	2734±547
Urea-nitrogen in pellets (µmol kg^{-1})	3170±323	7240±1687*
Urea-nitrogen in fluid phase (µmol l^{-1})	2019±206	4612±1074*

The concentrations in the fluid phase are also reported. See Materials and Methods and Results for details. Data are means±s.e.m. (N=12). Asterisks indicate a significant difference (*P<0.05) when compared with the value at 1 h incubation.

Journal of Experimental Biology (2025) 228, jeb249654. doi:10.1242/jeb.249654

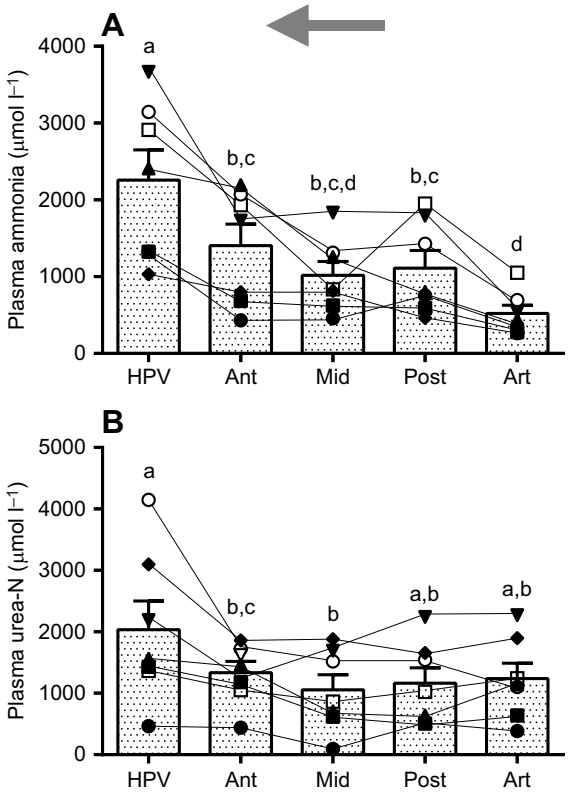

Fig. 1. Ammonia and urea-nitrogen in blood plasma in various parts of the gut circulation. (A,B) Ammonia (A) and urea nitrogen (urea-N) (B) concentrations in blood plasma sampled from the dorsal aorta (Art), the posterior (Post), mid- (Mid) and anterior (Ant) sub-intestinal vein, and the hepatic portal vein (HPV) in series 1. The arrow indicates the direction of blood flow *in vivo*. Data are means±s.e.m. (*N*=7). Points from each individual fish are connected to illustrate variability. Symbols for each fish are used consistently in Figs 1–4. Means sharing the same letter are not significantly different ($P<0.05$).

Plasma urea-N concentration (mean=1241 μmol l^{-1}) in arterial blood (Fig. 1B) was about twofold greater than total ammonia concentration (Fig. 1A). This urea-N concentration remained more or less unchanged at approximately 1150 μmol l^{-1} at all three sampling sites along the sub-intestinal vein, but then increased significantly to 2037 μmol l^{-1} in the HPV (Fig. 1B).

Plasma osmolality (Table S1) varied significantly among sampling sites according to the repeated-measures ANOVA ($P=0.046$). However, the *post hoc* test identified no significant differences. The trends were for a decrease from the dorsal aorta to the posterior sub-intestinal vein, a gradual increase moving in the cephalad direction along the vein from posterior to anterior, then a decrease from the anterior sub-intestinal vein to the HPV. Simple pair-wise comparisons with Student's *t*-test identified each of these changes as significant ($P=0.014$, $P=0.041$ and $P=0.011$, respectively).

Series 2: gut sac experiments

Gut metrics

Various gut metrics are summarized in Table 2. The gastro-intestinal tracts of these satiation-fed trout contained approximately 41 g kg^{-1} of chyme, with the largest amount in the stomach (44%), followed by the anterior intestine and pyloric caecae (31%), posterior intestine (18%) and mid intestine (7%). The total surface area of the tract was about 246 cm^2 kg^{-1}, with by far the largest amount in the anterior intestine and pyloric caecae (71%) owing to the numerous caecae, averaging 39 in number in a 1 kg trout. The stomach accounted for 15%, the posterior intestine for 9% and the mid-intestine for 5% of the total surface area. The total tissue mass of the tract was approximately 39 g kg^{-1}, with the anterior intestine and pyloric caecae accounting for 64%, followed by the stomach (27%), the posterior intestine (6%) and the mid-intestine (3%). The overall length of the tract was relatively short (~32 cm kg^{-1}), with a gut length to fork length ratio of only 0.86, which is typical of a strictly carnivorous fish. This length was more or less equally divided among the sections, with 28% in the posterior intestine, 27% in the stomach, 24% in the anterior intestine (excluding the lengths of the pyloric caecae) and 22% in the mid-intestine.

Chyme ammonia, urea-N and osmolality

Although there was substantial variation for both ammonia and urea-N concentrations in chyme, the patterns were qualitatively consistent among fish (Fig. 2A,B). The concentration of total ammonia in the fluid phase in the stomach (mean=5476 μmol l^{-1}) dropped by 40% to approximately 3250 μmol l^{-1} in the anterior intestine and pyloric caecae and mid-intestine, then increased significantly to 7775 μmol l^{-1} in the posterior intestine (Fig. 2A). Urea-N concentration in the fluid phase of the chyme exhibited a very different pattern, increasing more than 13-fold from the stomach (mean=1026 μmol l^{-1}) to the anterior intestine and pyloric caecae (13,552 μmol l^{-1}) (Fig. 2B). Thereafter, it decreased to 6631 μmol l^{-1} in the mid-intestine and to 1980 μmol l^{-1} in the posterior intestine, the latter representing a significant change relative to the peak in the anterior intestine and pyloric caecae. The mean concentrations of ammonia and urea-N in the fluid phase of trout pellets incubated with a physiologically realistic amount of fluid for 1 h (from Table 1) have been plotted as dashed lines for comparison in Fig. 2A,B. Clearly, they are sufficient to explain the measured concentrations in the fluid levels of stomach chyme for both nitrogenous products.

Table 2. Metrics of the gastrointestinal tract of satiation-fed rainbow trout determined from the gut sac preparations of series 2

	Total	Stomach	Anterior intestine and pyloric caecae	Mid-intestine	Posterior intestine
Body mass (kg)	1.223±0.070				
Fork length (cm)	44.37±1.60				
Gut length/fork length	0.863±0.047				
Number of pyloric caecae	39.22±3.06				
Gut area (cm^2 kg^{-1})	245.94±25.42	36.53±2.56^{b}	173.68±23.08^{a}	12.39±1.65^{d}	23.34±1.97^{c}
Gut tissue mass (g kg^{-1})	39.09±4.58	10.63±0.77^{b}	24.93±4.28^{a}	1.20±0.05^{d}	2.33±0.20^{c}
Gut length (cm kg^{-1})	31.81±2.30	8.60±0.47^{a,b}	7.55±0.60^{a,b}	6.81±0.83^{b}	8.85±0.82^{a}
Chyme mass (g kg^{-1})	40.87±7.93	17.85±6.85^{a,b,c}	12.82±1.99^{b}	3.01±0.35^{c}	7.68±1.17^{b}
Total ammonia (μmol kg^{-1})	239.11±76.91	128.46±72.33^{a}	37.81±10.54^{a,b}	10.82±2.15^{b}	62.02±12.42^{a,b}
Total urea-nitrogen (μmol kg^{-1})	206.25±49.28	11.76±2.81^{b}	160.19±46.93^{a}	18.08±5.50^{b}	16.23±4.45^{b}

Data are means±s.e.m. (*N*=9). Section means sharing the same letter are not significantly different at $P<0.05$.

Journal of Experimental Biology (2025) 228, jeb249654. doi:10.1242/jeb.249654

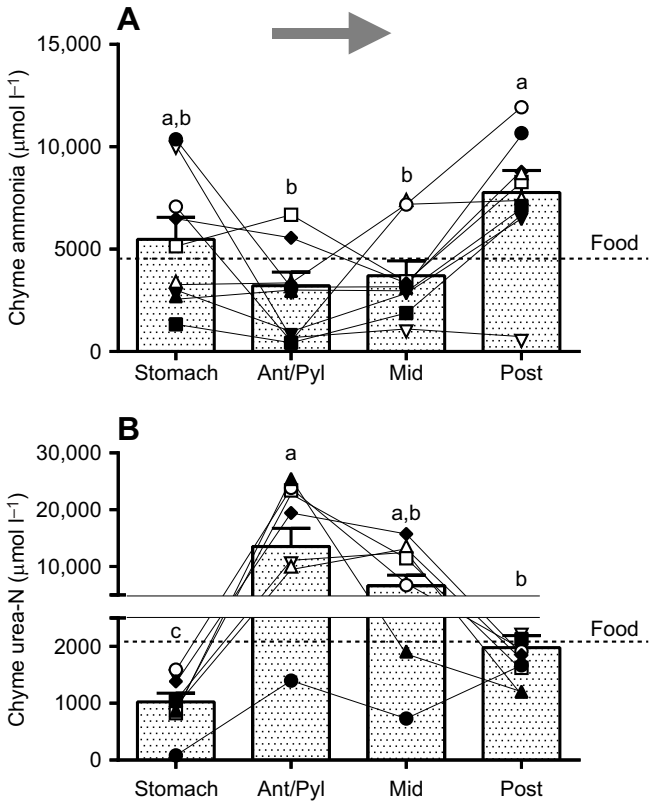

Fig. 2. Chyme ammonia and urea-nitrogen in various parts of the digestive tract. (A,B) Ammonia (A) and urea nitrogen (urea-N) (B) concentrations in the fluid phase of chyme sampled from the stomach, the anterior intestine and pyloric caecae (Ant/Pyl), the mid-intestine (Mid), and the posterior intestine (Post) gut sacs in series 2. The arrow indicates the direction of chyme flow *in vivo*. The dashed line indicates the mean concentrations of ammonia and urea-N measured in the fluid phase of incubated trout pellets (from Table 1). Data are means±s.e.m. (*N*=9). Points from each individual fish are connected to illustrate variability. Means sharing the same letter are not significantly different ($P<0.05$).

The total amount of ammonia and urea-N in chyme were estimated as the product of the concentrations and chyme masses in each section for each individual fish, assuming that fluid-phase concentrations were representative of whole-chyme concentrations. These amounted to means of 239 μmol kg^{-1} body mass for ammonia and 206 μmol kg^{-1} body mass for urea-N (Table 2). Most of the ammonia-N was in the stomach and posterior intestine, whereas most of the urea-N was in the anterior intestine and pyloric caecae. By way of contrast, based on the concentrations measured in food pellets in the 1 h incubations (Table 1), these trout on a 2% daily ration would have directly ingested approximately 147 μmol kg^{-1} body mass of ammonia and 63 μmol kg^{-1} body mass of urea-N each day.

The osmolality of the chyme increased progressively from a mean of 295 mOsm kg^{-1} in the stomach to approximately 495 mOsm kg^{-1} in the anterior intestine and pyloric caecae and mid intestine, and then to 610 mOsm kg^{-1} in the posterior intestine (see Fig. 4A). The osmolality of the external saline did not vary between the start and end of the gut sac flux experiments or among different sections, averaging 268±5 mOsm kg^{-1} overall (*N*=36 mean values from the start and end of the incubations of four gut sacs from each of the nine fish), as indicated by the horizontal dashed line in Fig. 4A. On a paired basis, chyme osmolality was significantly higher than saline osmolality in all sections, except the stomach.

Ammonia, urea-N and fluid transport rates in gut sacs

Both ammonia and urea-N were transported into the external saline (i.e. net absorption) in all four sections of the gastro-intestinal tract during the gut sac incubations (Fig. 3). The ammonia transport rate was greatest for the stomach (mean=16.9 μmol kg^{-1} h^{-1}), falling significantly to 7.9 μmol kg^{-1} h^{-1} in the anterior intestine and pyloric caecae and 3.6 μmol kg^{-1} h^{-1} in the mid-intestine, then rising again to 8.6 μmol kg^{-1} h^{-1} in the posterior intestine (Fig. 3A). Urea-N absorption rates were lower than ammonia transport rates and exhibited a different pattern, peaking at 3.9 μmol kg^{-1} h^{-1} in the anterior intestine and pyloric caecae, with mean rates between 0.7 and 1.9 μmol kg^{-1} h^{-1} in the other sections (Fig. 3). Total absorption rates for all sections were 37.0 ±6.7 (*N*=9) μmol kg^{-1} h^{-1} for ammonia and 8.1±2.3 (*N*=9) μmol kg^{-1} h^{-1} for urea-N.

Table S2 summarizes site-specific rates of ammonia and urea-N absorption expressed in other units that are sometimes reported – per unit surface area, per unit gut length and per unit gut tissue mass. When rates are expressed per unit surface area or per unit mass, relative values decrease greatly for the anterior intestine and pyloric caecae, and to some extent for the stomach, but tend to increase for the mid-intestine and posterior intestine. This reflects the much lower surface areas and tissue masses of these segments relative to the stomach and anterior intestine and pyloric caecae.

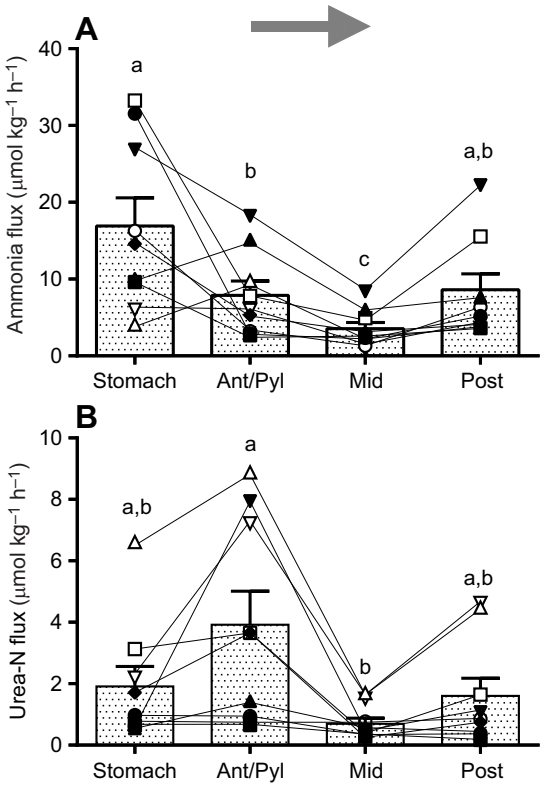

Fig. 3. Absorption of ammonia and urea-nitrogen in various parts of the digestive tract. (A,B) Absorptive flux rates (positive) of (A) ammonia and (B) urea nitrogen (urea-N) measured with gut sacs of the stomach, the anterior intestine and pyloric caecae (Ant/Pyl), the mid-intestine (Mid), and the posterior intestine (Post) in series 2. The arrow indicates the direction of chyme flow *in vivo*. Data are means±s.e.m. (*N*=9). Points from each individual fish are connected to illustrate variability. Means sharing the same letter are not significantly different ($P<0.05$).

Journal of Experimental Biology (2025) 228, jeb249654. doi:10.1242/jeb.249654

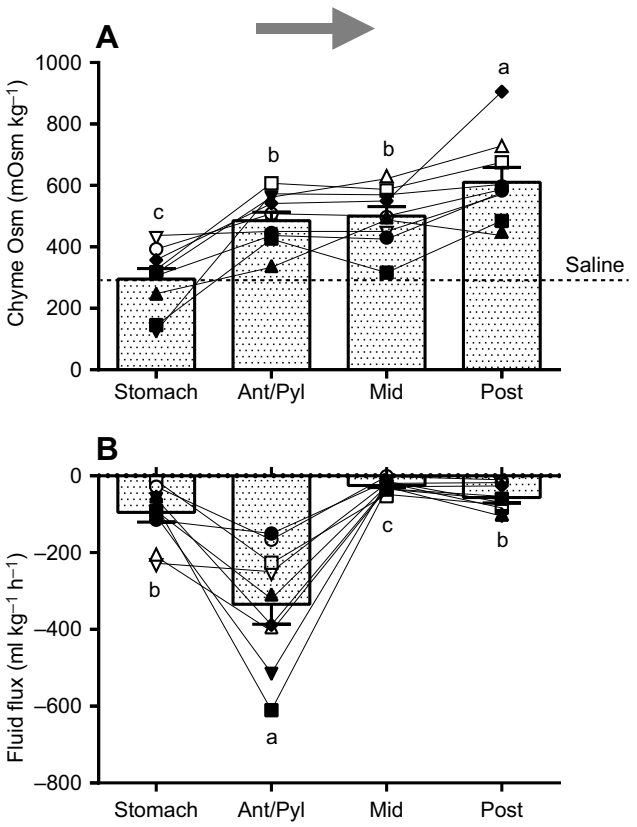

Fig. 4. Osmotic relationships and fluid fluxes in various parts of the digestive tract. (A,B) Osmolality of the fluid phase of chyme (A) and secretory flux rates (negative) (B) of fluid measured in gut sac preparations of the stomach, the anterior intestine and pyloric caecae (Ant/Pyl), the mid-intestine (Mid), and the posterior intestine (Post) in series 2. The arrow indicates the direction of chyme flow *in vivo*. The dashed line in A indicates the mean measured osmolality of the external saline in the serosal compartment. Data are means±s.e.m. (*N*=9). Points from each individual fish are connected to illustrate variability. Means sharing the same letter are not significantly different (P<0.05).

Net secretion of fluid (i.e. negative fluxes) occurred into the gut sac preparations of all four sections (Fig. 4B; Table S2). These increased significantly from −96 µl kg^{-1} h^{-1} in the stomach to −335 µl kg^{-1} h^{-1} in the anterior intestine and pyloric caecae, then decreased to −26 and −57 µl kg^{-1} h^{-1} in in the mid and posterior intestinal sacs. Overall, the intestine accumulated −514± 68 µl kg^{-1} h^{-1} (*N*=9) of fluid during the incubations.

Physico-chemical conditions in the gut sac experiments

Chyme was very acidic in the stomach (mean pH=3.87) but circumneutral in all sections of the intestine (Table 3). External saline remained at circumneutral pH for all sections, with no differences between start and end values. Chyme P_{NH_3} was essentially 0 µTorr in the stomach, reflecting the very low gastric pH, but increased progressively along the intestine. As a result, the P_{NH_3} gradient from the chyme to external saline was negative in the stomach but became increasingly positive moving caudad along the tract. Notably, the high rate of ammonia absorption by the stomach (Fig. 3A) occurred despite the fact that the P_{NH_3} gradient from chyme to external saline was in the opposite direction (i.e. negative), strongly favouring secretion (Table 3). The P_{NH_3} gradient was in favour of absorption (i.e. positive) in the other sections, especially in the posterior intestine. In view of the high pK of ammonia (Cameron and Heisler, 1983), at the pH values measured here, virtually all of it was present in the ionic form in both the chyme and external saline. Thus, NH_4^+ concentrations in chyme (Table 3) tracked the pattern for total ammonia concentrations shown in Fig. 2A. The NH_4^+ concentration gradient between chyme and external saline was positive in all sections, with the highest gradients in the posterior intestine and stomach, and lower values in the other two sections.

Chyme P_{O_2} dropped from approximately 38 Torr in the stomach to very low values of 5–11 Torr in the other three sections, despite strong P_{O_2} gradients from the external saline to the chyme (Table 3). Total CO_2 in the fluid phase of the chyme was negligible in the stomach but significantly higher in the other three gut segments, reaching about 35 mmol l^{-1} in the mid-intestine, and 25 mmol l^{-1} in the posterior intestine. Chyme HCO_3^- concentration closely followed the same profile as total CO_2 concentration, becoming two- to threefold higher than in the external saline (11.9 mmol l^{-1}) in both the mid- and posterior intestine. P_{CO_2} in the chyme was much higher than the 7.5 Torr set by continuous gassing in the external saline. Values ranged from 13 Torr in the stomach to a peak of approximately 58 Torr in the mid-intestine (Table 3). The osmolality gradient from chyme to external saline was not significantly different from 0 mOsm kg^{-1} in the stomach, but increased progressively and significantly down the tract. This reflected the constant osmolality in the saline and the gradually rising levels in the chyme from anterior to posterior intestine (Fig. 4A).

Table 3. Physico-chemical conditions in the gut sacs of satiation-fed rainbow trout of series 2

	Stomach	Anterior intestine and pyloric caecae	Mid-intestine	Posterior intestine
Chyme pH	3.87±0.32^{b}	6.85±0.09^{a}	6.97±0.20^{a}	7.19±0.14^{a}
Saline pH	6.90±0.38^{a}	6.96±0.28^{a}	7.16±0.13^{a}	7.12±0.31^{a}
Chyme P_{NH_3} (µTorr)	0.3±0.1^{c}	80.8±22.4^{b}	184.4±85.3^{b}	482.9±208.6^{a}
P_{NH_3} gradient (µTorr, chyme→saline)	−112.8±56.2^{c}	66.4±22.2^{b}	135.2±89.2^{b}	424.3±205.6^{a}
Chyme NH_4^+ (µmol l^{-1})	5732.7±1050.0^{a,b}	3211.3±658.8^{b}	3693.4±725.0^{b}	7748.0±1060.2^{a}
NH_4^+ gradient (µmol l^{-1}, chyme→saline)	5202.5±1081.5^{a,b}	2990.7±652.5^{b}	3541.9±734.6^{b}	7391.7±1066.3^{a}
Chyme P_{O_2} (Torr)	38.3±8.3^{a}	9.2±2.9^{b}	10.9±3.4^{b}	5.4±2.2^{b}
P_{O_2} gradient (Torr, saline→chyme)	98.6±9.4^{c}	118.7±4.2^{b,c}	124.4±4.5^{a,b}	131.8±3.1^{a}
Osmolality gradient (mOsm kg^{-1}, chyme→saline)	26±32^{c}	215±36^{b}	233±34^{b}	345±48^{a}
Chyme total CO_2 (mmol l^{-1})	0.9±0.1^{c}	17.0±3.9^{b}	34.6±6.7^{a}	26.9±6.8^{a,b}
Chyme HCO_3^- (mmol l^{-1})	0.1±0.04^{c}	14.3±3.5^{b}	30.8±6.3^{a}	24.1±6.5^{a,b}
Chyme P_{CO_2} (Torr)	13.0±2.3^{b}	40.7±9.3^{a,b}	57.7±15.9^{a}	42.9±16.5^{a,b}

Data are means±s.e.m. (*N*=9). Section means sharing the same letter are not significantly different at P<0.05.

Journal of Experimental Biology (2025) 228, jeb249654. doi:10.1242/jeb.249654

Table 4. Whole-animal rates of ammonia and urea-nitrogen excretion to the external water in fasted and fed rainbow trout of series 3

	Fasted (*N*=7)	Fed (*N*=8)
Ammonia excretion rate (μmol kg^{-1} h^{-1})	119.3±18.2	313.7±39.0*
Urea-nitrogen excretion rate (μmol kg^{-1} h^{-1})	26.7±4.7	63.6±7.5*
Urea-nitrogen fraction	0.176±0.030	0.181±0.029

Data are means±s.e.m. Asterisks indicate a significant difference (*P<0.05) compared with the value in fasted trout.

Series 3: whole-animal experiments

In both satiation-fed and fasted trout, there were no significant variations in whole-animal ammonia and urea-N flux rates over the four 1 h periods, so mean rates were compiled for each fish, then averaged in Table 4. Both ammonia and urea-N excretion rates were significantly greater by about 2.5-fold in fed fish. Urea-N accounted for about 18% of the total in both treatments. The increases in excretion rate associated with feeding were approximately 194 μmol kg^{-1} h^{-1} for ammonia and 37 μmol kg^{-1} h^{-1} for urea-N.

DISCUSSION

Overview

The primary objective of the present study was met, providing the first quantitative assessment of ammonia and urea-N production and absorption rates in all parts of the gastro-intestinal tract of the rainbow trout, as elaborated below. Using mature trout fed to satiation every day, we could examine a 'snapshot' of the entire tract under more or less steady-state conditions, and thereby estimate mean hourly transfer rates between compartments (Fig. 5). We confirmed our hypothesis that the stomach and pyloric caecae regions play quantitively important roles in ammonia and urea-N production and/or absorption. These two nitrogen products exhibited very different site-specific dynamics in different parts of the tract, with evidence of a surprisingly high rate of urea-N production in anterior intestine and pyloric caecae region (Fig. 2B), whereas the posterior intestine dominated for ammonia synthesis (Fig. 2A). The stomach was the major site of ammonia absorption (Fig. 3A), and the anterior intestine and pyloric caecal region was the dominant section of urea-N absorption (Fig. 3B). We have also provided the first measurements of ammonia and urea-N concentrations in commercial trout pellets (Table 1). They were high enough to make a significant contribution to the overall budget calculations, though dwarfed by the protein-N content of the food. These budget calculations suggest that 50% or more of the extra ammonia and urea-N excretion associated with satiation feeding is produced in the anaerobic gut. This challenges standard metabolic theory used in fuel use calculations. Our novel gut sac preparations filled with their own chyme proved successful in maintaining physico-chemical conditions in the mucosal fluid *in vitro* (Table 3) close to those measured recently *in vivo* (compare with table 1 of Bucking et al., 2024). Finally, in contrast to all previous reports on gut sac preparations filled with isosmotic saline as the mucosal solution, the present chyme-filled gut sacs gained rather than lost fluid during incubation, especially in the anterior intestine and pyloric caecae region (Fig. 4B). This was due to hyperosmolality in the chyme (Fig. 4A), and suggests that satiation feeding with commercial pellets is beneficial to the water balance of freshwater trout.

Ammonia and urea-N concentrations in food

We are not aware of any previous measurements of ammonia and urea-N concentrations in commercial trout pellets. The present values (Table 1) are very high, in the general range of concentrations measured in stomach chyme (Fig. 2A,B), so it is likely that most of the ammonia and urea-N measured in this section originates directly by leaching into the fluid phase from the pellets. The certified protein content of 6.0 mm Oregon Biotrout™ pellets is 43%, and it is likely that most of this originates from fish meal. Rudman et al. (1973) reported that the ammonia content of various processed meats was also in this range. Although these concentrations were high enough to be important in the balance calculations of Fig. 5, they are miniscule relative to the nitrogen provided by the protein content of the food. Using the standard conversion of 0.16 g nitrogen per gram of fish protein (Cho, 1990), the protein-N content of the pellets is 4,910,000 μmol kg^{-1}, or approximately 470 times the sum of the ammonia and urea-N content (Table 1). Interestingly, the significant rise in urea-N concentration and equimolar fall in ammonia-N concentration after 18 h of *in vitro* incubation suggested that the food itself may contain microbes (e.g. *Helicobacter pylori*; Mendz and Hazell, 1996) capable of carrying out this conversion.

Ammonia and urea-N concentrations in blood plasma

Plasma total ammonia concentrations (Fig. 1A) were generally high relative to the many measurements from fasted fish in the literature, but typical of the few measurements that have been reported from satiation-fed salmonids (reviewed by Wood, 1993, 1995). Plasma urea-N concentrations (Fig. 1B), which are generally less responsive to feeding, were in the general range reported by others in both fasted and fed salmonids (e.g. Wood, 1993; Knoph and Måsøval, 1996; Karlsson et al., 2006). We cannot eliminate the possibility that the stress of anaesthesia and maintenance of the fish on an operating table affected the measurements, because stress, and in particular cortisol mobilization, is known to elevate metabolic ammonia production, while downregulating gut function (Barton et al., 1987; Wood, 2001). In future studies of this nature, it will be useful to measure plasma cortisol levels.

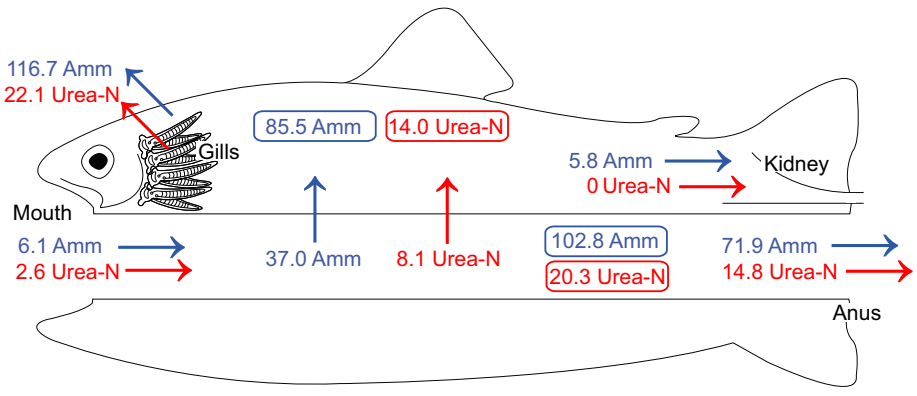

Fig. 5. A quantitative model of the origins and flux rates of the exogenous ammonia and exogenous urea nitrogen (urea-N) excretion (i.e. excretion above fasting levels, from Table 4) associated with feeding in satiation-fed rainbow trout. Values with arrows represent net flux rates. Values in boxes represent net metabolic production rates. Units are μmol kg^{-1} h^{-1}. Ammonia is in blue, urea-N in red. Please see Discussion for additional information.

Journal of Experimental Biology (2025) 228, jeb249654. doi:10.1242/jeb.249654

Blood moves in the cephalad direction through the tissue of the gastro-intestinal tract. The vasculature carrying this blood flow is extremely complex in salmonids, as detailed by Thorarensen et al. (1991). In brief, the major arterial supply is via branches of the coelo-mesenteric artery, which itself originates from the dorsal aorta, with additional smaller arteries branching directly from the dorsal aorta and serving more posterior regions. Thus, by sampling from the dorsal aorta, we obtained representative measurements of ammonia, urea-N and osmolality in the arterial blood flowing into the gut. Venous drainage of the intestine occurs via both the ventral sub-intestinal vein and the dorsal supra-intestinal vein, which have multiple anastomosing connections. Therefore, our sampling technique, which occluded the sub-intestinal vein in the caudad direction, would have allowed venous drainage to continue via the supra-intestinal vein. However, because of the anastomoses, the posterior, mid- and anterior sub-intestinal vein samples may have received some minor contamination from the other areas via the supra-intestinal vein. The anterior sub-intestinal samples, which were taken from a site just caudad to the entry point of the pyloric caecae, would likely have reflected influences from both the anterior intestine itself, as well as from the caecae. However, the major venous drainage from the caecae is via two major vessels that join the HPV. Additionally, all the drainage from the stomach via gastric veins and all the drainage from the intestine via the sub-intestinal and supra-intestinal veins, as well as from some other sites such as the spleen, are funnelled into the HPV. Therefore, differences in concentration between the anterior sub-intestinal vein and the HPV samples could reflect transport processes in the caecae or the stomach, or both.

With these caveats in mind, the doubling of plasma total ammonia concentration between the dorsal aorta and the posterior sub-intestinal vein points strongly to the posterior intestine as a major site of ammonia absorption (Fig. 1A). The further increases seen in the anterior sub-intestinal vein and particularly the HPV identify the anterior intestine and pyloric caecae and/or the stomach as additional major sites of ammonia uptake. Urea-N concentrations did not differ between the dorsal aorta, posterior and mid-sub-intestinal vein samples, but started to rise in the anterior and especially in the HPV samples (Fig. 1B). These responses point to the anterior intestine and pyloric caecae and/or stomach as important sites for urea-N uptake. In general agreement with these results, Karlsson et al. (2006) reported large increases in total ammonia and lesser increases in urea-N (relative to arterial concentrations) in the HPV of rainbow trout force-fed a single meal. These workers did not sample elsewhere along the intestine.

Ammonia and urea-N concentrations in chyme

Chyme moves through the tract in the caudad direction, from stomach to posterior intestine, opposite to the direction of blood flow. The total ammonia concentrations in chyme (Fig. 2A) were generally higher, by 2.5- to 6-fold, than those reported previously, where only single meals were given to fasted fish (see summary of six studies in table 1 of Bucking et al., 2024). However, the section-specific patterns were similar to those of previous investigations, with highest concentrations in the stomach and posterior intestine. This quantitative difference likely reflected the effects of daily satiation feeding, as well as perhaps the high ammonia concentrations in the present feed (Table 1). The drop in ammonia levels from the stomach to the anterior intestine and pyloric caecae section suggests that the latter is an important site for ammonia uptake or metabolic transformation (see below), whereas the 2-fold rise from mid-intestine to posterior intestine identifies the posterior intestine as an important site for ammonia synthesis (Fig. 2A). This could be catalysed by de-aminating enzymes secreted by the tissue, or as a result of microbial metabolism.

To our knowledge, there have been no previous measurements of urea-N in salmonid chyme. As for ammonia, the urea-N concentrations in the stomach could be explained by direct leaching of endogenous urea-N from the feed (Fig. 2B, Table 1). However, the remarkable 13-fold higher urea-N levels in the anterior intestine and pyloric caecae indicate that this section serves as a site of vigorous urea-N synthesis. The lowered ammonia concentrations in this section suggest that some metabolic transformation of ammonia to urea-N may occur, but clearly there must be additional input of nitrogen from other sources to provide the necessary nitrogen. Again, it is unclear whether this occurs by enzymes endogenous to the tissue or by microbes such as such as *Helicobacter pylori* (Mendz and Hazell, 1996). If the synthesis is via tissue enzymes, the ornithine–urea cycle (OUC) pathway seems unlikely, as general belief holds that expression of the enzymes is incomplete or at such a low level in most adult teleosts as to be non-functional. Although a few nitrogen-metabolism enzymes (carbamoyl phosphate synthetase III, glutamine synthetase, glutamate dehydrogenase, glutaminase and arginase) have been measured in the trout digestive tract (Bucking and Wood, 2012; Rubino et al., 2014; Jung et al., 2021), we are not aware of any studies that have specifically looked for OUC activity in the pyloric caeca. Other possibilities include urea-N generation by uricolysis, arginolysis or the gamma-guanido hydrolase pathway (Wood, 1993; Walsh and Mommsen, 2001). Clearly, further studies are needed to profile all the key enzymes of nitrogen metabolism in all sections of the tract, including the pyloric caeca.

Ammonia and urea-N transport in gut sac preparations

These *in vitro* experiments, in combination with the *in situ* plasma (Fig. 1) and chyme measurements (Fig. 2) confirmed our hypothesis as to the importance of the stomach and pyloric caecae in ammonia and urea-N handling. The highest rates of ammonia absorption were by the stomach (Fig. 3A). This fits with the highest concentrations of total ammonia in the plasma of the HPV (Fig. 1A) as well as the high ammonia concentration in gastric chyme (Fig. 2A). It also confirms the report of Jung et al. (2023b) that was based on only the rate of disappearance of ammonia from the mucosal medium. The second highest ammonia transport rate was in the posterior intestine, which fits with the highest total ammonia concentration in this section (Fig. 2A), as well as the sharp rise in total ammonia concentration of blood plasma between the dorsal aorta and posterior sub-intestinal vein (Fig. 1A). The expressions of two well known ammonia transporters – Rh glycoproteins (Nawata et al., 2007; Bucking and Wood, 2012; Rodela et al., 2012; Bucking et al., 2013a,b; Jung et al., 2023b) and the sodium, potassium, two chloride co-transporter (NKCC) – have been recorded throughout the gastro-intestinal tract of the rainbow trout (Nawata et al., 2007; Rubino et al., 2019; Jung et al., 2023b). However, in a pharmacological study on trout intestine, Rubino et al. (2019) attributed most of the absorptive transport of ammonia to barium-sensitive K^+ channels on the mucosal surface of the intestine. Although Rhcg2 (apical) glycoproteins are expressed in the trout stomach, at least at the message level (Jung et al., 2023b), it is difficult to see how they could transport ammonia out of the highly acidic gastric chyme. This is because trout Rh proteins are facilitated diffusion channels that transport NH_3 rather than NH_4^+ (Nawata et al., 2010). At pH 3.87, essentially all ammonia exists as NH_4^+, NH_3 is negligible and the P_{NH_3} gradient is in the wrong direction (Table 3). Perhaps, NH_4^+ is absorbed in substitution for K^+ from the gastric lumen, either on the NKCC, or via the K^+ channels that facilitate apical K^+ uptake for the action of the H^+/K^+ATPase pump (Shin et al., 2009).

The highest rates of urea-N absorption occurred in the anterior intestine and pyloric caecae (Fig. 3B), in accordance with the very high urea-N levels in the chyme of this section (Fig. 2B) and with the highest concentrations of urea-N in the blood plasma of the HPV, followed by the second highest concentrations of urea-N in the anterior sub-intestinal vein (Fig. 1B). The second highest chyme urea-N level was in the stomach (Fig. 2B), but this was not reflected in the urea-N transport rate, which was very low in this section (Fig. 3B). Urea transport mechanisms have not yet been identified in the gut of salmonids, although in the midshipman and the gulf toadfish (*Opsanus beta*), the facilitated diffusion urea transporter (UT) is expressed in the intestine and its levels increase after feeding in the midshipman (Bucking et al., 2013a,b).

Assessment of the novel chyme-filled gut sac preparations

Comparison of the data in Table 3 with the summary of *in vivo* measurements on chyme from multiple studies presented in table 1 of Bucking et al. (2024) illustrates that these novel preparations were very successful in duplicating the extreme conditions measured in the chyme of live trout. Particularly notable are the very high levels of P_{NH_3}, P_{CO_2}, HCO_3^- and osmolality, which are all close to *in vivo* measurements and are very different from the levels in the Cortland saline used as the mucosal medium in previous gut sac preparations. Chyme P_{O_2} was low, but not as low as the nearly anoxic P_{O_2} (<1 Torr) measured *in vivo* by Jung et al. (2022). The correct acidic pH was maintained in the stomach chyme, but the rise in pH along the intestine in the caudad direction was not as great as in many *in vivo* studies. It is unclear whether these discrepancies are due to the incubation *in vitro* or because the present chyme was from satiation-fed trout versus trout fed single meals in earlier studies.

Do the ammonia and urea-N flux rates measured with the present preparations differ from those determined previously with saline-filled gut sacs? The question is confounded by the fact that all the previous studies have expressed flux rates per unit of gut surface area rather per whole gut section, as we have done in Fig. 3. Therefore, in Table S2, the current rates have been expressed per unit of surface area. On this basis, our ammonia flux rates are about 1.5- to 2-fold greater than those reported by Rubino et al. (2014, 2015, 2019) and Jung et al. (2021) for mid-and posterior gut sacs, which are the only valid comparisons that can be made. Our urea-N flux rates are about the same as those reported by Jung et al. (2021). A further complication is the much greater size of our fish: 1.2 kg versus approximately 0.2 kg in the above-cited studies. The allometric scaling constant for nitrogen metabolism is about 0.74 (Cai and Summerfelt, 1992; C.M.W., unpublished observations). Applying this value to the data, the present ammonia flux rates become about 3-fold greater and the urea-N flux rates about 1.6-fold greater than those in the previous reports. Whether this is because of the generally higher concentrations of ammonia and urea-N in the mucosal fluid or the more realistic conditions in chyme-filled gut sacs remains to be determined. Regardless, it should be kept in mind that absolute transport rates are likely underestimated, owing to a lack of blood flow in the tissues, which is a problem with all *in vitro* gut preparations (Bucking et al., 2024). Another limitation of gut sac preparations in the current context is the absence of hormonal signalling along the gut and via the gut–brain axis resulting from stimulation of amino acid receptors within the tract itself (Calo et al., 2021, 2023).

Modelling the ammonia and urea-N budget of satiation-fed trout

Both whole-body ammonia and urea-N excretion rates were elevated by about 2.5-fold above 1-week fasting levels by daily satiation feeding (Table 4). This can be compared with 1.5-fold (Kajimura et al., 2004), 4-fold (Fromm, 1963) and 6-fold increments (Alsop and Wood, 1997) in other studies using satiation feeding, where smaller trout had larger increments. Therefore, in the present study, the exogenous fraction of nitrogen excretion (i.e. the rate above the endogenous fraction needed for body maintenance, see Wood, 1993) was 194.4 μmol kg^{-1} h^{-1} for ammonia and 36.9 μmol kg^{-1} h^{-1} for urea-N.

If we assume approximately steady-state conditions, the present data, together with some values from the literature, allow the construction of an ammonia and urea-N budget (Fig. 5) for these satiation-fed rainbow trout. The daily 2% ration feeding, averaged over 24 h, provided 6.1 μmol kg^{-1} h^{-1} of ammonia and 2.6 μmol kg^{-1} h^{-1} of urea-N by ingestion (from Table 1). The total absorption rates through the gastro-intestinal tract, were 37.0 μmol kg^{-1} h^{-1} for ammonia and 8.1 μmol kg^{-1} h^{-1} for urea-N (from Table S2), showing that both were produced in the tract, especially considering the amount passed out with faeces. Kajimura et al. (2004) used rectal ligation experiments to estimate how much ammonia and urea-N were excreted via the anus after feeding. Their data can be calculated in various ways, but if we use the most conservative approach, rectal excretion still accounted for 37% of the exogenous ammonia excretion and 44% of exogenous urea-N excretion to the water in fed trout. From Table 3, this would amount to 71.9 μmol kg^{-1} h^{-1} of ammonia and 14.8 μmol kg^{-1} h^{-1} of urea-N passing out through the anus. Thus, the total metabolic production rates in the tract would be 102.8 μmol kg^{-1} h^{-1} for exogenous ammonia and 20.3 μmol kg^{-1} h^{-1} for exogenous urea-N. According to Fromm (1963) and Bucking et al. (2010), the exogenous ammonia passing out in the urine after feeding, as measured by urinary catheter, is only about 3% of the total and exogenous urea-N excretion via the kidney is negligible. This means that the exogenous ammonia excretion rate via the urine is only about 5.8 μmol kg^{-1} h^{-1}. Therefore, in total, branchial excretion accounts for 116.7 μmol kg^{-1} h^{-1} of exogenous ammonia and 22.1 μmol kg^{-1} h^{-1} of exogenous urea-N efflux. The exogenous ammonia production rate in the body (i.e. outside the gut) is 85.5 μmol kg^{-1} h^{-1}, and the exogenous urea-N production rate in the body is 14.0 μmol kg^{-1} h^{-1}.

Impact on metabolic theory

A key conclusion is that 56.0% of the exogenous ammonia excreted by satiation-fed trout was either produced in the tract or ingested with the feed. For urea-N, the fraction is 62%. This fits very well with the 47% estimate of Rubino et al. (2014) for the contribution of the gut to ammonia excretion after feeding. The consequences of this for the application of metabolic theory to fuel use calculations are immense. Standard metabolic theory originally developed for mammals (Kleiber, 1992), assuming that that all fuels are oxidized aerobically, holds that the nitrogen quotient (NQ, the molar ratio of nitrogen excretion to O_2 consumption) provides a quantitative index of the fraction of metabolism fuelled by protein (see Lauff and Wood, 1996, and Wood, 2001 for a detailed explanation). For fish, NQ=0.27 represents 100% protein burning, so the equation X=(NQ/0.27)×100 gives the percentage of fuel use based on protein oxidation in any circumstance. If this and the respiratory quotient (RQ) are known, then the simultaneous contribution of lipid and carbohydrate fuels can be calculated. Clearly, if 50% or more of the exogenous nitrogen excretion after feeding is produced under the almost completely anaerobic conditions in the gut (Jung et al., 2020, 2022), whether by anaerobic microbes or anaerobic processes in the gut tissue, and/or comes from the ingestion of preformed ammonia and urea-N, the theory breaks down. This may explain why NQ

values greater than 0.27 have been reported in some feeding studies on fish (e.g. Kajimura et al., 2004; Wood et al., 2023). Wood (2001) reviewed many long-term feeding studies where high NQs (but still less than 0.27) lead to the conclusion that protein oxidation was very high. Even when the post-feeding NQ increases were in a lower range (e.g. Linton et al., 1997; Ferriera et al., 2019), the conclusions become suspect. We recommend that this otherwise very valuable approach be applied only in studies where the fish are not fed.

Fluid transport *in vivo* and in the novel chyme-filled gut sac preparations

The blood plasma osmolality dropped from the dorsal aorta to the posterior sub-intestinal vein, then progressively increased again as it flowed in the cephalad direction along the tract (Table S1). This likely reflected the delivery of nutrients to the tract by the arterial blood, and the subsequent progressive uptake of nutrients into the venous blood. As outlined below, the secretion of water from the blood into the hyper-osmotic chyme was another contributory factor. The drop between the anterior sub-intestinal vein and the HPV would be due to dilution with less nutrient-rich blood draining from other areas (Thorarensen et al., 1991).

A universal finding of previous trout gut sac studies using isosmotic saline as the mucosal medium is vigorous transport of fluid to the serosal media (e.g. Nadella et al., 2007, 2014; Rubino et al., 2015). This occurs because water osmotically follows the active absorption of Na^+ and nutrients. Whether net water absorption occurs *in vivo* probably depends on the nature of the diet. An unexpected bonus of the present experiments was confirmation of the finding of Bucking and Wood (2006) that, in trout fed commercial pellets, there was a net secretion of water from the fish into the chyme *in vivo* (Fig. 4B). Bucking and Wood (2006) reported a net fluid secretion rate of 0.24 ml kg^{-1} h^{-1} in trout fed a single meal, whereas in our chyme-filled gut sacs from trout fed daily to satiation, the net secretion rate was 0.51 ml kg^{-1} h^{-1} (Table S2). In both studies, there were substantial osmotic gradients from the nutrient-rich chyme to the external saline or blood plasma (Fig. 4A; Tables S1 and S2) along most of the tract. In addition, in both studies the dominant intestinal section for water secretion was the anterior intestine and pyloric caecae region (Fig. 4B), even though the osmotic gradient was not the highest there (Fig. 4A). The extremely high surface area in this region (Table 2) was the likely explanation. A chronic problem for freshwater fish is the continual osmotic influx of water across the gills and body surface. The osmotic secretion of water out of the body into the gastro-intestinal tract accompanying satiation-feeding with commercial pellets provides some beneficial compensation, and may be important in aquaculture.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: C.M.W., J.E.; Data curation: C.M.W., J.E.; Formal analysis: C.M.W., J.E.; Funding acquisition: C.M.W.; Investigation: C.M.W., J.E.; Methodology: C.M.W., J.E.; Project administration: C.M.W.; Resources: C.M.W.; Software: C.M.W.; Supervision: C.M.W.; Validation: C.M.W.; Visualization: J.E.; Writing – original draft: C.M.W.; Writing – review & editing: J.E.

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Data availability

Data are available from the authors upon reasonable request.

Special Issue

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