© 2019. Published by The Company of Biologists Ltd | Journal of Experimental Biology (2019) 222, jeb194787. doi:10.1242/jeb.194787

RESEARCH ARTICLE

Nitrogen handling in the elasmobranch gut: a role for microbial urease

Chris M. Wood[1,2,3,*], Hon Jung Liew[1,4], Gudrun De Boeck[1,5], J. Lisa Hoogenboom[1,6] and W. Gary Anderson[1,6]

ABSTRACT

Ureotelic elasmobranchs require nitrogen for both protein growth and urea-based osmoregulation, and therefore are probably nitrogen-limited in nature. Mechanisms exist for retaining and/or scavenging nitrogen in the gills, kidney, rectal gland and gut, but as yet, the latter are not well characterized. Intestinal sac preparations of the Pacific spiny dogfish shark (*Squalus acanthias suckleyi*) incubated *in vitro* strongly reabsorbed urea from the lumen after feeding, but mucosal fluid ammonia concentrations increased with incubation time. Phloretin (0.25 mmol l^{-1}, which blocked urea reabsorption) greatly increased the rate of ammonia accumulation in the lumen. A sensitive [^{14}C]urea-based assay was developed to examine the potential role of microbial urease in this ammonia production. Urease activity was detected in chyme/intestinal fluid and intestinal epithelial tissue of both fed and fasted sharks. Urease was not present in gall-bladder bile. Urease activities were highly variable among animals, but generally greater in chyme than in epithelia, and greater in fed than in fasted sharks. Comparable urease activities were found in chyme and epithelia of the Pacific spotted ratfish (*Hydrolagus colliei*), a ureotelic holocephalan, but were much lower in ammonotelic teleosts. Urease activity in dogfish chyme was inhibited by acetohydroxamic acid (1 mmol l^{-1}) and by boiling. Treatment of dogfish gut sac preparations with acetohydroxamic acid blocked ammonia production, changing net ammonia accumulation into net ammonia absorption. We propose that microbial urease plays an important role in nitrogen handling in the elasmobranch intestine, allowing some urea-N to be converted to ammonia, which is then reabsorbed for amino acid synthesis or reconversion to urea.

KEY WORDS: Ammonia, Urea, Amino acids, Phloretin, Acetohydroxamic acid, Intestine, Spiral valve, Gut sacs

INTRODUCTION

Ureotelic elasmobranchs require nitrogen not just for protein growth, but also for urea-based osmoregulation (Smith, 1929, 1936), and therefore are probably nitrogen-limited in nature (Wright and Wood, 2016). Indeed, Ballantyne (2016) has characterized them as the most nitrogen-limited aquatic vertebrates. Studies using the spiny dogfish shark (*Squalus acanthias*) as a model system have described the retention and/or scavenging of nitrogen in the gills (Boylan, 1967; Wood et al., 1995; Pärt et al., 1998; Fines et al., 2001; Hill et al., 2004; Wood et al., 2013; Nawata et al., 2015; Wood and Giacomin, 2016), kidney (Kempton, 1953; Forster and Berglund, 1957; Schmidt-Nielsen and Rabinowitz, 1964; Schmidt-Nielsen et al., 1972; Boylan, 1972; Hays et al., 1977; Wood et al., 1995), rectal gland (Burger and Hess, 1960; Zeidel et al., 2005) and gut (Wood et al., 2007b; Anderson et al., 2012; Liew et al., 2013; Anderson et al., 2015). The intestine (also known as the spiral valve in elamobranchs because of its unique structure) appears to be particularly important in this regard. During the processing of a natural meal of teleost fish, the intestine receives an input of urea originating from secretion by the stomach compartments, addition from biliary and pancreatic secretions, and likely secretion across the intestinal epithelium early in the digestive process (Wood et al., 2007b). This addition of urea from the shark's body is sufficient to raise levels in the chyme almost equal to those (350–400 mmol l^{-1}) in the bloodstream, thereby essentially equilibrating osmotic pressure between the chyme and the bloodstream. The amount of urea added may depend on whether the prey items are teleosts (strongly hypotonic to shark blood) or invertebrates (closer in osmolality to shark blood). The diet is reported to vary seasonally in *Squalus acanthias suckleyi* – teleosts in winter and invertebrates in summer (Jones and Geen, 1977). Additional information on the feeding ecology of this species was reviewed by Wood et al. (2007b), and on the feeding ecology of sharks in general by Cortés et al. (2008). Regardless, all of this valuable urea-N that has been secreted appears to be subsequently reabsorbed in the intestine rather than excreted (Wood et al., 2007b). Two recent *in vitro* studies have characterized the urea-reabsorbing mechanism of the intestine which is activated only after feeding: it is saturable, sensitive to competition by urea analogues, and involves apparent active transport (Liew et al., 2013; Anderson et al., 2015). Evidence for the latter includes its occurrence against strong urea gradients, reduction by ouabain and Na^+ removal, and strong inhibition by the general transport blocker phloretin (Liew et al., 2013; Anderson et al., 2015).

In contrast, essentially nothing is known about ammonia handling in the intestine. Based on only one study (Wood et al., 2009) that measured ammonia concentrations (1–3 mmol l^{-1}) in gastric chyme (not intestinal chyme) of *Squalus acanthias*, ammonia levels are only approximately 1% of urea levels. Nevertheless, the gills are capable of scavenging ammonia from even lower concentrations in the external water (Nawata et al., 2015; Wood and Giacomin, 2016), so it seems likely that the intestine would be able to absorb ammonia from the chyme, especially because evidence for carrier-mediated ammonia uptake has recently been presented for the teleost intestine (Rubino et al., 2014; Rubino et al., 2015).

With this background in mind, we revisited the *in vitro* gut sac study of Liew et al. (2013) and analyzed archived samples for ammonia concentrations, thereby allowing calculations of ammonia

[1]Bamfield Marine Sciences Centre, 100 Pachena Road, Bamfield, BC V0R 1B0, Canada. [2]Department of Zoology, University of British Columbia, Vancouver, BC V6T 1Z4, Canada. [3]Department of Biology, McMaster University, 1280 Main St. West, Hamilton, ON L8S 4K1, Canada. [4]Institute of Tropical Aquaculture, Universiti Malaysia Terengganu, 21030 Kuala Terengganu, Terengganu, Malaysia. [5]Systemic Physiological and Ecotoxicological Research, Department of Biology, University of Antwerp, Groenenborgerlaan 171, BE-2020 Antwerp, Belgium. [6]Department of Biological Sciences, University of Manitoba, Winnipeg, MB, Canada R3T 2N2.

*Author for correspondence (woodcm@zoology.ubc.ca)

C.M.W., 0000-0002-9542-2219

Received 16 October 2018; Accepted 3 December 2018

Journal of Experimental Biology (2019) 222, jeb194787. doi:10.1242/jeb.194787

flux rates. The results, which are reported here for the first time, proved very surprising, showing ammonia accumulation over time, with marked differences between fed and fasted animals. This led to the hypothesis that there must be significant urease activity in the gut of *S. acanthias*, which increases with feeding. Urease is an enzyme normally restricted to microbes, plants, fungi and invertebrates (Mobley and Hausinger, 1989), so it is very likely that, if present, it would be of microbial origin, contained in bacteria nutrified by the urea-rich environment in the intestinal lumen. A recent report based on pyrosequencing (Givens et al., 2015) reported that three shark species (not *Squalus* spp.) shared a similar core microbiome, comprising three operational taxonomic units. This was similar to but less diverse than the microbiomes in many teleosts. In order to test the idea that microbial urease is present in the intestine of *S. acanthias* and increases after feeding, we developed a sensitive radioisotopic assay based on the hydrolysis of [^{14}C]urea to [^{14}C]CO_2, and used it to quantify urease activity in freshly sampled chyme or intestinal fluid, intestinal epithelia and bile from fed and fasted dogfish. We also measured ammonia and urea concentrations in these fluids. We then hypothesized that blockade of urease activity in the gut would reduce ammonia accumulation in the gut sac preparations, possibly revealing the presence of an ammonia uptake pathway. To this end, we evaluated several potential inhibitors of urease activity in the chyme, and used one of these to examine the effect of urease inhibition on ammonia dynamics in intestinal sac preparations from fed dogfish.

In order to evaluate the generality of our findings, we also looked for the presence of urease activity in the chyme/intestinal fluid and intestinal epithelia of Pacific spotted ratfish (*Hydrolagus colliei*) freshly collected from the wild. These holocephalic chimaerids are also members of the Class Chondrichthyes, though only distantly related to the elasmobranch sharks. Nevertheless, they are similarly ureotelic and employ a urea-based mechanism of osmoregulation (Hyodo et al., 2007; Anderson et al., 2012). Samples from several marine ammoniotelic teleosts were also assayed for comparison. We hypothesized that urease activities in the ratfish would be comparable to those in the dogfish, and much higher than those in the teleosts. Based on all these results, we propose that microbial urease plays an important role in producing ammonia from urea in the gut, and that subsequent ammonia absorption, together with direct urea reabsorption, facilitates nitrogen conservation in ureosmotic and ureotelic Chondrichthyes.

MATERIALS AND METHODS

Experimental animals

Male spiny dogfish sharks (*Squalus acanthias suckleyi* Linnaeus, 0.7–3.6 kg) were collected from Barkley Sound, British Columbia, by commercial trawling in July 2011, and by angling in July–August 2016 and July–August 2018. Pacific spotted ratfish (*Hydrolagus colliei* Lay & Bennett 1839, 0.2–1.1 kg), assorted flatfish [*Lepidopsetta bilineata* (Ayres 1855) (rock sole); *Parophrys vetulus* Girard 1854 (English sole); *Lyopsetta exilis* (Jordan and Gilbert 1880) (slender sole); all 0.1–0.5 kg] and staghorn sculpins (*Leptocottus armatus* Girard 1854, 0.1–0.4 kg) all of indeterminate sex, were collected from Barkley Sound by angling in August 2016. Collection permits were issued by Fisheries and Oceans Canada. At Bamfield Marine Science Centre (BMSC), the fish were held in running seawater at the experimental temperature (12±1°C), salinity (30±2 ppt) and pH (7.95). Ammonia and urea-N levels were routinely measured in the holding tanks, and were usually undetectable, and always less than 5 µmol l^{-1}. Procedures were in accord with Canada Council for Animal Care Guidelines, and were approved by BMSC, McMaster University, University of Manitoba and University of British Columbia Animal Care Committees.

The ratfish, flatfish and sculpins were not fed, and were sampled within 24 h of capture. The dogfish were held in a large group (30+ fish) in a 151,000-liter tank, where they were entrained to a feeding regime comprising a 3% ration of dead hake (*Merluccius productus*) given every fourth day in 2011 and 2016, and every second day in 2018. The 2011 experiments on dogfish focused on *in vitro* gut sac studies. For fed treatments, dogfish were sampled at 24–48 h after feeding, and feeding was confirmed at autopsy. For fasted treatments, dogfish were moved to a smaller 1000-liter tank, where they were not fed for at least 7 days. The 2016 experiments focused on measurements of urease activity and intestinal fluid ammonia and urea concentrations, and the 2018 experiments on inhibitor studies in both chyme and the intestinal sac. Different experiments were performed on each of the three years, so data from different years were not combined in any means. In all studies, animals were euthanized by an overdose of neutralized MS-222 (Syndel Laboratories, Nanaimo, Canada), and for most of them, the samples were taken from animals that were being euthanized for several studies.

Dogfish gut sac preparations

These experiments were described in detail by Liew et al. (2013). In brief, in 2011, gut sacs were made from fed (N=10) and fasted (N=12) dogfish using the cardiac stomach (stomach 1, filling volume ∼12 ml), the pyloric stomach (stomach 2, ∼15 ml), the intestine (spiral valve, ∼20 ml) and the colon (∼3 ml). An additional series, using only intestinal sacs from fed fish (N=6), was performed in which 0.25 mmol l^{-1} phloretin (Sigma-Aldrich, St Louis, MO, USA) was included in the mucosal saline. In 2016, additional gut sac experiments were performed on fed dogfish (N=6) with identical methods, specifically for measurements of urease activity in the mucosal solutions. In 2018, larger fed dogfish were available (N=6), so it was possible to make two gut sacs from each intestine, facilitating a paired design. In one of the pair, 1 mmol l^{-1} acetohydroxamic acid (Sigma-Aldrich) was included in the mucosal saline, while control saline was used in the other one. The treatments were alternated between the anterior and posterior sacs in different animals. In all studies, the composition (in mmol l^{-1}) of the saline used in both the lumen (mucosal side) and the external bath (serosal side, 150 ml) for each sac was: NaCl=257; Na_2SO_4=7; $NaHCO_3$=6; Na_2HPO_4=0.1; KCl=4; $MgSO_4.7H_2O$=3; $CaCl_2.2H_2O$=2; glucose=5; TMAO=85; urea=350. Although no ammonia salts were used in the preparation of the saline in 2011 and 2016, the measured starting background concentration of total ammonia was approximately 0.5 mmol l^{-1}. In the inhibitor studies of 2018, 2 mmol l^{-1} of NH_4Cl was added to bring the starting background concentration to approximately 2.5 mmol l^{-1}, so as to reveal the potential presence of an ammonia uptake mechanism. In order to maximize O_2 supply and set P_{CO_2} (2.3 torr=307 Pa) and pH (8.1) to realistic *in vivo* levels, salines were pre-equilibrated with a precision gas mixture of 99.7% O_2 and 0.3% CO_2, and gassing of the external (serosal) bath was continued throughout the 3-h incubation period at 12°C. Samples of the initial and final mucosal and serosal solutions were frozen immediately in liquid N_2, transferred to a −80°C freezer, and stored for approximately 1 year prior to ammonia analyses in the 2011 experiments, and for up to 2 weeks in the 2016 and 2018 experiments. Tests in our laboratories have shown that ammonia is stable under these conditions, as long as assays are performed on the first thawing. Samples for measurement of urease activity were not frozen and were assayed immediately, as described below.

Journal of Experimental Biology (2019) 222, jeb194787. doi:10.1242/jeb.194787

The net ammonia flux rate was calculated from the initial and final mucosal ammonia concentration and fluid volumes, the latter measured gravimetrically, and expressed per unit gross surface area measured graphically, as described by Liew et al. (2013):

$$\text{Flux rate} = \frac{[V_i \times C_i] - [V_f \times C_f]}{T \times A}, \quad (1)$$

where C_i and C_f are the initial and final total ammonia concentrations (in μmol ml^{-1}) of the mucosal solutions, V_i and V_f are the initial and final volumes (in ml) of the mucosal solutions, A is surface area (in cm^2) and T is time (in h). Positive values represent net absorption rate, and negative values represent net accumulation rate. Because of the much higher volume in the external bath, changes in serosal ammonia concentrations could not be detected.

Intestinal chyme, fluids and tissue sampling

In 2016, samples of intestinal chyme (from fed dogfish) or intestinal fluid (from fasted dogfish), as well as bile (from the gall bladder) and epithelial tissue were obtained from freshly euthanized animals. In some fasted animals, there appeared to be no free intestinal fluid so the sample could not be taken, and in many fed animals, bile could not be obtained. In 2018, only intestinal chyme from fed dogfish was collected for inhibitor studies. As feeding was not controlled for ratfish, flatfish or sculpins, there were no distinct fed or fasted treatments; most of these freshly angled fish had small amounts of food in their tracts, and bile was not available. For ratfish, the intestinal fluid and epithelial tissues were taken as for dogfish, whereas for flatfish and sculpins, only intestinal fluid or chyme was collected. A disposable syringe of appropriate size (usually 5 ml with a 19-gauge needle) was used to obtain the chyme and fluid samples. Epithelial tissue was collected by first rinsing (with urease assay saline; see below), then gently blotting and scraping the surfaces of three to four intestinal folds with a glass slide onto aluminum foil. The slurry obtained was then gently mixed. Samples of chyme, intestinal fluid, bile and epithelial tissue were stored in sealed vials for a few hours at 4°C prior to urease assay, while samples of chyme, intestinal fluid and bile were frozen immediately in liquid N_2, transferred to a −80°C freezer and stored for up to 2 weeks prior to assay for total ammonia and urea concentrations.

Urease activity assay

Theoretically, urease activity can be measured by the disappearance of the substrate (urea) or the appearance of either of the products (CO_2 and 2× ammonia). However, it is difficult to measure small changes in urea (a few mmol l^{-1}) against the high background starting concentration of intestinal fluid or chyme (~350 mmol l^{-1}), especially when there is a high protein background, as in chyme or slurries of intestinal epithelial cells. Similarly, increases in ammonia concentration can only be measured enzymatically by the glutamic dehydrogenase/NAD method (Mondzac et al., 1965) when substantial protein is present, and this assay is less precise than the colorimetric assays traditionally used for water and salines. An additional problem is that urea and ammonia could be produced metabolically by the fish cells or microbial flora. Therefore, we developed a method based on the hydrolysis of added [^{14}C]urea to [^{14}C]CO_2. The [^{14}C]CO_2 was captured in an alkaline trap, using a setup modeled after that of Wood and Perry (1991), which was developed to measure the production of [^{14}C]CO_2 from [^{14}C]HCO_3 by blood. We included an acidification step (described below) to ensure that the [^{14}C]CO_2 produced, much of which would be in the form of [^{14}C]HCO_3 at the pH (6.6) of the assay medium, would be evolved into the overlying atmosphere and captured by the alkaline trap. The acidification lowered the pH to <2.0, thereby stopping the urease reaction and driving off the [^{14}C]CO_2 from solution. In preliminary tests, we spiked the assay medium with 0.1 μCi ml^{-1} (3.7 kBq ml^{-1}) of sodium [^{14}C]bicarbonate (Amersham, Little Chalfont, UK) to check the efficiency of trapping. [^{14}C]CO_2 trapping was independent of time after acidification (15, 30 and 60 min), with essentially all radioactivity removed from the assay medium and captured in the trap (average efficiency=104.5±7.6%, N=9). Therefore a post-acidification trapping period of 30–45 min was routinely used, and no correction was made for trapping efficiency. We also confirmed that this acidification step in itself caused no hydrolysis of [^{14}C]urea to [^{14}C]CO_2.

Commercial 'jack bean' urease (Type III powder; Sigma-Aldrich) was employed to evaluate the time course of the assay, and to compare the rate of urea lysis measured by [^{14}C]CO_2 trapping with that measured simultaneously by colorimetric assays of urea disappearance (Rahmatullah and Boyde, 1980) or ammonia appearance (2 ammonia per urea; Verdouw et al., 1978) in the assay medium. Final assay conditions as outlined below were used, but with the substitution of 100 μl of assay medium containing nominally 1 unit of urease activity for the experimental sample. The assay was stopped at 15, 30, 60 and 120 min. As shown in Fig. 1, the [^{14}C]urea assay was essentially linear up to 120 min, and showed better linearity than the delta urea or delta ammonia measurements, though there was reasonable overall agreement between the three.

In the final assay protocol employed, 1 ml of a simple saline (350 mmol l^{-1} urea, 300 mmol l^{-1} NaCl, pH≈6.6), duplicating the normal levels of these components measured in dogfish intestinal fluid or chyme (Wood et al., 2007b), was employed as the assay medium, and 20-ml glass scintillation vials were used as the assay vessels. The vials were incubated in a 12°C water bath mounted on top of a rotating platform shaker set at 100 cycles min^{-1}. Each vial

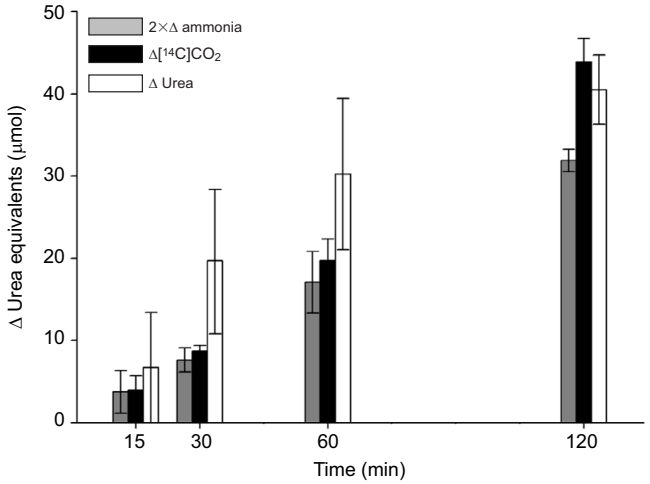

Fig. 1. A validation test of the [^{14}C]urea-based assay for urease activity. Commercial 'jack bean' urease (nominally 1 unit of activity) was run under the same conditions as the experimental samples. Production of [^{14}C]CO_2 at various times up to 120 min is compared with production of 2× ammonia (because of 2 ammonias produced per urea degraded) and with disappearance of urea measured in the same assay samples. Means and ranges of three independent samples for production of [^{14}C]CO_2, and of two independent samples for ammonia production and urea disappearance are shown. Note the greater linearity and proportionality of [^{14}C]CO_2 production in comparison to the other two indices.

could be sealed with a rubber septum from which was suspended a plastic well, containing a Whatman GF/A 2.4-cm glass micro-fiber filter (GE Health Care Life Sciences) folded into a fluted cone and saturated with 150 µl of 1 mol l^{-1} NaOH to serve as a [^{14}C]CO_2 trap. The assay medium had been air-saturated prior to use, and was spiked with 1 µCi ml^{-1} of [^{14}C]urea (37 kBq ml^{-1}). The added radiolabeled urea (Perkin Elmer) was of very high specific activity (57 mCi $mmol^{-1}$), so this addition caused negligible change in the total urea concentration.

Chyme, intestinal fluid and bile samples were quickly mixed by vortexing, and then 100 µl of the test fluid was added to the assay vial, which was then capped and thermostatted to 12°C. For intestinal epithelia, 100–200 mg of slurry (exact mass measured) was added to the assay vial. For blanks, 100 µl of non-labelled assay medium was added. Each sample was assayed in triplicate (i.e. three separate vials) and the results were averaged. For each vial, the assay started with the addition of 1 ml of the radiolabeled assay medium, and then the cap was replaced by the rubber septum with the suspended [^{14}C]CO_2 trap. Starts were staggered by 2-min intervals to allow time for processing of each vial, and the assay ran for 60 min. At the end of 60 min, 100 µl of 8% $HClO_3$ was injected through the septum to acidify the solution and abolish the urease activity, and then the samples were incubated with continued shaking for a further 30–45 min to ensure that all [^{14}C]CO_2 was trapped. At final sampling, the filters were removed from all vials and assayed immediately for [^{14}C] radioactivity. Additionally, multiple 100-µl aliquots of the common assay medium were also assayed.

Urease activity (µmol urea hydrolyzed ml^{-1} h^{-1} or µmol urea g^{-1} h^{-1}) was calculated from the cpm measured in the filter trap (cpm_t), corrected for the cpm measured in the filter blank (cpm_b), divided by the initial specific activity (SA=cpm µmol^{-1} urea) of the assay solution, the volume (V, in ml) of the sample and time (T, in h). The SA was based on the measured radioactivity of the assay medium and the assumption, using measured urea concentrations in the various fluids, that the concentration of urea in the assay medium (350 mmol l^{-1}) was proportionately changed by the small sample addition (10–20%). Therefore, urease activity was expressed as urea hydrolysis per hour per milliliter of chyme, intestinal fluid, bile or intestinal epithelial cells:

$$\text{Urease activity} = \frac{[\text{cpm}_t - \text{cpm}_b]}{\text{SA} \times V \times T}. \quad (2)$$

The detection limit of the assay was approximately 0.03 µmol ml^{-1} h^{-1}.

In 2018, three potential treatments for urease inhibition were evaluated, using intestinal chyme freshly collected from fed animals. In one series (N=8), using a repeated-measures design, chyme from each animal was tested under control conditions and immediately after addition of 1 mmol l^{-1} acetohydroxamic acid or 1 mmol l^{-1} silver nitrate (both from Sigma-Aldrich). In a second series, again using a paired design, urease activity of chyme from a different set of dogfish (N=6) was measured under control conditions and after 10 min of boiling of the sample in a sealed vial, followed by cooling to 12°C prior to test.

Analytical techniques

All [^{14}C] radioactivity samples (either filters or 100 µl aliquots of fluid) were added to 5 ml of Ultima Gold AB fluor (Perkin Elmer), allowed to settle to minimize chemiluminescence, and then counted on either a Triathler portable counter (Hidex, Helsinki, Finland) or a Tri-Carb 2900TR Liquid Scintillation Analyzer (PerkinElmer, Waltham, MA, USA). Tests showed that quench was constant.

Ammonia concentrations in the mucosal and serosal salines of the *in vitro* gut sac experiments of 2011 and 2018 were measured using a commercial kit (Raichem, San Diego, CA, USA) based on the glutamate dehydrogenase/NAD reaction (Mondzac et al., 1965). This is the preferred method when protein is present in solution. However, the kit was not available at the time of the intestinal fluid sampling studies in 2016, so ammonia was measured using the colorimetric assay of Verdouw et al. (1978) on samples that had been deproteinized with 8% $HClO_3$. Urea was similarly measured on deproteinized samples by the colorimetric assay of Rahmatullah and Boyde (1980).

Statistical analyses

Data have been generally expressed as means±1 s.e.m. (N), where N is the number of animals, though for some parameters, individual values have also been shown to indicate the variability in the data, and ranges are shown in Fig. 1. Data were checked for normality and homogeneity of variance, and where necessary were log or square root transformed to achieve these before one-way ANOVA followed by a Tukey's *post hoc* test. When these could not be achieved, the Kruskal–Wallis ANOVA on ranks was used, with Dunn's *post hoc* test. Specific comparisons employed the Mann–Whitney U-test or Student's two-tailed t-test, paired or unpaired as appropriate, with the Bonferroni correction where necessary for multiple comparisons. GraphPad Prism (La Jolla, CA, USA) was used for all analyses. A significance level of P<0.05 was used throughout.

RESULTS

Dogfish gut sac preparations

To enable comparison with urea handling, the ammonia data (Fig. 2) from the 2011 experiments, which are reported here for the first time, have been plotted in a comparable format to the urea data measured in the same experiments (cf. fig. 1 of Liew et al., 2013). In fasted fish, there were no significant changes in the mucosal ammonia concentration over the 3-h incubation period in gut sacs of stomach 1, stomach 2 and colon (Fig. 2A), and therefore the apparent ammonia flux rates for these sections were negligible (Fig. 2B). In intestinal sacs of fasted fish, mucosal ammonia concentration increased by approximately 1 mmol l^{-1} (Fig. 2A), yielding a small accumulation rate of ammonia (approximately −0.11 µmol cm^{-2} h^{-1}) in the lumen (Fig. 2B). Note that negative values represent accumulation in the lumen, while positive values represent absorption from the lumen, from Eqn 1. In preparations from fed animals, mucosal ammonia concentrations increased significantly over 3 h by 0.5–1.0 mmol l^{-1} in stomach 1, stomach 2 and colon, and by approximately 3 mmol l^{-1} in the intestine (Fig. 2A). Thus in all sections, there was an accumulation of ammonia in the lumen for fed dogfish, which was significantly greater than for fasted dogfish, and this accumulation rate (approximately −0.35 µmol cm^{-2} h^{-1}) was by far the greatest in the intestine (Fig. 2B). When phloretin (0.25 mmol l^{-1}) was included in the mucosal saline of intestinal sac preparations from fed animals, the changes in mucosal concentration (Fig. 2A) and the resulting accumulation rates of ammonia in the lumen over 3 h more than doubled (Fig. 2B).

At face value, the accumulation of ammonia in the mucosal solution would be interpreted as an apparent secretion of ammonia by the gut tissue. However, a more complex explanation is possible. As phloretin is a general blocker of transport processes, these results raised the possibility that the observed increases in mucosal ammonia concentration and apparent secretory fluxes might in fact

Journal of Experimental Biology (2019) 222, jeb194787. doi:10.1242/jeb.194787

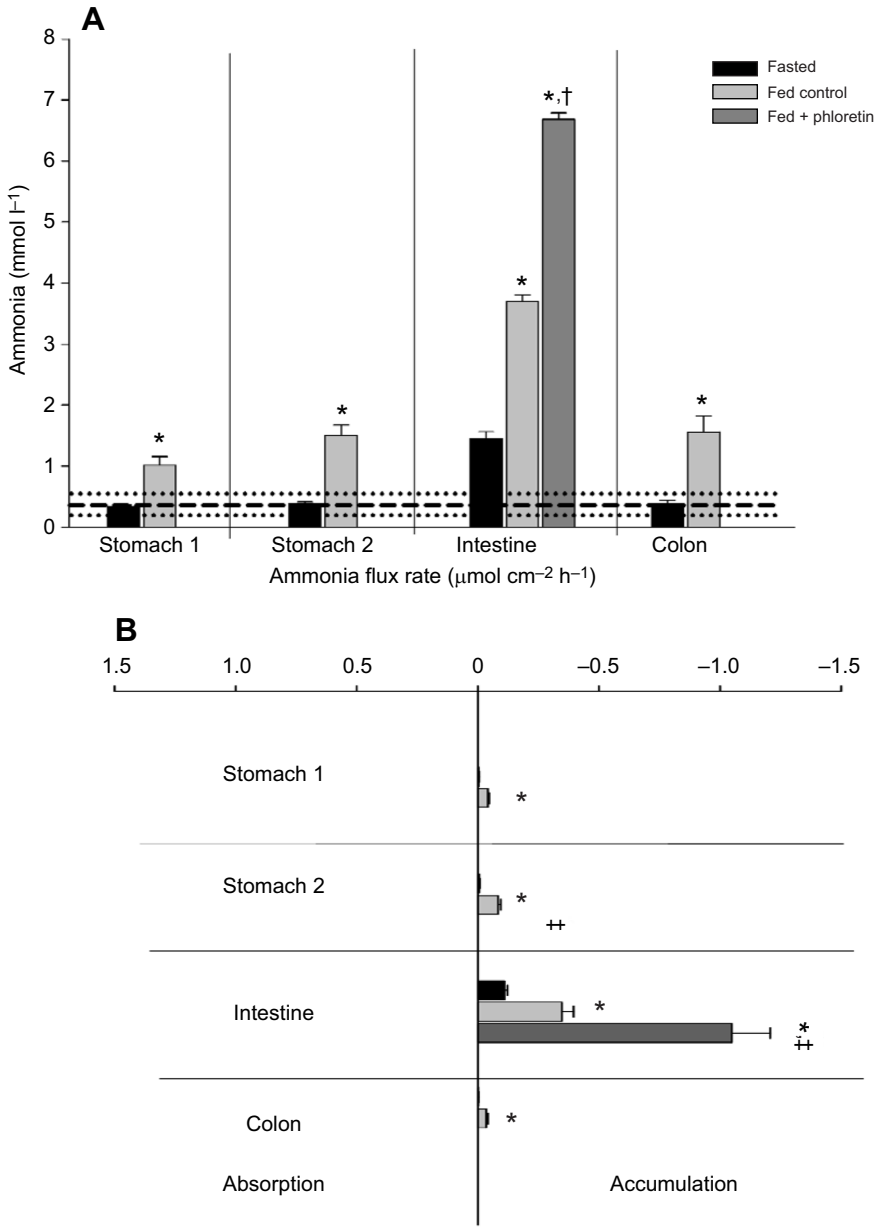

Fig. 2. Apparent ammonia transport by *in vitro* gut sac preparations of the dogfish shark (means±1 s.e.m.) for fasted (*N*=10), fed (*N*=12) and fed preparations treated with 0.25 mmol l^{-1} phloretin in the mucosal saline (*N*=6). Note that phloretin was tested only in the intestine, and only in fed preparations. (A) Final measured mucosal concentrations (mmol l^{-1}) relative to the starting (=serosal) concentration (dashed horizontal line represents mean, dotted horizontal lines represent 1 s.e.m.), indicating the concentration gradients achieved over 3 h. (B) Apparent flux rates (μmol cm^{-2} h^{-1}) per unit surface area (means±1 s.e.m.). Positive flux rates represent net absorption out of the mucosal saline, negative values indicate accumulation rates in the mucosal saline. Asterisk indicates a significant difference (P<0.05) from the fasted mean; double dagger indicates that the fed+phloretin mean is significantly different (P<0.05) from the fed mean. Compare with fig. 1 of Liew et al. (2013), showing a comparable representation of urea transport in these same preparations.

reflect the balance of two simultaneous processes, one that added ammonia to the lumen, and another (sensitive to phloretin) that removed ammonia from the lumen. One possible explanation for the process of adding ammonia could be ammonia production by the activity of microbial urease in the mucosal compartment. Therefore, in 2016, gut sac preparations were made from six additional fed animals, and the urease activity in mucosal fluid after 3 h of incubation was analyzed. While variable, urease activity was detectable in most of the mucosal fluid samples from all sections (Fig. 3A), and tended to be greatest in the intestine, with a mean value of approximately 0.4 μmol ml^{-1} h^{-1} (Fig. 3B). Over 3 h, this would be sufficient to convert 1.2 μmol ml^{-1} of urea to 2.4 μmol ml^{-1} of ammonia. This is in the same range as the increases in ammonia measured in the mucosal fluid in the 2011 experiments, and therefore could explain the accumulation of ammonia in the lumen (Fig. 2A). However, when the measured change in ammonia concentration was regressed against the measured urease activity in the mucosal fluid of the same gut sacs, there was no significant relationship, either for individual sections or for all sections (data not shown: overall r^2=0.06, N=24, n.s.).

Urease activity in freshly collected *in vivo* samples

The 2016 series focused on measurements of urease activity, as well as ammonia and urea concentrations, in freshly collected samples from live animals. Urease activity ranging from 0.1 to 6.3 μmol ml^{-1} h^{-1} was detected in intestinal chyme from 11 fed dogfish (Fig. 4A), with an overall average of approximately 2 μmol ml^{-1} h^{-1} (Fig. 4B). This was significantly higher by approximately 8-fold than the 0.25 μmol ml^{-1} h^{-1} (range 0–0.60 μmol ml^{-1} h^{-1}) recorded in intestinal fluid from eight fasted dogfish. Epithelial tissue also exhibited measurable urease activity in five fed (~0.65 μmol g^{-1} h^{-1}) and 11 fasted dogfish (~0.25 μmol g^{-1} h^{-1}). In a pair-wise comparison, epithelial urease activity was significantly lower than in the chyme of these same fed animals, whereas there was no difference in the fasted animals. There was no detectable urease activity in bile samples from three fed and nine fasted dogfish (data not shown).

Journal of Experimental Biology (2019) 222, jeb194787. doi:10.1242/jeb.194787

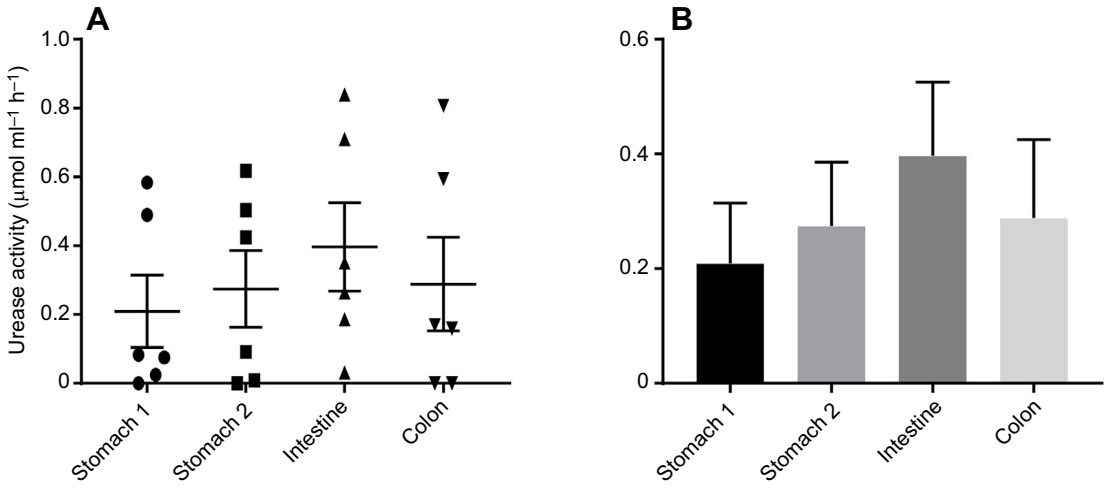

Fig. 3. Urease activity measured in the mucosal saline of gut sac preparations from fed dogfish (*N*=6) after 3 h of incubation *in vitro*. (A) Individual data points. (B) Means±1 s.e.m. There were no significant differences between the gut sections.

Measurements of urease activity in other species have been compared with the dogfish urease data in Fig. 5. As there were no distinct fed or fasted treatments in these other species, the fasted and fed data for dogfish have been pooled in Fig. 5. In the ratfish, which, like the dogfish, is ureotelic, urease activities were detected in intestinal fluid and intestinal epithelia at levels similar to those in the dogfish. Again, the mean activity in the fluid (~1.9 μmol ml^{-1} h^{-1}, *N*=6) was greater than in the intestinal epithelium (~0.45 μmol ml^{-1} h^{-1}, *N*=10), a difference that was significant in a pair-wise comparison. There was no detectable activity in the bile of ratfish (*N*=4; data not shown). In the teleosts, which are ammoniotelic, urease activities were measured only in intestinal fluid, and were extremely low in both flatfish (~0.20 μmol ml^{-1} h^{-1}, *N*=6) and sculpins (~0.30 μmol ml^{-1} h^{-1}, *N*=6).

Inhibition of urease activity

The 2018 series focused on potential blockers of urease activity in dogfish chyme, and the consequences for ammonia handling. Two widely used blockers of urease were employed, as well as simply boiling, the latter to show that the activity being measured originated from an enzyme. Acetohydroxamic acid (10^{-3} mol l^{-1}) significantly reduced urease activity by 51% on a pair-wise basis in chyme from fed dogfish, whereas silver nitrate (10^{-3} mol l^{-1}) had no significant effect (Table 1). Boiling the chyme for 10 min significantly reduced urease activity by 79%; this was not statistically distinguishable from 100% inhibition.

Based on these results, acetohydroxamic acid (10^{-3} mol l^{-1}) was chosen for use in intestinal sac studies. Under control conditions, the increase in ammonia concentration (about 2.4 mmol l^{-1}) in the mucosal fluid of the lumen (Fig. 6A) was very similar to that measured in the earlier studies (cf. Fig. 2A) and therefore the apparent accumulation flux rate (−0.46 μmol cm^{-2} h^{-1}) of ammonia into the lumen (Fig. 6B) was also very similar to that measured earlier (cf. Fig. 2B). However, the presence of 10^{-3} mol l^{-1} acetohydroxamic acid resulted in a net decrease in ammonia concentration in the lumen of approximately 1.5 mmol l^{-1}, and therefore a changeover to an apparent absorption flux of approximately 0.38 μmol cm^{-2} h^{-1}; both changes were highly significant.

Note that these conclusions were based on *N*=5. The results from one pair of preparations were considered as outliers. The intestine of this animal was greatly infected with tapeworms, which were removed in making the sac preparations. In this pair, ammonia accumulation in the mucosal fluid under control conditions was 4-fold higher (−1.80 μmol cm^{-2} h^{-1}) and was greatly inhibited but not reversed by acetohydroxamic acid (−0.22 μmol cm^{-2} h^{-1}).

Urea and ammonia concentrations in intestinal chyme and fluids

Urea concentrations (~385 mmol l^{-1}) in chyme or intestinal fluid did not differ between fed and fasted dogfish (Table 2). These values were more than two orders of magnitude greater than in ammoniotelic flatfish and sculpins (<1 mmol l^{-1}), but were lower than in ureotelic

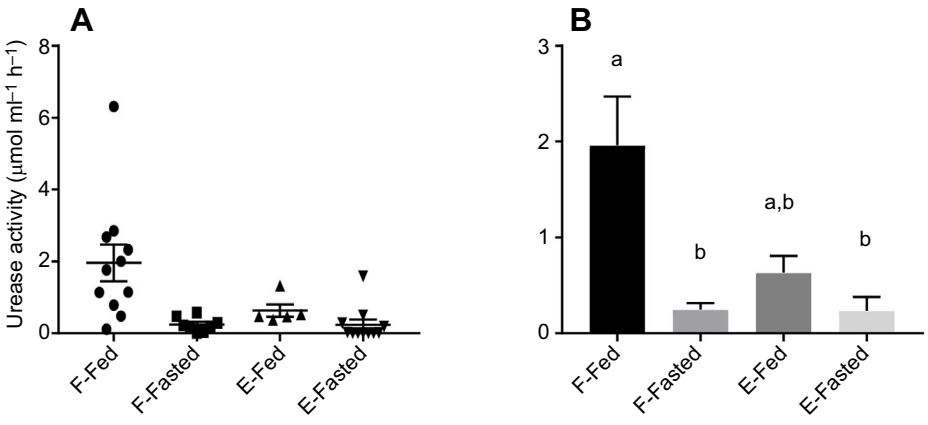

Fig. 4. A comparison of urease activity measured in the chyme/intestinal fluid (F) and in epithelial tissue (E) from fasted and fed dogfish, sampled *in vivo*. *N*=11 for fluid from fed dogfish, *N*=8 for fluid from fasted dogfish, *N*=5 for epithelia from fed dogfish, and *N*=8 for epithelia from fasted dogfish. (A) Individual data points. (B) Means±1 s.e.m. Means sharing the same letter are not significantly different from one another.

Journal of Experimental Biology (2019) 222, jeb194787. doi:10.1242/jeb.194787

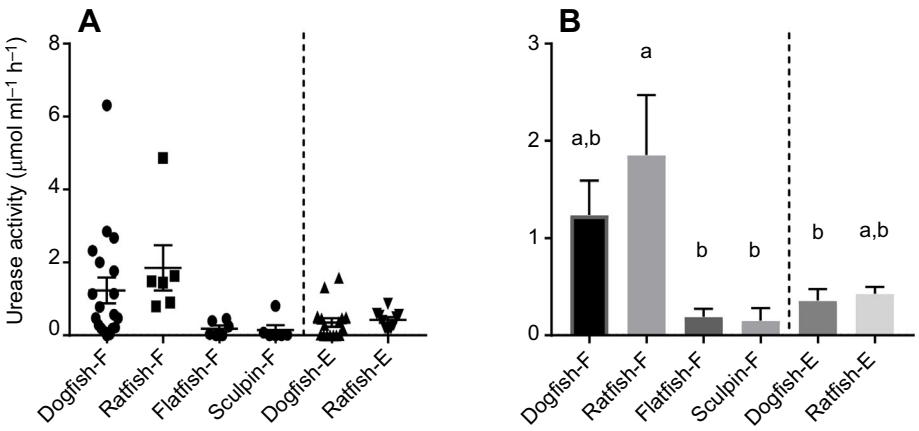

Fig. 5. A comparison of urease activity measured in the chyme/intestinal fluid (F) and in epithelial tissue (E) from dogfish, ratfish, flatfish and sculpin, sampled *in vivo*. As feeding was not controlled for ratfish, flatfish and sculpins, data for fed and fasted dogfish have been combined for comparison. *N*=19 for fluid from dogfish, *N*=6 for fluid from ratfish, *N*=6 for fluid from flounder, *N*=6 for fluid from sculpins, *N*=13 for epithelia from dogfish, and *N*=10 for epithelia from ratfish. (A) Individual data points. (B) Means±1 s.e.m. Means sharing the same letter are not significantly different from one another.

ratfish (~540 mmol l^{-1}). Total ammonia concentrations in intestinal fluids of both dogfish and ratfish (6–9 mmol l^{-1}) were significantly higher than in the teleosts (1–2 mmol l^{-1}). Interestingly, ammonia concentrations were 30% lower in fluids from fed versus fasted dogfish, a significant difference (Table 1). Urea concentrations in dogfish bile (~500 mmol l^{-1}) were higher than in intestinal fluid, whereas ammonia concentrations were much lower (~2 mmol l^{-1}).

DISCUSSION

In total, the present results indicate that there is significant urease activity in the intestine of both the elasmobranch *S. a. suckleyi* and a distant relative, the holocephalan chimaerid *H. colliei* (Figs 4 and 5), and that it produces ammonia from urea for absorption in the digestive tract. This possibility has been proposed previously (Perlman and Goldstein, 1988; Anderson et al., 2010, 2012; Wright and Wood, 2016; Doucette, 2016), and there is some old preliminary evidence that it may occur in elasmobranchs, but this was published only in grey literature abstracts, where the methods are not well described (Lloyd and Goldstein, 1969; Goldstein and DeWitt-Harley, 1972). The possible contribution of intestinal urease activity is generally overlooked in reviews of the feeding dynamics and digestive physiology of elasmobranchs (e.g. Holmgren and Nilsson, 1999; Evans et al., 2004; Cortés et al., 2008; Bucking, 2016). To our knowledge, the present study is the first experimental demonstration. As urease is not thought to occur in any vertebrate (Mobley and Hausinger, 1989), it is very likely that the activity, both in the fluid and in the epithelial tissues, is of microbial origin. What better environment for a ureolytic bacteria than the urea-rich digestive tracts of the Chondrichthyes? Notably, gut urease activity was very variable amongst these wild-caught animals (Figs 3, 4 and 5). The occasional presence of tapeworms (noted in the Results) may have contributed, but variations in the time since feeding and the extent of feeding probably played a more important role. Very likely, the microbial population increases greatly after feeding owing to the input of additional nutrients in the chyme, explaining the significantly increased urease activity at this time, both in the fluid and in the epithelial tissue (Fig. 4). In this regard, fasting for ≥7 days was sufficient to ensure that intestinal absorption processes were complete in *S. a. suckleyi*, based on our previous studies (Wood et al., 2007b, 2010), but we cannot eliminate the possibility that results may have been different had a longer fasting period been employed (Kajimura et al., 2008).

Table 1. The influence of potential inhibitors on urease activity (μmol ml^{-1} h^{-1}) measured in freshly collected chyme from fed dogfish

Treatment	Control	Inhibitor	% Inhibition
Acetohydroxamic acid (10^{-3} mol l^{-1}) (*N*=8)	3.88±0.33	2.02±0.43*	50.8±7.2*
Silver nitrate (10^{-3} mol l^{-1}) (*N*=8)		3.37±0.42	12.7±7.3
Boiling (*N*=6)	2.15±0.18	0.67±0.37*	79.3±15.8*

Means±1 s.e.m. (*N*). Asterisks indicate that inhibitor means are significantly different ($P<0.05$) from respective control means in pair-wise comparisons (Student's *t*-test, with Bonferroni correction where necessary). Note that the boiling tests were performed in a separate trial.

There are previous reports of the occurrence of ureolytic bacteria in several tissues of apparently healthy sharks with a particular focus on their presence in the liver (Grimes et al., 1985; Knight et al., 1988). Therefore, we suspected that the gall-bladder bile, which is synthesized in the liver, might harbour a resident population of microbes. The bile is discharged into the intestine after feeding (Wood et al., 2007b), so this might initiate rapid population growth of microbes in the chyme at this time. However, urease activity was not detected in gall-bladder bile from either dogfish or ratfish, so instead, proliferation of the resident population of microbes associated with the epithelial tissue seems more probable, although input of microbe-rich chyme from the stomach is another possible source (Wood et al., 2007b; Cortés et al., 2008; Bucking, 2016).

Inhibition by boiling (Table 1) was used to demonstrate that the observed ureolytic activity measured in dogfish chyme was of enzymatic origin, and acetohydroxamic acid was employed to more securely identify the activity as urease. Acetohydroxamic acid is a well-known, relatively specific inhibitor of microbial urease (Kobashi et al., 1962; Bremner and Douglas, 1971; Mobley and Hausinger, 1989; Upadhyay, 2012). The reason why it caused only 51% inhibition in the 1-h urease assays (Table 1) may be because its mechanism of action is competitive (i.e. 1 mmol l^{-1} inhibitor versus 350 mmol l^{-1} urea in the assay medium) and time-dependent. Indeed, its inhibitory actions may have been greater in the 3-h intestinal sac incubations. The ineffectiveness of silver nitrate, another fairly potent blocker of microbial urease (Ambrose et al., 1951; Mobley and Hausinger, 1989; Upadhyay, 2012), was undoubtedly due to the high Cl^- concentration (300 mmol l^{-1}) in the assay medium. Only the free silver ion (Ag^+) is involved in urease inhibition, and geochemical speciation analysis using Visual MINTEQ 3.1 (KTH, Stockholm, Sweden) indicates that 1 mmol l^{-1} $AgNO_3$ yields only 0.35 nmol l^{-1} of free Ag^+ ion in solution when 300 mmol l^{-1} Cl^- is present, insufficient to cause significant urease inhibition. Indeed, a visible precipitate formed in the assay medium, identified by Visual MINTEQ as cerargyrite, a silver chloride complex.

We propose that the increase in ammonia concentration in mucosal fluid of gut sac preparations (Fig. 2A), which manifested as

Journal of Experimental Biology (2019) 222, jeb194787. doi:10.1242/jeb.194787

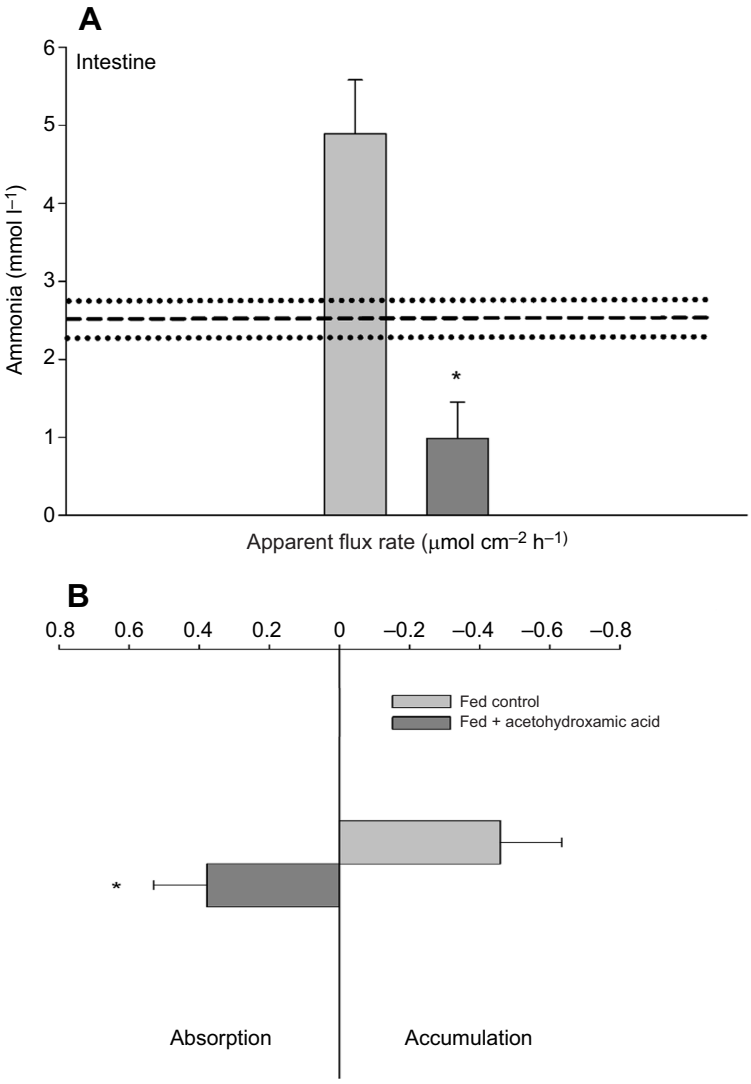

Fig. 6. The effect of the specific urease inhibitor acetohydroxamic acid (10^{-3} mol l^{-1}) on ammonia transport by *in vitro* gut sac preparations of fed dogfish shark (means±1 s.e.m., *N*=5). (A) Final measured mucosal concentrations (mmol l^{-1}) relative to the starting (=serosal) concentration (dashed horizontal line represents mean, dotted horizontal lines represent 1 s.e.m.), indicating the concentration gradients achieved over 3 h. (B) Apparent flux rates (μmol cm^{-2} h^{-1}) per unit surface area (means ±1 s.e.m.). Positive flux rates represent net absorption out of the mucosal saline, negative values indicate accumulation rates in the mucosal saline. Asterisk indicates that the fed+acetohydroxamic acid mean is significantly different (P<0.05) from the fed control mean (N=5, paired design). Compare with Fig. 2.

an apparent secretory flux of ammonia by the gut tissue (Fig. 2B), in fact reflected the conversion of urea to ammonia by urease. There was detectable urease activity in the mucosal fluid of gut sac preparations after 3 h of incubation (Fig. 3), even though the original chyme or intestinal fluid had been rinsed away. This activity probably resulted from proliferation of microbes from the epithelial tissue. The reason why there was no significant relationship between measured urease activity and measured ammonia accumulation amongst gut sac preparations was probably because both ammonia production (by urease) and ammonia absorption were occurring, and that the relative rates and balance points of these two opposing processes varied amongst preparations. We further propose that the marked increase in this ammonia accumulation rate caused by 0.25 mmol l^{-1} phloretin (Fig. 2) in fact resulted from the blockade of an ammonia-absorptive mechanism by this relatively non-specific inhibitor. Indeed, when a specific blocker (acetohydroxamic acid) of urease activity was applied to the intestinal sac preparation in the 2018 incubations, the accumulation rate of ammonia was reversed to an apparent absorption flux. Therefore, the ammonia production rate by urease would have exceeded the ammonia absorption rate by the phloretin-sensitive transporter in the 2011 incubations, and the ammonia absorption rate was revealed by the blockade of urease activity in the 2018 incubations. The original purpose for using phloretin in these experiments was to exploit its well-known ability to block urea transport (Levine et al., 1973; Kato and Sands, 1998; Walsh and Smith, 2001). However phloretin is a relatively non-specific blocker of the transport of a wide variety of other substrates (e.g. K^+, Ca^{2+}, glucose). Recently, it has been shown that ammonia can actually

Table 2. Concentrations of urea and total ammonia in intestinal fluid or chyme samples taken from freshly sampled dogfish, ratfish, flatfish and sculpins

Intestinal fluid or chyme	Urea (mmol l^{-1})	Ammonia (mmol l^{-1})
Dogfish fed (*N*=8)	384.5±18.9[b]	6.03±0.48[a]
Dogfish fasted (*N*=8)	384.7±38.5[b]	8.71±0.39[a,*]
Ratfish (*N*=6)	540.3±36.1[a]	7.33±0.95[a]
Flatfish (*N*=5)	0.70±0.17[c]	1.49±0.22[b]
Sculpins (*N*=6)	0.37±0.04[c]	2.05±0.22[b]
Dogfish bile (*N*=7)	500.9±23.5[a]	1.73±0.29[b]

Bile samples from dogfish are also shown.
Means±1 s.e.m. (*N*). Means not sharing the same letter are significantly different (P<0.05) by one-way ANOVA plus Tukey's test. The data from fed and fasted dogfish were pooled for this test, but are significantly different from each other in a pair-wise comparison (P<0.05, Student's *t*-test) as indicated by the asterisk.

move via the human urea transporter B (UT-B)-facilitated diffusion urea transporter, and that phloretin is actually more effective in blocking this ammonia flux than urea flux through UT-B (Geyer et al., 2013). Facilitated diffusion UT transporters are expressed at the mRNA level in the intestinal tracts of elasmobranchs (also of holocephalans; Anderson et al., 2012) but interestingly, expression levels of Rh glycoproteins (putative selective ammonia transporters) are more than an order of magnitude greater (Anderson et al., 2010). Thus, the proposed blockade of ammonia absorption by phloretin could have resulted from inhibition of UT or Rhesus channels or both.

The present experiments have demonstrated that both ammonia production by urease and ammonia absorption occur in dogfish gut sacs *in vitro*. *In vivo*, ammonia concentrations were 30% lower in intestinal fluids from fed versus fasted dogfish *in vivo* (Table 2). This observation, coupled with the higher urease activity measured in these same samples from fed dogfish (Fig. 4), suggests that the ammonia absorption rate normally keeps pace with or exceeds the ammonia production rate in the intestinal chyme. The lack of blood flow and reduced convective mixing in the lumen in gut sac preparations may have slowed ammonia absorption *in vitro*. Plasma ammonia concentrations increased after feeding in *S. a. suckleyi* (Wood et al., 2005), providing additional evidence of ammonia absorption *in vivo*.

Concentrations of urea measured in the intestinal fluids of both *S. a.s suckleyi* and *H. collei* (as well as in bile of the former) were generally consistent with previous measurements in these same species (Wood et al., 2007b; Anderson et al., 2012), though the present levels of urea in ratfish chyme were somewhat higher (Table 2). The reason for this difference is unknown. Ammonia concentrations in these same fluids from the ureotelic species were substantially higher than in the ammoniotelic teleosts (Table 2), undoubtedly reflecting the production of ammonia from urea. The low ammonia concentration in the gall-bladder bile of dogfish demonstrates that this was not the source of the high intestinal ammonia levels. The low ammonia and urea concentrations in the intestinal fluids of the marine teleosts are in agreement with similar measurements in another marine teleost, the plainfin midshipman (Bucking et al., 2013), as well as in the freshwater rainbow trout (Bucking and Wood, 2012; Rubino et al., 2014). We are aware of no previous reports of ammonia concentrations in intestinal chyme of Chondrichthyes, but the present values (Table 1) are 3- to 4-fold higher than previous measurements in gastric chyme of the dogfish (Wood et al., 2009). Again, this likely reflects ammonia generation by intestinal urease activity.

The relationship between the ureolytic microbes and their host is very likely both symbiotic and mutualistic. The benefits to the microbes is obvious, the benefits to the host more subtle. Urea is a metabolic dead-end in vertebrates; microbial urease circumvents this problem. Urea cannot be used for amino acid synthesis and protein growth, whereas ammonia can be trapped by glutamine synthetase and glutamate dehydrogenase, and in turn, the amino groups can be used to synthesize other amino acids by transamination. Enzymatic activities of glutamine synthetase, glutamate dehydrogenase and alanine aminotransferase are very high in the intestine of *S. a. suckleyi* relative to other tissues (Kajimura et al., 2006), and the affinity of elasmobranch glutamine synthetase for ammonia is particularly high (Shankar and Anderson, 1985). Additionally, the key enzymes of the ornithine urea cycle, which is the pathway by which elasmobranchs synthesize urea, are also expressed in the intestine, as well as in the muscle and liver (Kajimura et al., 2006). The presence of separate pathways for synthesizing both amino acids and urea in the intestine gives the host the flexibility of apportioning scavenged ammonia into each pathway according to needs.

Quantitatively, the amount of nitrogen reabsorbed as ammonia by this mechanism may be very important. Based on the data in Fig. 4, urease activity in the chyme fluid of a fed animal would create 4 μmol ml^{-1} $h^{-1}$of ammonia in the intestinal chyme fluid, where the volume *in vivo* is typically approximately 7 ml kg^{-1} (Wood et al., 2007b), i.e. 28 μmol kg^{-1} h^{-1}. To this we can add about the same amount generated by urease activity in the intestinal epithelial tissue. Therefore, the intestine alone can reabsorb ammonia-N at a rate equivalent to approximately half the normal loss rate of ammonia-N across the gills after feeding (Kajimura et al., 2006; Wood et al., 2007a), and urease activity in other sections of the tract (Fig. 3) may further add to this total. By way of comparison, the estimated rate of recycling of urea-N by direct reabsorption across the intestine in fed dogfish is approximately 750 μmol urea-N kg^{-1} h^{-1} (Liew et al., 2013), approximately 13-fold greater than the estimated 56 μmol ammonia-N kg^{-1} h^{-1} recycled by absorption in the present study. *Squalus acanthias suckleyi* is capable of surviving long periods of fasting during which muscle protein stores are degraded in order to maintain internal urea concentrations for osmotic homeostasis (Kajimura et al., 2008; Wood et al., 2010). These absorptive mechanisms, and precise regulation of how much ammonia-N goes back into urea synthesis versus into amino acid synthesis, may become particularly important at this time. Thus ammonia appears to be scavenged at the gut, as well as at the gills of dogfish sharks (Nawata et al., 2015; Wood and Giacomin, 2016). It is interesting that these primitive ureotelic fishes appear to use exactly the same mechanism in their digestive tract to conserve nitrogen and maintain amino acid synthesis as do hibernating frogs (Wiebler et al., 2018) and bears (Stenvinkel et al., 2013). Indeed, Singer (2003) has proposed that this may be a universal strategy for nitrogen conservation in ureotelic vertebrates.

The results of the present study suggest several future directions. Although microbial counts alone may not be particularly useful because ureolytic activity will depend on the species and physiological state of the bacteria, a metagenomic analysis of the microbial population in the dogfish digestive tract and how it changes with feeding and fasting may prove to be very informative. The impact on microbial urease activity of experimentally enriching the digestive tract with urea in elasmobranchs, as well as in teleosts, will be interesting. The strategy of 'hunt warm, rest cool' has been shown to be energetically advantageous in many aquatic ectothermic predators, and may explain the diel vertical migrations of a variety of freshwater and marine organisms, including some elasmobranchs such as the European dogfish (*Scyliorhinus canicula*) (e.g. Sims et al., 2006; Di Santo and Bennett, 2011). It is not known whether it applies to the two Chondrichthyes species of the present study, but in this regard, it would be interesting to investigate whether the thermal sensitivity (i.e. Q_{10}) of the microbial urease activity is co-ordinated with that of intestinal absorption. Additionally, as the present investigation focused exclusively on male dogfish, in future it would be of interest to investigate whether nitrogen recycling in the intestine is qualitatively or quantitatively different in females, especially prenatal ones, which have special osmoregulatory needs (Kormanik, 1992). Finally, elasmobranchs exhibit a wide variety of dietary habits ranging from strict carnivory to omnivory and planktivory (Cortés et al., 2008; Bucking, 2016), which likely differ in their ability to provide nitrogen. A comparative examination of the role of intestinal urease across these different trophic strategies would be of great interest.

Acknowledgements
We thank the research co-ordinators at BMSC, Dr Bruce Cameron (2011) and Dr Eric Clelland (2016, 2018), without whose help this research would have been impossible. Dr Katie Gilmour kindly loaned materials. Marina Giacomin, Ellen Jung, Dr Lygia Noguiera, Dr Anne Cremazy, Dr Courtney Deck, Justin Feilberg, Dr Sunita Nadella and Dr Ora Johannsson are also thanked for their assistance.

Competing interests
The authors declare no competing or financial interests.

Author contributions
C.M.W. designed the study. C.M.W. and H.J.L. performed the experiments. H.J.L., J.L.H., G.D.B. and W.G.A. obtained the samples and provided advice. C.M.W. wrote the manuscript, and all authors edited it.

Funding
Supported by Natural Sciences and Engineering Research Council of Canada (NSERC) Discovery Grants to C.M.W. (RGPIN-2017-03843, RGPIN/473-2012) and W.G.A. (RGPIN-2015-05348), an International Collaboration Grant (IWS-BOF) issued by the Research Council of the University of Antwerp to G.D.B. and C.M.W., and by an award (104519-011) from the International Development Research Centre (IDRC, Ottawa, Canada) to C.M.W. and Dr Adalto Bianchini. H.J.L. was supported by the Ministry of Higher Education, Malaysia, and Universiti Malaysia Terengganu. J.H.L. was supported by an NSERC Postgraduate Scholarship D in 2018. C.M.W. was supported by the Canada Research Chairs Program (950-203776).

References

Ambrose, J. F., Kistiakowsky, G. B. and Kridl, A. G. (1951). Inhibition of urease by silver ions. *J. Am. Chem. Soc.* **73**, 1232-1236.
Anderson, W. G., Dasiewicz, P. J., Liban, S., Ryan, C., Taylor, J. R., Grosell, M. and Weihrauch, D. (2010). Gastro-intestinal handling of water and solutes in three species of elasmobranch fish, the white-spotted bamboo shark, *Chiloscyllium plagiosum*, little skate, *Leucoraja erinacea* and the clear nose skate *Raja eglanteria*. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **155**, 493-502.
Anderson, W. G., Nawata, C. M., Wood, C. M., Piercey-Normore, M. D. and Weihrauch, D. (2012). Body fluid osmolytes and urea and ammonia flux in the colon of two chondrichthyan fishes, the ratfish, *Hydrolagus colliei*, and spiny dogfish, *Squalus acanthias*. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **161**, 27-35.
Anderson, W. G., McCabe, C., Brandt, C. and Wood, C. M. (2015). Examining urea flux across the intestine of the spiny dogfish, *Squalus acanthias*. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **181**, 71-78.
Ballantyne, J. S. (2016). Some of the most interesting things we know, and don't know, about the biochemistry and physiology of elasmobranch fishes (sharks, skates and rays). *Comp. Biochem. Physiol.* **199**, 21-28.
Boylan, J. W. (1967). Gill permeability in *Squalus acanthias*. In *Sharks, Skates, and Rays* (ed. P. W. Gilbert, R. F. Mathewson and D. P. Rall), pp. 197-206. Baltimore: John Hopkins Press.
Boylan, J. W. (1972). A model for passive urea reabsorption in the elasmobranch kidney. *Comp. Biochem. Physiol. A. Physiol.* **42**, 27-30.
Bremner, J. M. and Douglas, L. A. (1971). Inhibition of urease activity in soils. *Soil Biol. Biochem.* **3**, 297-307.
Bucking, C. (2016). Feeding and digestion. In *Physiology of Elasmobranch Fishes* (Fish Physiology Series, Vol. 34B) (ed. R. Shadwick, A. P. Farrell and C. J. Brauner), pp. 347-394. San Diego: Elsevier Inc.
Bucking, C. and Wood, C. M. (2012). Digestion of a single meal affects gene expression of ion and ammonia transporters and glutamine synthetase activity in the gastrointestinal tract of freshwater rainbow trout. *J. Comp. Physiol. B* **182**, 341-350.
Bucking, C., LeMoine, C. M. R., Craig, P. M. and Walsh, P. J. (2013). Nitrogen metabolism of the intestine during digestion in a teleost fish, the plainfin midshipman (*Porichthys notatus*). *J. Exp. Biol.* **216**, 2821-2832.
Burger, J. W. and Hess, W. N. (1960). Function of the rectal gland in the spiny dogfish. *Science* **131**, 670-671.
Cortés, E., Papastamatiou, Y. P., Carlson, J. K., Ferry-Graham, L., Wetherbee, B. M., Cyrino, J. E. P., Bureau, D. P. and Kapoor, B. G. (2008). An overview of the feeding ecology and physiology of elasmobranch fishes. In *Feeding and Digestive Functions of Fishes* (ed. J. E. P. Cyrino, D. Bureau and B. G. Kapoor), pp. 393-443, Boca Raton: CRC Press.
Di Santo, V. and Bennett, W. A. (2011). Is post-feeding thermotaxis advantageous in elasmobranch fishes? *J. Fish Biol.* **78**, 195-207.
Doucette, K. K. (2016). The elasmobranch–microbe relationship: trimethylamine oxide synthesis, urea hydrolysis, and microbe–osmolyte interactions in the Atlantic stingray, *Dasyatis sabina*. *MSc thesis*, University of Southern Mississippi, USA.
Evans, D. H., Piermarini, P. M. and Choe, K. P. (2004). Homeostasis: osmoregulation, pH regulation, and nitrogen excretion. In *Biology of Sharks and their Relatives* (ed. J. C. Carrier, J. A. Musick and M. R. Heithaus), pp. 247-268. Boca Raton: CRC Press.
Fines, G. A., Ballantyne, J. S. and Wright, P. A. (2001). Active urea transport and an unusual basolateral membrane composition in the gills of a marine elasmobranch. *Am. J. Physiol.* **280**, R16-R24.
Forster, R. P. and Berglund, F. (1957). Contrasting inhibitory effects of probenecid on the renal tubular excretion of p-aminohippurate and on the active reabsorption of urea in the dogfish, *Squalus acanthias*. *J. Cell. Comp. Physiol.* **49**, 281-285.
Geyer, R. R., Musa-Aziz, R., Enkavi, G., Mahinthichaichan, P., Tajkhorshid, E. and Boron, W. F. (2013). Movement of NH_3 through the human urea transporter B: a new gas channel. *Am. J. Physiol.* **304**, F1447-F1457.
Givens, C. E., Ransom, B., Bano, N. and Hollibaugh, J. T. (2015). Comparison of the gut microbiomes of 12 bony fish and 3 shark species. *Mar. Ecol. Prog. Ser.* **518**, 209-223.
Goldstein, L. and Dewitt-Harley, S. (1972). The role of intestinal bacteria in urea metabolism by the skate, *Raja erinacea*. *Bull. Mt. Desert Is. Biol. Lab.* **12**, 37-39.
Grimes, D. J., Brayton, P., Colwell, R. R. and Gruber, S. H. (1985). Vibrios as autochthonous flora of neritic sharks. *Syst. Appl. Microbiol.* **6**, 221-226.
Hays, R. M., Levine, S. D., Myers, J. D., Heinemann, H. O., Kaplan, M. A., Franki, N. and Berliner, H. (1977). Urea transport in the dogfish kidney. *J. Exp. Zool.* **199**, 309-315.
Hill, W. G., Mathai, J. C., Gensure, R. H., Zeidel, J. D., Apodaca, G., Saenz, J. P., Kinne-Saffran, E., Kinne, R. and Zeidel, M. L. (2004). Permeabilities of teleost and elasmobranch gill apical membranes: evidence that lipid bilayers alone do not account for barrier function. *Am. J. Physiol.* **287**, C235-C242.
Holmgren, S. and Nilsson, S. (1999). Digestive system. In *Sharks, Skates, and Rays* (ed. W. C. Hamlett), pp. 144-173. Baltimore: Johns Hopkins University Press.
Hyodo, S., Bell, J. D., Healy, J. M., Kaneko, T., Hasegawa, S., Takei, Y., Donald, J. A. and Toop, T. (2007). Osmoregulation in elephant fish *Callorhinchus milii* (Holocephali), with special reference to the rectal gland. *J. Exp. Biol.* **210**, 1303-1310.
Jones, B. C. and Geen, G. H. (1977). Food and feeding of spiny dogfish (*Squalus acanthias*) in British Columbia waters. *J. Fish. Res. Board Can.* **34**, 2056-2066.
Kajimura, M., Walsh, P. J., Mommsen, T. P. and Wood, C. M. (2006). The dogfish shark (*Squalus acanthias*) increases both hepatic and extrahepatic ornithine urea cycle enzyme activities for nitrogen conservation after feeding. *Physiol. Biochem. Zool.* **79**, 602-613.
Kajimura, M., Walsh, P. J. and Wood, C. M. (2008). The spiny dogfish *Squalus acanthias* L. maintains osmolyte balance during long-term starvation. *J. Fish. Biol.* **72**, 656-670.
Kato, A. and Sands, J. M. (1998). Active sodium-urea counter-transport is inducible in the basolateral membrane of rat renal initial inner medullary collecting ducts. *J. Clin. Invest.* **102**, 1008-1015.
Kempton, R. T. (1953). Studies on the elasmobranch kidney II. Reabsorption of urea by the smooth dogfish, *Mustelus canis*. *Biol. Bull.* **104**, 45-56.
Knight, I. T., Grimes, D. J. and Colwell, R. R. (1988). Bacterial hydrolysis of urea in the tissues of carcharhinid sharks. *Can. J. Fish Aquat. Sci.* **45**, 357-360.
Kobashi, K., Hase, J. and Uehara, K. (1962). Specific inhibition of urease by hydroxamic acid. *Biochem. Biophys. Acta,* **65**, 380-383.
Kormanik, G. A. (1992). Ion and osmoregulation in prenatal elasmobranchs: evolutionary implications. *Am. Zool.* **32**, 294-302.
Levine, S., Franki, N. and Hays, R. M. (1973). Effect of phloretin on water and solute movement in the toad bladder. *J. Clin. Invest.* **52**, 1435-1442.
Liew, H. J., De Boeck, G. and Wood, C. M. (2013). An *in vitro* study of urea, water, ion, and CO_2/HCO_3^- transport in the gastrointestinal tract of the dogfish shark (*Squalus acanthias*): the influence of feeding. *J. Exp. Biol.* **216**, 2063-2072.
Lloyd, K. W. and Goldstein, L. (1969). Permeability and metabolism of urea in the intestine of the elasmobranch, *Squalus acanthias*. *Bull. Mt. Desert Is. Biol. Lab.* **9**, 22-23.
Mobley, H. L. and Hausinger, R. P. (1989). Microbial ureases: significance, regulation, and molecular characterization. *Microbiol. Rev.* **53**, 85-108.
Mondzac, A., Ehrlich, G. E. and Seeg-Miller, J. E. (1965). An enzymatic determination of ammonia in biological fluids. *J. Lab. Clin. Med.* **66**, 526-531.
Nawata, C. M., Walsh, P. J. and Wood, C. M. (2015). Physiological and molecular responses of the spiny dogfish shark (*Squalus acanthias*) to high environmental ammonia: scavenging for nitrogen. *J. Exp. Biol.* **218**, 238-248.
Pärt, P., Wright, P. A. and Wood, C. M. (1998). Urea and water permeability in dogfish (*Squalus acanthias*) gills. *Comp. Biochem. Physiol.* **119A**, 117-123.
Perlman, D. F. and Goldstein, L. (1988). Nitrogen metabolism. In *Physiology of Elasmobranch Fishes* (ed. T. J. Shuttleworth), pp. 253-275. New York: Springer-Verlag.
Rahmatullah, M. and Boyde, T. R. C. (1980). Improvements in the determination of urea using diacetyl monoxime; methods with and without deproteinisation. *Clin. Chim. Acta.* **107**, 3-9.
Rubino, J. G., Zimmer, A. M. and Wood, C. M. (2014). An *in vitro* analysis of intestinal ammonia handling in fasted and fed freshwater rainbow trout (*Oncorhynchus mykiss*). *J. Comp. Physiol. B.* **184**, 91-105.

Rubino, J. G., Zimmer, A. M. and Wood, C. M. (2015). Intestinal ammonia transport in freshwater and seawater acclimated rainbow trout (*Oncorhynchus mykiss*): evidence for a Na+ coupled uptake mechanism. *Comp. Biochem. Physiol. A* **183**, 45-56.

Schmidt-Nielsen, B. and Rabinowitz, L. (1964). Methylurea and acetamide: active reabsorption by elasmobranch renal tubules. *Science* **146**, 1587-1588.

Schmidt-Nielsen, B., Truniger, B. and Rabinowitz, L. (1972). Sodium-linked urea transport by the renal tubule of the spiny dogfish, *Squalus acanthias*. *Comp. Biochem. Physiol.* **42A**, 13-25.

Shankar, R. A. and Anderson, P. M. (1985). Purification and properties of glutamine synthetase from liver of *Squalus acanthias*. *Arch. Biochem. Biophys.* **239**, 248-259.

Sims, D. W., Wearmouth, V. J., Southall, E. J., Hill, J. M., Moore, P., Rawlinson, K., Hutchinson, N., Budd, G. C., Righton, D., Metcalfe, J. D. et al. (2006). Hunt warm, rest cool: bioenergetic strategy underlying diel vertical migration of a benthic shark. *J. Anim. Ecol.* **75**, 176-190.

Singer, M. A. (2003). Do mammals, birds, reptiles and fish have similar nitrogen conserving systems? *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* **134**, 543-558.

Smith, H. W. (1929). The composition of body fluids of elasmobranchs. *J. Biol. Chem.* **81**, 407-419.

Smith, H. W. (1936). The retention and physiological role of urea in the elasmobranchii. *Biol. Rev.* **11**, 49-82.

Stenvinkel, P., Jani, A. H. Johnson, R. J. (2013). Hibernating bears (Ursidae): metabolic magicians of definite interest for the nephrologist. *Kidney Int.* **83**, 207-212.

Upadhyay, L. S. B. (2012). Urease inhibitors: a review. *Ind. J. Biotechnol.* **11**, 381-388.

Verdouw, H., van Echteld, C. J. A. and Dekkers, E. M. J. (1978). Ammonia determination based on indophenol formation with sodium salicylate. *Water Res.* **12**, 399-402.

Walsh, P. J. and Smith, C. P. (2001). Physiology of urea transport in fish. In *Nitrogen Excretion, Fish Physiology*, Vol. 20 (ed. P. A. Wright and P. M. Anderson), pp. 289-295. New York: Academic Press.

Wiebler, J. M., Kohl, K. D., Lee, R. E. and Costanzo, J. P. (2018). Urea hydrolysis by gut bacteria in a hibernating frog: evidence for urea-nitrogen recycling in Amphibia. *Proc. R. Soc. B* **285**, 20180241.

Wood, C. M. and Giacomin, M. (2016). Feeding through your gills and turning a toxicant into a resource: how the dogfish shark scavenges ammonia from its environment. *J. Exp. Biol.* **219**, 3218-3226.

Wood, C. M. and Perry, S. F. (1991). A new *in vitro* assay for carbon dioxide excretion by trout red blood cells: effects of catecholamines. *J. Exp. Biol.* **157**, 349-366.

Wood, C. M., Pärt, P. and Wright, P. A. (1995). Ammonia and urea metabolism in relation to gill function and acid-base balance in a marine elasmobranch the spiny dogfish (*Squalus acanthias*). *J. Exp. Biol.* **198**, 1545-1558.

Wood, C. M., Kajimura, M., Mommsen, T. P. and Walsh, P. J. (2005). Alkaline tide and nitrogen conservation after feeding in an elasmobranch (*Squalus acanthias*). *J. Exp. Biol.* **208**, 2693-2705.

Wood, C. M., Bucking, C., Fitzpatrick, J. and Nadella, S. R. (2007a). The alkaline tide goes out and the nitrogen stays in after feeding in the dogfish shark, *Squalus acanthias*. *Respir. Physiol. Neurobiol.* **159**, 163-170.

Wood, C. M., Kajimura, M., Bucking, C. P. and Walsh, P. J. (2007b). Osmoregulation, ionoregulation and acid-base regulation by the gastrointestinal tract after feeding in the elasmobranch (*Squalus acanthias*). *J. Exp. Biol.* **210**, 1335-1349.

Wood, C. M., Schultz, A. G., Munger, R. S. and Walsh, P. J. (2009). Using omeprazole to link the components of the post-prandial alkaline tide in the spiny dogfish, *Squalus acanthias*. *J. Exp. Biol.* **212**, 684-692.

Wood, C. M., Walsh, P. J., Kajimura, M., McClelland, G. B. and Chew, S. F. (2010). The influence of feeding and fasting on plasma metabolites in the dogfish shark (*Squalus acanthias*). *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **155A**, 435-444.

Wood, C. M., Liew, H. J., De Boeck, G. and Walsh, P. J. (2013). A perfusion study of the handling of urea and urea analogues by the gills of the dogfish shark (*Squalus acanthias*). *Peer J* **1**, e33.

Wright, P. A. and Wood, C. M. (2016). Regulation of ions, acid-base, and nitrogenous wastes in elasmobranch fishes. In: *Fish Physiology, Vol 34B: Physiology of Elasmobranch Fishes: Internal Processes* (ed. R. E. Shadwick, A. P. Farrell and C. J. Brauner), pp. 279-345. San Diego: Academic Press.

Zeidel, J. D., Mathai, J. C., Campbell, J. D., Ruiz, W. G., Apodaca, G. L., Riordan, J. and Zeidel, M. L. (2005). Selective permeability barrier to urea in shark rectal gland. *Am. J. Physiol.* **289**, F83-F89.