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RESEARCH ARTICLE

Using ^{15}N to determine the metabolic fate of dietary nitrogen in North Pacific spiny dogfish (*Squalus acanthias suckleyi*)

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ABSTRACT

Marine elasmobranchs are ureosmotic, retaining large concentrations of urea to balance their internal osmotic pressure with that of the external marine environment. The synthesis of urea requires the intake of exogenous nitrogen to maintain whole-body nitrogen balance and satisfy obligatory osmoregulatory and somatic processes. We hypothesized that dietary nitrogen may be directed toward the synthesis of specific nitrogenous molecules in post-fed animals; specifically, we predicted the preferential accumulation and retention of labelled nitrogen would be directed towards the synthesis of urea necessary for osmoregulatory purposes. North Pacific spiny dogfish (*Squalus acanthias suckleyi*) were fed a single meal of 7 mmol l^{-1} $^{15}NH_4Cl$ in a 2% ration by body mass of herring slurry via gavage. Dietary labelled nitrogen was tracked from ingestion to tissue incorporation and the subsequent synthesis of nitrogenous compounds (urea, glutamine, bulk amino acids, protein) in the intestinal spiral valve, plasma, liver and muscle. Within 20 h post-feeding, we found labelled nitrogen was incorporated into all tissues examined. The highest $\delta^{15}N$ values were seen in the anterior region of the spiral valve at 20 h post-feeding, suggesting this region was particularly important in assimilating the dietary labelled nitrogen. In all tissues examined, enrichment of the nitrogenous compounds was sustained throughout the 168 h experimental period, highlighting the ability of these animals to retain and use dietary nitrogen for both osmoregulatory and somatic processes.

KEY WORDS: $\delta^{15}N$, Elasmobranch, Intestine, Nitrogen, Osmoregulation, Spiral valve

INTRODUCTION

Nitrogen is an important element essential to life on this planet. It is the backbone of DNA and RNA, the building block for amino acids and proteins, and is a biologically limiting nutrient. As such, organisms are concerned with the acquisition of nitrogen, and those unable to fix atmospheric nitrogen must possess mechanisms necessary to obtain it from other sources. This can occur through the ingestion and catabolism of dietary amino acids and proteins that are absorbed along the gastrointestinal (GI) tract (Fauconneau and Michel, 1970; Wu et al., 2014), with excess nitrogen generally excreted rather than retained (Wright, 1995).

Marine elasmobranchs are ureosmotic and retain much of their excess 'waste' nitrogen rather than excreting it. In addition to its use for somatic processes, such as the synthesis of amino acids, the retained nitrogen is also used to synthesize large concentrations of urea (>300 mmol l^{-1}) necessary to balance the osmotic pressure of the marine environment (Smith, 1936). Importantly, despite their carnivorous feeding strategy that provides dietary nitrogen through the catabolism of ingested amino acids and proteins, marine elasmobranchs are considered nitrogen limited because of their putative intermittent feeding behaviours and their need to synthesize and retain urea, as well as amino acids (Bucking, 2015; Wood, 2001). Therefore, these animals must possess physiological mechanisms necessary for both nitrogen acquisition and retention, especially between meals when exogenous nitrogen uptake is limited.

Research on the overall nitrogen homeostasis of marine elasmobranchs has historically focused on urea and the osmotic organs involved in its retention and excretion (i.e. the gills and kidney) (Boylan, 1967, 1972; Fines et al., 2001; Hays et al., 1977; Hyodo et al., 2004; Morgan et al., 2003a,b; Pärt et al., 1998; Schmidt-Nielsen et al., 1972; Walsh and Smith, 2001; Wood et al., 1995). More recently, research has focused on the role of the intestinal spiral valve and its ability to acquire and retain nitrogen (Hoogenboom et al., 2020, 2023; Kajimura et al., 2006; Liew et al., 2013; Weinrauch et al., 2020; Wood et al., 2005). From these studies, we know the spiral valve in fed North Pacific spiny dogfish (*Squalus acanthias suckleyi*) moves urea from the lumen to the serosal medium, as demonstrated by *in vitro* intestinal gut sac and Ussing chamber flux studies (Anderson et al., 2015; Liew et al., 2013). In contrast, *in vitro* flux studies on fasted dogfish demonstrated a net efflux (accumulation) of urea on the lumenal side (Anderson et al., 2015; Liew et al., 2013). The bidirectional movement of urea demonstrated by these *in vitro* experiments highlights the ability of the spiral valve to traffic urea both into and out of the intestinal lumen.

The primary pathway by which marine elasmobranchs synthesize urea is the ornithine urea cycle (OUC) (Schooler et al., 1966). Operating primarily in the liver (Anderson, 1995, 2001; Walsh and Smith, 2001), OUC enzymes have also been identified in the muscle and spiral valve of *S. acanthias suckleyi* (identified as *S. acanthias*; Kajimura et al., 2006). In contrast to the mammalian OUC, the marine elasmobranch OUC uses glutamine as the main nitrogen-donating substrate, which is formed by glutamine synthetase (GS) transferring nitrogen from ammonia to glutamate (Anderson, 1981). The activity of GS within the elasmobranch liver has been shown to be correlated to the retention of urea, as obligate freshwater elasmobranchs do not retain urea and have no detectable levels of GS in their liver (Webb and Brown, 1980). Glutamine is also a functional amino acid, essential in various metabolic and nutritional processes including amino acid and protein synthesis, and can act as a reservoir for nitrogen storage (Wu, 2009). The final enzyme that facilitates the synthesis of urea via the OUC is arginase (ARG), which transfers nitrogen from arginine to urea (Anderson, 1991).

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GS activity increased in the liver of *S. acanthias suckleyi* within 6 h post-feeding, and ARG increased within 20 h, but both decreased to pre-fed levels by 30 h (Kajimura et al., 2006). In the intestine, ARG showed no change, while GS showed a significant decrease at 30 h post-feeding (Kajimura et al., 2006). In the muscle, GS showed no change in activity, while ARG significantly decreased at 30 h, but significantly increased by 48 h post-feeding (Kajimura et al., 2006).

To understand the dynamics of ecosystem interactions and animal movements, stable isotope analysis can be used to determine the trophic level and/or the prey of consumers (Deniro and Epstein, 1978, 1981). Laboratory diet-switching experiments have been used to calculate isotopic turnover rates (i.e. the addition of new material or the replacement of exported material; Fry and Arnold, 1982) and diet-discrimination factors (i.e. the difference between the consumer's tissues and the prey; Martínez del Rio et al., 2009) of elasmobranchs. In these diet-switching studies, the liver and blood were shown to have faster turnover rates than muscle (Kim et al., 2012; Logan and Lutcavage, 2010; MacNeil et al., 2006; Malpica-Cruz et al., 2012). Understanding the rate of stable isotope incorporation into tissues with fast turnover rates, such as blood, can provide insight into recent diet ingestion (hours or days prior to sampling), while examining tissues with slow turnover rates, such as muscle, allows for an understanding of prey consumption several months prior to sampling (Fry, 2006). Stable isotopes can also be used in studies to investigate digestion and nutrient acquisition over a short time period, such as the use of ^{15}N to determine the metabolic fate of dietary nitrogen in ruminants (Archibeque et al., 2001; Bunting et al., 1987; Lapierre and Lobley, 2001), and $^{15}NH_4Cl$ to show the uptake of environmental ammonia and the subsequent synthesis of urea, glutamine, amino acids and proteins in Gulf toadfish (*Opsanus beta*) (Rodicio et al., 2003).

The objective of our study was to understand the short-term fate of dietary nitrogen following a meal. To do this, we tracked the uptake and incorporation of ^{15}N (labelled nitrogen) into nitrogenous compounds important for the synthesis of urea (ammonia and glutamine), osmoregulation (urea) and general somatic processes (bulk amino acids and protein). We focused on the main site of dietary nitrogen acquisition (intestinal spiral valve), the route of circulation of dietary nitrogen and post-prandial synthesized nitrogenous compounds (plasma), and two of the known sites of urea synthesis (liver, muscle). To understand the role of dietary nitrogen in the synthesis of urea in various tissues, we also examined the activity of two OUC enzymes, GS and ARG. We hypothesized that dietary nitrogen acquired across the spiral valve would be preferentially directed toward the synthesis of urea for osmoregulation, and then directed towards the synthesis of nitrogenous compounds necessary for somatic processes. We predicted the importance of nitrogen for these animals would necessitate the retention of the labelled nitrogen and its incorporation into all tissues we examined, as evidenced by an enrichment of ^{15}N in all samples.

MATERIALS AND METHODS

Animals

Male North Pacific spiny dogfish, *Squalus acanthias suckleyi* Girard 1855, were captured by rod-and-reel in Barkley Sound, BC, Canada, in July and August 2018 and 2019 (n=36; body mass 2.12 ±0.05 kg). Animals were held at Bamfield Marine Sciences Centre in 1500 l outdoor covered flow-through circular tanks in groups of 4–6. Sea water was held at a constant temperature (12±1.0°C) and salinity (30±2.0 ppt) with a natural photoperiod. All protocols were approved by the Animal Care Committee at Bamfield Marine Sciences Centre (RS-18-03, RS-19-03) within the guidelines of the Canadian Council on Animal Care and appropriate collection permits for scientific research as issued by Fisheries and Oceans Canada (DFO) (XR 149 2018, XR 99 2019). We focused on the collection of males only, as female dogfish are typically pregnant at this time of year and therefore, if captured by rod-and-reel, they are immediately released.

Feeding

All dogfish were fasted for 7 days prior to experimentation to ensure complete evacuation of previous meals from their GI tracts and ensure all animals were in a similar metabolic state with similar internal resource demands (Jones and Geen, 1977; Kajimura et al., 2006; Wood et al., 2007a). Following light anaesthesia (tricaine methanesulfonate, MS-222, 100 ppm; Syndel Labs, Vancouver, BC, Canada), dogfish were weighed and fed according to previously published methods (Hoogenboom et al., 2020): frozen Atlantic herring (*Clupea harengus*; Rhys Davis, Sidney, BC, Canada) were thawed and blended into a slurry with minimal filtered seawater (no more than 5% seawater by mass) and $^{15}NH_4Cl$ (99% $^{15}NH_4Cl$; Sigma-Aldrich) to a final concentration of 7 mmol l^{-1}. We chose a biologically relevant concentration of ammonia (7 mmol l^{-1}) based on concentrations previously reported for the herring slurry (7.59 mmol l^{-1}) (Hoogenboom et al., 2020), and for the intestinal fluids of fed (6.03 mmol l^{-1}) and fasted (8.71 mmol l^{-1}) dogfish (Wood et al., 2019). Following the 7 day fast, a single meal of the enriched slurry was force fed at a 2% body mass ration via gavage directly into the cardiac stomach (enriched fed). The dogfish were returned to the 1500 l holding tanks (in groups of 4–6) and allowed to recover and digest for 20, 48, 72 or 168 h (n=6 per digestion time point). To ensure any changes in the $\delta^{15}N$ values of the enriched fed dogfish were the result of the $^{15}NH_4Cl$ and not the single herring meal, a control group was fed the herring slurry with no $^{15}NH_4Cl$, in the manner described above, and allowed to digest for 20 h (non-enriched fed; n=6). To quantify the $\delta^{15}N$ values of the dogfish prior to feeding, a second control group was euthanized after the 7 days with no food (fasted; n=6).

Samples

Following the allotted digestive time periods of the enriched (20, 48, 72, 168 h) and non-enriched (20 h) fed dogfish, the dogfish were immersed in a terminal dose of anaesthetic (MS-222; 250 ppm) and blood was drawn from the caudal sinus and immediately centrifuged; plasma was collected and frozen at −80°C until analysis. The fasted dogfish were treated in the same manner following 7 days without food. The following tissues were also collected: anterior (ASV), mid (MSV) and posterior (PSV) intestinal folds from the spiral valve, liver, and white skeletal muscle (muscle) collected adjacent to the vertebrae midway along the length of the shark. For collection of the spiral valve folds, when a longitudinal incision is made along the length of the spiral valve of *S. acanthias suckleyi*, the internal structure consists of 14–15 internal folds (Fig. 1) that slow the passage of food and increase the surface area for nutrient absorption (Bucking, 2015; Chatchavalvanich et al., 2006; Leigh et al., 2021). We classified the anterior section of the spiral valve as the first 4–5 folds (ASV), the mid-section as the middle 4–5 folds (MSV) and the posterior as the remaining 4–5 folds (PSV) before the colon (Fig. 1). The folds were cut from the underlying outer intestinal wall and rinsed in ammonia-free elasmobranch Ringer's solution (in mmol l^{-1}: 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 chyme or

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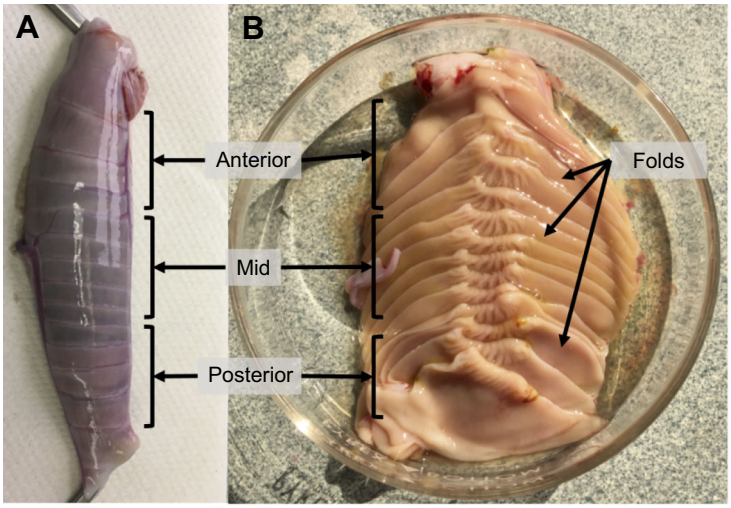

Fig. 1. Intestinal spiral valve of North Pacific spiny dogfish (*Squalus acanthias suckleyi*). (A) External view of an excised spiral valve, showing the anterior, mid and posterior regions (ASV, MSV, PSV). (B) Internal view of spiral valve showing 14 intestinal folds. This spiral valve is from a 7 day fasted dogfish and is devoid of digestive chyme. Photo credit: J.L.H.

residual blood. Control animals (20 h non-enriched fed and 7 day fasted) were treated in the same manner. Samples of the enriched and non-enriched herring slurry (before being fed to the animals), and the corresponding digestive fluids/chyme from the intestinal lumen at 20 h post-feeding were also collected and used as a comparison for ^{15}N-tissue enrichment. All samples were stored at −80°C prior to analysis.

^{15}N analysis

δ^{15}N values are the ratio of ^{15}N (heavier isotope) to ^{14}N (lighter isotope) defined as the deviation from a standard reference material in parts per thousand (‰) relative to atmospheric air and calculated as follows:

$$\delta X = \left[\left(\frac{R_{\text{sample}}}{R_{\text{standard}}}\right) - 1\right] \times 1000, \quad (1)$$

where X is ^{15}N, R_{sample} is the ratio of ^{15}N/^{14}N in the samples and R_{standard} is the ratio of the standard. Samples processed at the Trophic Ecology Laboratory at the University of Windsor's Great Lakes Institute for Environmental Research (GLIER; University of Windsor, Windsor, ON, Canada) were analysed using a Delta Advantage isotope-ratio mass spectrometer (Thermo Finnigan, San Jose, CA, USA) coupled to a Thermo Isolink elemental analyser (Thermo Finnigan). Every thirteenth sample was run in triplicate. The accuracy was based on certified values of USGS40 (within runs: −0.05‰) and IVA33802174 urea (within runs: 0.08‰). Precision was assessed by the standard deviation of replicate analyses of four standards (NIST1577c, internal lab fish standard of tilapia muscle, USGS 40, urea; n=18 for all) measured <0.19‰ for δ^{15}N. Instrumentation accuracy was periodically checked based on National Institute of Standards and Technology (NIST, Gaithersburg, MD, USA) standards (8573: δ^{15}N=−4.68±0.03‰; 8547: δ^{15}N=0.4±0.06‰; 8574: δ^{15}N=47.52±0.22‰; n=10 for all); the mean difference from the certified values was −0.16‰, −0.03‰ and −0.04‰, respectively. Samples processed at the Freshwater Institute, DFO (Winnipeg, MB, Canada) were analysed using a continuous flow isotope ratio mass spectrometer (Thermo-Delta 5 Plus) equipped with a zero blank auto sampler and an elemental analyser (Costech, Valencia, CA, USA). Every tenth sample was run in duplicate. Samples were normalized against certified reference materials USGS40 (within runs: δ^{15}N=−4.46– −4.66±0.01–0.18‰; among runs: δ^{15}N=−4.53±0.09‰) and USGS41a (within runs: δ^{15}N=47.50–47.57±0.02–0.19‰; Among runs: δ^{15}N=47.54±0.12‰) obtained from the US Geological Service (Reston, VA, USA). An in-house fish standard (58 replicates: δ^{15}N=12.95±0.10‰) and IAEA-N-2 standard (Nitrogen Isotopes in Ammonium Sulfate from the NIST; within runs: δ^{15}N=20.27–20.75±0.01–0.18‰; among runs: δ^{15}N=20.51±0.12‰) were also analysed periodically during each sample run.

Whole tissue analysis

To ensure incorporation of ^{15}N from the enriched herring slurry was detectable at the chosen concentration (7 mmol l^{-1}), we initially compared whole tissue, herring slurry, digestive fluids and plasma samples from the ^{15}N-enriched fed dogfish with the whole tissues and plasma from the control animals (20 h non-enriched fed and 7 day fasted). In our study, whole tissue represents all of the nitrogenous compounds contained within the tissue prior to its separation into ammonia, urea, glutamine, amino acids and protein. Samples were weighed (tissue 0.3 g; plasma 0.3 ml), lyophilized and sent for initial isotope ratio mass spectrometry to GLIER. All other samples were analysed at the Freshwater Institute, DFO.

Nitrogen compound analysis

Upon confirmation of ^{15}N incorporation into the whole tissues at 20 h post-feeding (Fig. 2B), the target nitrogenous compounds (ammonia, urea, glutamine, amino acids, protein) were extracted for all digestion time points (20, 48, 72, 168 h), using methods modified from previously published protocols (Rodicio et al., 2003). To maximize collection, two sets of samples were used: one set to isolate urea and the second set to isolate glutamine and amino acids; ammonia and protein were collected from both sample sets. Samples were weighed (0.2–0.3 g), treated with perchloric acid (PCA; 7%), and centrifuged to separate the protein and non-protein components. The non-protein supernatant was carefully removed for subsequent separation and analyses. The protein component was rinsed 3 times with deionized water to remove residual PCA, then lyophilized and processed for ^{15}N analysis (see below). The use of water during tissue preparation for marine elasmobranch stable isotope analysis has been shown to remove urea without altering the protein composition of muscle (Kim and Koch, 2012). Therefore, in addition to removing residual acid, the water was also used to remove residual urea from the protein precipitate, ensuring only protein-nitrogen remained in the protein pellet.

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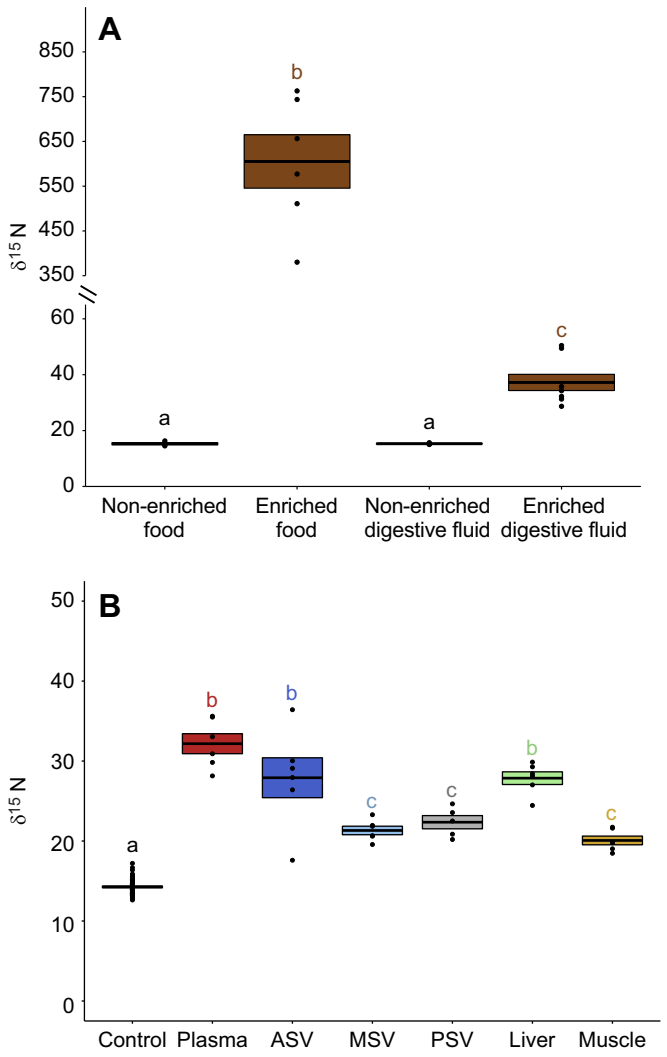

Fig. 2. ^{15}N-enriched herring slurry method validation. (A) δ^{15}N values (ratio of ^{15}N to ^{14}N isotope) of herring slurry without (non-enriched food) and with 7 mmol l^{-1} $^{15}NH_4Cl$ (enriched food), collected prior to being fed via gavage to North Pacific spiny dogfish, and the corresponding enriched/non-enriched digestive fluids collected after 20 h digestion (n=4–6). (B) δ^{15}N values of whole tissue and plasma collected from dogfish following 20 h digestion of $^{15}NH_4Cl$ herring slurry (n=6). Control represents tissues (all three spiral valve regions, liver, muscle) and plasma from control animals (data for 7 day fasted and 20 h non-enriched fed dogfish combined; n=72). The horizontal line within the jitter boxplots indicates the mean, and the upper and lower box boundaries indicate the s.e.m., with individual data points shown as black dots. Means not sharing the same letter are significantly different (one-way ANOVA, P<0.05).

Ammonia

The non-protein supernatants were transferred to 25 ml glass Erlenmeyer flasks and sealed with rubber stoppers, from which a basket was suspended (Fig. S1). To release the ammonia from the samples, 0.15 ml of 10 mol l^{-1} sodium hydroxide (NaOH; Sigma-Aldrich) was added to the supernatant in the flask via syringe through the rubber stopper. To trap the ammonia, 0.5 ml of 0.1 mol l^{-1} hydrochloric acid (HCl; Sigma-Aldrich) was added to the plastic basket via syringe through the rubber stopper. The flasks were incubated in an oscillating water bath (New Brunswick C76, Edison, NJ, USA) at 85 rpm for 24 h at 37°C. After 24 h, the acid trap was collected as 'ammonia', lyophilized, and processed for ^{15}N analysis (see below).

Urea

After the removal of ammonia, the fluid remaining in the Erlenmeyer flask was neutralized to pH 6.5–8 with 0.1 mol l^{-1} HCl, and diluted with 2 ml of 10 mmol l^{-1} Hepes, pH 6.5, and 3 mmol l^{-1} EDTA. To release the urea as ammonia, 6 U urease (from *Canavalia ensiformis*; Sigma-Aldrich) suspended in 0.1 ml deionized H_2O, were added to the supernatant fluid via syringe through the rubber stopper. The flasks were incubated in the oscillating water bath at 85 rpm at 25°C. After 24 h, 0.5 ml of 0.1 mol l^{-1} HCl was added to the basket and 0.15 ml of 10 mol l^{-1} NaOH was added to the flask contents, as described above. The samples were incubated in the oscillating water bath at 85 rpm at 37°C. After 24 h, the acid trap, now containing the urea as ammonia, was collected as 'urea', lyophilized, and processed for ^{15}N analysis (see below).

Glutamine

The non-protein supernatants from a duplicate set of samples were transferred to 25 ml glass Erlenmeyer flasks. The flasks were sealed and the ammonia was removed and collected, as described above. The remaining supernatant was then adjusted to pH 5 with HCl. To release glutamine as ammonia, 0.1 ml of 2 U glutaminase dissolved in 5 mmol l^{-1} sodium acetate, pH 6 (adjusted with acetic acid), was added via syringe through the rubber stopper. The samples were incubated in the oscillating water bath at 85 rpm and 37°C. After 24 h, 0.5 ml of 0.1 mol l^{-1} HCl was added to the basket, and 0.15 ml of 10 mol l^{-1} NaOH was added to the flask contents, as described above. The samples were incubated in the oscillating bath at 85 rpm and 37°C. After 24 h the acid trap was collected as 'glutamine', lyophilized, and processed for ^{15}N analysis (see below).

Bulk amino acids

Following the removal of glutamine, the fluid remaining in the flask was adjusted to pH 5 with HCl, and 1 ml of 3 mol l^{-1} sodium acetate, pH 5 (adjusted with acetic acid), and 2 ml of 3% ninhydrin solution was added. Ninhydrin was used to release the amino groups as ammonia (Kennedy, 1965), collectively referred to as 'bulk amino acids', with the exception of the glutamine previously removed. Samples were incubated at 100°C for 10 min in a water bath before 0.25 ml of 30% hydrogen peroxide solution was added and samples were incubated again at 100°C for 10 min. Samples were allowed to cool, collected as 'amino acids', lyophilized, and processed for ^{15}N analysis (see below).

Lyophilization

The whole tissue and protein samples were lyophilized (Labconco Benchtop Freeze-dry System), ground with mortar and pestle and weighed (0.0006–0.0008 g) on a microbalance (XP2U, Mettler Toledo). The nitrogen compounds within the acid traps did not lyophilize well and resulted in a hard white residue that adhered to the vials, so glass fibre filters (GFFs) were used to absorb the analyte of interest prior to lyophilization. The GFFs were pre-weighed and 0.03 ml sample was added and lyophilized. GFFs were re-weighed and additional 0.03 ml sample added and lyophilized again. This was repeated until GFFs contained between 0.0006 and 0.0008 g of lyophilized sample.

Enzyme assays

The activity of two OUC enzymes (GS and ARG) was examined in whole tissue from the ASV, MSV, PSV, liver and muscle. To

examine whether an animal's metabolic state affects urea synthesis, tissues from fasted dogfish were compared with those of 20 h non-enriched and 72 h enriched fed dogfish. The 20 h post-fed dogfish were examined owing to the documented significant influx of urea from the lumen into the plasma that occurs at 20 h post-feeding (Kajimura et al., 2008; Wood et al., 2007b, 2010). The later digestion time point of 72 h was chosen as the spiral valve has considerably less food/chyme than at 20 h, but is not yet empty (J.L.H., personal observation), and presumably the animal still has access to dietary/lumenal nitrogen for uptake. Activity levels in the liver from the 72 h post-fed dogfish were not determined because of limited tissue availability. Frozen samples were removed directly from −80°C and ground to a fine powder with mortar and pestle in liquid N_2. The ground tissues were homogenized with 4 volumes of ice-cold homogenization buffer (50 mmol l^{-1} Hepes, pH 8.0) in a bead mill (VWR Mini Bead Mill Homogenizer), centrifuged and the resulting supernatants used in the respective assays (see below), following published methods (Kajimura et al., 2006; Mommsen and Walsh, 1989).

GS activity

GS activity was measured by the formation of L-glutamic acid γ-monohydroxamate. Supernatant was incubated in a GS reaction cocktail (in mmol l^{-1}: 60 glutamine, 15 hydroxylamine, 0.4 ADP, 20 KH_2ASO_4, 50 Hepes, 3 $MnCl_2$; pH 6.7) for 10 min. Reactions were terminated by the addition of ferric chloride reagent (FCR; in a 1:1:1 ratio, 50% HCl:24% trichloroacetic acid:10% $FeCl_3$ in 0.2 mol l^{-1} HCl), and centrifuged, transferred to a microtitre plate, and absorbance was read at 540 nm (Powerwave XS, BioTek). Assay controls were handled in the same manner but the reaction was terminated with FCR prior to the addition of the GS reaction cocktail. A standard curve was generated from a serial dilution series of L-glutamic acid γ-monohydroxamate (Sigma-Aldrich) incubated in GS cocktail and terminated with the FCR, as described above. GS activity was calculated as the formation of L-glutamic acid γ-monohydroxamate in μmol min^{-1} g^{-1} of tissue:

$$\text{GS activity} = \frac{\Delta A}{S} \times \frac{d}{VT}, \quad (2)$$

where ΔA is the absorbance of the sample minus the absorbance of the corresponding assay control; S is the standard curve of the L-glutamic acid γ-monohydroxamate; d is the dilution factor of tissue to the homogenization buffer; V is the volume of supernatant divided by the total volume of assay placed in the microtitre plate; and T is the assay incubation time.

ARG activity

ARG activity was measured by the formation of urea. Supernatant was preincubated in 9 volumes of preincubation reagent (5 mmol l^{-1} $MnCl_2$ in 50 mmol l^{-1} Hepes) for 10 min to activate the enzyme, then incubated in ARG reaction cocktail (in mmol l^{-1}: 250 arginine, 1 $MnCl_2$, 50 Hepes, pH 8.0) for 60 min. The reaction was terminated by the addition of 70% perchloric acid, and assayed for the production of urea using the diacetyl monoxime method (Rahmatullah and Boyde, 1980). Briefly, the ARG reaction mix was added to deionized water and urea reagent [a 2:1 mix of Solution A (60 ml H_2O, 30 ml H_2SO_4, 10 ml 85% H_3PO_4, 0.01 g $FeCl_3$) and Solution B (100 ml H_2O, 0.01 g thiosemicarbazide, 0.5 g 2,3 butanedionemonoxime)], and boiled in the dark at 100°C for 10 min. Samples were transferred to a microtitre plate and absorbance was read at 540 nm (Powerwave XS, BioTek). Assay controls were handled in the same manner, but the ARG reaction was terminated with 70% perchloric acid prior to the addition of the reaction cocktail. A standard curve was generated from a serial dilution series of urea (Sigma-Aldrich), assayed using the diacetyl monoxime method, as described above. ARG activity was calculated as the production of urea in μmol min^{-1} g^{-1}:

$$\text{ARG activity} = \frac{\Delta A}{S} \; x \; \frac{V_{\text{ARG}} V_{\text{u}} d_{\text{h}} d_{\text{p}}}{V_{\text{s}} V_{\text{p}} T}, \quad (3)$$

where ΔA is the absorbance of the sample minus the absorbance of the corresponding assay control; S is the standard curve of the urea; V_{ARG} is the ARG assay volume (preincubate, perchloric acid and ARG reaction cocktail); V_{urea} is the urea assay volume (ARG reaction and water); d_{h} is the dilution of tissue to homogenization buffer; d_{p} is the dilution of homogenized supernatant to preincubation reagent; V_{s} is the volume of the homogenized supernatant used in preincubation reagent; V_{p} is volume of the preincubate used in the ARG assay; and T is the ARG assay incubation time.

Statistics and figures

Statistical analysis was conducted using Posit (formerly *RStudio*: https://posit.co/) and figures were produced using the package *ggplot2* (https://CRAN.R-project.org/package=ggplot2). Data were checked for normality (Shapiro–Wilks) and homogeneity of variance (Levene's test). One-way or two-way analysis of variance (ANOVA) was performed and Tukey's *post hoc* test was used to detect significant differences. Where only two samples were available (e.g. enzyme activity in the liver), Student's *t*-test was used. Differences were accepted as significant when $P<0.05$. Data are expressed as means±s.e.m within the text, tables and figures.

RESULTS

There was a significant difference between the non-enriched ($\delta^{15}N$=15.3±0.4; n=4) and ^{15}N-enriched ($\delta^{15}N$=605.0±59.7; n=6) herring slurry collected prior to being fed to the dogfish ($F_{3,18}$=81.8, $P<0.001$) (Fig. 2A). The digestive fluids/chyme collected from the spiral valve of the 20 h non-enriched fed ($\delta^{15}N$=15.3±0.1; n=4) and 20 h enriched fed dogfish ($\delta^{15}N$=37.2±2.9; n=6) were also significantly different ($P<0.001$) (Fig. 2A). All tissue and plasma samples from the 7 day fasted ($\delta^{15}N$=14.2±0.1, n=36) and 20 h non-enriched fed ($\delta^{15}N$=14.4±0.2, n=36) dogfish showed similar $\delta^{15}N$ values, with no significant difference between the groups ($P>0.3$) (Table 1). The non-enriched fed and fasted groups were thus combined and collectively referred to as control ($\delta^{15}N$=14.3±0.1; n=72), and used as the baseline $\delta^{15}N$ signal from which to compare the dietary incorporation (i.e. enrichment) of labelled nitrogen (Fig. 2B).

To validate the use of the ^{15}N-enriched herring slurry and the method of administration via gavage, whole tissues were initially collected from the 20 h enriched fed dogfish and compared with the control baseline values (Fig. 2B). One-way ANOVA showed a significant enrichment in the $\delta^{15}N$ values of the 20 h enriched fed animals ($F_{6,100}$=174.4, $P<0.001$). There were also significant differences in the $\delta^{15}N$ values between the tissues; plasma, ASV and liver levels were all significantly higher compared with MSV, PSV and muscle levels ($P<0.05$), but not compared with each other ($P>0.05$).

Whole tissue

For whole tissue ^{15}N enrichment, two-way ANOVA showed a significant interaction between time (20, 48, 72, 168 h) and tissue

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Table 1. $\delta^{15}N$ values for whole tissues from control North Pacific spiny dogfish (*Squalus acanthias suckleyi*)

Tissue	$\delta^{15}N$ control		$\delta^{15}N$ 20 h enriched fed
	7 day fasted	20 h non-enriched fed	
ASV	13.2±0.1	13.3±0.2	27.9±2.5*
MSV	13.7±0.1	13.8±0.3	23.3±0.5*
PSV	13.9±0.1	14.1±0.3	22.4±0.8*
Plasma	14.6±0.2	14.6±0.2	32.2±1.3*
Liver	15.4±0.4	15.7±0.3	27.9±0.8*
Muscle	14.3±0.4	14.4±0.3	20.1±0.5*
Mean $\delta^{15}N$ of all tissues	14.2±0.1	14.4±0.2	25.4±0.9*

Control animals were 7 day fasted and 20 h non-enriched fed dogfish; $\delta^{15}N$ values for whole tissues from 20 h enriched fed dogfish are included for comparison (n=6). Data are means±s.e.m. *P<0.05 versus both control groups (two-way ANOVA and Tukey *post hoc* test). For statistical significance of ^{15}N enrichment between tissues, see Figs 2 and 3. ASV, anterior spiral valve; MSV, mid spiral valve; and PSV, posterior spiral valve.

(ASV, MSV, PSV, plasma, liver, muscle) ($F_{20,175}$=3.5, P<0.001). All whole tissues were enriched in ^{15}N within 20 h (P<0.01) compared with control, and remained enriched throughout all time points (P<0.001) (Fig. 3, Table 1; Fig. S2). There were no significant differences in ^{15}N enrichment within the individual tissues across the four time points (P>0.07), but there were differences between the tissues at each time point. At 20 h post-feeding, the $\delta^{15}N$ values of the ASV, plasma and liver were significantly higher than those of the MSV and muscle (P<0.02), but only the plasma levels were significantly higher than those of the PSV (P<0.001). At 48 h post-feeding, the $\delta^{15}N$ values of the plasma were significantly higher than those of all other tissues (P<0.02) (Fig. 3B; Fig. S2B), and the muscle levels were significantly lower than the ASV levels (P<0.2). At 72 h, the $\delta^{15}N$ values of the plasma were significantly higher than those of the muscle (P<0.05), but not the liver (P=0.06). At 168 h, the $\delta^{15}N$ values of the plasma were significantly higher than those of the MSV, liver and muscle (P<0.05).

Ammonia

For ^{15}N-ammonia, two-way ANOVA showed a significant interaction between time and tissue ($F_{20,169}$=23.5, P<0.001). Ammonia was significantly enriched in ^{15}N at all time points and in all tissues compared with control (P<0.01) (Fig. 4; Fig. S3). The ASV had significantly higher $\delta^{15}N$ values at 20 h compared with all other time points (P<0.001), and all other tissues (P<0.001). The $\delta^{15}N$ values of the PSV were significantly higher at 20 h than at 72 and 168 h post-feeding (P<0.02). There were no significant differences in $\delta^{15}N$ values across the time points for the MSV, plasma, liver or muscle (P>0.08).

At all time points, the ASV had significantly higher $\delta^{15}N$ values compared with the MSV, PSV and muscle (P<0.01), and higher than the plasma at 20, 48 and 72 h post-feeding (P<0.01), and the liver at 20 h (P<0.001). The liver had significantly higher $\delta^{15}N$ values than the muscle at 20 and 48 h post-feeding (P<0.05).

Glutamine

For ^{15}N-glutamine, two-way ANOVA showed a significant interaction between time and tissue ($F_{20,161}$=4.4, P<0.001). Glutamine was significantly enriched in the ASV and plasma at all time points compared with control (P<0.001) (Fig. 5; Fig. S4).

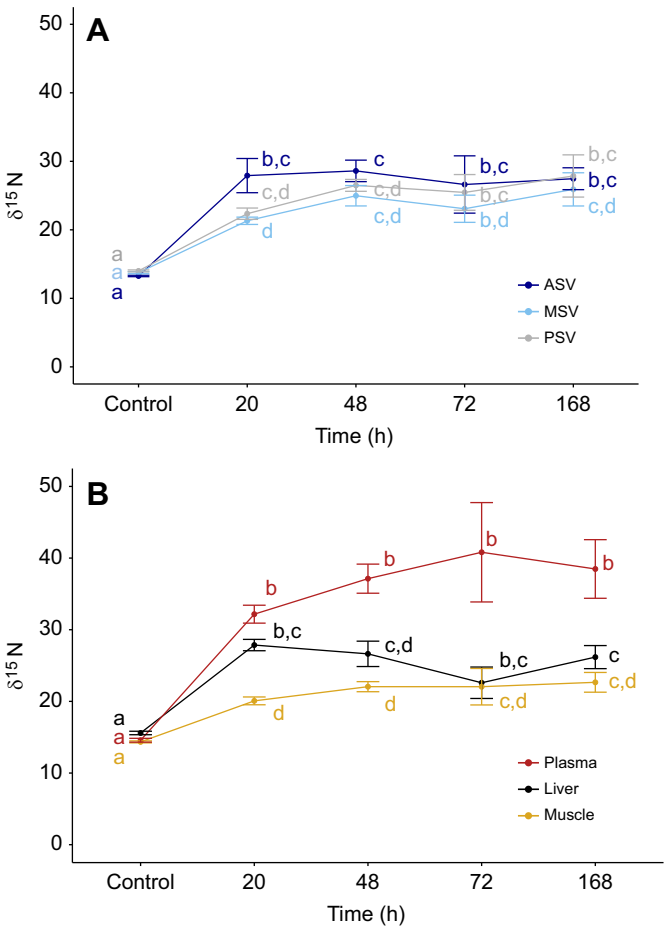

Fig. 3. $\delta^{15}N$ values from whole tissues and plasma of North Pacific spiny dogfish following ingestion of ^{15}N-enriched herring slurry. Dogfish were fed herring slurry enriched with 7 mmol l^{-1} $^{15}NH_4Cl$ via gavage, and tissues and plasma were collected after 20, 48, 72 or 168 h digestion (n=6 animals per time point). Also shown are the $\delta^{15}N$ values of the whole tissues and plasma of control animals (see Fig. 2; n=12). (A) ASV, MSV and PSV. (B) Plasma, liver and muscle. Data are presented as means ±s.e.m.; means not sharing the same letter are significantly different (two-way ANOVA, P<0.05).

The MSV was significantly enriched at 20, 48 and 168 h (P<0.01), while the PSV (P>0.09), muscle (P>0.06) and liver (P>0.2) were not significantly enriched at any time point.

At 20 h, the $\delta^{15}N$ value of the ASV was significantly higher than that of the PSV and muscle (P<0.05). At 48 h, the ASV levels were significantly higher than those of the MSV and muscle (P<0.05), and at 72 h, the ASV levels were significantly higher than those of the MSV, PSV and muscle (P<0.05). There were no significant differences in $\delta^{15}N$ values at 168 h post-feeding (P>0.3).

Urea

For ^{15}N-urea, there was a significant interaction between time and tissue ($F_{20,175}$=3.0, P<0.001). All tissues were significantly enriched at all time points (P<0.001) (Fig. 6; Fig. S5). At 20 h, the $\delta^{15}N$ values in the ASV were significantly higher than those in the MSV, PSV and muscle (P<0.05), but not the plasma or liver (P>0.2). There were no significant differences between the tissues at 48, 72 or 168 h (P>0.05), and no significant differences within the tissues across the time points (P>0.05).

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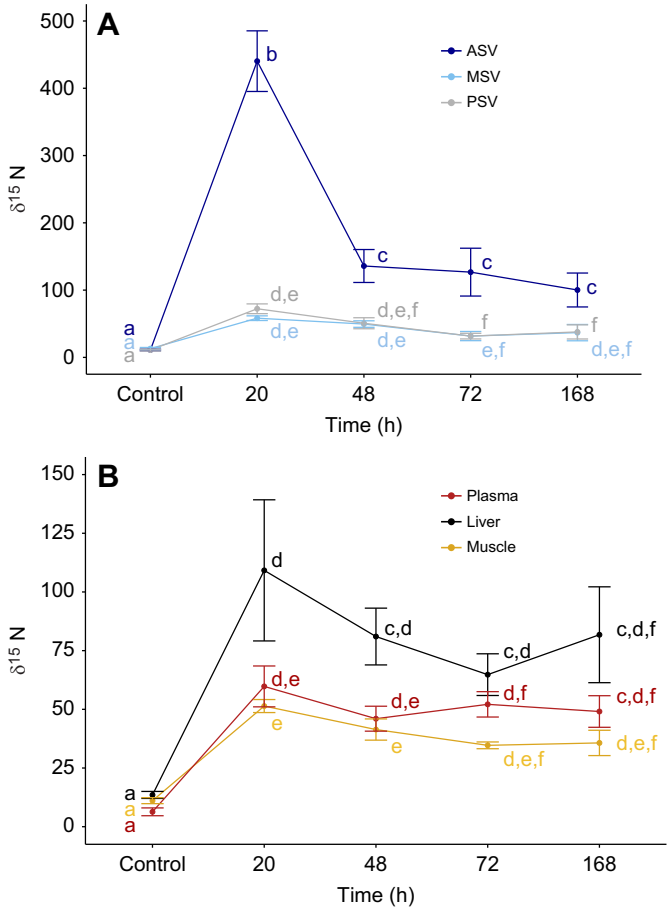

Fig. 4. δ^{15}N values for ammonia within tissues and plasma of North Pacific spiny dogfish following ingestion of ^{15}N-enriched herring slurry. Dogfish were fed herring slurry enriched with 7 mmol l^{-1} $^{15}NH_4Cl$ via gavage, and tissues and plasma were collected after 20, 48, 72 or 168 h digestion (n=6 animals per time point). Also shown are the δ^{15}N values of the ammonia component of the control animals (n=12). (A) ASV, MSV and PSV. (B) Plasma, liver and muscle. Data are presented as means±s.e.m.; means not sharing the same letter are significantly different (two-way ANOVA, P<0.05).

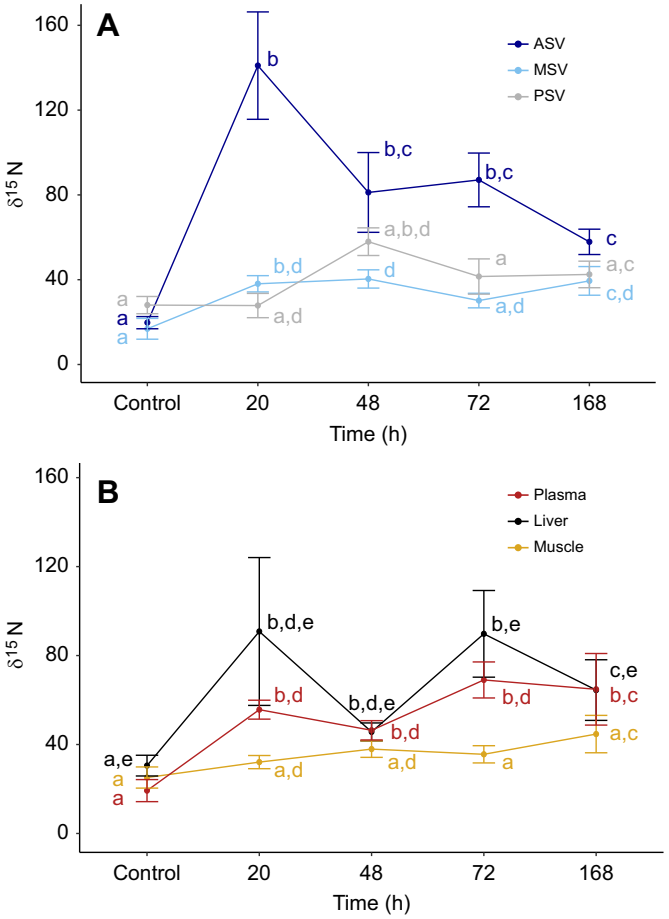

Fig. 5. δ^{15}N values for glutamine within tissues and plasma of North Pacific spiny dogfish following ingestion of ^{15}N-enriched herring slurry. Dogfish were fed herring slurry enriched with 7 mmol l^{-1} $^{15}NH_4Cl$ via gavage, and tissues and plasma were collected after 20, 48, 72 or 168 h digestion (n=6 animals per time point). Also shown are the δ^{15}N values of the glutamine component of the control animals (n=12). (A) ASV, MSV and PSV. (B) Plasma, liver and muscle. Data are presented as means±s.e.m.; means not sharing the same letter are significantly different (two-way ANOVA, P<0.05).

Bulk amino acids

For bulk ^{15}N-amino acids following the removal of glutamine, two-way ANOVA showed no significant interaction between time and tissue ($F_{20,164}$=0.9, P=0.6). The ASV, MSV, PSV, plasma and muscle were all significantly enriched with bulk ^{15}N-amino acids at all time points compared with control (P<0.01) (Fig. 7; Fig. S6), except the liver, which was significantly enriched at 48, 72 and 168 h post-feeding (P<0.01). At 20 h, the plasma δ^{15}N values were significantly higher than those of the liver (P<0.05). There were no other significant differences in δ^{15}N values between the tissues at any time point (P>0.05).

Protein

For ^{15}N-protein, two-way ANOVA showed a significant interaction between time and tissue ($F_{20,174}$=8.5, P<0.001). The tissues were all significantly enriched with ^{15}N-protein at all time points (P<0.01) (Fig. 8; Fig. S7), except the muscle, which was significantly enriched at 48, 72 and 168 h (P<0.05). The δ^{15}N values for the ASV were significantly higher at 20 and 48 h than at 72 and 168 h (P<0.05). In the PSV, the δ^{15}N values were significantly lower at 72 h than at 48 h (P<0.05), while the δ^{15}N values in the liver were significantly lower at 168 h than at 20 and 48 h (P<0.05).

At 20 and 48 h, the ASV, plasma and liver all had significantly higher δ^{15}N values than the MSV, PSV and muscle (P<0.001). At 48 h, the ASV levels were also significantly higher than those of the liver (P<0.01), but not the plasma (P>0.8), and there was no difference between the liver and plasma (P>0.05). At 72 and 168 h, there were no differences in δ^{15}N values across the three spiral valve regions (P>0.1), the liver or the muscle (P>0.05), but the plasma levels were significantly higher than those of the MSV, PSV and muscle (P<0.01).

Enzyme activity

For both GS and ARG, there were no statistical differences between the 20 h non-enriched fed and 72 h enriched fed dogfish (P>0.05), except for ARG activity in the MSV (see below). For GS activity, two-way ANOVA showed a significant interaction between treatment (fasted, non-enriched fed, 72 h enriched fed) and tissue (ASV, MSV, PSV, liver, muscle) ($F_{7,48}$=3.0, P=0.01). Within the tissues, treatment had no effect on GS activity levels, except for an

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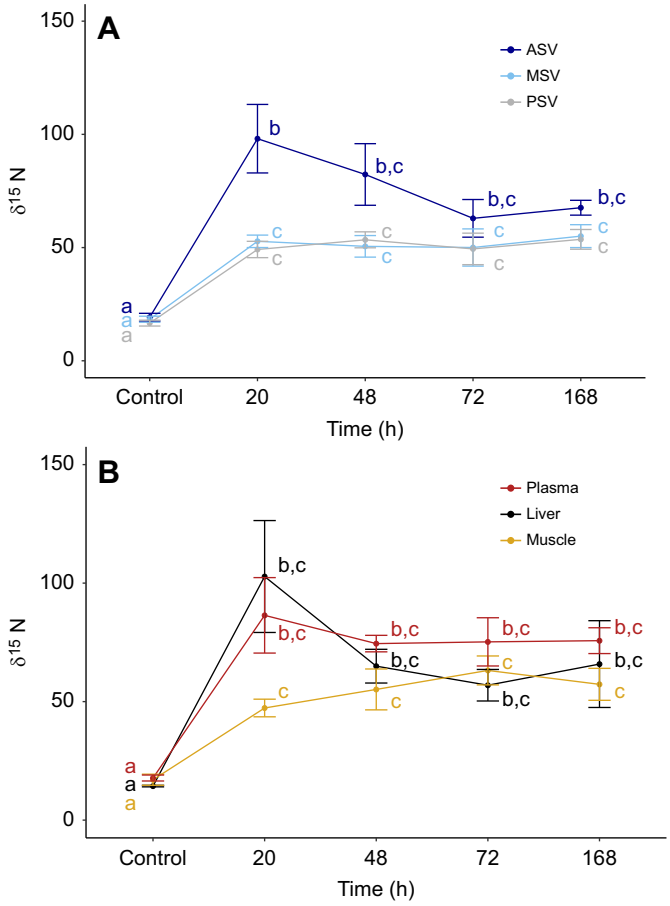

Fig. 6. δ^{15}N values for urea within tissues and plasma of North Pacific spiny dogfish following ingestion of ^{15}N-enriched herring slurry. Dogfish were fed herring slurry enriched with 7 mmol l^{-1} $^{15}NH_4Cl$ via gavage, and tissues and plasma were collected after 20, 48, 72 or 168 h digestion (n=6 animals per time point). Also shown are the δ^{15}N values of the urea component of the control animals (n=12). (A) ASV, MSV and PSV. (B) Plasma, liver and muscle. Data are presented as means±s.e.m.; means not sharing the same letter are significantly different (two-way ANOVA, P<0.05).

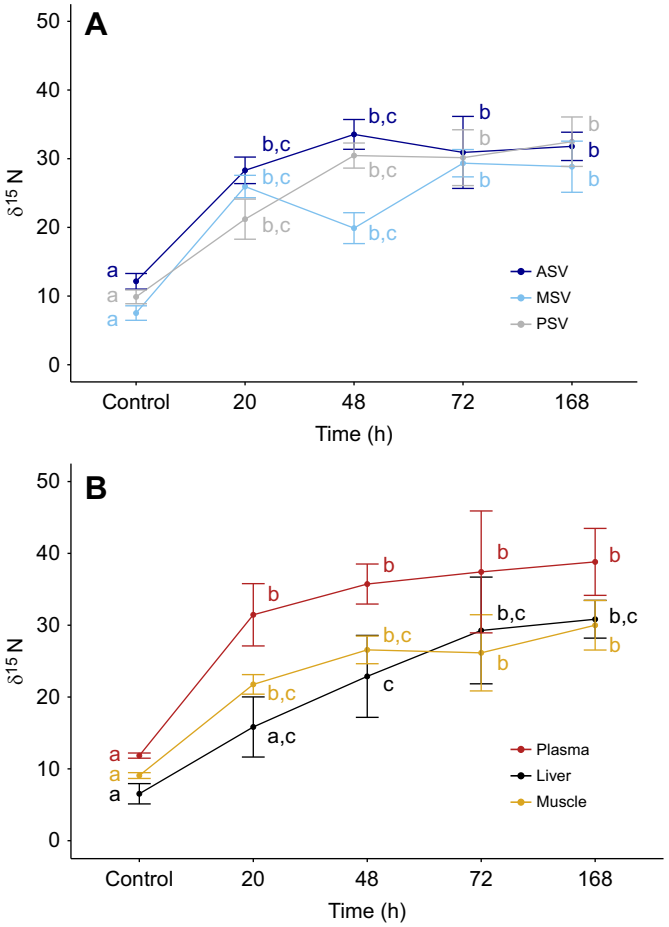

Fig. 7. δ^{15}N values for bulk amino acids (excluding glutamine) within tissues and plasma of North Pacific spiny dogfish following ingestion of ^{15}N-enriched herring slurry. Dogfish were fed herring slurry enriched with 7 mmol l^{-1} $^{15}NH_4Cl$ via gavage, and tissues and plasma were collected after 20, 48, 72 or 168 h digestion (n=6 animals per time point). Also shown are the δ^{15}N values of the bulk amino acid component of the control animals (n=12). (A) ASV, MSV and PSV. (B) Plasma, liver and muscle. Data are presented as means±s.e.m.; means not sharing the same letter are significantly different (two-way ANOVA, P<0.05).

increase in activity in the ASV of fasted dogfish compared with that of 72 h enriched fed dogfish (P<0.05) (Table 2). Among the tissues, GS activity in all three treatments was significantly higher in the ASV and MSV than in all other tissues (P<0.05), except between the ASV and the liver (P>0.08). GS activity in the liver was significantly higher than that in the PSV and muscle in the fasted animals (P<0.05), and significantly higher than that in the muscle in the 20 h post-fed animals (P<0.001).

For ARG activity, there was a significant interaction between treatment and tissue ($F_{7,54}$=3.5, P<0.01). Within the tissues, treatment only had a significant effect on ARG activity in the MSV of the 20 h non-enriched fed dogfish compared with the 72 h enriched fed dogfish (P<0.05) (Table 2). In the fasted and 20 h non-enriched fed dogfish, ARG activity was significantly higher in the liver than in all other tissues (P<0.01). The ASV and MSV also had significantly higher ARG levels in all three treatments compared with the PSV and muscle (P<0.05).

DISCUSSION

This is the first study to comprehensively track dietary labelled nitrogen in a nitrogen-limited marine elasmobranch, in an effort to better understand how ingested nitrogen is incorporated and used to synthesize nitrogenous compounds. Our results show exogenous labelled nitrogen acquired across the GI tract was mobilized throughout the whole animal within 20 h post-feeding and retained over the 168 h experimental period. During this period, the δ^{15}N values for ^{15}N-urea in the ASV (range of mean δ^{15}N values: 59.3–113.5; Fig. 6; Fig. S5) were 2–5 times higher than those for the ^{15}N-amino acids (range of mean δ^{15}N: 28.3–33.3; Fig. 7; Fig. S6) and ^{15}N-protein (range of mean δ^{15}N: 19.6–29.5; Fig. 8; Fig. S7). Our data indicate the preferential use of dietary nitrogen to synthesize the obligate osmolyte, urea, as well as its prerequisite, glutamine (range of mean δ^{15}N: 57.9–141.5) shortly after feeding. The dietary labelled nitrogen was also used to synthesize somatic compounds (amino acids and protein), but at apparent lower rates. Our data are presented as δ^{15}N, which is the ratio of ^{15}N to ^{14}N within a sample; therefore, we cannot say that the enrichment of ^{15}N within the tissues or plasma corresponds to an increase in concentration, only that a significant amount of dietary ^{15}N was incorporated into the tissue or nitrogenous compound of interest.

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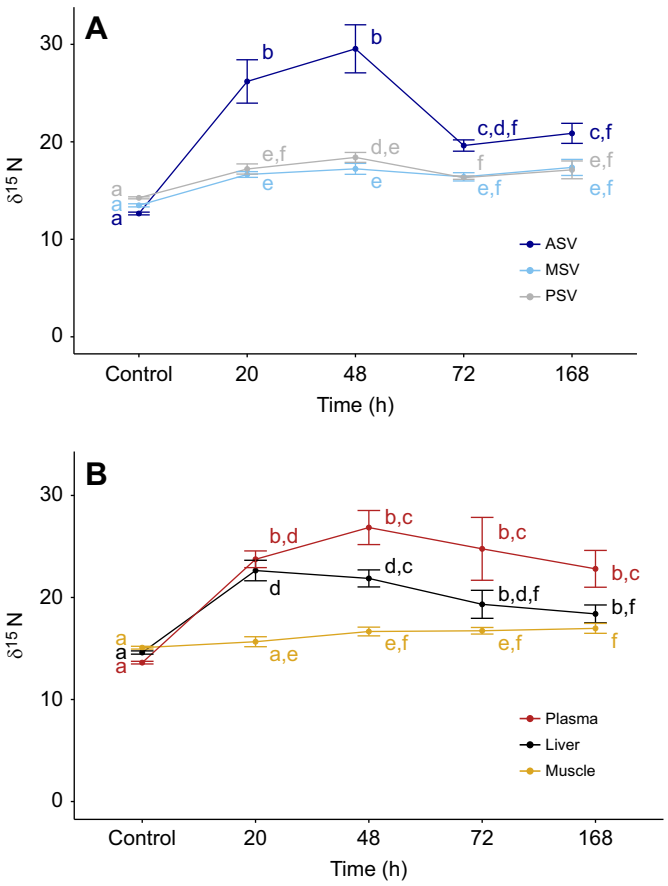

Fig. 8. $\delta^{15}N$ values for protein within tissues and plasma of North Pacific spiny dogfish following ingestion of ^{15}N-enriched herring slurry. Dogfish were fed herring slurry enriched with 7 mmol l^{-1} $^{15}NH_4Cl$ via gavage, and tissues and plasma were collected after 20, 48, 72 or 168 h digestion (n=6 animals per time point). Also shown are the $\delta^{15}N$ values of protein component of the control animals (n=12). (A) ASV, MSV and PSV. (B) Plasma, liver and muscle. Data are presented as means±s.e.m.; means not sharing the same letter are significantly different (two-way ANOVA, P<0.05).

Table 2. Glutamine synthetase (GS) and arginase (ARG) activity within fasted and fed North Pacific spiny dogfish

	7 day fasted	20 h non-enriched fed	72 h enriched fed
GS activity (μmol g^{-1} min^{-1})			
ASV	24.6±6^{a,c,*}	14.2±2^{a,c,*,‡}	10.0±2^{a,‡}
MSV	27.3±6^{a}	23.6±4^{a}	13.1±2^{a}
PSV	2.3±1^{b}	3.8±0.9^{b}	2.3±0.4^{b}
Liver	10.6±2^{c}	7.4±0.7^{b,c}	n/a
Muscle	0.5±0.1^{d}	0.4±0.1^{d}	0.8±0.1^{d}
ARG activity (μmol g^{-1} min^{-1})			
ASV	1.7±0.3^{a}	1.3±0.2^{a}	1.2±0.1^{a}
MSV	1.0±0.1^{a,*,‡}	1.3±0.1^{a,*}	0.9±0.1^{a,‡}
PSV	0.3±0.2^{b,d}	0.6±0.1^{b}	0.3±0.1^{b,d}
Liver	8.8±0.1^{c}	5.6±0.9^{c}	n/a
Muscle	0.3±0.1^{d}	0.2±0.1^{d}	0.5±0.1^{d}

Data are means±s.e.m (n=3–6). Means not sharing the same symbol within the same tissue across treatments are significantly different from one another; means not sharing the same letter within the same treatment and the same enzyme are significantly different from one another (two-way ANOVA and Tukey *post hoc* test, P<0.05). Statistical comparisons were not made between enzymes.

Whole tissue

Analysis of the whole tissues from the 7 day fasted ($\delta^{15}N$=14.2±0.1, n=36) and 20 h non-enriched fed dogfish ($\delta^{15}N$=14.4±0.2, n=36) showed similar $\delta^{15}N$ values (Table 1). Previous studies have demonstrated higher $\delta^{15}N$ values in animals following a period of fasting/starvation (Doucett et al., 1999; Gaye-Siessegger et al., 2007; Hertz et al., 2015), due to the preferential excretion of ^{14}N over ^{15}N (Gannes et al., 1997; Hobson et al., 1993). In our study, the 7 day fasting period was not long enough to induce a significant difference in $\delta^{15}N$ values between the fasted and 20 h non-enriched fed animals (Table 1); therefore, we combined the two groups into a baseline control group ($\delta^{15}N$=14.3±0.1, n=72) to examine the incorporation of dietary labelled nitrogen in the $^{15}NH_4Cl$-fed dogfish. We found a significant enrichment above control values in all whole tissues (Fig. 2B).

Throughout the 168 h experimental period, the plasma $\delta^{15}N$ values were significantly higher than those of the MSV and the muscle, as well as the ASV at 48 h, the liver at 48 and 168 h, and the PSV at 20 and 48 h (Fig. 3; Fig. S2). Captive feeding studies on elasmobranchs demonstrated the blood and liver had higher metabolic rates and faster turnover rates than the muscle (Logan and Lutcavage, 2010; MacNeil et al., 2006; Malpica-Cruz et al., 2012), indicative of faster rates of nitrogen addition and/or replacement. While we did not analyse whole blood, the dogfish plasma $\delta^{15}N$ values during the 168 h experimental period remained relatively high ($\delta^{15}N$=37.2±1.8; Fig. 3B; Fig. S2B) compared with those of the other tissues (mean $\delta^{15}N$ of all whole tissues=20.8±0.6; Fig. 3; Fig. S2), indicating the continual circulation of labelled nitrogen via the plasma. Additionally, despite a slow turnover rate in the muscle (Logan and Lutcavage, 2010), labelled nitrogen was incorporated within 20 h post-feeding, albeit at lower values ($\delta^{15}N$=21.7±0.6) than in most of the other tissues, but at levels not significantly different from those in the MSV ($\delta^{15}N$=23.8±1.0) (P>0.9). These captive feeding studies (Logan and Lutcavage, 2010; MacNeil et al., 2006; Malpica-Cruz et al., 2012) aid in our ecological understanding of elasmobranch foraging and movement. In contrast, our study provides $\delta^{15}N$ values in various tissues following the ingestion of a single ^{15}N-enriched meal, with the goal of understanding the physiological assimilation of dietary nitrogen throughout the body in the short-term immediate post-feeding period. Therefore, care should be taken when extrapolating from our data to infer any ecological trophic structures, food-web dynamics, or tissue turnover rates for marine elasmobranchs.

An important finding of this study is the capacity of these animals to maintain enriched levels of labelled nitrogen in all tissues examined (Fig. 3; Fig. S2). Previous studies have reported digestion within *S. acanthias suckleyi* requires approximately 5 days (120 h) (Jones and Geen, 1977; Wood et al., 2007a). In our study, food was withheld from the dogfish for 7 days, at which point their spiral valves were virtually clear of digestive material (Fig. 1B). Therefore, retention of the dietary labelled nitrogen throughout the 168 h experimental period indicates the ability of these animals to regulate their nitrogen stores, both during times of active digestion and once their GI tract is devoid of food. *Squalus acanthias suckleyi* have been shown to be capable of surviving at least 56 days without food, while maintaining plasma urea concentrations and osmolality (Kajimura et al., 2008; Wood et al., 2010). Coupling the ingestion of labelled nitrogen with a long-term fasting study would allow for a better understanding of their putative intermittent feeding behaviours and long-term reliance on dietary nitrogen, as well as an understanding of how these animals recycle and retain nitrogen, and when in their fasted state they are compelled

to catabolize protein stores to recycle protein-nitrogen into urea-nitrogen to maintain osmolality.

Ammonia

The ^{15}N-enriched diet allowed us to examine potential regional differences of nitrogen incorporation along the length of the spiral valve over a short-term post-feeding period. Our data demonstrated the ASV had significantly higher δ^{15}N values than the MSV and PSV (Fig. 4A; Fig. S3A), indicating dietary ammonia uptake was greatest in the anterior region. The spiral valve has the capacity to transport ammonia from the lumen into the intestinal villi via Rhp2 and/or Rhbg (Anderson et al., 2010; Hoogenboom et al., 2023; Nawata et al., 2015a), with apically expressed Rhp2 increasing in the PSV of fed cloudy catsharks (*Scyliorhinus torazame*) compared with levels in the ASV (Hoogenboom et al., 2023). In our study, the consumption of $^{15}NH_4Cl$ would negate the need to catabolize the ^{15}N-ammonia before transport; in contrast, when consuming whole prey, it is possible the digestive processes may not catabolize dietary amino acids and proteins into transportable ammonia until further along the spiral valve, in which case an increase in distal ammonia transporters would be necessary, as seen in the catsharks (Hoogenboom et al., 2023). We are not aware of any study that has investigated the affinity or capacity for intestinal ammonia transport proteins within any fish species, and investigations into this would provide a better understanding of the transport capabilities of ammonia across intestinal tissues.

The enrichment of ^{15}N-ammonia throughout the tissues highlights the importance of dietary ammonia acquisition across the spiral valve. Because of their ureosmotic nature, investigations into ammonia handling in marine elasmobranchs have focused heavily on excretion and/or retention via branchial routes (for a review, see Weihrauch et al., 2009). Only recently have studies begun to focus on the capabilities of these ureosmotic animals to traffic ammonia for purposes other than excretion (Anderson et al., 2010; Hoogenboom et al., 2020, 2023; Nawata et al., 2015a,b; Wood and Giacomin, 2016; Wood et al., 2019). Our study demonstrates that ammonia within the spiral valve is an important source of nitrogen for these animals, as it is clear they possess mechanisms to acquire and retain lumenal ammonia and circulate via the plasma. Marine elasmobranchs are considered ureotelic, with ammonia excretion accounting for <3% of total nitrogen loss (Wood et al., 1995), and following feeding, ammonia excretion via the gills increased only slightly (Wood et al., 2005). We did not examine nitrogen excretion in this study; however, the δ^{15}N values for the ASV at 20 h post-feeding (440.3±45.1) and the enriched food (605.0±59.7) prior to being fed to the dogfish were not significantly different from one another (P>0.05). Therefore, although nitrogen excretion rates in our study are unresolved, the data indicate ammonia acquisition across the spiral valve tissues is an important component of whole-body nitrogen homeostasis in these animals.

Glutamine

The enrichment of ^{15}N-glutamine in the ASV closely matched the acquisition of lumenal ^{15}N-ammonia at 20 h (Figs 4A and 5A; Figs S3A and S4A). These data, along with the detection of GS activity (Table 2; Kajimura et al., 2006) demonstrate that dietary nitrogen is used to synthesize glutamine within the spiral valve tissues. The synthesis of glutamine in the intestinal tissues would also be partially responsible for maintaining an inwardly directed concentration gradient of ammonia and facilitate its uptake from the lumen. The decreasing trend of δ^{15}N values in the ASV from 48 to 168 h may be the result of the glutamine being transported to the plasma and/or being used to synthesize other compounds, including urea (Fig. 5A; Fig. S4A). Marine elasmobranchs primarily synthesize urea via the OUC and use glutamine as the nitrogen-donating substrate (Anderson, 1981, 1991; Casey and Anderson, 1983). The presence of ^{15}N-glutamine, ^{15}N-urea, and GS and ARG enzyme activity (Table 2) provide contributing evidence that the OUC operates within the spiral valve of *S. acanthias suckleyi*, as previously proposed (Kajimura et al., 2006). It was also proposed that the 20 h post-feeding increase in plasma urea concentration, demonstrated in *S. acanthias suckleyi*, was due to the reabsorption of lumenal urea that had been moved pre-prandially into the GI tract to balance the osmotic pressure of the chyme with the internal osmotic pressure (Kajimura et al., 2006, 2008; Wood et al., 2007b, 2010). Our data, along with those of Kajimura et al. (2006) suggest that the post-prandial increase in plasma urea may in part be from urea synthesized in the intestinal tissues using dietary nitrogen, which is then moved to the plasma.

In the muscle and liver, there was no significant enrichment in ^{15}N-glutamine (Fig. 5B; Fig. S4B). However, in the muscle, there was an increasing trend, although not significant, of δ^{15}N values from 20 h (δ^{15}N=32.1±3.0) to 168 h (δ^{15}N=44.7±8.4), indicating dietary labelled nitrogen was circulated and incorporated into the muscle. The δ^{15}N values within the liver at 20, 48 and 168 h (δ^{15}N=90.8±33.2, 45.6±4.0 and 64.5±13.6, respectively) would also suggest that glutamine synthesized with the labelled nitrogen was present, but the high variation between samples negated any statistical significance from the control animals. Although the liver is the primary organ for urea synthesis in marine elasmobranchs (Schooler et al., 1966), when whole-body mass is considered, the muscle may contribute more to the production of urea than the liver (Kajimura et al., 2006; Steele et al., 2005). Therefore, it is possible that the non-significant signal of ^{15}N-glutamine in the muscle and liver may be due to the ^{15}N being quickly donated to the OUC for synthesis of urea. Alternatively, as our data are presented as δ^{15}N values (the ratio of ^{15}N to ^{14}N), the lack of a significant signal in the liver and muscle may also be due to the large abundance of ^{14}N-glutamine present within the tissues, which may mask the signal of ^{15}N-glutamine synthesized from the dietary ^{15}N.

Urea

Within 20 h post-feeding, there was a significant enrichment of ^{15}N-urea in all tissues, which persisted throughout the 168 h experimental period (Fig. 6; Fig. S5). The use of labelled nitrogen allowed us to examine how quickly and, to some extent, in what tissues marine elasmobranchs use dietary nitrogen to synthesize urea. Similar to ^{15}N-ammonia and ^{15}N-glutamine, the δ^{15}N values for urea at 20 h post-feeding were significantly higher in the ASV than in the MSV or PSV. There was also a significant enrichment of ^{15}N-urea in the liver and muscle by 20 h post-feeding. This is not surprising, as both tissues are known to synthesize urea (Kajimura et al., 2006; Schooler et al., 1966; Steele et al., 2005). Our data cannot resolve the total contribution that each tissue made to the whole-body urea budget, but the presence of ^{15}N-ammonia, ^{15}N-glutamine, ^{15}N-urea, and both GS and ARG activity indicate the synthesis of urea occurs in the liver, muscle and spiral valve, and is circulated through the plasma.

One notable aspect of our data is the significant enrichment of ^{15}N-urea at all time points. Urea contains two nitrogen molecules and is generally considered ^{15}N depleted, which creates a large pool of ^{14}N that can mask ^{15}N within the samples (Hussey et al., 2012; Li et al., 2016). Therefore, because of the presumably large quantity of

^{14}N already present within the endogenous urea, as well as the dietary ^{14}N ingested as part of the herring slurry, it is worth noting that a significant enrichment in ^{15}N-urea was demonstrated against the large pool of endogenous ^{14}N-urea, indicating that a large portion of dietary labelled nitrogen was used to synthesize urea, enough to show against an already substantial ^{14}N pool of endogenous urea-nitrogen.

An important omission in our study was an investigation into the potential role of the microbiome in nitrogen recycling within the elasmobranch gut. The presence of urease, an enzyme responsible for catabolizing urea to ammonia, has been demonstrated within the epithelial tissue and digestive fluids of *S. acanthias suckleyi*, and is thought to be microbial in origin (Wood et al., 2019). In this study, we provided readily available nitrogen in the form of labelled $^{15}NH_4Cl$. Under normal digestion, the microbiome would be involved in processing and providing a large quantity of nitrogen to the host. The possible impact of the microbiome on the distribution and use of the labelled nitrogen would be interesting to investigate, particularly the synthesis of essential, non-essential and trophic amino acids. Additionally, the persistence of the ^{15}N signal within the spiral valve throughout the 168 h experimental period may partially be the result of microbial-generated nitrogen recycling.

Bulk amino acids

All tissues were significantly enriched in bulk ^{15}N-amino acids by 20 h, and remained enriched throughout all time points, with the exception of the liver, which was not significantly enriched until 48 h post-feeding (Fig. 7; Fig. S6). All spiral valve regions showed similar $\delta^{15}N$ values for the bulk amino acids at all time points, indicating a comparable contribution to amino acid synthesis. These data also indicate that not all free amino acids moving from the spiral valve to the plasma are from the hydrolyzation of dietary proteins (i.e. herring slurry); rather, some were synthesized using the dietary labelled nitrogen. In *S. acanthias suckleyi* that fed voluntarily on a non-enriched meal, both essential and non-essential amino acid concentrations increased in the plasma within 6 h post-feeding, and remained elevated over the 60 h sampling period, with the exception of a return back to pre-fed levels at 30 h (Wood et al., 2010). In our study, the persistent ^{15}N-amino acid enrichment at all time points, with the exception of the liver at 20 h, would indicate either the continual synthesis of ^{15}N-amino acids or the continued recycling of ^{15}N through synthesis and catabolism to maintain a pool of enriched ^{15}N-amino acids.

In addition to using amino acids as building blocks for protein synthesis, marine elasmobranchs can also use them, especially glutamine, as a source of aerobic fuel (Chamberlin and Ballantyne, 1992; Speers-Roesch and Treberg, 2010). Elasmobranchs have a higher proportion of glutaminase, an enzyme responsible for catabolizing glutamine to ammonia, than GS in their muscle, and, presumably, greater glutamine catabolism than synthesis (Chamberlin and Ballantyne, 1992). The low capacity of marine elasmobranchs to oxidize lipids in their muscle, and the high rates of glutamine catabolism, indicate the use of glutamine as an alternative energy source in red muscle used for locomotion (Chamberlin and Ballantyne, 1992). Therefore, it is not surprising that *S. acanthias suckleyi* use dietary nitrogen to synthesize amino acids necessary both for somatic processes and as a metabolic energy source. Future work to identify which individual amino acids are synthesized from dietary nitrogen will allow us to gain a better understanding of the partitioning of exogenous nitrogen into osmoregulatory, somatic or metabolic energy routes.

Protein

All tissues were significantly enriched in ^{15}N-protein by 20 h, and remained enriched throughout all time points, with the exception of the muscle, which was not significantly enriched until 48 h post-feeding (Fig. 8). Similar to the significant enrichment of ^{15}N-ammonia, ^{15}N-glutamine and ^{15}N-urea in the ASV, ^{15}N-protein was also significantly enriched at 20 h compared with that in the MSV and PSV (Fig. 8A; Fig. S7A). These data highlight the role that the ASV played in the acquisition of the labelled nitrogen, and suggest there are regional differences along the length of the spiral valve in terms of lumenal ammonia uptake and subsequent nitrogenous compound synthesis, at least around the onset of digestion (i.e. 20 h).

Interestingly, in contrast to the ^{15}N-protein enrichment, the ^{15}N-amino acid enrichment in the anterior region did not differ significantly from that in the other regions (Fig. 7A; Fig. S6A). To our knowledge, post-feeding protein synthesis has not been investigated within a marine elasmobranch species. In carnivorous teleosts, the maximum rate for whole-body protein synthesis occurred between 12 and 18 h post-feeding in cod (*Gadus morhua*), reaching a maximum synthesis rate within 6 h in the liver and stomach (Lyndon et al., 1992), and within 3 h in the liver and 6 h in the white muscle of rainbow trout (*Oncorhynchus mykiss*) (McMillan and Houlihan, 1989, 1992). A protein synthesis study in which ^{15}N-protein was fed to rainbow trout showed that these fish were capable of synthesizing 0.83 g of protein per gram ingested, and complete protein turnover occurred within 60 h post-feeding (Carter et al., 1994). It is unclear whether the protein synthesis rates of a teleost fish are comparable to those of a marine elasmobranch, as teleosts are not concerned with retaining nitrogen for urea synthesis, and therefore, a larger portion of dietary nitrogen is presumably available to the teleost for protein synthesis. Further research investigating the marine elasmobranch compartmentalization of dietary nitrogen into either osmoregulatory or somatic processes, as well as the protein synthesis rates, would allow us to better understand exogenous nitrogen usage by these animals.

Enzyme activity

To better understand the role of nitrogen acquisition from the spiral valve in the synthesis of urea, we examined two OUC enzymes: GS, which synthesizes glutamine, and ARG, which catalyses the synthesis of urea and ornithine (Anderson, 1991). There were no statistical differences in enzyme activity between treatments, except for GS activity in the ASV, and ARG activity within the MSV (Table 2). Therefore, it appears that the synthesis of urea was not greatly affected by the consumption of enriched dietary nitrogen, or by digestion time. As urea is an obligate osmolyte, and critical for the osmoregulatory strategy of marine elasmobranchs, it is not surprising that the mechanisms to synthesize this compound would function regardless of the metabolic state of the animal.

Conclusions

This is the first study to track dietary nitrogen uptake and incorporation in a marine elasmobranch. Our data show the ability of *S. acanthias suckleyi* to circulate dietary nitrogen throughout their whole body within 20 h post-feeding, and use it to synthesize glutamine, urea, amino acids and protein. We also showed the preferential usage of dietary nitrogen for the synthesis of urea over amino acid and protein synthesis necessary for somatic processes. Based on the data collected for this study, we propose the ASV was responsible for the majority of ^{15}N-ammonia uptake from the lumen, and that all spiral valve regions were involved in glutamine

and urea synthesis. The persistent enrichment of all of the examined nitrogenous compounds throughout the 168 h period highlights the ability of these animals to retain dietary nitrogen and utilize it in various tissues to synthesize the compounds necessary for both somatic and osmoregulatory processes.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: J.L.H., W.G.A.; Methodology: J.L.H.; Formal analysis: J.L.H.; Investigation: J.L.H.; Resources: W.G.A.; Writing - original draft: J.L.H.; Writing - review & editing: J.L.H., W.G.A.; Visualization: J.L.H.; Supervision: W.G.A.; Funding acquisition: W.G.A.

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Data availability
All relevant data can be found within the article and its supplementary information.

ECR Spotlight
This article has an associated ECR Spotlight interview with Lisa Hoogenboom.

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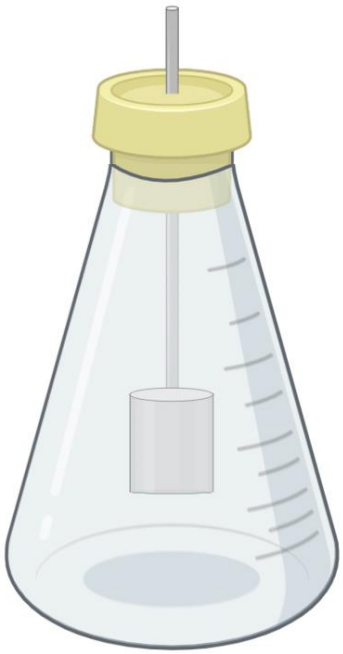

Fig. S1. Schematic of an Erlenmeyer flask and suspended basket used to separate and collect nitrogenous compounds. Image created with BioRender.com (2022).

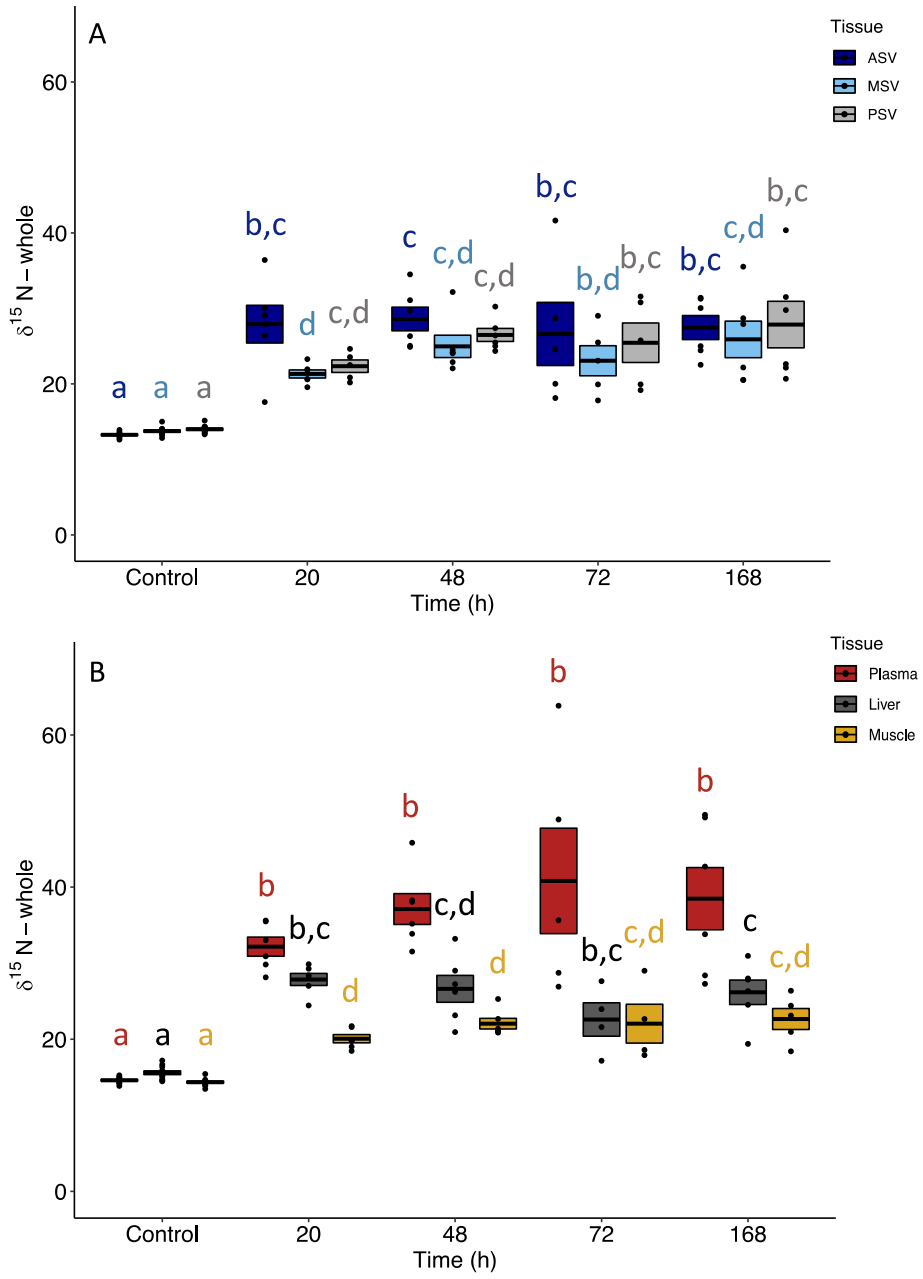

Fig. S2. δ^{15}N values for whole tissues and plasma of North Pacific spiny dogfish (*Squalus acanthias suckleyi*) following the ingestion of ^{15}N-enriched herring slurry. Dogfish were fed herring slurry enriched with 7 mmol l^{-1} $^{15}NH_4Cl$ via gavage, and tissues and plasma were collected after 20, 48, 72, or 168 h digestion (n = 6 animals per time point). Also shown are the δ^{15}N values of the whole tissues of control animals (n = 12). A) ASV, MSV, PSV, B) plasma, liver, and muscle. The horizontal line within the boxplots indicate means and the upper and lower box boundaries indicate sem with individual data points represented as black dots. Means not sharing the same letter are significantly different (two-way ANOVA, $P < 0.05$).

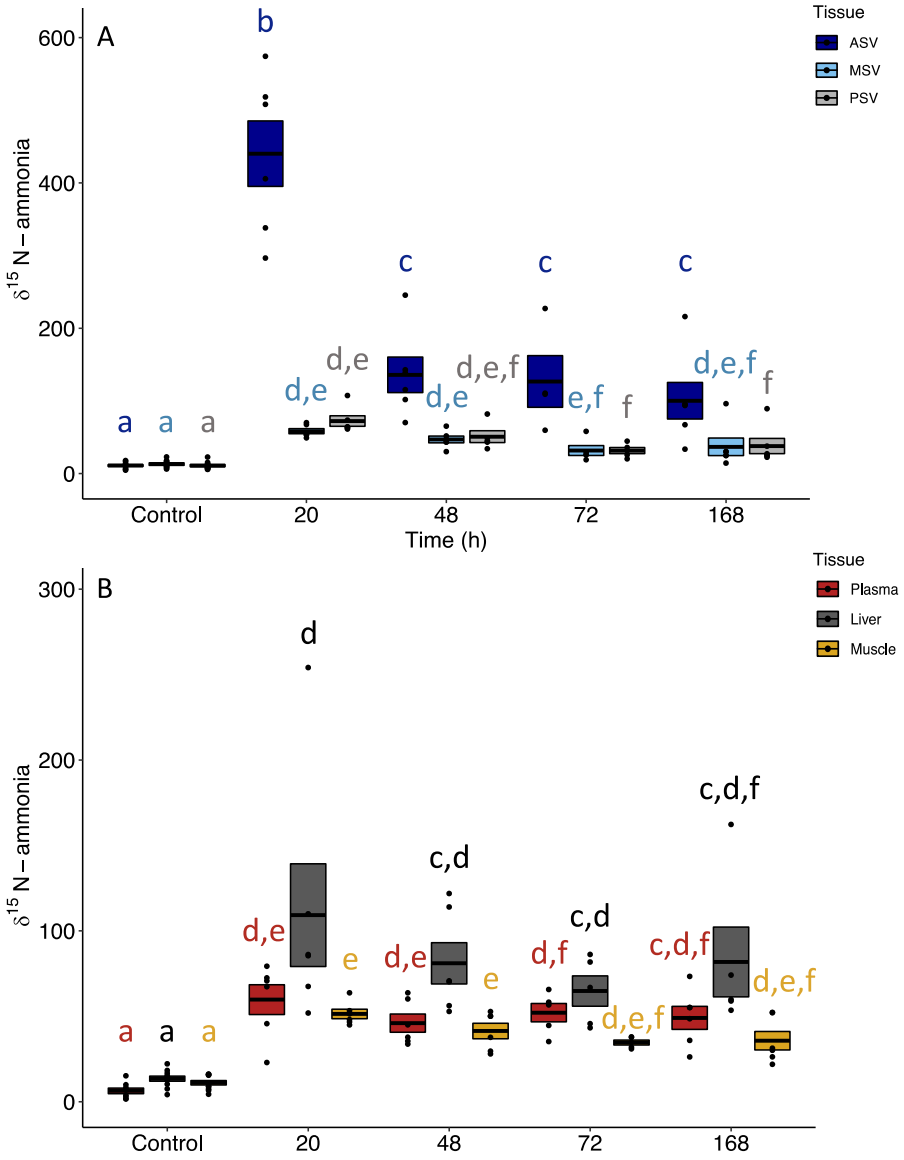

Fig. S3. δ^{15}N values for ammonia within tissues and plasma of North Pacific spiny dogfish (*Squalus acanthias suckleyi*) following the ingestion of ^{15}N-enriched herring slurry. Dogfish were fed herring slurry enriched with 7 mmol l^{-1} $^{15}NH_4Cl$ via gavage, and tissues and plasma were collected after 20, 48, 72, or 168 h digestion (n = 6 animals per time point). Also shown are the δ^{15}N values of the ammonia component of the control animals (n = 12). A) ASV, MSV, PSV; B) plasma, liver, and muscle. The horizontal line within the boxplots indicate means and the upper and lower box boundaries indicate sem with individual data points represented as black dots. Means not sharing the same letter are significantly different (two-way ANOVA, $P < 0.05$).

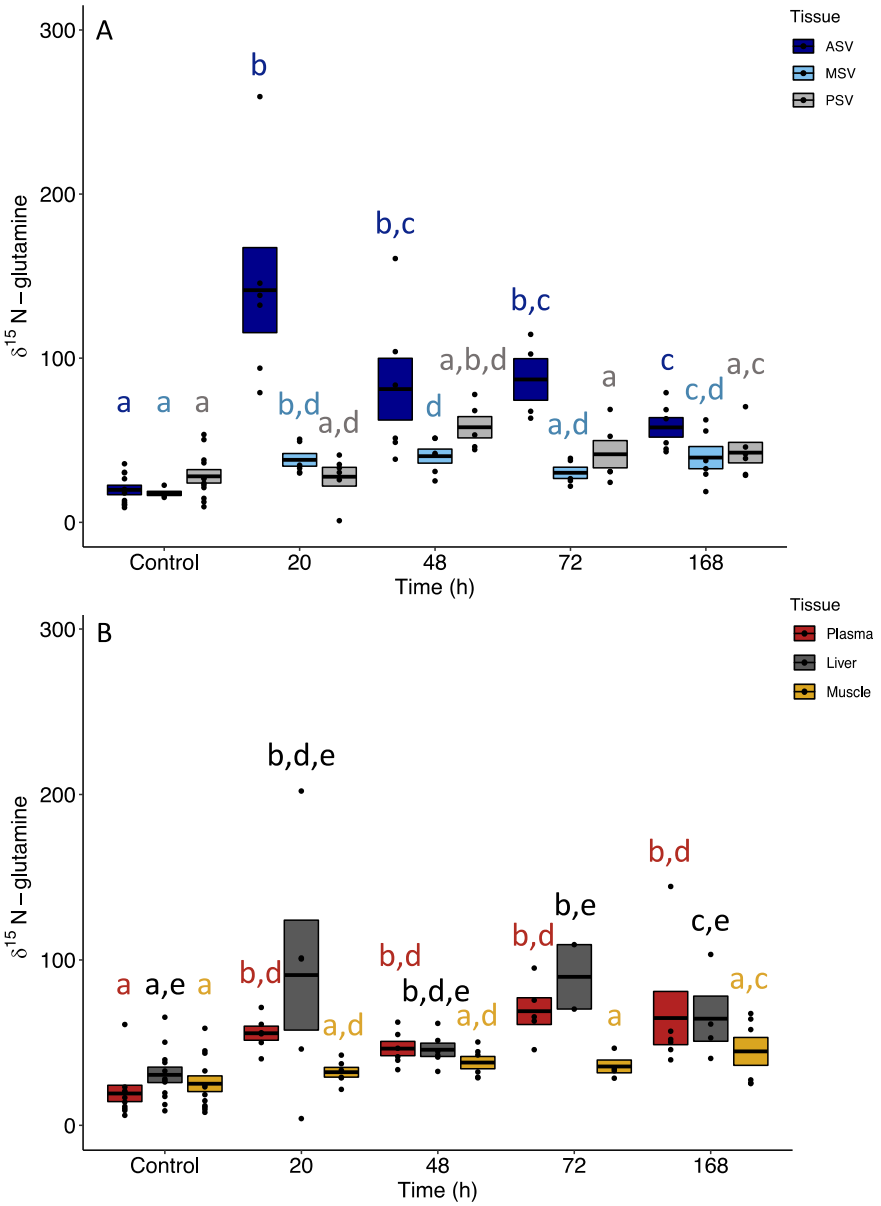

Fig. S4. δ^{15}N values for glutamine within tissues and plasma of North Pacific spiny dogfish (*Squalus acanthias suckleyi*) following the ingestion of ^{15}N-enriched herring slurry. Dogfish were fed herring slurry enriched with 7 mmol l^{-1} $^{15}NH_4Cl$ via gavage, and tissues and plasma were collected after 20, 48, 72, or 168 h digestion (n = 6 animals per time point). Also shown are the δ^{15}N values of the glutamine component of the control animals (n = 12). A) ASV, MSV, PSV; B) plasma, liver, and muscle. The horizontal line within the boxplots indicate means and the upper and lower box boundaries indicate sem with individual data points represented as black dots. Means not sharing the same letter are significantly different (two-way ANOVA, $P< 0.05$).

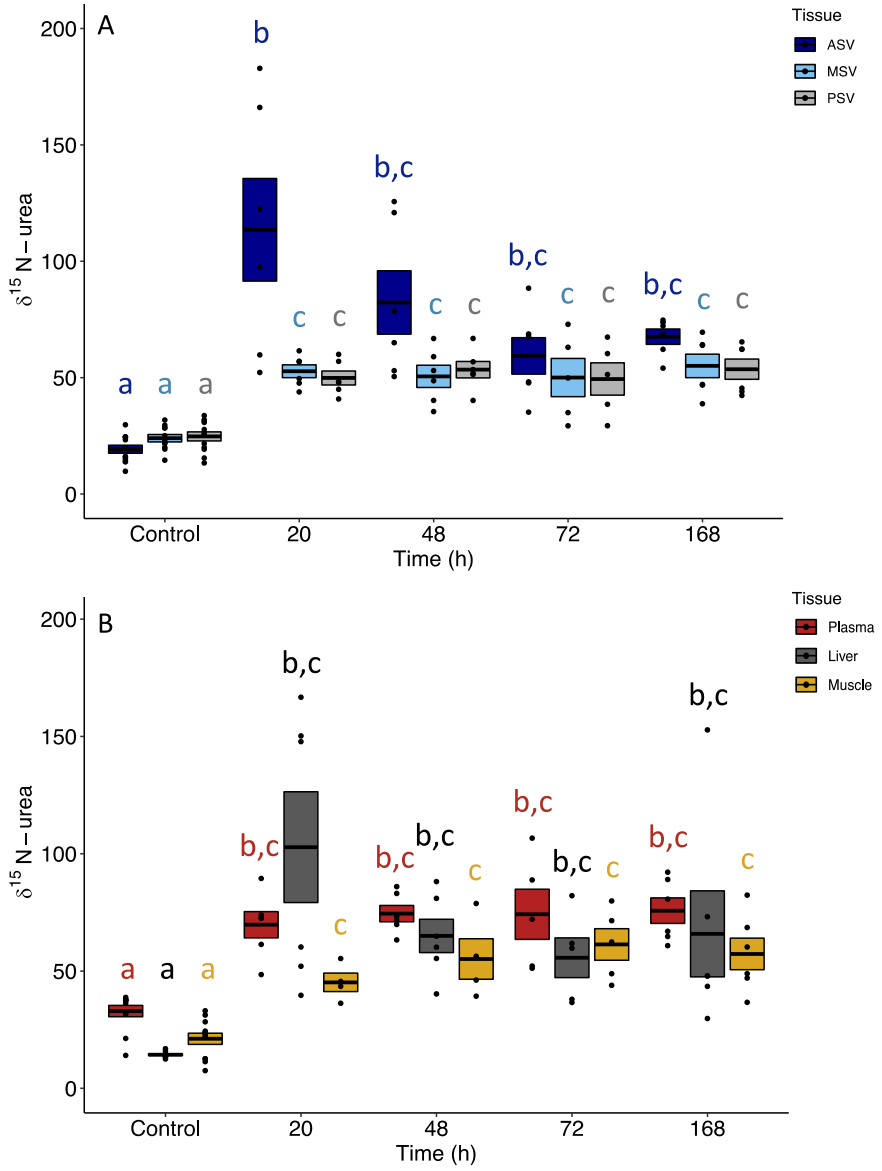

Fig. S5. δ^{15}N values for urea within tissues and plasma of North Pacific spiny dogfish (*Squalus acanthias suckleyi*) following the ingestion of ^{15}N-enriched herring slurry. Dogfish were fed herring slurry enriched with 7 mmol l^{-1} $^{15}NH_4Cl$ via gavage, and tissues and plasma were collected after 20, 48, 72, or 168 h digestion (n = 6 animals per time point). Also shown are the δ^{15}N values of the urea component of the control animals (n = 12). A) ASV, MSV, PSV; B) plasma, liver, and muscle. The horizontal line within the boxplots indicate means and the upper and lower box boundaries indicate sem with individual data points represented as black dots. Means not sharing the same letter are significantly different (two-way ANOVA, $P < 0.05$).

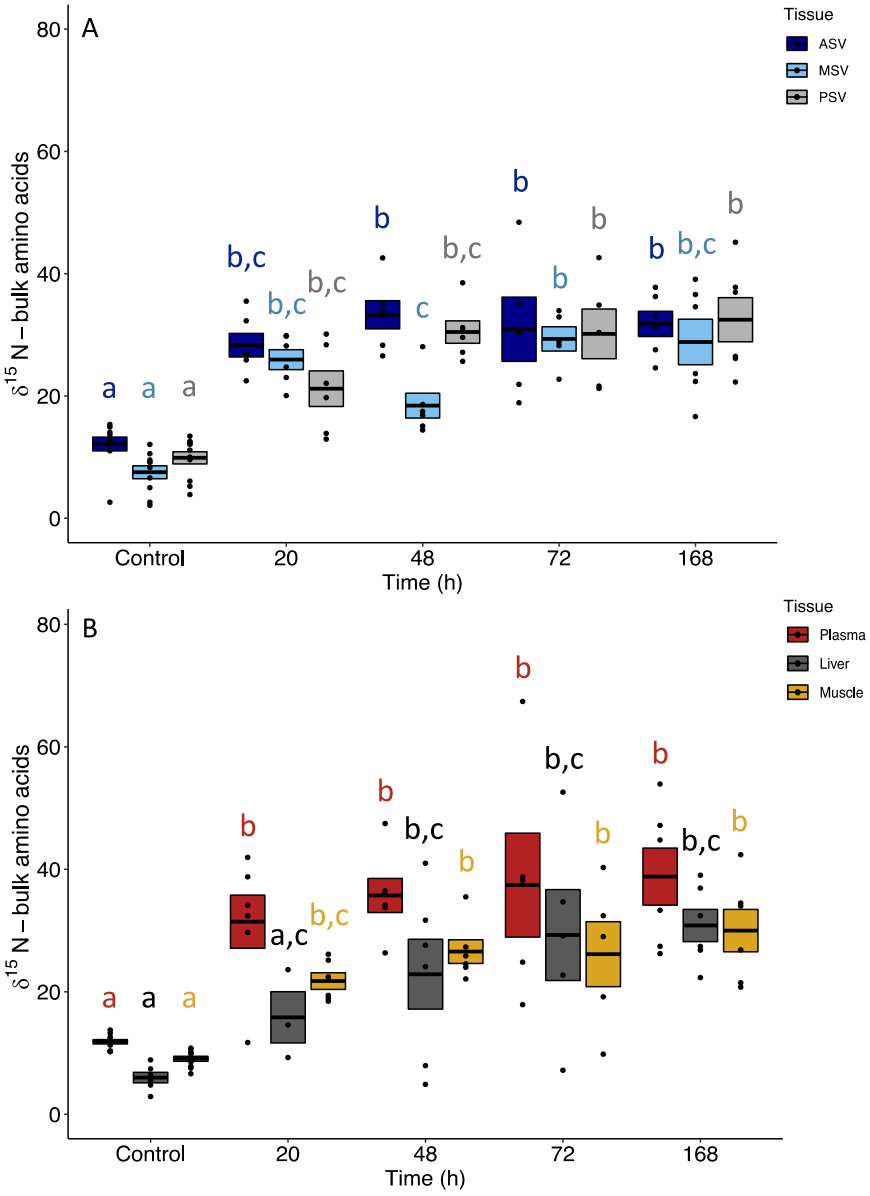

Fig. S6. δ^{15}N values for bulk amino acids within tissues and plasma of North Pacific spiny dogfish (*Squalus acanthias suckleyi*) following the ingestion of ^{15}N-enriched herring slurry. Dogfish were fed herring slurry enriched with 7 mmol l^{-1} $^{15}NH_4Cl$ via gavage, and tissues and plasma were collected after 20, 48, 72, or 168 h digestion (n = 6 animals per time point). Also shown are the δ^{15}N values of the bulk amino acid component of the control animals (n = 12). A) ASV, MSV, PSV; B) plasma, liver, and muscle. The horizontal line within the boxplots indicate means and the upper and lower box boundaries indicate sem with individual data points represented as black dots. Means not sharing the same letter are significantly different (two-way ANOVA, $P < 0.05$).

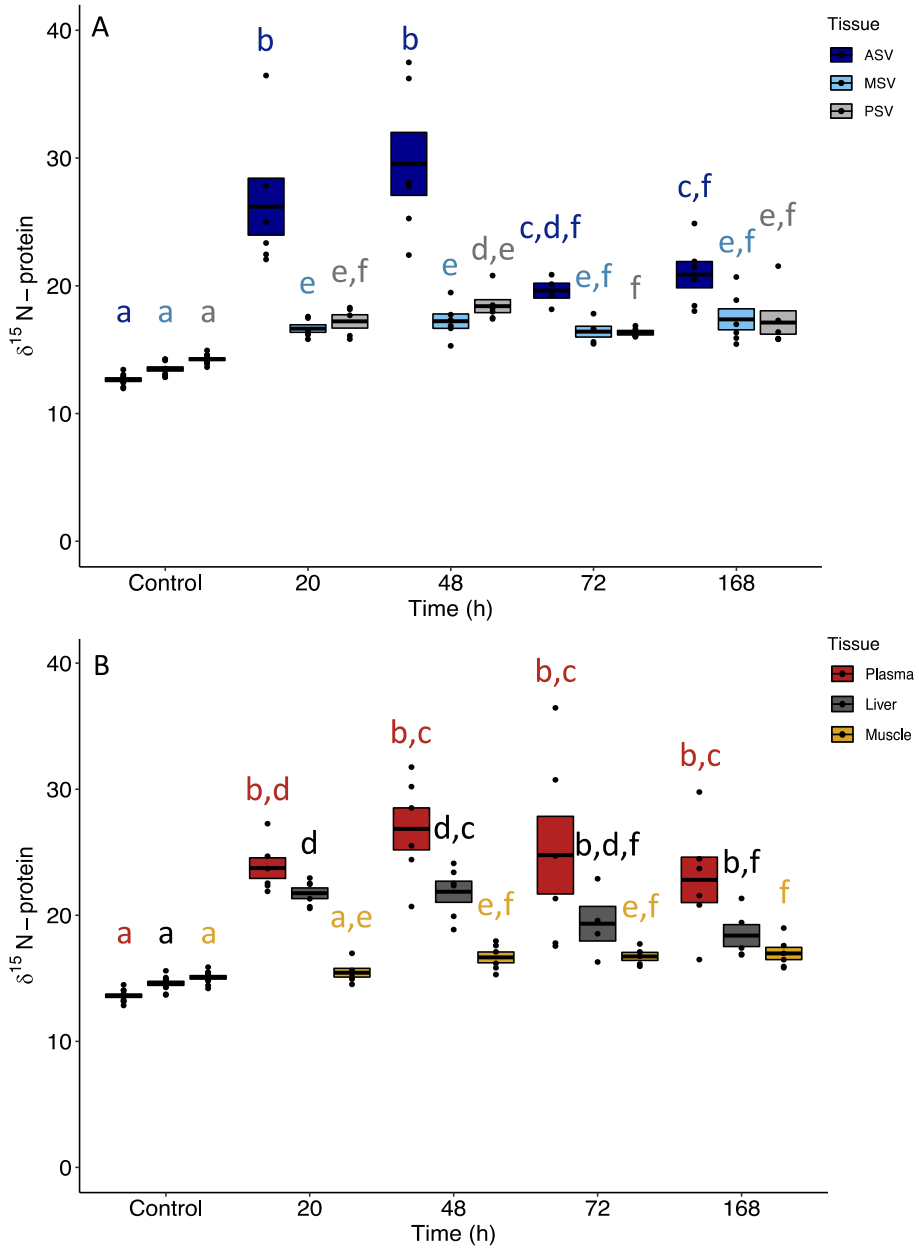

Fig. S7. δ^{15}N values for protein within tissues and plasma of North Pacific spiny dogfish (*Squalus acanthias suckleyi*) following the ingestion of ^{15}N-enriched herring slurry. Dogfish were fed herring slurry enriched with 7 mmol l^{-1} $^{15}NH_4Cl$ via gavage, and tissues and plasma were collected after 20, 48, 72, or 168 h digestion (n = 6 animals per time point). Also shown are the δ^{15}N values of the protein component of the control animals (n = 12). A) ASV, MSV, PSV; B) plasma, liver, and muscle. The horizontal line within the boxplots indicate means and the upper and lower box boundaries indicate sem with individual data points represented as black dots. Means not sharing the same letter are significantly different (two-way ANOVA, $P < 0.05$).