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Gastro-intestinal handling of water and solutes in three species of elasmobranch fish, the white-spotted bamboo shark, *Chiloscyllium plagiosum*, little skate, *Leucoraja erinacea* and the clear nose skate *Raja eglanteria*☆

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ABSTRACT

The present study reports aspects of GI tract physiology in the white-spotted bamboo shark, *Chiloscyllium plagiosum*, little skate, *Leucoraja erinacea* and the clear nose skate, *Raja eglanteria*. Plasma and stomach fluid osmolality and solute values were comparable between species, and stomach pH was low in all species (2.2 to 3.4) suggesting these elasmobranchs may maintain a consistently low stomach pH. Intestinal osmolality, pH and ion values were comparable between species, however, some differences in ion values were observed. In particular Ca^{2+} (19.67 ± 3.65 mM) and Mg^{2+} (43.99 ± 5.11 mM) were high in *L. erinacea* and Mg^{2+} was high (130.0 ± 39.8 mM) in *C. palgiosum* which may be an indication of drinking. Furthermore, intestinal fluid HCO_3^- values were low (8.19 ± 2.42 and 8.63 ± 1.48 mM) in both skates but very high in *C. plagiosum* (73.3 ± 16.3 mM) suggesting ingested seawater may be processed by species-specific mechanisms. Urea values from the intestine to the colon dropped precipitously in all species, with the greatest decrease seen in *C. plagiosum* (426.0 ± 8.1 to 0 mM). This led to the examination of the molecular expression of both a urea transporter and a Rhesus like ammonia transporter in the intestine, rectal gland and kidney in *L. erinacea*. Both these transporters were expressed in all tissues; however, expression levels of the Rhesus like ammonia transporter were orders of magnitude higher than the urea transporter in the same tissue. Intestinal flux rates of solutes in *L. erinacea* were, for the most part, in an inward direction with the notable exception of urea. Colon flux rates of solutes in *L. erinacea* were all in an outward direction, although absolute rates were considerably lower than the intestine, suggestive of a much tighter epithelia. Results are discussed in the context of the potential role of the GI tract in salt and water, and nitrogen, homeostasis in elasmobranchs.

1. Introduction

The gastrointestinal (GI) tract is an integral component of homeostatic regulation of acid-base, salt and water and nitrogen in all vertebrates. However, the extent the GI tract plays in these homeostatic mechanisms varies between vertebrates with post prandial acid base balance being most notable in ambush reptilian predators (Coulson et al., 1950; Secor and Diamond, 1995); osmoregulation in teleost fish (Grosell et al., 2005; Wilson et al., 2002); and nitrogen balance, which is relevant to all vertebrates. Furthermore, nitrogen balance is perhaps of greatest concern to those animals that are nitrogen limited and require high levels, not only for somatic growth, but also osmoregulation. The marine elasmobranch fishes are an obvious example as they retain >300 mM urea in extracellular fluids as part of their osmoregulatory strategy (Smith, 1936). However, the role of the GI tract in osmoregulation and acid base balance in elasmobranchs is still of importance, indeed given the intermittent nature of feeding in many elasmobranch species (Wetherbee and Cortes, 2004), it has been suggested that stomach pH is high in times of starvation and only drops following ingestion of a meal (Papastamatiou and Lowe, 2004; Sullivan, 1905). Alternatively, given the opportunistic nature of feeding in many predatory sharks it has been suggested that stomach pH is kept consistently low to allow for the instantaneous onset of digestion after feeding (Papastamatiou and Lowe, 2005; Papastamatiou et al., 2007; Wood et al., 2007b). To our knowledge stomach pH has not been reported in the more sedentary species of elasmobranchs, such as those in the order rajiforme.

The role of the GI tract following feeding in elasmobranch fish, in particular the spiny dogfish, *Squalus acanthias*, has received considerable

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attention in recent years with evidence indicating that the GI tract is involved in all three of the major homeostatic mechanisms (Kajimura et al., 2006; Wood and Walsh, 2008; Wood et al., 2007a,b; 2005, 2009). A post prandial alkaline tide has been reported in *S. acanthias*, following both involuntary and voluntary feeding (Wood et al., 2007b, 2005). Further, the mechanisms for the post prandial metabolic alkalosis observed in the blood stream of the spiny dogfish was recently determined to be analogous to what has been observed in higher vertebrates (Niv and Fraser, 2002). That is, the apical secretion of acid by the oxyntincopeptic (acid and enzyme synthesising) cells (Hogben, 1967) in the gastric mucosa of the dogfish, is balanced by basolateral HCO_3^- secretion into the blood stream, likely through the coordinated actions of apical H^+,K^+ATPase and basolateral Cl^-/HCO_3^- exchange (Wood et al., 2007b). Using the specific H^+,K^+ATPase inhibitor, omeprazole, Wood et al. (2009) provided compelling evidence to support this mechanism in the spiny dogfish.

The intestine has become well recognised as a major osmoregulatory organ, in seawater (SW) teleost fish (Grosell, 2006; Grosell et al., 2005; Wilson et al., 2002). Briefly, volume challenged SW teleosts drink copious amounts of SW to offset water lost to the environment across semi-permeable membranes. This imbibed water is then reabsorbed across the intestine along with Na^+ and Cl^- ions. Excess Na^+ and Cl^- are excreted by the mitochondrial rich cells at the gill with the water being retained (Marshall and Grosell, 2006). The cellular mechanisms driving Na^+, Cl^- and water absorption across the intestine of SW teleosts include apical Cl^-/HCO_3^- anion exchange (Grosell et al., 2009) which concomitantly decreases luminal fluid osmolality through precipitation of divalent ions (most notably calcium) with HCO_3^- (Wilson and Grosell, 2003). The net result is more favourable conditions for the absorption of water across the intestine into the animal.

More recently focus has shifted to examining the mechanistic transport properties of the teleost fish intestine, Taylor and Grosell (2006) have shown the Cl^-/HCO_3^- exchange system to be far more widespread and suggest its utilisation by all animals that may drink SW to maintain osmotic homeostasis (Taylor and Grosell, 2006). The notion that elasmobranch fish drink SW goes against Homer Smith's (1936) original hypothesis; however, drinking has now been demonstrated in at least 3 different elasmobranch species (De Boeck et al., 2001; Hazon et al., 1989; Taylor and Grosell, 2006). Further, following a hyperosmotic challenge elasmobranch fish will increase drinking rates much in the same way as teleost fish (Anderson et al., 2002; Taylor and Grosell, 2006). This suggests a link between the intestine and osmoregulation in elasmobranchs. Further evidence to support this notion is provided by the high HCO_3^- levels (up to 73 mM), reported in the intestine of the white spotted bamboo shark, *Chiloscyllium plagiosum* (Anderson et al., 2007; Taylor and Grosell, 2006). Conversely, much lower intestinal fluid [HCO_3^-] (approx. 5 mM) has been reported in the spiny dogfish, *S. acanthias* despite evidence of drinking SW (Wood et al., 2007b).

The high levels of urea in the plasma of elasmobranch fish render them ureotelic with the vast majority of urea being lost across the branchial epithelia (Pärt et al., 1998). With this high rate of loss and the reliance on urea for osmoregulatory purposes, nitrogen conservation is of the utmost importance to all marine elasmobranchs. Recently, Wood and colleagues demonstrated that this dependence was so strong that even after a meal there was minimal additional nitrogen loss and what was lost was in the form of ammonia (Wood et al., 2007a, 2005). However, it is not known how urea-*N* and ammonia-*N* are absorbed across the intestine in the elasmobranch fish. A specific urea transporter (UT) may be involved and was first identified in the gill of the spiny dogfish (Smith and Wright, 1999) and subsequently characterised in 5 other elasmobranch species (Hyodo et al., 2004; Janech et al., 2008; Morgan et al., 2003b). Research has focused on the role of this transporter in the kidney and gills of elasmobranchs (Fines et al., 2001; Morgan et al., 2003a,b), and to date there are no reports on the potential role of the UT in urea transport across the intestine of an elasmobranch fish. An alternative route for nitrogen conservation could be through the transport of ammonia. The discovery of some Rhesus glycoproteins being involved in the transport of ammonia in humans (Marini et al., 2000) has led to the Rhesus-like ammonia transporters being examined in teleost fish where they have also been identified as playing a crucial role in ammonia transport across epithelia (Braun et al., 2009; Nakada et al., 2007; Nawata et al., 2007). For elasmobranchs, however, little is known regarding the role these glycoproteins may play in nitrogen balance in these ureotelic fish.

As is perhaps evident from the above introduction much of our knowledge on feeding ecology and gastrointestinal physiology of elasmobranch fish has been focussed on actively foraging predators belonging to the charcharinid or squaliform orders. In the present study we examined basic gastrointestinal parameters in two species of rajiformes, the little skate, *Leucoraja erinacea*, and the clear nose skate, *Raja eglanteria* as well as body fluid osmolality and solute concentration in *C. plagiosum*. The ecology of these animals is somewhat different to the charcharinids and Squaliformes and therefore it is of interest to determine if their basic GI function is similar to the more active elasmobranchs. Body fluid analyses of the plasma, stomach, intestine and colon were conducted in all three species to examine changes in osmolality, pH and ion concentration along the GI tract. Ion and water flux rates in the intestine and colon of *L. erinacea* were examined to determine any potential role of the GI tract in osmoregulation and for comparison with ion and water flux rates in the intestine of *C. plagiosum* (Anderson et al., 2007). Finally, molecular expression of a urea transporter and a Rhesus like ammonia transporter was investigated to determine a potential role in nitrogen conservation across the intestine.

2. Materials and methods

2.1. Animals

L. erinacea and *R. eglanteria* of mixed sex were obtained from the Huntsman Marine Science Centre, St. Andrews, New Brunswick. *R. eglanteria* were caught in June/July of 2008 by Otter trawl and were maintained for a minimum of 5 days post capture at the Huntsman Marine Science Centre in flow through aquaria at 12 °C under ambient light conditions and were not fed throughout this time period. *L. erinacea* were also caught by Otter trawl from April to August in 2006, 2007 and 2008 and transported to the University of Manitoba where they were held in re-circulating marine aquaria (Instant Ocean™) at 16 °C under a 12 h:12 h light:dark cycle for a minimum of 4 weeks prior to the onset of experimentation. *L. erinacea* were fed *ad libitum* a diet of squid and whitefish, however, food was withheld from *L. erinacea* for 3 days prior to experimentation. *C. plagiosum* were obtained and held under identical conditions as previously reported (Anderson et al., 2007). All experiments were conducted under animal protocol F04-041 approved by the University of Manitoba's animal protocol management review committee as outlined in the guidelines for the care and use of animals by the Canadian Council for Animal Care. In the experiments where *L. erinacea* was adapted to varying salinities the animals were immediately transferred to 75% or 125% SW and allowed to adapt to these environments for a minimum of 2 weeks prior to experimentation. Different salinities were made by proportional alteration of the volume of water used to dissolve the unit mass of Instant Ocean™ required for 100% SW.

2.2. Measurement of body fluids

Animals were sacrificed by immersion in a SW bath containing 350 ppm tricaine methanesulfonate (Syndel Labs, Vancouver, BC, Canada) followed by transection of the spinal column just posterior to

the brain. The abdominal cavity was then exposed taking great care not to disrupt any internal organ, particularly the colon, as this often caused fluid to be voided from that area. Blood samples were taken by cardiac puncture and immediately centrifuged at 4 °C and 5000 g for 3 min. The plasma was then frozen and stored at −80 °C for further analyses. However, for the bamboo shark, pH and total CO_2 values were measured in freshly taken samples. It is important to note that freezing of the sample may have influenced the pH and total CO_2, therefore HCO_3^-, values presented in this study. That said trends in these values are comparable between species, with the exception of the intestinal HCO_3^- values in the bamboo shark (see below). Stomach fluid samples were taken by carefully opening the cardiac portion of the stomach and with a blunted 16 gauge needle removing any fluid present. Intestinal fluid samples were obtained by removing the intestine. In this study the intestine was the portion of the GI tract from the posterior end of the pylorus, close to the pancreatic duct, to the sphincter at the posterior end of the spiral valve where it empties into the colon. The intestine was clamped at each end and blotted dry to avoid contamination of fluids. Gentle squeezing of the intestine, with the posterior clamps removed, allowed for collection of any fluid that was present in the intestine. Colonic fluid samples were taken by inserting a blunted 16 gauge needle in through the anus of the fish and withdrawing any fluid in the colon. In this study the colon was considered the region of the GI tract that ran from the posterior intestinal sphincter to the anus. Stomach, intestinal and colon fluid samples were then centrifuged at 4 °C and 5000 g for 3 min. The supernatant was removed and stored at −80 °C for further analyses.

Plasma osmolality was measured using a vapor pressure osmometer (Vapro 5520, Wescor, Inc., Logan, UT, USA). Plasma Na^+, K^+, Ca^{2+}, Mg^{2+}, Cl^-, SO_4^{2-} and PO_4^{3-} were measured by ion-exchange chromatography (Metrohm-Peak, Herisau, Switzerland). The cation eluent was 4 mM tartaric acid and 0.75 mM dipicolinic acid, and the anion eluent was 3.6 mM Na_2CO_3 with CO_2 suppression by 100 mM H_2SO_4 followed by CO_2 free air. Total CO_2 was measured using a Corning 965 carbon dioxide analyser (Olympic Analytical, Malvern, UK), and pH was measured using an Accumet micro pH probe connected to an Accumet AB15 pH meter (Fisher Scientific). In *L. erinacea*, trimethylamine oxide (TMAO) was measured using the spectrophotometric method described by (Wekell and Barnett, 1991). All chemicals used in the present study were obtained from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated.

2.3. Flux rate measurements

In vitro gut sacs of the intestine and colon of *L. erinacea* were prepared in a manner similar to those previously described for teleost fish (Grosell et al., 2005) and *C. plagiosum* (Anderson et al., 2007). Briefly, the intestine and colon were dissected from the fish, as described above, and a small flared piece of P.E. 90 cannula (approximately 5 cm) was inserted into both ends of the preparation and tied in place using silk suture thread. For the colon preparation a tight ligature was placed around the rectal gland duct that empties into the colon. Ringers solutions used for bathing and filling the preparations were based on the measured concentrations of the plasma (for bathing) and luminal fluids (for filling) for each tissue (Table 2). The preparations were flushed at least 3 times to ensure adequate mixing. A 20 mL syringe was then filled with the appropriate Ringers solution that contained 0.25 mCi mL^{-1} of ^3H labelled Polyethylene glycol (PEG −4000 MW) (Perkin Elmer, Waltham, MA, USA) and weighed. The preparations were then filled and a small start sample, which was also weighed, was collected at the posterior end. The posterior cannula was then sealed and the preparations were filled, sealed at the anterior end, and incubated in plasma Ringers solutions (composition in mM: NaCl 240, KCl 7, $MgCl_2$ 2, Na_2HPO_4 0.5, Na_2SO_4 2.5, urea 360, trimethylamine-N-oxide 40, $CaCl_2$ 10, $NaHCO_3$ 2.3) bubbled with a 95%:5% O_2:CO_2 gas mix for a minimum of 3 h. The filling syringe was again weighed and a total filling volume was calculated as the start syringe minus the end syringe and start sample assuming a specific gravity of 1. At the end of the incubation period samples were taken from both the luminal and incubation fluid for osmolality and ion analysis.

Water flux rates were calculated based on the concentration of the non-absorbable marker ^3H-PEG at the start and end of each preparation. A 20 μL sample was added to a scintillation vial and 4 mL of scintillation cocktail (Ultima gold, Perkin Elmer, Waltham, MA, USA) was added prior to counting each sample in triplicate on a Beckman LS6500 liquid scintillation counter (Beckman Coulter, Fullerton, CA, USA). Solute flux rates were calculated from the measured concentrations before and after the incubation period. Because the architecture of the intestine in elasmobranchs inhibits accurate determination of the total surface area of the tissue, final units for water reabsorption and flux rates of solutes are expressed in units of body mass for both tissues in order to allow for a more direct comparison between tissues in *L. erinacea.*

2.4. Quantitative RT-PCR

For total RNA extraction the intestine of *L. erinacea* was divided into 3 regions based on the total number of folds in the spiral valve. The anterior section comprised of the first 1–2 folds in the spiral valve, the mid-section was the mid 1–2 folds in the spiral valve, and posterior section was the last 1–2 folds in the spiral valve. Prior to scraping the epithelial layer all undigested material was removed. Intestinal scrapings, rectal gland and renal tissue samples were initially placed in RNAlater (Ambion, Austin, TX, USA) and stored at −80 °C until RNA extraction was conducted under RNase-free conditions using TRIZOL reagent (Invitrogen, Carlsbad, CA, USA). After DNase treatment (DNase 1, Invitrogen, Carlsbad, CA, USA) RNA was tested for purity by polymerase chain reaction (PCR) (40 cycles) using 2 μg DNase treated RNA as a template and *L. erinacea* specific primers Raja GAPDH-F/Raja GAPDH-R (product size 313 bp, see Table 1) designed to amplify DNA coding for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Genbank accession no. DQ382343). RNA samples showing no PCR-products (DNA free) were reverse transcribed into cDNA using oligo (dT) primers and *Thermoscript* reverse transcriptase (Invitrogen) employing 1 μg of DNase treated total RNA. The quality of the generated cDNAs from all tissues was assessed by employing the primer pair GAPDH-F/Raja GAPDH-R in PCR (60 ºC annealing temperature, 30 cycles, see Table 1) after gel-electrophoresis and ethidium bromide/UV visualization.

All primers to be employed in quantitative Real-Time PCR targeting GAPDH, the urea transporter (UT) and the Rhesus like ammonia transporter (Rhbg) were designed based on published sequences from *L. erinacea* (see Table 1). PCR products were evaluated

Table 1
Primers employed in quantitative Real-Time PCR targeting glyceraldehyde-3-phosphate dehydrogenase (GAPDH), urea transporter (UT) and Rhesus like ammonia transporter Rhbg from *L. erinacea.*

Primer	Nucleotide sequence (5′→3′)	Annealing temperature (°C)	Product size (bp)
GAPDH			
Raja GAPDH-F	CTGTTTTCCAGGAGCGAGAC	60	313
Raja GAPDH-R	TGGACCGTGGTCATTAGTCC	60	
Urea transporter			
Raja UT-FA	GTTGTGCAGAATCCATGGTG	63	146
Raja UT-RB	GCCAATAAGAGACCGACCAA	63	
Rhbg			
Raja Rhbg-F1	CGTCCACGTCATGATATTCG	60	426
Raja Rhbg-R2	GGTGATGGTCAGTCCGAAAT	60	

The primer pairs Raja GAPDH-F/Raja GAPDH-R, Raja UT-FA/Raja UT-RB and Raja Rhbg-F1/Raja Rhbg-R2 were designed based on published sequences GenBank Accession No. DQ382343, AY161305 and GD273979, respectively.

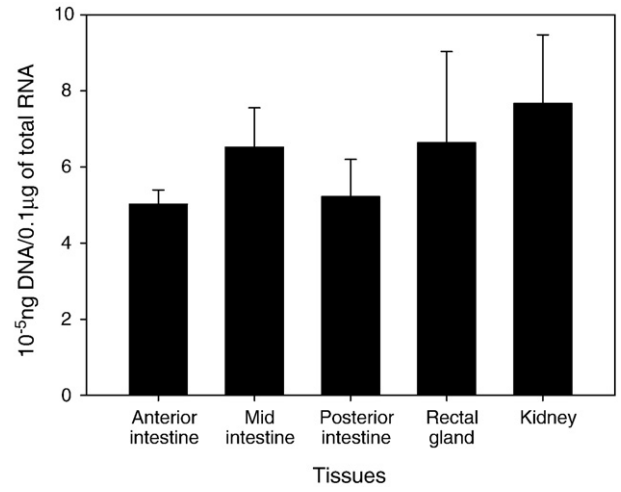

Fig. 1. Quantitative mRNA expression of internal real time PCR control, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in anterior, mid and posterior intestine, rectal gland and kidney tissue from 100% SW adapted *L. erinacea*. Values are expressed as a mean ng DNA/0.1 µg DNase treated total RNA ± 1 sem, ($n \geq 3$).

for correctness by sequencing using a Hitachi 3130 Genetic Analyzer with ABDNA Sequencing Analyzing Software (Applied Biosystems, Foster City, CA, USA).

For quantitative PCR (Miniopticon, Biorad, Mississauga, Ontario, Canada) standard curves were generated employing a dilution series of known quantities (10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7} ng DNA) of the respective purified PCR product (QIAquick Gel Extraction Kit, Qiagen Inc, Mississauga, Ontario, Canada) of the target gene. For the standard curve a minimum r^2 value of 0.98 was required. Real-time PCR were performed employing cDNA transcribed from 100 ng total RNA, 1 µM of each primer and iQS SYBR green supermix (Biorad, Mississauga, Ontario, Canada) in an 20 µL assay. Single product PCR was verified performing a melting curve analysis as well as gel electrophoresis and ethidium bromide/UV visualization of the final Real-Time PCR product. For relative RNA expression analysis GAPDH served as an internal standard. Suitability of GAPDH was evaluated by quantitative PCR showing similar expression levels of the gene in all tissues employed (Fig. 1).

2.5. Statistical analyses

Statistical significance for osmolarity, ion concentration and pH within species between body fluids was assessed using analysis of variance with a Tukey's post hoc test (Tables 2 and 3; Fig. 5). A similar test was performed to determine statistical significance between water flux rates in different salinity treatments in colon gut sac preparations. Values were accepted as significant where $p < 0.05$.

Table 3
Body fluid osmolality, pH and ion concentrations from the plasma, stomach, intestine and colon in *R. eglanteria* adapted to 100% SW.

	Plasma	Stomach	Intestine	Colon
Osmolality	966.43 ± 14.27^{a}	961.58 ± 20.18^{a}	885.38 ± 19.46^{b}	926.00 ± 8.47^{ab}
pH	7.50 ± 0.04^{a}	3.36 ± 0.44^{b}	7.75 ± 0.12^{a}	6.95 ± 0.30^{a}
Na^+	274.99 ± 7.29^{ab}	232.71 ± 42.72^{b}	218.98 ± 31.06^{b}	371.08 ± 17.42^{a}
Cl^-	278.65 ± 7.62^{a}	436.32 ± 14.55^{b}	290.73 ± 13.91^{a}	488.17 ± 14.13^{b}
Urea	271.28 ± 23.79^{a}	90.07 ± 26.25^{b}	185.77 ± 20.26^{a}	10.29 ± 4.61^{b}
HCO_3^-	3.87 ± 0.46^{a}	0.11 ± 0.11^{a}	8.63 ± 1.48^{b}	0.85 ± 0.26^{a}
SO_4^{2-}	7.71 ± 1.98	32.54 ± 11.54	27.78 ± 3.93	20.87 ± 6.25
PO_4^{3-}	1.07 ± 0.17	9.16 ± 4.21	0.08 ± 0.09	8.05 ± 3.15
K^+	4.51 ± 0.41^{a}	20.22 ± 2.19^{b}	18.98 ± 1.82^{b}	12.34 ± 1.69^{b}
Mg^{2+}	5.58 ± 1.42	4.57 ± 0.70	4.74 ± 1.34	2.96 ± 0.54
Ca^{2+}	4.56 ± 0.40^{a}	9.75 ± 2.70^{ab}	5.31 ± 1.04^{a}	1.01 ± 0.29^{ac}

Values are expressed as a mean osmolality (mOsm kg^{-1}), and molarity for the ions (mmol L^{-1}) ± sem. Different letters denote a significant difference between fluids for osmolality, pH and each of the measured ions ($n \geq 4$).

3. Results

In *L. erinacea*, osmolality of stomach and intestinal fluid was similar but the colon was significantly lower than the intestine (Table 2). In *R. eglanteria* intestinal fluid osmolality was significantly lower than the stomach but similar to the colon (Table 3). In *C. plagiosum* body fluid osmolality did not differ across all body fluids. pH values followed similar trends in all three species across all body fluids, however, the lowest pH was seen in the colon of *C. plagiosum*. In *L. erinacea*, Na^+ values were lowest in the intestine and highest in the colon, a trend also seen in *C. plagiosum* and *R. eglanteria* except the lower intestinal Na^+ value was not significant in *R. eglanteria*. Cl^- values showed a similar pattern in all three species peaking in the stomach and colon fluid (Tables 2, 3 and 4). Likewise trends for urea and HCO_3^- values were comparable between both species of rajiforme with higher values in the plasma and intestinal fluid, but the HCO_3^- values in intestinal fluid of *C. plagiosum*, as previously reported (Anderson et al., 2007) were approximately 6 fold higher. The absolute amount of urea was much lower in intestinal fluids of *R. eglanteria* compared to *L. erinacea* and *C. plagiosum* (Tables 2, 3 and 4). SO_4^{2-}, PO_4^{3-}, and K^+ values again showed similar trends between species in the different body fluids, however, peak values for SO_4^{2-} were found in the stomach of *L. erinacea* and the intestine of *R. eglanteria* and *C. plagiosum*. Mg^{2+} and Ca^{2+} showed the largest differences in trends between the body fluids of the species examined.

Table 2
Body fluid osmolality, pH and ion concentrations from the plasma, stomach, intestine and colon in *L. erinacea* adapted to 100% SW.

	Plasma	Stomach	Intestine	Colon
Osmolality	946.8 ± 4.69^{a}	971.73 ± 11.83^{abc}	999.72 ± 14.28^{b}	949.52 ± 13.58^{ac}
pH	7.58 ± 0.02^{a}	3.12 ± 0.29^{b}	7.50 ± 0.15^{c}	6.38 ± 0.32^{a}
Na^+	263.10 ± 6.42^{a}	269.03 ± 19.24^{a}	168.49 ± 14.19^{b}	356.45 ± 22.53^{c}
Cl^-	244.03 ± 5.05^{a}	403.74 ± 11.15^{b}	207.97 ± 14.44^{a}	369.46 ± 16.24^{b}
Urea	385.79 ± 15.86^{a}	142.00 ± 25.20^{b}	375.48 ± 21.66^{a}	21.80 ± 5.39^{c}
TMAO	35.88 ± 3.18^{a}	4.62 ± 1.10^{b}	8.19 ± 2.42^{b}	5.73 ± 2.67^{b}
HCO_3^-	4.19 ± 0.31^{a}	1.46 ± 0.30^{a}	12.94 ± 3.99^{b}	1.43 ± 0.67^{ab}
SO_4^{2-}	3.35 ± 0.35^{a}	11.73 ± 1.92^{a}	38.32 ± 5.44^{b}	13.26 ± 2.61^{a}
PO_4^{3-}	0.97 ± 0.20^{a}	2.92 ± 1.11^{a}	0.07 ± 0.04^{a}	7.20 ± 2.50^{b}
K^+	4.64 ± 0.11^{a}	18.72 ± 1.57^{b}	13.94 ± 4.22^{bc}	7.50 ± 1.48^{ac}
Mg^{2+}	1.31 ± 0.15^{a}	24.57 ± 2.42^{b}	43.99 ± 5.11^{c}	27.98 ± 4.86^{b}
Ca^{2+}	3.11 ± 0.40^{a}	16.20 ± 2.31^{b}	19.67 ± 3.65^{b}	27.89 ± 6.95^{b}

Values are expressed as a mean osmolality (mOsm kg^{-1}), and molarity for the ions (mmol L^{-1}) ± 1 sem. Different letters denote a significant difference between fluids for osmolality, pH and each of the measured ions ($n \geq 6$).

Table 4
Body fluid osmolality, pH and ion concentrations from the plasma, intestine and colon in *C. plagiosum* adapted to 100% SW.

	Plasma	Stomach	Intestine	Colon
Osmolality	983.7 ± 12.7	972.0 ± 3.8	1039.5 ± 1.5	997.4 ± 11.0
pH	7.6 ± 0.1^{a}	2.2 ± 0.3^{b}	n.m.	5.5 ± 0.2^{a}
Na^+	212.0 ± 15.9^{a}	283.1 ± 19.6^{b}	105.1 ± 26.0^{c}	313.7 ± 10.7^{b}
Cl^-	211.6 ± 13.8^{a}	422.8 ± 18.6^{b}	168.6 ± 13.4^{a}	407.3 ± 14.0^{b}
Urea	331.6 ± 6.4^{a}	84.3 ± 16.9^{b}	426.0 ± 8.1^{c}	0^{d}
HCO_3^-	4.3 ± 0.3^{a}	0^{b}	73.3 ± 16.3^{c}	0.1 ± 0.1^{b}
SO_4^{2-}	0	18.1 ± 0.8	30.7 ± 18.5	29.0 ± 8.7
K^+	4.5 ± 0.3	19.7 ± 1.3	4.8 ± 0.6	42.6 ± 7.4
Mg^{2+}	1.2 ± 0.1^{a}	83.3 ± 2.3^{b}	130.0 ± 39.8^{b}	75.0 ± 17.9^{b}
Ca^{2+}	3.9 ± 0.1^{a}	11.7 ± 1.0^{b}	3.9 ± 1.1^{a}	7.3 ± 1.6^{b}

Values are expressed as a mean osmolality (mOsm kg^{-1}), and molarity for the ions (mmol L^{-1}) ± sem. Different letters denote a significant difference between fluids for osmolality, pH and each of the measured ions ($n \geq 4$), n.m = not measured. Values for plasma and intestinal fluids are taken from (Anderson et al., 2007).

In *L. erinacea* and *C. plagiosum* Mg^{2+} values were orders of magnitude higher in the GI tract fluids compared to the plasma, yet showed no differences in *R. eglanteria* across all body fluids. Similarly large differences between plasma and GI tract fluid values for Ca^{2+} were observed in *L. erinacea* and to a lesser extent *C. plagiosum* but not *R. eglanteria* (Tables 2, 3 and 4).

Fig. 2 shows the flux rates of the major solutes in the intestine of *L. erinacea* adapted to 100% SW. Urea, K^+, PO_4^{3-} and HCO_3^- all showed an efflux into the lumen of the preparation with the greatest being urea. Conversely, Na^+, Cl^-, TMAO, Mg^{2+}, SO_4^{2-} and Ca^{2+} all showed an influx with the greatest being Cl^-. In the colon preparation all measured ions showed an efflux from the serosa to the lumen (Fig. 3).

Molecular expression of the Rhbg-like transporter and the UT transporter in various tissues is presented in Fig. 4. Both transporters were highly expressed in renal tissue and the Rhbg like transporter was also highly expressed in the rectal gland (Fig. 4). Expression of both transporters was orders of magnitude lower in the intestinal scrapings, however, both showed an increasing trend in expression from the anterior to the posterior regions of the intestine. UT expression in the rectal gland was comparable to the low expression levels observed in the intestinal scrapings. Perhaps most interestingly, relative expression levels of the Rhbg like transporter were orders of magnitude higher in all tissues examined compared to UT expression (Fig. 4).

Salinity adaptation to 75, 100 and 125% SW influenced osmolality and ion concentration of the stomach, intestine and colon in *L. erinacea*. Not surprisingly the trend of a low, medium and high osmolality in 75, 100 and 125% SW was observed in all 3 GI tract fluids. Cl^- was significantly higher in the stomach and intestine of the 125% SW-adapted fish whereas urea was only significantly higher in the intestinal fluids of that treatment group (Fig. 5). The response of other ions was variable with only Mg^{2+} and Ca^{2+} concentrations being significantly influenced by changes in salinity. Osmolality and ion concentrations for 125% SW-adapted fish are not reported as these samples were not collected due to lack of available fluid. Salinity adaptation had a significant effect on water movement in both the intestine and interestingly the colon of *L. erinacea*. In 125% SW-adapted fish there was a significant reversal from influx to efflux of water movement in the intestine gut sac preparation. In the colon, 75% and 100% SW-adapted fish had no difference in water movement across the colon, however, adaptation to 125% SW significantly increased water influx rate across the colon (Fig. 6).

4. Discussion

The present study has for the first time described in detail the body fluid solutes of two rajiformes and augmented previously published data on a hemiscyllidae, and is to our knowledge, the first report of colonic fluid solutes in any elasmobranch species. Plasma osmolality, pH and ion concentrations compared favourably between all species with the exception of urea where plasma values in *R. eglanteria* were at least 100 mM less than the other species and plasma levels of Na^+ and Cl^- were marginally elevated. The shortfall in total plasma osmolality due to the reduced urea in *R. eglanteria* can likely be accounted for by other organic osmolytes. While the contribution of methylamines to the overall plasma osmolality of elasmobranchs has been recognised for a number of decades (Yancey and Somero, 1979, 1980), it has only recently been shown that these levels vary considerably between species and that the reliance on particular methylamines such as TMAO and or β amino acids is related to the animals ability to osmoregulate in reduced salinities or their ability to synthesise TMAO *de novo* (Treberg et al., 2006). Levels of TMAO or other methylamines, for that matter, were not measured in *R. eglanteria* or *C. plagiosum*. Furthermore, whilst the duration of starvation was controlled in *L. erinacea* and *C. plagiosum*, due to a longer time in captivity, the feeding history of *R. eglanteria* in the present study was less well understood as the fish were obtained from the wild and held in captivity for 5 days prior to sampling. As a consequence these animals may have been under a greater nitrogen imbalance and this is reflected in the reduced plasma urea and the slightly elevated Na^+ and Cl^- levels. It has been shown in the European dogfish, *Scyliorhinus canicula*, that if the diet is nitrogen limited there is a compensatory response with NaCl making up the shortfall in plasma osmolality due to the reduced availability of nitrogen for urea synthesis (Armour et al., 1993).

Previous examination of gastric fluid in elasmobranchs has indicated that either the pH of gastric fluid rose during times of starvation, which is dependent upon species, and fell during times of feeding (Papastamatiou and Lowe, 2004; Sullivan, 1905) or gastric pH remained consistently low to keep the gastric juice in a constant state

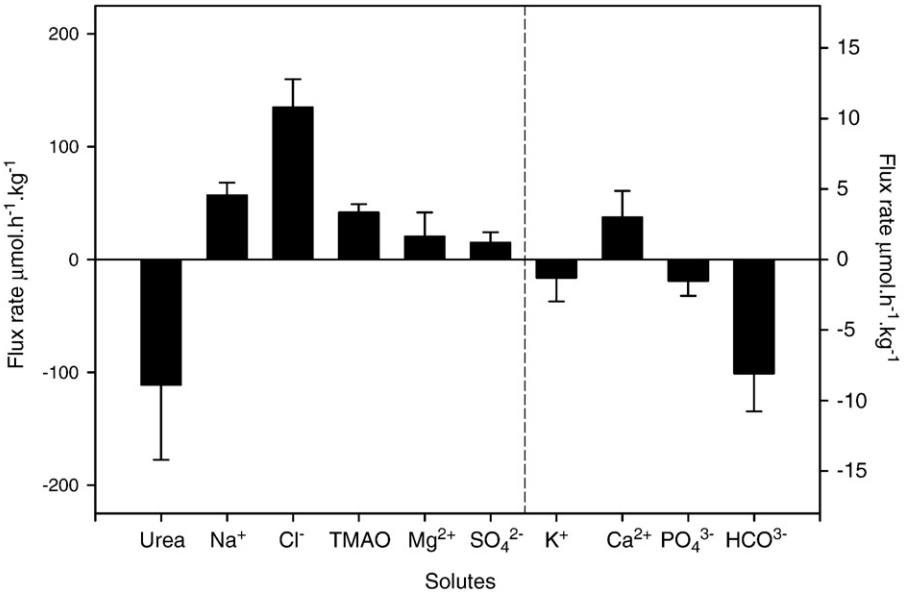

Fig. 2. Flux rates of solutes in the isolated intestine of 100% SW adapted *L. erinacea*. Values are expressed as mean µmol of solute/kg of fish/h of incubation ± 1 sem. For values to the right of the dotted line, use the right-hand scale. A negative value equates to a net efflux of the solute into the lumen of the intestine, ($n \geq 6$).

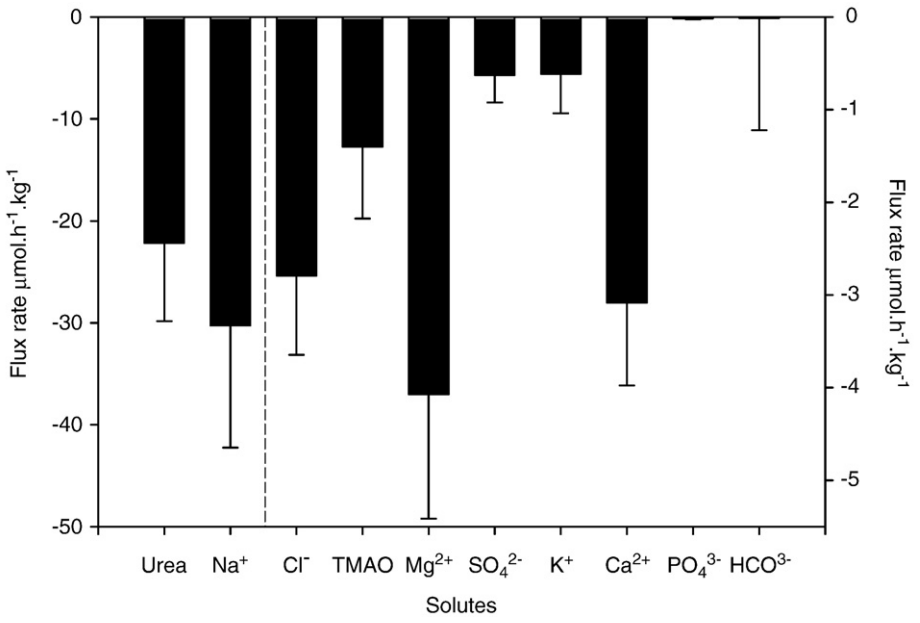

Fig. 3. Flux rates of solutes in the isolated colon of 100% SW adapted *L. erinacea*. Values are expressed as mean µmol of solute/kg of fish/h of incubation ± 1 sem. For values to the right of the dotted line, use the right-hand scale. A negative value equates to a net efflux of the solute into the lumen of the colon, ($n \geq 6$).

of readiness possibly related to the opportunistic feeding behaviour of many elasmobranchs (Papastamatiou and Lowe, 2005; Papastamatiou et al., 2007; Wood et al., 2007b). In the present study, gastric fluid pH in all three species was consistently acidic in nature and thereby supports the latter hypothesis.

Intestinal fluid solute concentration in *L. erinacea* compare favourably with recently published values in *S. acanthias* at 360 h post feeding (Wood et al., 2007b). The reduced urea levels in both the stomach and intestine of *R. eglanteria* are likely a reflection of the lower plasma urea levels in this species. The increased levels of Mg^{2+} and Ca^{2+} in the stomach of *L. erinacea* and *C. plagiosum* in comparison to *R. eglanteria* may indicate SW drinking in these species as suggested in the spiny dogfish following analysis of gastric chyme post forced feeding (Wood et al., 2009); however, these increases were only accompanied by an increase in stomach Na^+ in *C. plagiosum*.

Previous reports of intestinal HCO_3^- in *C. plagiosum* showed levels comparable to marine teleost fish (46–73 mM) (Anderson et al., 2007), however, in *S. acanthias* levels rarely rose above 5 mM even after a feeding event (Wood et al., 2007b). In the present study intestinal HCO_3^- was 12.94 ± 3.99 and 8.63 ± 1.48 in *L. erinacea* and *R. eglanteria* respectively, which are more comparable to *S. acanthias*. One possible explanation for the increased intestinal HCO_3^- levels in *C. plagiosum* may be a temperature induced increase in drinking rate. In the present study animals were maintained at 12 ± 1 °C for *R. eglanteria* and 16 ± 0.5 °C for *L. erinacea*. In the studies conducted by Wood and colleagues *S. acanthias* were held at 11 ± 1 °C, and *C. palgiosum* were held at 28 ± 2 °C in this and the previous study (Anderson et al., 2007). In teleost fish there is a clear effect of temperature on drinking rate (Carroll et al., 1994) which may be related to the temperature dependant permeability of the gill to water and ions (Maetz and Evans, 1972; Motais and Isaia, 1972). However, this temperature-dependent gill permeability was not shown in the Japanese eel, *Anguilla japonica* (Takei and Tsukada, 2001), despite temperature-dependent drinking rates in this species. Consequently, the increased levels of intestinal HCO_3^- in *C. plagiosum* are as yet unexplained but there is a clear species-specific difference to be explored.

In addition to the cause of the increase in intestinal levels of HCO_3^- in *C. plagiosum* the source of intestinal HCO_3^- is of interest. In teleosts the major source is transport of either endogenous or serosal HCO_3^- across the apical membrane of the intestine epithelium, in exchange for Cl^- uptake (Wilson et al., 2002). However, isolated intestinal sacs of *C. plagiosum* give very low efflux rates of HCO_3^- (4.22 ± 0.96 µmol h^{-1} kg^{-1}) (Anderson et al., 2007) which were marginally lower than those found using the same methodology in *L. erinacea* (8.08 ± 2.69 µmol h^{-1} kg^{-1}) (Fig. 2) where intestinal HCO_3^- concentration was also much lower (Table 2). It is unlikely therefore the source of HCO_3^- is secretion by the intestinal epithelium. As indicated by Wood et al. (2007b) two other avenues include the bile fluid or pancreatic juice. Bilary contribution to intestinal HCO_3^- is conflicting even within a single species; in the fed spiny dogfish, bile HCO_3^- concentration was low (0.61 mM) (Wood et al., 2007b) whereas in the starved spiny dogfish bilary HCO_3^- concentration was high (5.8 mM) (Boyer et al., 1976). In the present study animals were starved and intestinal HCO_3^- values were comparable to the spiny dogfish studies conducted by Wood et al. (2007b). However, intestinal HCO_3^- values were extremely high in *C. plagiosum* and these animals were also starved (Anderson et al., 2007). A final source of HCO_3^- is the secretion of pancreatic juice from acinar cells. Few studies have examined the regulation of pancreatic secretion in elasmobranchs, and those that have, have demonstrated a very slow and intermittent rate of secretion from the pancreas in response to *in vivo* administration of a 0.49% solution of HCl (Babkin, 1929). Clearly our current understanding of HCO_3^- secretion into the intestine of elasmobranch fish is conflicting and warrants further investigation.

Perhaps the most striking results in the present study is the lack of urea in colonic fluid (Tables 2, 3 and 4) in all species examined. This precipitous decline in urea is despite an efflux of urea into the lumen of the intestinal sac preparations in *L. erinacea* (Fig. 3) and *C. plagiosum* (Anderson et al., 2007) in addition to a modest efflux of urea in the colon of *L. erinacea* (Fig. 3). Coupled with this, the very slight increase in nitrogen loss in *S. acanthias* following feeding (Wood et al., 2007a) suggests that there must be significant reabsorption of urea across the intestinal epithelia in elasmobranch fish. One possible route for reabsorption could be through facilitated transport by way of the UT transporter first identified in the spiny dogfish (Smith and Wright, 1999). However, Fig. 4 shows that while there is a modest increase in mRNA expression of the UT transporter from anterior to posterior regions of the intestine, the level of expression is at least 2 orders of magnitude lower than seen in the

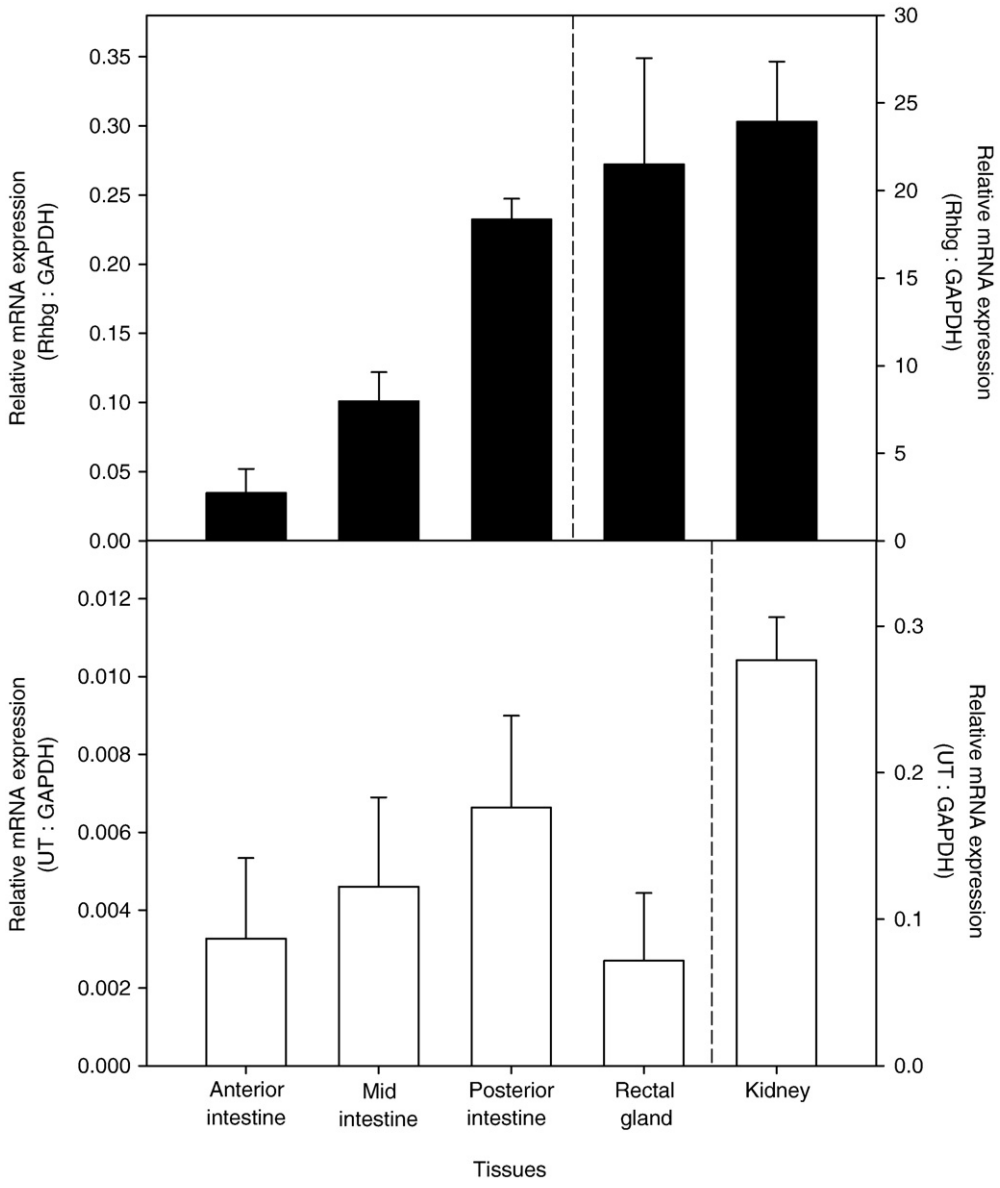

Fig. 4. mRNA expression of Rhesus like ammonia transporter (Rhbg) (filled bars) and urea transpoter (UT) (open bars) relative to the internal standard of GAPDH in, anterior, mid and posterior intestine, rectal gland and kidney tissue from 100% SW adapted *L. erinacea*. Values are expressed as a mean ± 1 sem, for values to the right of the dotted line, use the right-hand scale, ($n \geq 3$).

kidney, a tissue that is also responsible for significant urea reabsorption from the primary urine (Boylan, 1972). A second route of transport may be via a sodium linked urea transport mechanism as hypothesised in the kidney of the spiny dogfish (Schmidt-Nielsen et al., 1972), however, molecular identification of this transporter has yet to be reported. A third possibility is the transport of nitrogen, not in the form of urea but as ammonia.

In the present study we evaluated mRNA expression levels of a Rhesus like ammonia transporting protein using primers directed against Rhbg, the rhesus protein that is predominantly expressed on the basolateral membrane of epithelial cells (Handlogten et al., 2005; Nakada et al., 2007; Weiner and Hamm, 2007). This family of proteins have only recently been identified as ammonia transporters in mammals (Marini et al., 2000) and fishes (Braun et al., 2009; Nakada et al., 2007; Nawata et al., 2007), however, their role in the transport of ammonia across epithelia is becoming increasingly recognised in a variety of aquatic animals (Weihrauch et al., 2009; Wright and Wood, 2009). Interestingly, Fig. 4 shows that the level of relative mRNA expression of this protein is orders of magnitude higher than the UT transporter in the same tissues. The question then arises as to why the expression levels for the transcripts of an ammonia transporter are so much higher than the urea transporter despite the high concentrations of urea in the intestinal lumen of all elasmobranch species examined.

The answer may lie in the presence of ureolytic bacteria in the tissues of elasmobranch fish (Knight et al., 1988). Knight et al. (1988) demonstrated high levels of ureolytic acivity in the liver but not the blood of two species of charcarhinid sharks, the tiger shark, *Galeocerdo cuvieri*, and the lemon shark, *Negaprion brevirostris*. Further, significant amounts of bacteria have been demonstrated in the kidney, muscle, liver, spleen and GI tract in a number of different species of shark (Grimes et al., 1985). While entirely speculative at this stage the most parsimonious explanation for the high mRNA expression of Rhbg like ammonia transporter in the intestine, rectal gland and kidney is the presence of ureolytic bacteria responsible for hydrolysis of urea into ammonia, means that the elasmobranch is forced to reabsorb

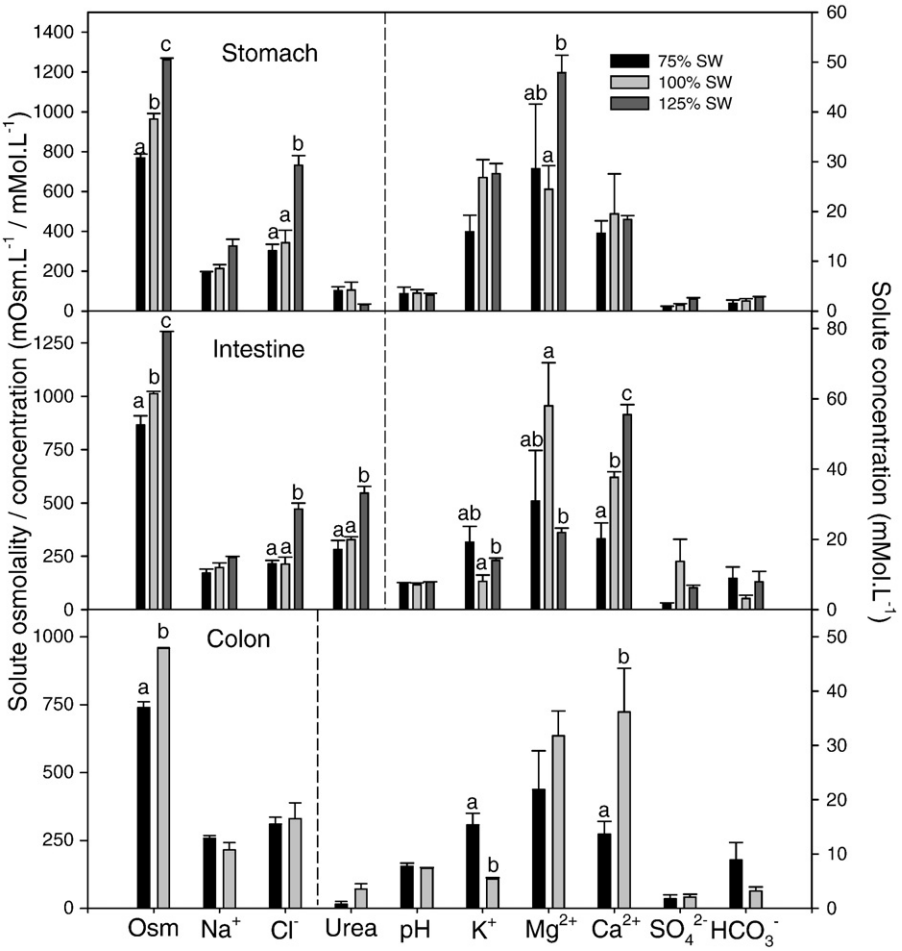

Fig. 5. Body fluid osmolality, pH and ion concentrations from the plasma, stomach, intestine and colon in *L. erinacea* adapted to 75% (top panel), 100% (middle panel) and 125% (bottom panel) SW. Values are expressed as a mean ± 1 sem. Different letters denote a significant difference between fluids for osmolality, pH and each of the measured ions between salinities. For values to the right of the dotted line, use the right-hand scale, ($n \geq 6$).

nitrogen in the form of ammonia; after all what better environment for a ureolytic bacteria than in an animal that retains urea in high concentrations. Although the presence of ureolytic bacteria is evident, it is not known if they exist intracellularly or in the interstitial space. Evidence to support the latter is found in the Rhbg expression shown in the present study and the location and activity of ornithine urea cycle (OUC) enzymes as well as the enzyme glutamine synthetase throughout a variety of tissues, including the intestine, of the dogfish (Kajimura et al., 2006).

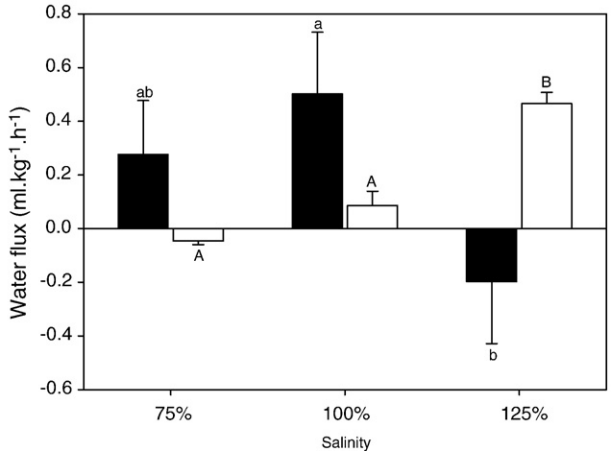

Fig. 6. Water flux rates in isolated intestine (filled bars) and colon (open bars) preparations from *L. erinacea*. Values are expressed as a mean mL/kg of fish body mass/hour of incubation ± 1 sem. Different letters denote significant differences within tissues (lower case for intestine and upper case for colon), ($n \geq 6$).

The OUC is the cellular mechanism responsible for urea synthesis. Previously it was thought that urea synthesis predominated in the liver of elasmobranch fishes, however, recent evidence indicates that urea synthesis is far more widespread and can be found in both muscle and to a lesser extent the GI tract of elasmobranch fish (Kajimura et al., 2006; Steele et al., 2005; Tam et al., 2003). Furthermore, it has been hypothesised that the presence of key enzymes involved in both the trafficking and conversion of ammonia-N to urea-N may assist in the retention of nitrogen after a meal (Wood et al., 2009). Evidence to support this is found in the presence of OUC enzymes in the intestinal epithelial of the spiny dogfish, in addition to the highest levels of glutamine synthetase being found in the intestine (Kajimura et al., 2006). Glutamine synthetase is the enzyme responsible for the conversion of ammonia to glutamine which acts as the main nitrogen donor for the OUC in elasmobranch fish (Anderson, 2001). Wood et al (2009) report ammonia levels in the gastric chyme of *S. acanthias* around 1–3 mM, and suggest that the presence of the OUC enzymes in addition to glutamine synthetase may be to detoxify the neutralised chyme as it enters the intestine. Clearly, there is an exciting avenue of research to untangle the interaction between ureolytic bacteria, urea synthesis and the expression and function of key nitrogen trafficking proteins in the intestine of elasmobranch fish. Among other things, results found for the mRNA expression of Rhbg and the urea transporter

need to be verified at the protein level together with accompanying localization studies in future investigations.

In the present study salinity transfer of *L. erinacea* significantly altered osmolality of stomach, intestinal and colonic fluids with a decrease in 75% SW adapted animals and an increase in 125% SW-adapted animals compared to the 100% SW control (Fig. 5). This result may simply be a reflection of similar changes that would be expected in the plasma of these animals. The observed differences were largely attributable to similar directional changes in urea and chloride levels. Although the magnitude of change of any one solute does not account for the observed differences in osmolality, the combined changes in all the solutes do. Furthermore, the results follow similar trends seen in *C. plagiosum* after transfer from 100% to 140% SW with the exception of Mg^{2+} where intestinal fluid values ranged from 130 ± 39.8 mM in 100% SW to 112 ± 52.4 in 140% SW. In the present study Mg^{2+} levels reached a maximum of 58.02 ± 12.27 mM in 100% SW adapted *L. eriniacea* and are more comparable to reported values for *S. acanthias* (Wood et al., 2007b) than *C. plagiosum*. The high levels of Mg^{2+} levels in the intestine of *C. plagiosum* may be further indication of an increased baseline drinking rate in these fish.

This study presents for the first time data on fluid and ion movements in the colon of any elasmobranch species, with all solutes demonstrating a net efflux in 100% SW adapted *L. erinacea*. However, the magnitude of these fluxes was low suggestive of a tighter epithelia than the intestine (Fig. 3). Furthermore, adaptation to different environmental salinities significantly influenced water movement in the intestine and colon with a significant intestinal efflux in 125% SW-adapted animals in comparison to 75 and 100% SW adapted animals (Fig. 6). However, water movement in the colon of 125% SW adapted animals was in a significant inward direction suggesting that the colon of *L. erinacea* may be involved in water conservation under hyperosmotic conditions. Interestingly the rectal region or colon has been implicated in water reabsorption in the Japanese eel, *Anguilla japonica* (Ando, 1980) albeit at much lower levels than found in the mid gut region of the same species.

In conclusion the present study has identified a number of similarities in body fluid solute concentration of the two rajiforme species compared with other elasmobranch species, suggesting comparable mechanisms for fluid and ion transport across species. However, some anomalies are present such as the extraordinarily high levels of HCO_3^- in the intestinal fluids of *C. plagiosum*. The lack of urea in the colonic fluid of *L. erinacea*, *R. eglanteria* and *C. plagiosum* further supports the strong need for nitrogen conservation in elasmobranch fish, an idea first put forward by Wood and colleagues. However, the mechanism by which nitrogen is conserved across the gut remains enigmatic. Ureolytic bacteria may play a role in this balancing act, although the extent and nature of their contribution remains to be elucidated. Finally, data presented on ion and fluid movement in the colon are suggestive of a potential role for the colon in water reabsorption under hyperosomotic conditions, with further work being required to clarify the extent of this role.

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