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The effects of digesting a urea-rich meal on North Pacific spiny dogfish (*Squalus acanthias suckleyi*)

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ABSTRACT

Marine elasmobranchs are nitrogen-limited owing to the requirement of nitrogen for both somatic growth and urea-based osmoregulation, and due to the loss of urea across the gills and kidney as nitrogenous waste. In this study we used *in vitro* stomach and intestinal gut sacs to investigate the effects of consuming a urea-rich meal (700 mM within a 2% body-mass ration of food-slurry) on nitrogen movement across the gastrointestinal (GI) tract of North Pacific spiny dogfish (*Squalus acanthias suckleyi*). Plasma urea concentrations did not differ between fasted (359 ± 19 mM), urea-poor fed (340 ± 16 mM), and urea-rich fed (332 ± 24 mM) dogfish. Interestingly, *in vitro* gut sacs of urea-rich fed dogfish showed no net urea absorption from the lumen over 3 h incubation, which contrasts previously published data on urea-poor fed dogfish that absorb urea from the lumen. In addition, ammonium (NH_4^+) concentration within the gut sac intestinal lumen significantly increased from 0.62 to 4.35 mM over 3 h. This is likely due to a combination of tissue production and microbial urease activity in the intestine. The overall results highlight the ability of *S. a. suckleyi* to regulate and maintain internal nitrogen concentrations despite the addition of excess dietary urea.

1. Introduction

Central to feeding in vertebrates is the involvement of the gastrointestinal (GI) tract and its role in absorbing nutrients. Recent marine elasmobranch feeding studies have examined post-prandial uptake across the GI tract and the systemic physiological consequences in both voluntary and force-fed elasmobranchs (Anderson et al., 2015, 2012; Kajimura et al., 2006; Liew et al., 2013; Wood et al., 2019, 2010, 2007b, 2007a, 2005). Cumulatively, these studies have shed light on the role the GI tract plays in the homeostatic control of acid-base balance, osmoregulation, and the uptake of nutrients such as nitrogen (Bucking, 2015).

As sporadic and opportunistic feeders marine elasmobranchs are considered nitrogen-limited due to obligatory nitrogen requirements for both somatic growth and urea-based osmoregulation; this nitrogen-limited nature is compounded by urea loss across the gills and kidney as nitrogenous waste (Ballantyne, 2016; Smith, 1931; Wood et al., 2005). To counterbalance the loss it has been hypothesized that the elasmobranch GI tract plays a critical role in nitrogen acquisition and conservation (Kajimura et al., 2006; Wood et al., 2005). In North Pacific spiny dogfish (*Squalus acanthias suckleyi*) feeding studies (*i.e.* nitrogen acquisition), post-prandial urea and ammonia levels peaked in plasma samples collected after 20 h digestion (Kajimura et al., 2008, 2006; Wood et al., 2010, 2007b). Also after feeding, the GI tract of *S. a. suckleyi* absorbs urea from the lumen, as assayed using *in vitro* gut sac preparations of the cardiac and pyloric stomachs, colon, and particularly the intestine (Liew et al., 2013). This net intestinal urea absorption in fed dogfish has also been confirmed in Ussing chamber flux studies with ^{14}C-urea (Anderson et al., 2015). In contrast, intestinal gut sacs from fasted dogfish showed a net efflux (*i.e.* accumulation) of urea into the lumen (Liew et al., 2013). This efflux was mirrored using intestinal tissue from fasted little skate (*Leucoraja erinacea*) and *S. a. suckleyi* mounted in Ussing chambers, which showed a net accumulation of ^{14}C-urea in the lumen (Anderson et al., 2015, 2010). In contrast, colonic tissue mounted in Ussing chambers showed resistance to ^{14}C-urea movement (Anderson et al., 2012). *In vivo* urea concentrations (< 25 mM) in the colon of three elasmobranch species (*L. erinacea*; white-spotted bamboo shark, *Chiloscyllium plagiosum*; and, clear nose skate, *Raja eglanteria*) were shown to be significantly less than the urea concentrations of the intestinal fluid (> 185 mM) and plasma

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(> 270 mM) of all three species (Anderson et al., 2010). These data indicate the ability of the colon to prevent urea loss despite a large concentration gradient between the blood plasma and the chyme and/or colonic fluid. Similar to urea, ammonia has also been shown to accumulate within the intestinal lumen of fasted *S. a. suckleyi* gut sacs, but no net flux was evident across the gut sacs of the cardiac and pyloric stomachs or the colon (Wood et al., 2019). After feeding, ammonia accumulated in the lumen of all GI sections and was significantly elevated in the intestine (Wood et al., 2019). Clearly, urea and ammonia are capable of moving across GI tissues, but the direction appears to be dependent on the metabolic state of the animal, the nitrogenous compound under investigation, and the section of GI tract being examined.

The natural diet of marine elasmobranchs can include urea-poor prey (*e.g.* teleosts, crustaceans, cephalopds, ctenophores, molluscs) and urea-rich prey (*e.g.* sharks, skates, rays, ratfish) (Hanchet, 1991; Jones and Geen, 1977; Wetherbee et al., 1990; White et al., 2004). However, feeding studies have generally examined the effects of a diet dominated by teleost fish which are largely devoid of urea (Kajimura et al., 2006; Wood et al., 2007a, 2007b, 2005). To our knowledge no study has investigated the effects of a urea-rich meal on nitrogen balance in an elasmobranch, nor the role the GI tract may play in response to such a diet.

Large elasmobranch species such as lemon (*Negaprion brevirostris*), hammerhead (*Sphyrna mokarran*), and bull sharks (*Carcharhinus leucas*) are known to prey upon other chondrichthyans but are virtually impossible to study within a laboratory setting (Wetherbee et al., 1990). Analyses of the stomach contents of North and South Pacific spiny dogfish have shown the occasional consumption of other urea-rich species, including the cannibalism of juvenile dogfish (Hanchet, 1991; Jones and Geen, 1977), and the consumption of ratfish by *S. a. suckleyi* has been observed both in the lab and in the field by one of the authors (C. M. Wood, personal observations). Thus, the documented predation on ureosmotic chondrichthyes, despite the infrequency, suggests they are capable of handling a urea-rich meal. Existing literature on GI nitrogen transport in *S. a. suckleyi* allows for comparison of urea-rich fed dogfish with urea-poor fed and fasted dogfish (Anderson et al., 2015, 2012; Kajimura et al., 2006; Liew et al., 2013; Wood et al., 2019, 2010, 2007b, 2007a, 2005). Therefore, despite being a species that may only occasionally encounter a urea-rich meal, we used *S. a. suckleyi* to begin to explore the effects of a urea-rich meal on nitrogen uptake and homeostatic regulation across the GI tract.

In this study we examined the gastrointestinal handling of nitrogen (urea and ammonia) using *in vitro* gut sac preparations from dogfish fed a urea-rich meal, and provide a comparison to previously published data on gut sacs from fasted and teleost-fed (a urea-poor meal) dogfish (Liew et al., 2013; Wood et al., 2019). Knowing the nitrogen-limited nature of marine elasmobranchs, we set out to test if a urea-rich meal would influence nitrogen transport across the GI tract of *S. a. suckleyi*; this would be evident as a net uptake of nitrogen across *in vitro* gut sac preparations, increased plasma levels of urea and ammonia, and increased urea concentrations in the skeletal muscle reflective of increased available nitrogen. Alternatively, if *S. a. suckleyi* are capable of strong nitrogen-regulation the ingestion of excess-prandial urea will show no effect on the whole animal homeostatic nitrogen balance.

2. Material and methods

Male North Pacific spiny dogfish were captured by hook-and-line in Barkley Sound, British Columbia in July and August 2018 and 2019 (n = 20; 2.12 ± 0.05 kg). Following capture, animals were held at Bamfield Marine Sciences Centre in 1500 l outdoor covered flow-through circular tanks. Sea water was held at ambient temperature (12.0 ± 1.0 °C), salinity (30.0 ± 2.0 ppt), and natural photoperiod. All protocols were approved by the Animal Care Committee at Bamfield Marine Sciences Centre (RS-19-03) within the guidelines of Canadian Council of Animal Care and appropriate collection permits for scientific research as issued by Fisheries and Oceans, Canada.

2.1. Feeding

All dogfish used in this study were fasted for seven days to ensure previously ingested meals had passed completely through the GI system and ensure all animals were in a similar metabolic state (Jones and Geen, 1977; Kajimura et al., 2006; Wood et al., 2007a). Frozen Atlantic herring (*Clupea harengus;* Rhys Davis, Sidney B.C.) were thawed and blended with a minimal amount of filtered seawater (no more than 5% seawater by mass) and urea to make a final concentration of 700 mM urea, approximately double the concentration expected from the consumption of a marine elasmobranch. This was done in an attempt to push the physiological limits of nitrogen homeostasis in *S. a. suckleyi* during elevated prandial urea concentrations. Following light anesthesia (tricaine methanesulfonate, MS-222; 100 ppm, Syndel Labs, Vancouver, BC, Canada), dogfish (n = 7) were weighed and force-fed the urea-rich slurry at a 2% body-mass ration *via* gavage directly into the cardiac stomach, making the administered dose approximately 14 mmol urea kg^{-1}. A sample of the food-slurry was collected directly from the gavage and frozen at −80 °C for further analysis. The dogfish were returned to the holding tanks and allowed to recover for 20 h before immersion in a terminal dose of anaesthetic (MS-222; 250 ppm). A blood sample was collected immediately from the caudal artery and centrifuged for one minute at 13,000*g*. Plasma was collected and stored at −80 °C until further analysis. All fluid and plasma samples collected in this study were treated in a similar manner. Control animals were either fed the slurry with no added urea (urea-poor; n = 7) in the manner described above, or food was withheld for seven days prior to sacrifice (fasted; n = 6).

2.2. Gut sacs

Following a 20 h digestion period, *in vitro* gut sac preparations were made from the stomachs (cardiac and pyloric stomachs as one sac) and intestine (spiral valve as one sac) of dogfish fed the urea-rich slurry. Gut sac protocols followed previously published work by Liew et al. (2013). Briefly, the abdominal cavity was exposed with a midventral incision, taking care not to disrupt any internal organs. The stomachs and intestine were clamped (posterior to the esophagus and anterior to the rectal gland), removed from the body cavity and separated into two sacs at the pylorus. The sacs were emptied of chyme and digestive fluids, and a sample of these fluids were collected and stored at −80 °C for future analysis. The sacs were then rinsed three times with ammonia-free elasmobranch Ringer's solution (in mM: 400 Urea, 257 NaCl, 80 TMAO, 7 Na_2SO_4, 6 $NaHCO_3$, 5 glucose, 4 KCl, 3 $MgSO_4$, 2 $CaCl_2$, 0.1 $NaHPO_4$; pH 7.8) to remove any remaining fluids or chyme. Digestive fluids and plasma from the control animals were treated in the same manner, although fasted dogfish had little or no stomach and intestinal fluid to collect.

Double ligatures were secured at the posterior end of the each gut sac. The anterior openings were cannulated with a 0.7 ml polyethylene centrifuge tube, with the lid and curved bottom cut off. Plastic airline tubing was placed over the narrow end of the tube to facilitate filling the lumen of the sacs with 35 ml ammonia-free elasmobranch Ringer's solution, pre-equilibrated with speciality gas (99.7% O_2: 0.3% CO_2) for 30 min prior to beginning the experiment — 5 ml was subsequently removed from the lumen for future analysis, representing time zero (0 h). A rubber micro-bung was used to seal the tube. The filled sacs were carefully blotted dry of all exterior moisture, weighed to the nearest 0.0001 g, and placed in a beaker with a 200 ml bath of nominally ammonia-free elasmobranch Ringer's solution (representing the serosal side of the preparation) and continuously bubbled with speciality gas. The bath and preparations were kept at a constant 12 °C by standing the beakers in running seawater. After 3 h incubation, a sample of the bath was collected for pH and fluid composition analysis.

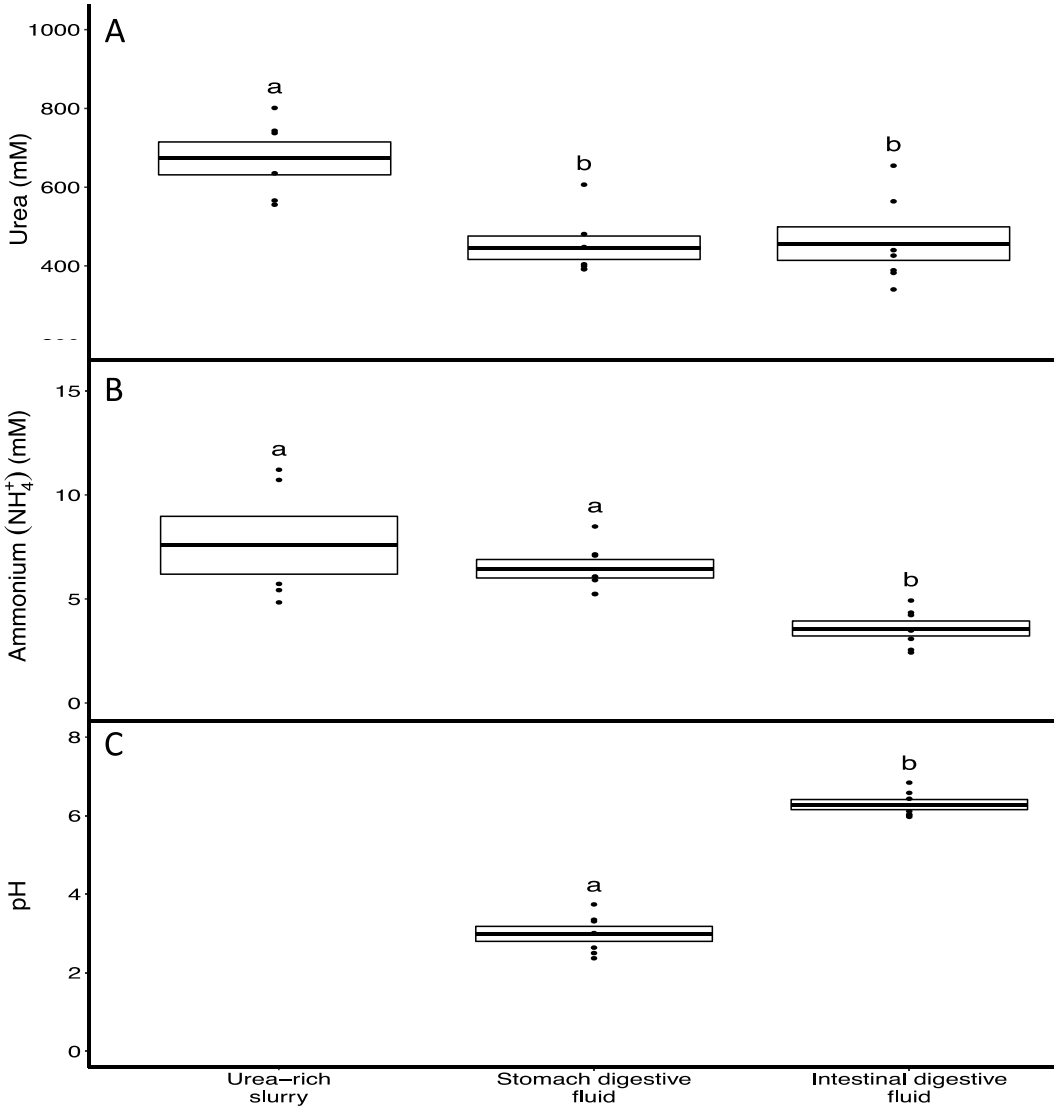

Fig. 1. (A) Urea and (B) ammonium (NH_4^+) concentrations (mM) and (C) pH of stomach and intestinal digestive fluids of North Pacific spiny dogfish (*S. a. suckleyi*) (n = 6–7) collected 20 h after being fed a urea-rich slurry. Also included are the urea and ammonium concentrations of the slurry. The horizontal line within the jitter boxplots indicates mean, and the upper and lower box boundaries indicate sem. Means not sharing the same letter are significantly different.

The sacs were carefully removed, blotted dry and weighed again before the contents were emptied and a sample collected. A longitudinal incision was made to open the sacs and the inside thoroughly blotted dry before being weighed a third time. The sacs were then placed on graph paper, pulled flat, and traced to determine gross surface area (cm^2).

2.3. Food and fluid analysis

Ammonia concentration of all collected fluids was measured as ammonium (NH_4^+) using an ion-specific ammonia electrode (Orion 9512, ThermoFisher). Urea concentration was measured by the diacetyl monoxime method (Rahmatullah and Boyde, 1980), after a 1:6000 dilution. Food samples were homogenized on ice three times for 10 s at 11,000 rpm (Polytron PT2100) with two parts deionized-water and centrifuged at 13,000*g* for 3 min, 4 °C; the resulting supernatant was used for ammonium and urea analysis as above. Osmolality of the collected fluids was measured by vapour pressure osmometry (Wescor 5520) and pH with a Mettler Toledo pH probe (Fisher Scientific, Accumet AE 150).

2.4. Skeletal muscle

Skeletal muscle samples were collected adjacent to the vertebral column midway down the length of the dogfish. Samples were blotted with paper towel to remove any residual blood before transfer to a collection tube and storage at −80 °C. Upon first thaw, samples were weighed and placed into centrifuge tubes with 5 parts deionized water (1:6 dilution) and homogenized in a chilled tissue lyser (TissueLyser II, Qiagen) at 30 Hz for 3 min with a steel ball-bearing, then centrifuged for 3 min at 13,000*g* , 4 °C. The supernatant was removed and further diluted (1:1000) giving a final dilution of 1:6000, and assayed for urea as above.

2.5. Flux calculations

Ammonia and urea flux calculations were based on Liew et al. (2013). The initial gut sac volume (*Vi*; ml) was calculated from the initial mass of the filled sac minus the preparation mass (tissue, ligatures, and tubing). The final volume (*Vf*; ml) was the mass of the filled sac after 3 h incubation minus the preparation mass. The flux rates were calculated as follows:

Urea and Ammonium

$$\frac{(Ci \times Vi) - (Cf \times Vf)}{T \times A}$$

Water

$$\frac{(Vi - Vf)}{T \times A} \times 1000$$

where *Ci* was the initial concentration and *Cf* was the final concentration in mM, *T* was time in hours and *A* was the gross surface area (cm^2) of the preparation. Positive flux values represent uptake from the gut sac lumen and negative values represent secretion into the lumen.

2.6. Statistics

All data are expressed as mean ± sem within the text and Figures. Statistical analyses were conducted using *R* (R Core Team, 2017) and Figures were produced using the package ggplot2 (Wickham, 2009). Data were checked for normality (Shapiro-Wilks) and homogeneity of variance (Levene's test). For single comparisons where data from only two variables were collected, a Two Sample *t*-test was used; for multiple comparisons, one-way analysis of variance (ANOVA) was performed and Tukey's *post hoc* test was used to detect significant differences. Differences were accepted as significant when $p < .05$.

3. Results

3.1. Food and fluids

After 20 h digestion of urea-rich slurry, the urea concentrations in the stomach (446 ± 30 mM) and intestinal (457 ± 42 mM) digestive fluids of *S. a. suckleyi* were significantly reduced compared to the slurry (673 ± 42 mM) ($F_{(2,17)} = 10.8$, $p < .001$, $n = 6–7$), but were not significantly different from one another (Fig. 1A). Ammonium concentrations in the intestinal fluid (3.58 ± 0.36 mM) were significantly lower than the food-slurry (7.59 ± 1.39 mM) and stomach fluid (6.46 ± 0.45 mM) ($F_{(2,16)} = 8.11$, $p < .004$, $n = 5–7$) (Fig. 1B). The pH of the stomach (pH 3.0) was significantly more acidic than that of the intestine (pH 6.3) ($t = 14.3$, df = 12, $p < .0001$, n = 6–7) (Fig. 1C).

3.2. Gut sacs

The lumenal fluids of the stomach and intestinal gut sacs from urea-rich dogfish showed no significant change in urea concentrations after 3 h incubation ($F_{(7,48)} = 0.50$, $p = .83$, $n = 7$) (Fig. 2A). In contrast, ammonium within the lumen of the intestine (4.35 ± 0.60 mM) accumulated to a significantly greater extent than the stomach (0.65 ± 0.11 mM) ($F_{(7,47)} = 42.5$, p < .001, n = 6–7) (Fig. 2B), but osmolality remained largely unchanged ($F_{(7,46)} = 3.86$, $p = .002$, n = 6–7), with the exception of a significant reduction in the stomach serosal bath compared to the stomach lumen ($p = .01$, Fig. 2C). The pH of all samples became significantly more acidic than the starting pH 7.8 ($F_{(7,44)} = 168$, p < .0001), but the stomach (pH 5.8) and intestinal (pH 6.1) lumen were not significantly different from one another ($p = .12$, Fig. 2D).

The net rate of accumulation of urea within the lumens of both the stomach (−1.69 ± 9.73 μmol cm^{-2} h^{-1}) and intestinal (−1.05 ± 7.76 μmol cm^{-2} h^{-1}) gut sacs did not differ significantly ($t = 0.05$, df = 12, $p = .96$, n = 7) (Fig. 3A). The intestinal lumen accumulated ammonium at a significantly faster rate (−1.06 ± 0.17 μmol cm^{-2} h^{-1}) than the stomach (−0.09 ± 0.02 μmol cm^{-2} h^{-1}) ($t = -5.64$, df = 12, $p = .0001$) (Fig. 3B). Water flux rates did not differ significantly between the stomach (0.40 ± 1.61 μl cm^{-2} h^{-1}) and intestinal (2.09 ± 1.08 μl cm^{-2} h^{-1}) gut sacs ($t = 0.87$, df = 12, $p = .40$) (Fig. 3C).

3.3. Plasma and muscle

Consuming the urea-rich slurry did not have a significant effect on plasma urea concentrations (332 ± 24 mM) compared to the fasted (359 ± 19 mM) and urea-poor (340 ± 16 mM) dogfish ($F_{(2,15)} = 0.45$, $p = .65$, $n = 5–7$) (Fig. 4A). A urea-rich meal also did not affect the plasma ammonium concentrations of the urea-rich dogfish (1.96 ± 0.23 mM) compared to the fasted dogfish (2.29 ± 0.41 mM), but the urea-poor dogfish showed significantly reduced ammonium (0.88 ± 0.17 mM) in the plasma compared to the other two groups ($F_{(2,14)} = 5.66$, $p = .02$, n = 5–6) (Fig. 4B).The fed state of the dogfish had no significant effect on the plasma osmolality between the urea-rich (887 ± 53 mOsm kg^{-1}), urea-poor (925 ± 34 mOsm kg^{-1}), and fasted dogfish (925 ± 35 mOsm kg^{-1}) ($F_{(2,17)} = 0.19$, $p = .83$, $n = 6–7$) (Fig. 4C). Likewise, the skeletal muscle urea concentrations of the urea-rich (336 ± 7 mM), urea-poor (323 ± 13 mM), and fasted animals (347 ± 15 mM) did not significantly differ among the treatments ($F_{(2,16)} = 0.99$, $p = .39$, n = 5–8) (Fig. 4D).

4. Discussion

To our knowledge this is the first study to examine the effects of a urea-rich meal on nitrogen movement across the GI tract of a marine elasmobranch. The results add to our understanding of the ability of *S. a. suckleyi* to regulate internal nitrogen concentrations for homeostatic balance, despite increased prandial nitrogen. From *in vitro* gut sac and Ussing chamber experiments we know the cardiac and pyloric stomachs, the intestine, and the colon are all capable of trafficking urea from the lumen to the serosal medium in both fed and fasted dogfish; the exception is the intestine of fasted dogfish which appears to accumulate urea in the lumen (Anderson et al., 2015, 2010; Liew et al., 2013).

In this study we saw no pronounced urea concentration gradients nor net loss of urea from the stomach or intestinal gut sac lumens; however, we did see a significant accumulation of ammonium (4.35 mM) within the lumen of the intestinal gut sac, which was comparable to the gut sac data from urea-poor fed dogfish reported by Wood et al. (2019). This accumulation may be the product of tissue-derived ammonia production moving down its concentration gradient from the tissue to the ammonia-free Ringer's solution. However, an alternative explanation may come from the significant urease activity identified in the epithelium and fluids of the pyloric and cardiac stomachs, intestine, and colon of *S. a. suckleyi* (Wood et al., 2019). Urease is an enzyme that hydrolyzes urea into ammonia and is likely microbial in origin (Mobley and Hausinger, 1989). Within the stomachs of ruminants, urease is responsible for recycling host-derived urea back into ammonia, which is then used as the major source of nitrogen for much of the bacterial community residing in the rumen. Supplementary exogenous urea is often added to ruminant diets in order to facilitate the hydrolysis of urea into ammonia to sustain the lumenal microbiome (Mobley and Hausinger, 1989). Within the elasmobranch GI tract, it has been suggested that the urease activity of the elasmobranch GI tract may function to recycle urea into ammonia for concomitant assimilation into other nitrogenous compounds such as amino acids and proteins. The microbiota of the elasmobranch GI tract is predominantly composed of *Proteobacteria*, with some species capable of nitrogen-fixation (Givens et al., 2015). Based on the probable presence of nitrogen-fixing bacteria within the elasmobranch GI tract, the recycling of urea into ammonia to sustain the microbiome should not be discounted, and is one possible explanation for the occurrence of this phenomenon in nitrogen-limited animals (Wood et al., 2019). In studies using *in vitro* intestinal gut sacs and intestinal tissue mounted in Ussing chambers, the net movement of urea in fasted animals was to the lumenal side of

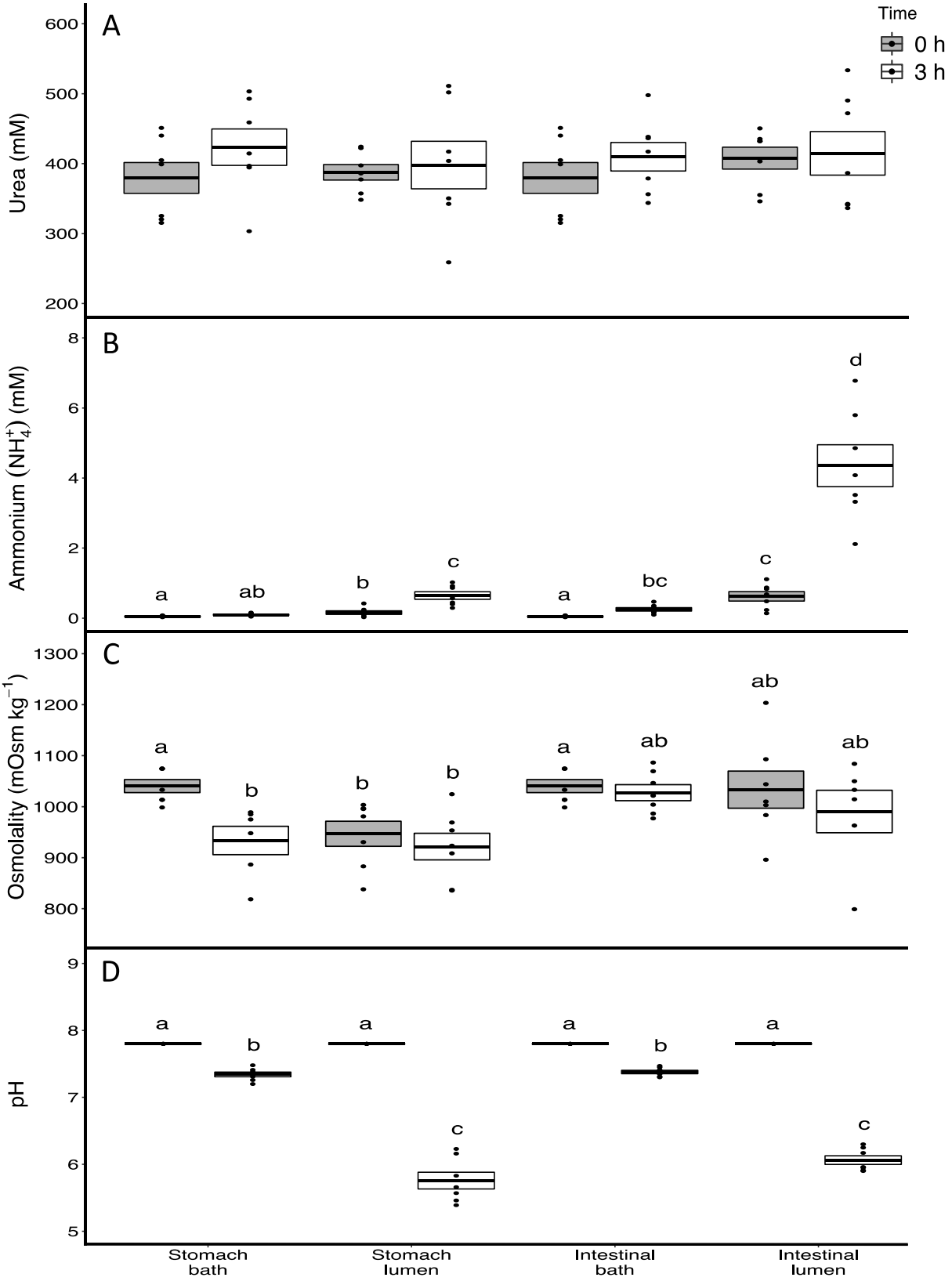

Fig. 2. (A) Urea and (B) ammonium (NH_4^+) concentrations (mM), (C) osmolality (mOsm kg^{-1}) and (D) pH of *in vitro* gut sac fluids collected at time zero (filled boxes) and after 3 h (empty boxes) incubation. Stomach and intestinal gut sacs collected from North Pacific spiny dogfish (*S. a. suckleyi*) fed a urea-rich slurry and allowed to digest 20 h (n = 6–7). The horizontal line within the jitter boxplots indicates mean, and the upper and lower box boundaries indicate sem. Means not sharing the same letter are significantly different.

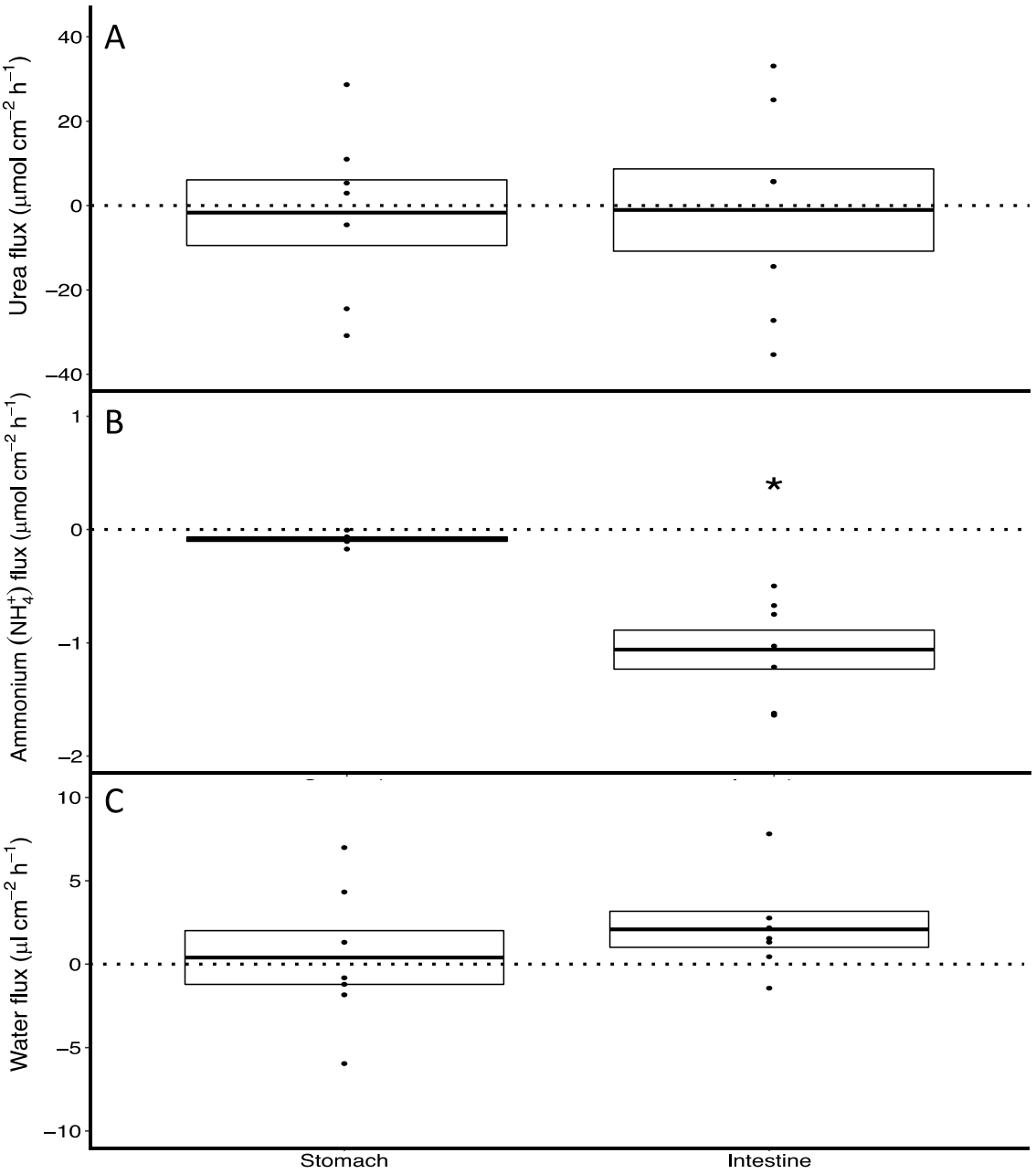

Fig. 3. (A) Urea and (B) ammonium (NH_4^+) (μmol cm^{-2} h^{-1}) and (C) water (μl cm^{-2} h^{-1}) flux across *in vitro* gut sacs from North Pacific spiny dogfish (*S. a. suckleyi*) fed urea-rich slurry ($n = 7$). Positive values indicate net uptake from the lumen and negative values indicate net accumulation in the lumen. The horizontal line within the jitter boxplots indicates mean, and the upper and lower box boundaries indicate sem. Significant difference denoted by *.

the preparations (Anderson et al., 2015, 2010; Liew et al., 2013). This fasted efflux appears counterintuitive for nitrogen-limited animals needing to retain nitrogen, especially considering the low concentrations of urea found within the colon of dogfish, indicative of very little urea excreted in colonic fluid (Anderson et al., 2012). Therefore, when viewed through a urease-recycling lens, this efflux of urea in fasted dogfish may be partially for the benefit and sustenance of the GI microbial community.

Plasma ammonia concentrations in *S. a. suckleyi* have been reported to range from 2.3 mM (this study) down to 81 μM, despite ammonia concentrations within intestinal fluids recorded as high as 8.7 mM (Wood et al., 2019, 2005, 1995). Transport to the liver and subsequent conversion of ammonia into amino acids and/or urea may account for the lower plasma concentrations compared to the lumenal concentrations (Bucking, 2015; Schooler et al., 1966). Significant ammonium accumulation within the *in vitro* stomach and intestinal gut sacs established a large concentration gradient between the lumenal and serosal media, supporting the idea of strong regulation over bulk ammonia uptake from the lumen. This regulation may be accomplished in part by transporters such as two Rhesus (*Rh*) glycoprotein ammonia transporters (*Rhbg* and *Rhp2*) that have been identified in the elasmobranch GI tract (Anderson et al., 2010; Nawata et al., 2015; Hoogenboom, unpublished) Their saturable properties may be partially responsible for establishing the concentration gradient and preventing large amounts of ammonia from flooding into the plasma. Indeed, plasma ammonia concentrations in the urea-rich fed dogfish at 20 h post-feeding did not significantly increase, and significantly decreased in the urea-poor fed dogfish, indicating any excess prandial-ammonia was not shown to be circulating in blood plasma at that time.

Wood (2001) hypothesized these nitrogen-limited animals would

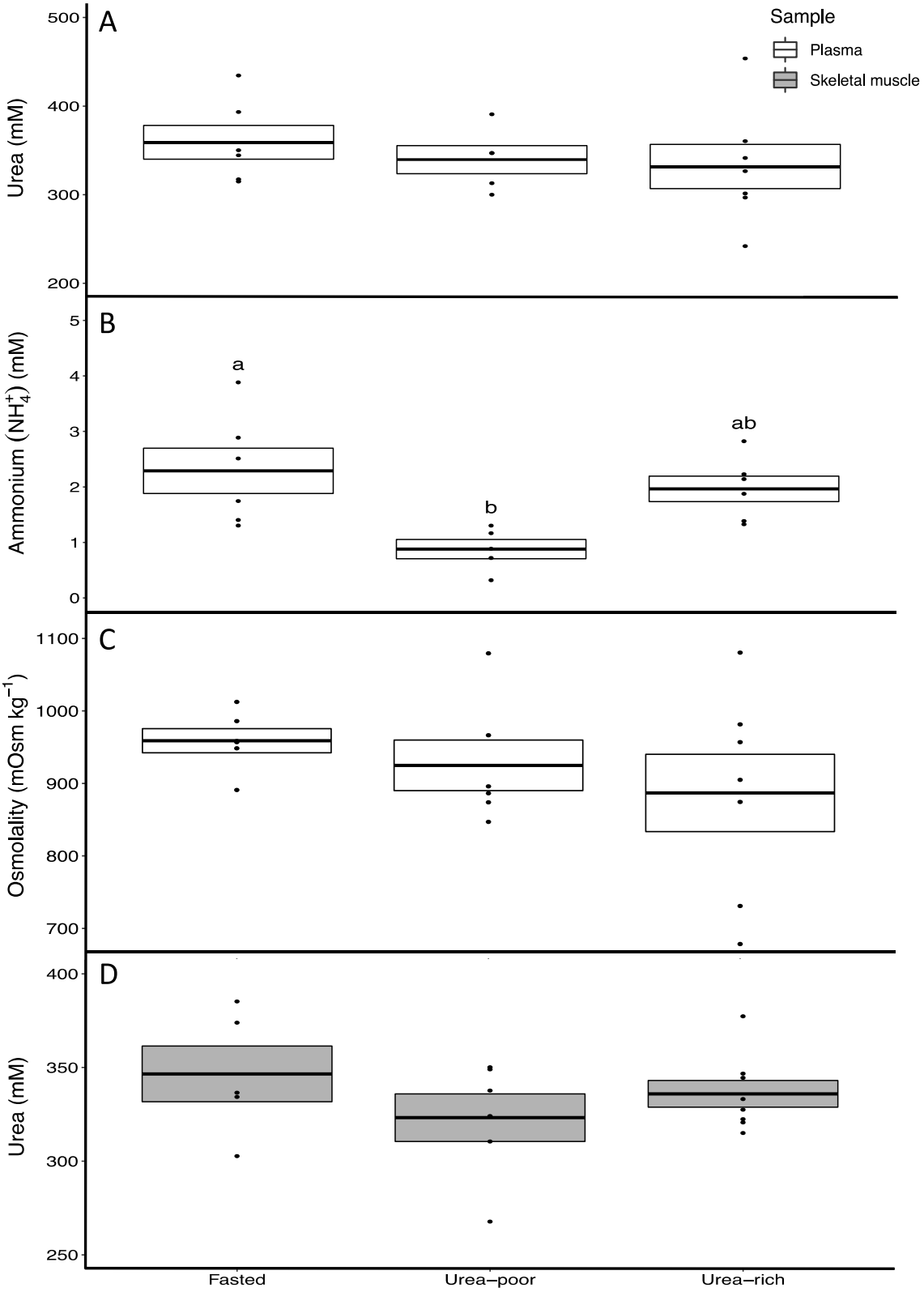

Fig. 4. (A) Urea and (B) ammonium (NH_4^+) concentrations (mM) and (C) osmolality (mOsm kg^{-1}) from plasma (open boxes, n = 6–7) and (D) skeletal muscle (closed boxes, n = 5–8) of North Pacific spiny dogfish (*S. a. suckleyi*). Samples were collected from fasted animals and those fed a urea-poor or a urea-rich slurry and allowed to digest 20 h; food was withheld from fasted animals for seven days. The horizontal line within the jitter boxplots indicates mean, and the upper and lower box boundaries indicate sem. Means not sharing the same letter are significantly different.

attempt to retain as much post-prandial ammonia as possible for future urea synthesis during periods of fasting or salinity challenges; therefore, prandial ammonia may be converted into urea *via* the ornithine urea cycle (OUC) within intestinal tissues (Kajimura et al., 2006; Liew et al., 2013) or transported to the liver and/or muscle for urea synthesis *via* the OUC (Casey and Anderson, 1982; Kajimura et al., 2006; Steele et al.,

2005). Following a urea-poor feeding event, two OUC enzymes (ornithine transcarbamoylase and arginase) showed increased activity in the liver and muscle of *S. a. suckleyi*, while glutamine synthetase, which catalyzes the hydrolysis of ammonia to glutamine (a prerequisite for the OUC), showed increased activity in the liver (Casey and Anderson, 1982; Kajimura et al., 2006). It has been suggested that OUC production in the liver may be regulated in response to urea demand (Wright and Wood, 2015). If the prandial nitrogen ingested by the urea-rich fed dogfish was in excess of the urea demands, OUC production may have been downregulated; however, further investigation into the OUC enzyme activity post-feeding is needed to determine if this is the case. It has also been shown that production of urea *via* the OUC in the elasmobranch skeletal muscle may contribute more to the overall production of urea than the liver when total body mass is considered (Kajimura et al., 2006; Steele et al., 2005). Therefore, we investigated the possibility of excess urea within the skeletal muscle, as the intracellular compartments of the muscle of little skate and southern stingray (*Dasyatis americana*) can retain urea concentrations at, or greater than, plasma concentrations (Forster and Goldstein, 1976; Withers et al., 1994; Yancey and Somero, 1980). In this study, the urea concentrations in the skeletal muscle were similar to those of plasma and did not differ between the fasted, urea-poor, or urea-rich dogfish, once again indicating homeostatic regulation of nitrogen throughout various tissues.

After 20 h digestion of a urea-rich meal, urea and ammonium plasma concentrations and osmolality did not differ from fasted dogfish nor those fed a urea-poor meal, which aligned with the alternative hypothesis of strong nitrogen regulation for these animals. In several feeding studies, urea and ammonia plasma concentrations were shown to increase above pre-fed levels by at least 20 h post-feeding, suggesting this to be a critical time point for the uptake of nitrogen in those studies (Kajimura et al., 2008, 2006; Wood et al., 2010, 2007b, 2005). However, the previously reported elevated post-prandial urea and ammonia plasma concentrations were not sustained for long periods, indicating probable homeostatic regulation of circulating nitrogen. The absence of a significant increase of circulating nitrogen levels in our study at 20 h may be a function of how the sharks were fed and the type of meal they received. The use of a blended slurry may have accelerated the digestive and absorptive process, as upon dissection the digestive fluids of the stomachs and intestine were mostly liquid and devoid of large pieces of chyme. Despite documentation of only occasional chondrichthyan consumption by *S. a. suckleyi*, the lack of elevated urea and ammonium concentrations in the plasma of urea-rich fed dogfish by 20 h highlights their apparent ability to regulate circulating nitrogen following the ingestion of a dietary urea load. It should be noted that although the fasted control animals were neither handled nor lightly anaesthetised 20 h prior to being euthanized the non-significant differences between the three treatment groups indicates that any potential handling and feeding stress the two fed groups may have experienced was likely negligible in terms of overall nitrogen homeostasis.

The nitrogen-limited nature of marine elasmobranchs is partially due to the loss of nitrogen across the gills and kidney. It appears that following a feeding event these animals are capable of slightly increasing ammonia excretion while suppressing urea excretion up to 39% (Wood et al., 2007a); however, in our study the final destination of excess nitrogen, whether stored or excreted, remains unclear and is an area for future investigation. In contrast to the previously reported reduction in urea excretion rates following a urea-poor meal, at 20 h post-feeding we did not observe the predicted increase in urea or ammonium in the plasma (Wood et al., 2007b). It is possible the excess nitrogen from the 700 mM urea meal, which was approximately 14 mmol urea kg^{-1}, was eliminated *via* the gills to maintain homeostatic levels. To answer this question, nitrogen excretion could be investigated by placing dogfish into individual isolation boxes, as used in several studies, to quantify nitrogen flux into the surrounding water (Kajimura et al., 2008, 2006; Wood et al., 2007a, 2005).

Apart from the possibility that excess nitrogen may have been excreted or used to synthesize urea, there are several internal destinations for prandial nitrogen, including amino acids and protein synthesis. Elasmobranchs rely on amino acids for aerobic fuel, especially in red muscle and liver, and as substrates for ketogenesis in the liver (reviewed by Speers-Roesch and Treberg, 2010). Some amino acids are also thought to be involved in intracellular osmotic balance (reviewed by Wright and Wood, 2015). Speers-Roesch and Treberg (2010) proposed a link between ketogenesis, ketone body oxidation, and urea synthesis, potentially linking amino acids to the homestatic regulation of nitrogen. Although amino acid synthesis was not investigated in this study, total amino acid concentrations in the plasma following a urea-poor feeding event were reported to increase in two phases, the first occurring within 20 h and the second within 60 h, while another study reported a single significant increase by 30 h (Kajimura et al., 2006; Wood et al., 2010). Knowing that amino acid concentrations can increase following feeding, it is likely some of the excess nitrogen in our study was used for amino acid synthesis. This would permit the retention of important nitrogen sources (*i.e.* amino acids and proteins) in a form that could be easily stored and accessed, particularly in dogfish that may feed infrequently and rely on internal nitrogen stores during periods of fasting.

Protein synthesis and catabolism are also important components of nitrogen homeostatic regulation. During a prolonged fasting study of 56 days, *S. a. suckleyi* maintained plasma urea concentrations, omolality, and a constant rate of urea excretion, despite a lack of dietary nitrogen intake (Kajimura et al., 2008; Wood et al., 2010). The persistence of endogenous nitrogen excretion was maintained through the degradation of proteins and equated to the loss of 70 g of protein per kg of muscle (Wood et al., 2010). Similarily, when challenged with increased salinity (130% seawater) small-spotted catsharks (*Scyliorhinus canicula*) fed a reduced protein diet were unable to compensate for the increased external osmolarity *via* urea synthesis, but compensated by retaining increased sodium and chloride (Armour et al., 1993). In contrast, high-protein fed catsharks were able to synthesize extra urea under the hypersaline conditions without retaining increased sodium and chloride. Taken together, these studies highlight the role that protein metabolism may play in nitrogen balance. Although not examined in this study, it would be interesting to investigate the synthesis of amino acids and proteins following repeated urea-rich feeding events over a prolonged period to determine if the uptake of nitrogen, beyond homeostatic demand, would be used for somatic growth.

5. Conclusions

Overall, our results indicate the ability of nitrogen-limited *S. a. suckleyi* to regulate internal urea and ammonia concentrations following the ingestion of a urea-rich meal; after 20 h digestion, urea and ammonium concentrations in the plasma and skeletal muscle, as well as plasma osmolality were all at pre-fed levels. When presented alongside two previous *in vitro* gut sac studies (Liew et al., 2013; Wood et al., 2019), ammonia appears to accumulate within the lumen of the stomach and intestinal gut sacs, regardless of metabolic state. In contrast, urea is taken-up across the stomach and intestinal tissues of urea-poor fed dogfish, and the stomach of fasted dogfish, but accumulated within the intestine of fasted dogfish and showed no apparent net movement across the stomach or intestine 20 h after a urea-rich meal. Recently identified urease activity within the GI tract of *S. a. suckleyi* likely plays a role in ammonia accumulation within the GI lumens as well as in the overall nitrogen homeostatic regulation. Despite the apparent infrequency of predation on other ureosmotic chondrichthyans, it seems the ingestion of a urea-rich meal does not adversely affect the nitrogen balance of *S. a. suckleyi*.

Declaration of Competing Interests

The authors declare that they have no known competing financial

interests or personal relationships that could have appeared to influence the work reported in this paper.

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