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Body fluid osmolytes and urea and ammonia flux in the colon of two chondrichthyan fishes, the ratfish, *Hydrolagus colliei*, and spiny dogfish, *Squalus acanthias*

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ABSTRACT

The present study has examined the role of the colon in regulating ammonia and urea nitrogen balance in two species of chondrichthyans, the ratfish, *Hydrolagus colliei* (a holocephalan) and the spiny dogfish, *Squalus acanthias* (an elasmobranch). Stripped colonic tissue from both the dogfish and ratfish was mounted in an Ussing chamber and in both species bi-directional urea flux was found to be negligible. Urea uptake by the mucosa and serosa of the isolated colonic epithelium through accumulation of ^{14}C-urea was determined to be 2.8 and 6.2 fold greater in the mucosa of the dogfish compared to the serosa of the dogfish and the mucosa of the ratfish respectively. Furthermore, there was no difference between serosal and mucosal accumulation of ^{14}C-urea in the ratfish. Through the addition of 2 mM NH_4Cl to the mucosal side of each preparation the potential for ammonia flux was also examined. This was again found to be negligible in both species suggesting that the colon is an extremely tight epithelium to the movement of both urea and ammonia. Plasma, chyme and bile fluid samples were also taken from the agastric ratfish and were compared with solute concentrations of equivalent body fluids in the dogfish. Finally molecular analysis revealed expression of 3 isoforms of the urea transport protein (UT) and an ammonia transport protein (Rhbg) in the gill, intestine, kidney and colon of the ratfish. Partial nucleotide sequences of the UT-1, 2 and 3 isoforms in the ratfish had 95, 95 and 92% identity to the equivalent UT isoforms recently identified in another holocephalan, the elephantfish, *Callorhinchus milii*. Finally, the nucleotide sequence of the Rhbg identified in the ratfish had 73% identity to the Rhbg protein recently identified in the little skate, *Leucoraja erinacea*.

1. Introduction

Marine adapted Chondrichthyan fishes, including the elasmobranchii and holocephali, are well recognised for the retention of high concentrations of urea (300–400 mM) within their body fluids (Krukenberg, 1888; Smith, 1931). The increased plasma concentration of urea serves to increase osmolality such that plasma osmolality is similar to, or slightly higher than that of the surrounding marine environment. While this ureosmotic strategy is common to both groups of fishes it is interesting to note that with the exception of two studies (Dakin, 1931; Hyodo et al., 2007), reports to date suggest that circulating levels of urea and sodium chloride (NaCl) in holocephali are lower and higher, respectively, than the corresponding solutes in the elasmobranch fish (Fange and Fugelli, 1962; Read, 1971). Although recent evidence in the holocephalan elephantfish, *Callorhinchus milii*, suggests that feeding results in circulating levels of urea similar to those described in elasmobranch fish (Hyodo et al., 2007), this indicates that holocephalans as a group of fish may rely more heavily on the regulation of NaCl to maintain the iso- or slightly hyper-osmoregulatory strategy than through the retention of urea, as evident in marine elasmobranchs (Hazon et al., 2003). However, depth may also play a role as evidenced recently with the interchangeable retention of organic osmolytes in the intra- and extracellular compartments of deep sea teleost and elasmobranch fish (Gillett et al., 1997; Treberg and Driedzic, 2002; Yancey, 2005).

The retention of high levels of urea in elasmobranchs renders them not only ureosmotic but also ureotelic, that is, the majority of nitrogenous loss is in the form of urea (Wood et al., 1995; Pärt et al., 1998). As a consequence, nitrogen conservation is critical in this group of fishes and it appears that the gastrointestinal (GI) tract is heavily invested in this process as even after a meal in the spiny dogfish, *Squalus acanthias*, additional nitrogen loss was low and the nitrogen that was lost was in the form of ammonia (Wood et al., 2005; Kajimura et al., 2006; Wood et al., 2007a). The role of the GI tract in nitrogen conservation in elasmobranch fish is further supported by urea concentrations in fluids from the different regions of the GI tract in three species of elasmobranch. In the white-spotted bamboo shark, *Chiloscyllium plagiosum*, little skate, *Leucoraja erinacea* and the clear nose skate *Raja eglanteria*, urea concentration in intestinal fluid was comparable to, or higher than, plasma concentrations, however, in the colonic fluid urea concentration

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fell to between 0 and 20 mM depending on the species examined (Anderson et al., 2010).

Our understanding of urea balance in the holocephali is not so clear, there are no published reports of urea levels in body fluids other than urine and plasma, and only a single study has examined plasma urea concentration following a salinity challenge (Hyodo et al., 2007). Furthermore, the significant anatomical differences in the agastric holocephali (Wilson and Castro, 2010) suggest that the mechanisms for nitrogen retention in the GI tract in this group of fishes may be distinctly different from those of the elasmobranch fish.

It is clear that there is a large capacity for urea retention in the intestine and colonic region of the elasmobranch gut, however, it is not known how this is achieved or if a similar mechanism is in place in the agastric holocephali. Transport of nitrogen in the form of urea and/or ammonia both provide plausible explanations in the dogfish as expression profiles of both the facilitative urea transport protein (UT) and the ammonia transport protein (Rhbg) exhibited increases in expression from the anterior to posterior end of the intestine in the little skate *L. erinacea*. Furthermore, the expression of the Rhbg protein was orders of magnitude higher than UT expression, suggesting a greater involvement of ammonia transport, and ultimately nitrogen conservation, than perhaps previously realised (Anderson et al., 2010).

In the present study we aimed to determine plasma solute concentrations in bile, intestinal chyme and plasma of the holocephalan ratfish, *Hydrolagus colliei*, in addition to molecular characterisation of specific urea and ammonia transport proteins. This provides a comparison with previously published values in these body fluids in a variety of elasmobranch species (Wood et al., 2005; Wood et al., 2007b; Anderson et al., 2010) and previously published molecular sequences of key nitrogen transport proteins in elasmobranchs and holocephalans (Smith and Wright, 1999; Hyodo et al., 2004; Janech et al., 2008; Kakumura et al., 2009; Anderson et al., 2010). Further, using an Ussing chamber setup, we aimed to examine urea and ammonia uptake rates in isolated stripped colonic epithelia and determine if these rates differed between representatives of the sister taxa, elasmobranchii and holocephali.

2. Materials and methods

2.1. Animals

Using frozen Pacific hake, *Merluccius productus*, as bait, ratfish, *H. colliei*, ($n = 7$; mean body mass 695 ± 75 g) and dogfish, *S. acanthias* ($n = 6$ mean body mass 2.10 ± 0.60 kg) of mixed sex were captured by rod and line in Barkley Sound, British Columbia, in June of 2010. All described procedures were conducted under approved animal care protocols at Bamfield Marine Sciences Centre under the guidelines of the Canadian Council for Animal Care.

When captured, fish were immediately transferred to holding tanks at Bamfield Marine Sciences Centre. The ratfish were held in small flow-through aquaria supplied with seawater at 975 mOsm L^{-1}, 12 ± 1 °C and ambient light conditions. Dogfish were maintained in a larger 151,000 L indoor holding tank under identical environmental conditions. All fish were held for a minimum of 24 h prior to experimentation. Following immersion in a terminal dose of tricaine methanesulfonate (250 ppm MS-222), a blood sample was taken from the caudal sinus of the ratfish and dogfish in a chilled heparinised (ammonium heparin, 100 IU mL^{-1}) 3 mL syringe with a 22 gauge needle. The blood was immediately centrifuged and the plasma was separated and stored at −80 °C for future analysis. In addition a sample of intestinal chyme and bile was taken from the dogfish and ratfish. Bilary fluid was taken directly from the gallbladder of each species using a 23-gauge needle and 1-mL syringe. Intestinal chyme was taken from the dogfish by clamping the pyloric sphincter and posterior end of the spiral valve. The spiral valve was then removed, blotted dry and the contents of the intestine were collected by removing the posterior clamp and gently squeezing the spiral valve to empty into a 1.5 mL tube. Any solid matter was removed and the tube was then centrifuged at 13,000 g for 5 min. The supernatant was then removed and stored at −80 °C for future analysis. A similar procedure was used to collect the contents of the ratfish intestine, however, in this case the entire gastrointestinal tract was removed and emptied into a collection tube. Efforts were made to sample colonic fluid from both animals however, these proved unsuccessful. With the exception of a single ratfish, all fish in these experiments had food in their gastrointestinal tract at the time of sacrifice.

2.2. Body fluid analysis

Body fluid osmolality was measured using a vapor pressure osmometer (Vapro 5520, Wescor, Inc., Logan, Utah, USA). Plasma sodium (Na^+), potassium (K^+), calcium (Ca^{2+}), magnesium (Mg^{2+}), trimethylamine oxide (TMAO), chloride (Cl^-), sulfate (SO_4^{2-}) and phosphate (PO_4^{3-}) were measured by ion-exchange chromatography (Metrohm-Peak, Herisau, Switzerland). The cation eluent was 1.7 mM nitric acid and 0.7 mM dipicolinic acid, and the anion eluent was 3.2 mM Na_2CO_3 and 1 mM $NaHCO_3$ with CO_2 suppression by 100 mM H_2SO_4 followed by CO_2 free air. Total CO_2, or bicarbonate (HCO_3^-), of body fluid samples 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, Ottawa, On, Canada). Urea concentration for all samples was measured using the spectrophotometric method as previously described (Rahmutallah and Boyd, 1980). Total ammonia was measured only in ratfish plasma using an enzymatic based assay that measures L-glutamate production following catalysis by glutamate dehydrogenase (Raichem, Cliniqa Corp, San Marcos, CA, USA).

2.3. Reverse transcribed PCR

For total RNA extraction the branchial and renal tissues (approximately 100 mg) were removed from a single ratfish and stored in RNAlater (Applied Biosystems, Austin TX, USA) at a ratio of approximately 1:10, tissue:RNAlater. Samples were then frozen and stored at −80 °C before being shipped to the University of Manitoba on dry ice. Anterior and posterior intestine and colonic tissues were removed from the gastrointestinal tract, gently rinsed in Ringers solution and scrapings of mucosal tissue were taken from each section and stored in RNAlater as described above. RNA extraction was conducted under RNase-free conditions using TRIZOL reagent (Invitrogen, Carlsbad, CA, USA).

Following DNase treatment (DNase 1, Invitrogen, Carlsbad, CA, USA), RNA was tested for purity by polymerase chain reaction (PCR) (40 cycles) using 1 μg DNase treated RNA as a template and elasmobranch specific primers shark β-actin-F/shark β-actin-R (product size 315 bp, see Table 1) designed to amplify DNA coding for shark β-actin. 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 PCR employing the primer pair shark β-actin-F/shark β-actin-R. PCR products were evaluated by gel-electrophoresis, ethidium bromide staining and UV visualization.

PCR products amplified by primers targeting shark β-actin (shark β-actin-F/shark β-actin-R), Rhbg (Raja Rhbg-F1/Raja Rhbg-R2), UT-1 (efUT1-F/efUT1-R), UT-2 (efUT2-F/efUT2-R) and UT-3 (efUT3-F/efUT3-R) were gel-purified and sequenced using a Hitachi 3130 Genetic Analyzer with ABDNA Sequencing Analyzing Software (Applied Biosystems, Foster City, CA, USA). Based on the obtained sequences ratfish specific primer pairs were designed to assess presence or absence of Rhbg, UT-1, UT-2 and UT-3 in various tissues. Also these PCR products were evaluated for correctness by sequencing. Nucleotide sequences were obtained by using BigDye ver 3.1 and a 3130 Genetic Analyser (Applied Biosystems) following the manufacturer's instructions.

Table 1
Primers employed in PCR targeting shark β-actin, Rhesus-like ammonia transporter — Rhbg and the urea transporters UT-1, UT-2 and UT-3 from the ratfish *Hydrolagus colliei*.

Primer	Nucleotide sequence (5 → 3)	Annealing temp (°C)	Product size (bp)
β-actin			
Shark actin-F	CACGAGCTGTCTTTCCTTCC	61	315
Shark actin-R	TTGCAGGGGTATTGAAGGTC	61	
Rh protein			
Raja-Rhbg-F1	CGTCCACGTCATGATATTCG	55	426
Raja-Rhbg-R2	GGTGATGGTCAGTCCGAAAT	55	
Ratfish Rh-F	CCAGTGCAACTGTTGGTCAT	61	125
Ratfish Rh-R	GCCCCAAACACATGGATTAC	61	
UT-1			
efUT1-F	CTTACGAGGAGCTGCTCAGGTG	50	930
efUT1-R	TCCTCTGGGTATGTCACTGTAT	50	
Ratfish UT1-F3	TTAATATATCTGTGGGCC	53	132
Ratfish UT1-R2	TTAGAAGCATTGGAATGC	53	
UT-2			
efUT2-F	GCAATTTCAGAAGGGCTCCA	50	583
efUT2-R	TATGTGTCTGCCATGTGAGG	50	
Ratfish UT2-F2	CTCCATGATGGAAGGAAT	52	146
Ratfish UT2-R1	ATGAGATGTTTTGTGCTACA	52	
UT-3			
efUT3-F	GATAACTTTTGGCCTTCTGT	50	459
efUT3-R	ATTTGTCTCATTTGATTGTGGC	50	

Primer pair sequences for efUT1-F/R, efUT2-F/R, and efUT3-F/R were taken from Kakumura et al. (2009) and sequences for Raja-Rhbg-F1/R2 were taken from Anderson et al. (2010).

Sequences were aligned with Biology WorkBench using the CLUSTAL sequence alignment tools (http://seqtool.sdsc.edu). Phylogenetic analysis using maximum parsimony (MP) was performed in PAUP* 4.0 (Wilgenbusch and Swofford, 2003) using a heuristic search with 1000 replicates of random addition of taxa, tree-bissection-reconnection (TBR) branch swapping, and MulTrees off. Bootstrap values were determined using 1000 bootstrap replicates (Felsenstein, 1985) with simple addition of taxa and TBR branch swapping. Bootstrap values greater than 90 are reported in the phylogenetic tree. A phylogenetic analysis using Neighbour Joining was also conducted to confirm the MP results.

2.4. Ussing chamber experiments

Following sacrifice and body fluid sampling, a small section of the post-valvular intestine, approximately 3 cm in length, was removed between the posterior end of the spiral valve and the rectum in both species, henceforth described as the colon. The colon was then opened longitudinally to expose the mucosal surface and the tissue was gently washed in ice-cold Ringers solution. For the dogfish this was composed of (in mM) NaCl, 250; Na_2SO_4 7; $MgSO_4$ 3; KCl 4; $CaCl_2 \cdot 2H_2O$ 2; $NaHCO_3$ 5; Na_2HPO_4 0.1, urea 450; trimethylamine-N-oxide 100; glucose 4; pH 7.8 and for the ratfish (in mM) NaCl, 300; Na_2SO_4 7; $MgSO_4$ 3; KCl 4; $CaCl_2 \cdot 2H_2O$ 2; $NaHCO_3$ 5; Na_2HPO_4 0.1, urea 300; trimethylamine-N-oxide 50; glucose 4; pH 7.8. The mucosal epithelium was then gradually separated from the muscular wall of the colon in the dogfish and from the musculature and the rectal gland tubules in the ratfish. It is important to note that as with the other Chimaeridae, the rectal gland tubules did not appear to be as developed as those described in the elephantfish (Hyodo et al., 2007).

Once dissected free, two sections from the same colon were mounted in identical tissue holders with an exposed surface area of 0.2 cm^2. The tissue holders were then inserted into water-jacketed Ussing chambers (Physiologic Instruments, San Diego, CA, USA) cooled to 12 ± 1 °C by a re-circulating chiller (Haake, Fisher Scientific, Hampton, NH, USA). Depending on the species, 4 mL of ratfish or dogfish Ringers solution was added to the serosal side of the preparation, and 4 mL of ratfish or dogfish Ringers with 2 mM NH_4Cl was added to the mucosal side of the preparation. Both the serosal and mucosal fluids were aerated with a 99.7:0.3% O_2:CO_2 gas mix throughout the experimental period. The tissue was then allowed to equilibrate for at least 30 min. At the end of the equilibration period, 10 μL of ^{14}C-urea, specific activity 2.04 MBq μmol^{-1} (Perkin Elmer, Waltham, MA, USA) was added to either the mucosal or serosal side of each preparation. This created a specific activity of approximately 3700 cpm per μmol of urea in the Ringer solution. Immediately following the addition of the ^{14}C-urea, a 20 μL sample, in duplicate, was removed from both the serosal and mucosal side of the preparations and added to 4 mL scintillation vials containing 1 mL of 0.1% sodium azide in MilliQ water. Sodium azide was added to negate any potential influence of ureolytic bacteria that may have been present in the tissue and therefore samples. Four mL of scintillation fluid (Ultima Gold, Perkin Elmer) was then added to the vials and radioactivity was counted on a LS6000 liquid scintillation counter (Beckman Coulter, Brea, CA, USA). Internal standardization ensured that quenching was uniform. At the same time additional 5 and 20-μL samples, both in duplicate, were removed from both the serosal and mucosal side for analysis of total urea and total ammonia respectively using the assays described above. Subsequent samples were taken in an identical fashion as described above every 30 min for the following 3 h.

At the end of the 3-h experimental period the tissue holders were removed from the Ussing chamber and both the mucosal and serosal sides were gently rinsed with Ringers solution *in situ* to remove any loosely bound ^{14}C-urea from the preparation. Using the tip of a glass Pasteur pipette, mucosal tissue was scraped from the tissue *in situ*. The tissue scrapings were added to a scintillation vial containing 1 mL of 0.1% sodium azide and samples were counted as above. The remaining serosal tissue was added to a second scintillation vial and counted in a similar manner. In this way a measure of ^{14}C-urea uptake rates in either the serosal or mucosal tissue of each preparation could be obtained in a manner similar to that previously described for gut sac preparations in rainbow trout, *Oncorhynchus mykiss* (Nadella et al., 2007) using the equation:

$$\text{Uptake rate} = \text{Tissue cpm}/(\text{SA X ISA X t})$$

where SA was the specific activity of ^{14}C-urea in the measured compartment (cpm/μmol), ISA was the intestinal surface area and t was the time in minutes, the final unit of measurement being μmol min^{-1} cm^2. Statistical significance between tissues was determined using one way analysis of variance with a Bonferroni post-hoc test.

3. Results

Table 2 shows the solute concentration of intestinal chyme, bile and plasma from the ratfish, *H. colliei*, and for comparison solute concentration from intestinal chyme, bile and plasma from the dogfish, *S. acanthias*. Osmolality and measured solute concentrations of the ratfish body fluids were generally equivalent to the comparable solutes from dogfish with some notable exceptions. In the ratfish, plasma osmolality, urea and TMAO concentrations were significantly lower than the corresponding values for the dogfish. In the intestinal chyme TMAO was undetectable in the ratfish, yet measurable in the dogfish. In the bile fluid HCO_3^- concentration, as measured by total CO_2, was significantly higher in the ratfish as opposed to the dogfish. Total ammonia levels were measured only in ratfish plasma using the described enzymatic approach and were 0.13 ± 0.02 mM for the ratfish.

Sequencing of the PCR product gained by employing the primer pair Raja Rhbg-F1/Raja Rhbg-R2 (Table 1) gave a 182 bp fragment (GenBank accession # **HQ852228**) that showed 73% identity to the Rhbg nucleotide sequence of the little skate *L. erinacea* (**FL670153**). The deduced amino acid sequence showed 75% identity to Rhcg in

Table 2
Concentration of various body fluid solutes in the plasma, intestinal chyme and bile of the ratfish and dogfish. Results are expressed as a mean ± 1 SEM (nd = not detected). Statistical analysis was conducted between the equivalent body fluids from both species and significance was accepted when *p<0.05; ***p<0.001.

	Ratfish, *H. colliei*			Dogfish, *S. acanthias*		
	Plasma	Chyme	Bile	Plasma	Chyme	Bile
Osmolality (mOsm kg^{-1})	898.8 ± 30.1*	987.0 ± 3.8	882.4 ± 29.1	988.1 ± 13.0	950.8 ± 19.2	938.4 ± 47.2
pH	7.68 ± 0.04	7.76 ± 0.4	8.11 ± 0.2	7.71 ± 0.1	7.48 ± 0.03	6.27 ± 0.2
Urea (mM)	338 1 ± 11.3*	296.7 ± 34.2*	289.8 ± 52.7	372.1 ± 8.3	453.2 ± 10.6	319.1 ± 50.5
Na^+ (mM)	259.8 ± 7.4	164.4 ± 34.6	255.4 ± 35.1	238.5 ± 9.6	136.0 ± 12.2	291.8 ± 35.9
K^+ (mM)	3.8 ± 0.1	4.3 ± 0.4	3.4 ± 0.7	2.9 ± 0.2	2.6 ± 0.7	3.9 ± 0.4
Ca^{2+} (mM)	3.2 ± 0.3	42.5 ± 9.8*	4.7 ± 0.9	3.5 ± 0.1	25.5 ± 3.5	9.1 ± 1.3
Mg^{2+} (mM)	3.0 ± 0.5	44.9 ± 24.0	3.1 ± 0.7	2.6 ± 0.4	72.2 ± 12.9	5.0 ± 0.6
TMAO (mM)	3.6 ± 0.8***	nd	nd	81.7 ± 8.7	7.3 ± 2.3	nd
Cl^- (mM)	275.3 ± 14.2	247.9 ± 57.7	208.4 ± 37.7	253.8 ± 15.5	270.2 ± 32.7	117.6 ± 39.3
SO_4^{2-} (mM)	2.4 ± 0.4	13.0 ± 5.4	5.2 ± 1.7	1.7 ± 0.2	20.1 ± 8.5	2.9 ± 0.5
PO_4^{3-}(mM)	1.0 ± 0.1	1.6 ± 0.5	nd	1.1 ± 0.1	3.2 ± 2.0	nd
HCO_3^-(mM)	5.6 ± 0.7	14.2 ± 4.1	11.3 ± 3.6	5.8 ± 0.4	15.4 ± 2.7	nd

rainbow trout (**AAU89494**) and 70% identity to RhCG in humans (**3HD6_A**), both proteins with confirmed ammonia transport capability (Weiner and Hamm, 2007; Nawata et al., 2010). As shown in Fig. 1 ratfish Rhbg is predominately expressed in gill tissues and in the colon. A very weak PCR signal was also detected in the posterior gut and kidney.

Sequencing of the PCR products gained by the use of UT-specific primers first employed in elephantfish (Table 1) gave three distinct sequences each with a high degree of identity to the urea transporters UT-1 (**HQ852229**), UT-2 (**HQ852230**) and UT-3 (**HQ852231**). For ratfish UT-1 an 814 bp fragment was obtained that showed 95% identity to elephantfish UT-1 (**AB470074**), 86% identity to the urea transporter ShUT from the dogfish *S. acanthias* (**AF257331**) and 80% identity to the predicted urea transporter from the little skate *L. erinacea* (**AY161305**). As shown in Fig. 1, ratfish UT-1 mRNA was only expressed in the kidney. For the ratfish UT-2 a 513 bp fragment was obtained using the primer combination efUT2-F/efUT2-F (Table1). This sequence revealed a 95% identity to the elephantfish UT-2 (**AB470076**), 80% identity to the elephantfish UT-1 and 74% identity to the elephantfish UT-3 (**AB470077**). Ratfish UT-3 was expressed predominately in the kidney and anterior gut in addition to low, but still detectable levels of expression in the gills, posterior gut and the colon (Fig. 1).

Ratfish UT-3 showed a very similar expression pattern to UT-2 and the partial sequence of 439 bp in the ratfish had a 92% identity to elephantfish UT-3 and 70% or less identity to published sequences of elasmobranch urea transporters. Phylogenetic analysis of the UT isoforms indicated high confidence for five major clades. Two clades are comprised of UT in teleost fishes, two clades each with UT-2 and UT-3 transporters in holocephalans, and one derived clade that contained UT sequences from holocephalans and elasmobranchs. The UT-1 isoform formed a sister clade to the UT transporter identified in elasmobranch fish, and together they formed a sister clade to the UT-2 transporter in holocephalans. The UT-3 transporter is more basal to the UT-1 and UT-2 isoforms reported in this tree. However, these taxonomic clades may also contain another ancestral form that diverged into teleost UT-C in addition to divergence of the teleost UT clade that is a sister clade to the UT-1 and UT-2 clade. The sea urchin, *Strongylocentotus purpuratus*, and the jewel wasp, *Nasonia vitripennis* were assigned as the outgroup taxon.

In regard to the Ussing chamber results, changes in total ammonia and urea measured from the serosal and mucosal fluid throughout the experimental period for dogfish and ratfish are presented in Figs. 3 and 4 respectively. It was evident from these data that movement of either ammonia or urea from the mucosal to serosal side was negligible during the 3 h experimental period. In support of these results addition of ^{14}C-urea to either the mucosal or serosal side of the preparation did not result in a significant appearance of ^{14}C-urea in the opposite side (data not shown). That is, the maximum cpm of ^{14}C-urea in the 20 μL sample following the 3 h incubation period was 91 and 116 in the mucosal and serosal fluids respectively. Accounting for background this equates to between 0.1 and 0.15 nmol min^{-1} of urea flux across the entire epithelium. This suggests that this epithelium was extremely resistant to the movement of urea and also ammonia across the tissue. However, uptake of ^{14}C-urea into the mucosal and serosal tissue during the 3-h experimental period for both the dogfish and ratfish is presented in Fig. 5. There was substantial uptake into the mucosal tissue

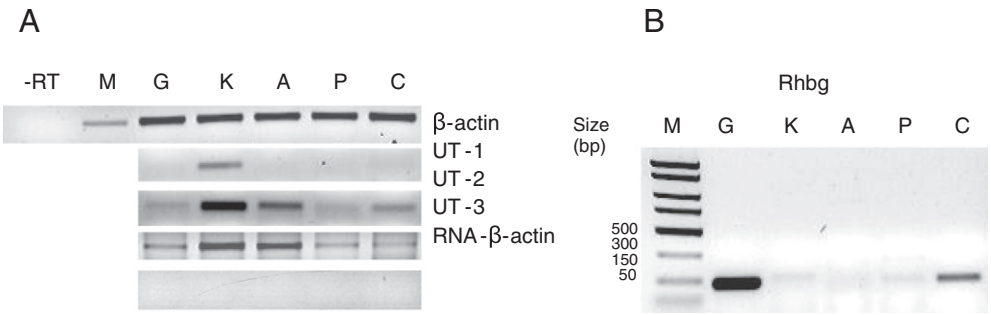

Fig. 1. PCR of β-actin, Rhbg, UT-1, UT-2 and UT-3 in different ratfish tissues. Panel A: For amplification of β-actin primers Shark actin-F/Shark actin-R and 30 cycles were employed. In the negative control (−RT), no cDNA was added to the reaction. For UT-1 the primers Ratfish UT1-F3/Ratfish UT1-R2, for UT-2 the primers Ratfish UT2-F2 Ratfish UT2-R1, and for UT-3 the primers efUT3-F/efUT3-R were employed using 32, 34, and 36 cycles respectively. Panel B: For amplification of Rhbg, the primers Ratfish Rh-F/Ratfish Rh-R were used to generate a 125 bp product. Purity of RNA was confirmed employing the following primers, Shark actin-F/Shark actin-R and 35 cycles. A = anterior gut; P = posterior gut; C = colon, G = gill; K = kidney; M, molecular weight marker (band in panel A shows 300 bp).

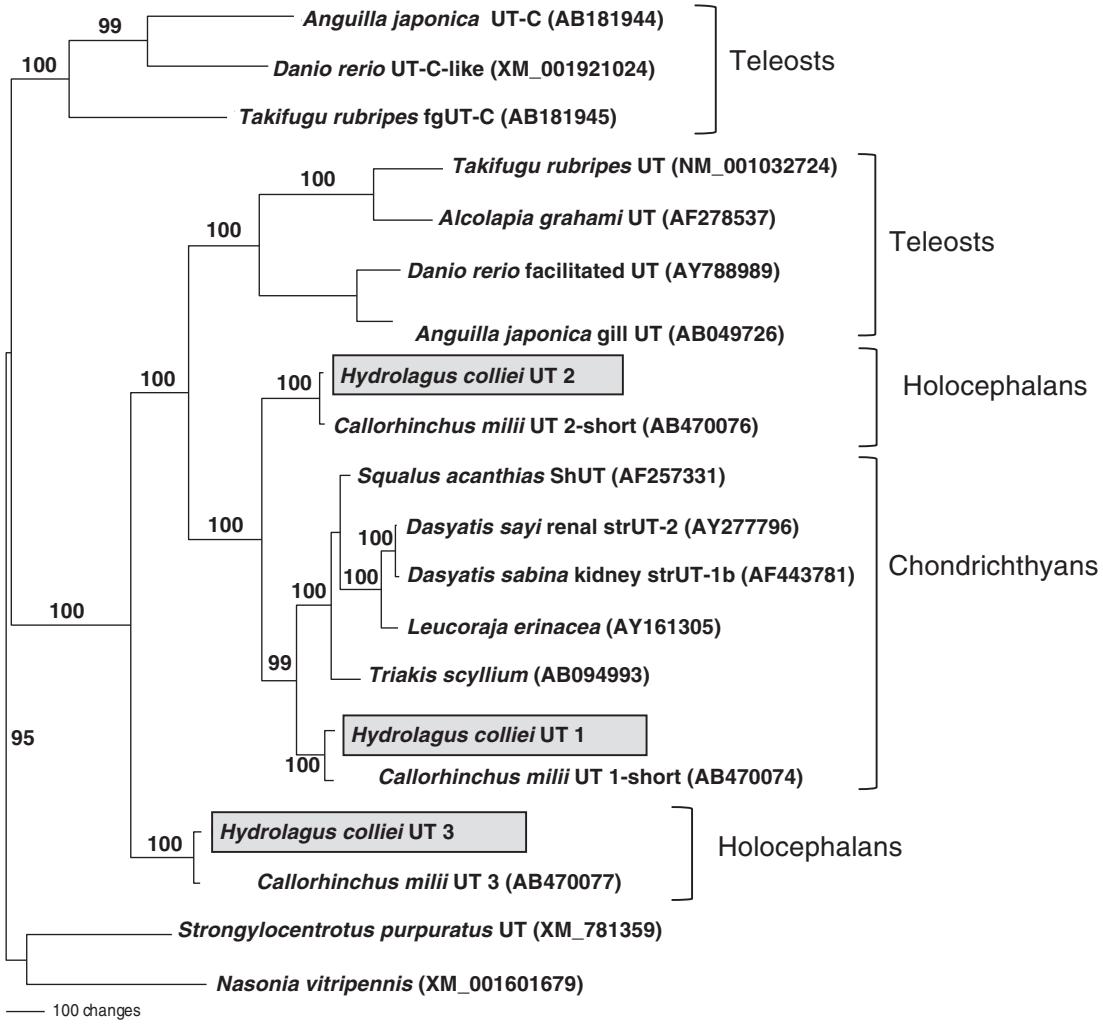

Fig. 2. One of two most parsimonious trees showing the evolutionary history of the urea transporter (UT-1, UT-2 and UT-3) isoforms in ratfish, *Hydrolagus colliei*, and other taxa. *Strongylocentrotus purpuratus* and *Nasonia vitripennis* are assigned as the outgroup taxa. Only those bootstrap values greater than 90 are shown on the tree. GenBank accession numbers are given in brackets. The tree has 4627 changes, a consistency index of 0.6250, a retention index of 0.5611, and 930 parsimony informative characters out of 2642 total characters.

of the dogfish colon, although this was very small in the serosal tissue of the dogfish, nor was there any difference in the equally low ^{14}C-urea uptake rates between serosal and mucosal tissue in the ratfish colon.

4. Discussion

In previous studies on holocephalans, plasma osmolality has been shown to be iso-osmotic to seawater in the rabbitfish, *Chimaera monstrosa* (Fange and Fugelli, 1962) and elephantfish, *C. milii* (Dakin, 1931; Hyodo et al., 2007). In the present study, plasma osmolality was slightly hyper-osmotic to seawater in the dogfish but slightly hypo-osmotic in the ratfish. Plasma urea concentration in the ratfish was significantly lower than in the respective fluids in the dogfish, however, plasma urea and Na^+ concentrations were equivalent to those previously reported in the ratfish (Urist and Van De Putte, 1967; Read, 1971).

It has been shown that a variety of methylamines and organic solutes, such as β amino acids and TMAO contribute to the overall intracellular and extra-cellular osmotic pressure in elasmobranch fishes (Boyd et al., 1977; King et al., 1980; Treberg and Driedzic, 2006; Treberg et al., 2006). From this and other studies it is evident that holocephalans are also ureosmotic and therefore it would be reasonable to assume that the ratfish should have at least measurable amounts of TMAO in their plasma. However, this was not the case and indeed low circulating levels of TMAO have previously been reported in ratfish plasma (Norris and Benoit, 1945; Read, 1967). In the elephantfish, plasma levels of TMAO were shown to be as high as 50 mM and free amino acids, with the exception of taurine, were found to be similar between the elephantfish and the dogfish (Bedford, 1983). Interestingly, in all reports to date, plasma urea concentration in the elephantfish is consistently higher than in any other representative holocephalan (Hyodo et al., 2007). These data suggest that the ureosmotic strategy so well-described in marine elasmobranch fishes is, among the holocephali, perhaps best developed in the elephantfish. Indeed when exposed to reduced salinity the elephantfish present an osmoregulatory strategy of off-loading urea that is analogous to that described in a number of marine elasmobranchs when exposed to reduced salinities (Sulikowski and Maginniss, 2001; Hazon et al., 2003; Sulikowski et al., 2003; Hyodo et al., 2007).

An alternative hypothesis for the observed discrepancy in urea levels between the ratfish and elephantfish could be depth. While the ratfish in the present study were caught at depths<30 m it has been shown that they may inhabit waters in excess of 900 m deep (Eschmeyer et al., 1983) whereas the elephantfish are typically found at water depths no greater than 300 m (Cox and Francis, 1997). In representative teleost and elasmobranch species there is an inverse

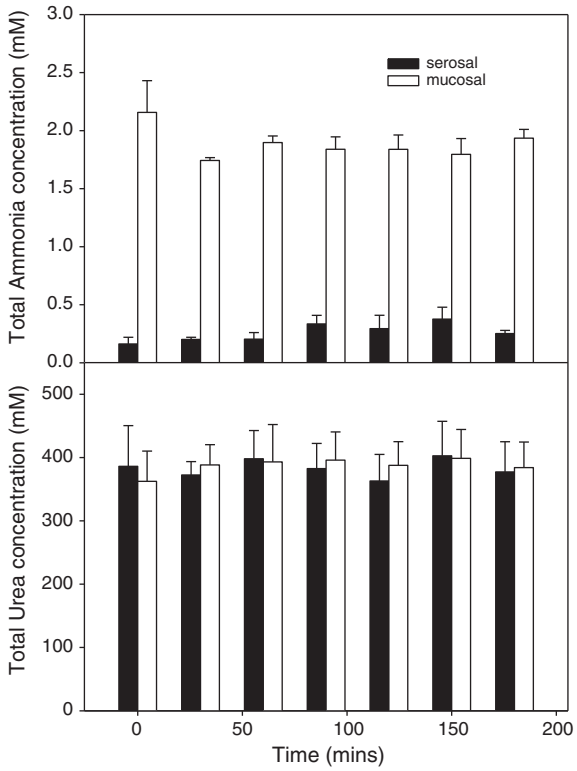

Fig. 3. Total ammonia (upper panel) and urea (lower panel) concentrations (mM) of serosal (filled bars) and mucosal (open bars) fluids throughout a 3-h incubation period of isolated stripped colonic epithelium from the dogfish, *S. acanthias* mounted in an Ussing chamber. Samples were collected every 30 min during the experiment and data are expressed as a mean ± 1 SEM, n = 6.

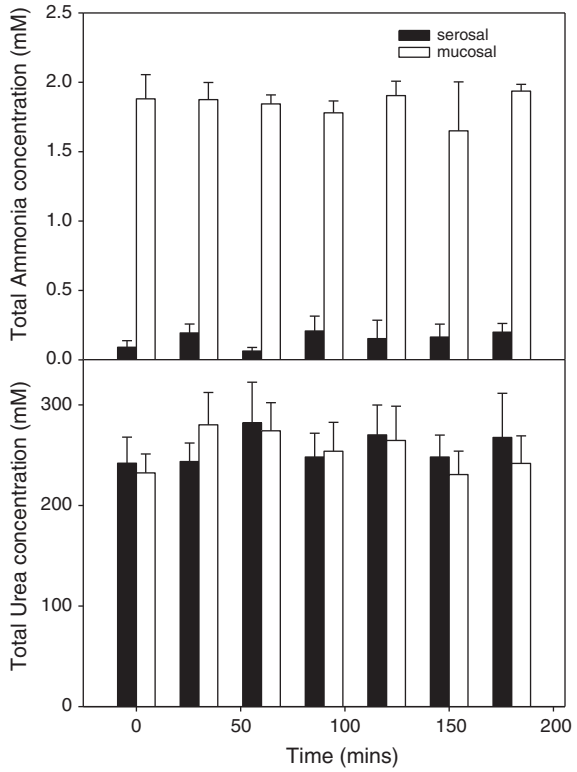

Fig. 4. Total ammonia (upper panel) and urea (lower panel) concentrations of serosal (filled bars) and mucosal (open bars) fluids throughout a 3-h incubation period of isolated stripped colonic epithelia from the ratfish, *H. colliei* mounted in an Ussing chamber. Samples were collected every 30 min during the experiment and data are expressed as a mean ± 1 SEM, n = 7.

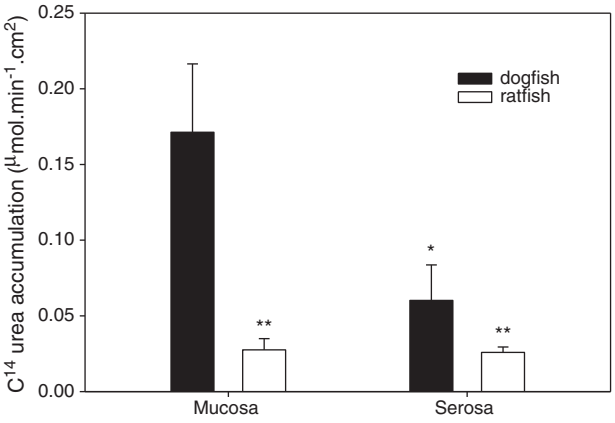

Fig. 5. ^{14}C-urea uptake rates (μmol min^{-1}) in the mucosa and serosa of isolated stripped colonic epithelia from the dogfish, *S. acanthias* (filled bars) and ratfish, *H. colliei* (open bars) mounted in an Ussing chamber for a period of 3-h. Data are expressed as a mean ± 1 SEM, n ≥ 6. Statistical analysis was conducted between the mucosal epithelium of the dogfish and all other tissues. Significance was accepted when $^{**}p<0.01$ and $^{*}p<0.05$.

relationship between urea concentration and depth (Yancey, 2005). Interestingly the rabbitfish, another deep sea holocephalan, has urea levels analogous to the ratfish (Robertson, 1976). Finally betaine is recognised as a critical counteractant for urea perturbation in the elephantfish (Bedford et al., 1998), however, TMAO appears critical as a cytoprotectant in deep water species (Yancey, 2005). Clearly further research is warranted to determine both the extra- and intra-cellular role of organic solutes in the ratfish, particularly during osmoregulatory challenges such as exposure to reduced or increased salinities.

To the authors' knowledge, the present study is the first to provide data on the solute make-up of the intestinal chyme and bile in a representative holocephalan fish. Solute levels in the chyme and bile of the dogfish compare favourably with previously published values in this species (Wood et al., 2007b) and furthermore, solute values were similar between species for both these fluids with the exception of urea, TMAO and calcium in the chyme and bicarbonate in the bile. In the present study the agastric nature of the ratfish GI tract (Wilson and Castro, 2010) led to the chyme comprising a mix of fluids from the entire length of the GI tract, with the exception of the colon. Therefore, it is difficult to explain the described differences between intestinal chyme collected in the ratfish and dogfish. Nonetheless, it is clear that the ratfish, has a greater osmolality in the intestinal chyme in comparison to the plasma ($p<0.001$), that one presumes would impede water reabsorption across the intestine. What solutes are contributing to the increased osmolality in the intestinal chyme of the ratfish are unknown, however, one would presume that pancreatic secretions required for the digestion of food are likely contributing factors. Collection of pancreatic secretions from the well developed pancreatic duct in the ratfish would help in determining this.

Further, drinking seawater as an osmoregulatory necessity is a well-described phenomenon in teleost fish (Marshall and Grosell, 2006), and has also been described in elasmobranch fish, including the spiny dogfish (Hazon et al., 1989; De Boeck et al., 2001), however, there are no reports on the drinking rate in any holocephalan species. Measurement of drinking rates in the ratfish would provide support for a role of the GI tract in osmoregulation in this species in addition to an evolutionary and adaptive significance of this physiological response in agastric fishes.

Further to the observed increase in intestinal fluid osmolality in the ratfish, there was also a significant level of urea within the intestinal chyme. High levels of urea have also been reported in intestinal chyme of elasmobranch fish (Wood et al., 2007b; Anderson et al.,

2010) although this all but disappears in the colonic fluid of some species, suggesting a significant amount of urea reabsorption across the intestinal epithelium (Anderson et al., 2010). It was therefore reasonable to assume that the colon may be involved in urea balance in the dogfish and also the ratfish given the observed ureosmotic strategy. Experiments utilising ^{14}C-urea showed that the uptake rate of urea was greatest in the mucosa of the dogfish colon, and negligible in the serosa. However, there was no appearance of urea or ammonia in the serosal side of the preparation for either the dogfish or ratfish. This may be the result of the experimental timeframe or lack of additional mechanisms in the stripped epithelium preparation, such as blood vessels, that are required for the uptake of urea into the animal. Nonetheless, the absence of any difference in serosal and mucosal uptake of ^{14}C-urea in the ratfish suggests that distinctly different mechanisms are in place between these two species despite both utilising a ureosmotic strategy. However, it is premature to conclude that the ratfish intestine is not involved in urea balance particularly in light of differential expression of key transport proteins along the length of the teleost fish intestine (Grosell, 2006). Therefore, it would be beneficial to conduct further comparisons of urea uptake by the intestinal epithelia in both species to determine if urea uptake is restricted to the colonic region or if there are differential rates of uptake throughout the GI tract in these species. In addition inhibitors such as phloretin, and competitors such as thiourea, could be used to further characterise the urea transport mechanisms in the GI tract of both species.

In elasmobranch fish a single UT transporter sensitive to phloretin and homologous to the mammalian facilitative UT-A2 transporter has been identified in batoids (rays and skates) and selachians (extant sharks) (Smith and Wright, 1999; Morgan et al., 2003; Hyodo et al., 2004; Janech et al., 2006; Janech et al., 2008; Anderson et al., 2010). In the present study 3 UT isoforms were identified in the ratfish, UT-1, UT-2 and UT-3. The identification and evolutionary hypothesis of these isoforms is consistent with the recent discovery of 3 efUT isoforms in the closely related holocephalan, the elephantfish *C. milii* (Kakumura et al., 2009). The efUT-1 isoform is most closely related to sequenced elasmobranch UT's. The ratfish UT-2 isoform is more distantly related to the ratfish UT-1 isoform than to the UT of elasmobranchs, and potentially arose from a gene-duplication event in chondrichthyan fishes (Kakumura et al., 2009) (Fig. 2). The efUT-3 isoform showed relatively low homology to the efUT-1 and 2 isoforms. The UT-3 isoform may have diverged into the teleost UT isoform and the UT-1/UT-2 isoforms, however, the possibility exists that the teleost UT isoform may exist as a pseudogene.

Homology of the UT-3 isoform in the elephantfish was lowest when compared to previously identified UT transporters in teleost fish including the UT-C isoform in the eel, *Anguilla japonica* (Mistry et al., 2001). It was concluded by Kakumura et al. (2009) that the phylogenetic positioning of the efUT-3 was uncertain and it may belong to the UT-C isoforms identified in teleost fish. Phylogenetic analysis in the present study revealed, however, that the UT-3 isoforms are not closely related to the UT-C isoforms. Indeed the UT-3 isoform identified in the ratfish and elephantfish may in fact be the ancestral form of this protein in chondricthyans given its relatedness to the UT isoforms identified in the sea urchin, *S. purpuratus*, and the jewel wasp, *N. vitripennis*.

Functional relevance of all 5 of the efUT isoforms has been demonstrated, as urea uptake was sensitive to the UT inhibitor phloretin, when the efUT's were inserted into *Xenopus* oocytes (Kakumura et al., 2009). From the perspective of tissue expression, the ratfish UT-3 isoform was expressed in all tissues examined (gill, kidney, anterior and posterior intestine and colon). The UT-2 isoform was also expressed in all tissues, in particular the kidney, but the UT-1 isoform was expressed only in the kidney. In contrast, in the elephantfish, all efUT isoforms were expressed in the kidney and interrenal gland. The UT-1 isoform was the most ubiquitously expressed isoform in the tissues examined for the elephantfish, however, none of the isoforms were expressed in the gill and only the UT-3 isoform was expressed in the intestine (Kakumura et al., 2009).

In marine elasmobranch fish, 90% of urea loss occurs across the gills (Pärt et al., 1998), reflecting the immense concentration gradient from the internal to the external environment (Boylan, 1967). That said, the gill epithelia, through a combination of differential permeabilities in the basolateral and apical membranes alongside a Na^+-urea countertransport mechanism that creates a urea "back transport" system (Fines et al., 2001), limits this loss (reviewed in McDonald et al., 2006). In holocephalans, basic mechanisms of whole body urea balance are not well understood yet it is interesting to note the expression of the UT-3 isoform in the gills of the ratfish. Indeed in elasmobranch fish the presence of a facilitative UT in the gills is limited to the original identification of UT's in the spiny dogfish (Smith and Wright, 1999). Clearly there is a need to further explore urea balance in the holocephalans to better explain the functional significance and presence of the UT-3 isoform in the gills. Further, comparative expression studies of both the UT transporters and Rhbg proteins along the entire length of the GI tract in the dogfish and ratfish would provide further information on the role of the gut in nitrogen balance in these two species of chondricthyans.

Since the discovery in the late nineties (Marini et al., 1997) that proteins belonging to the rhesus (Rh) glycoprotein family play a role in the transcellular transport of ammonia there has been a re-thinking of the proposed paradigms for nitrogen balance, with aquatic organisms receiving significant attention (Weihrauch et al., 2009; Wright and Wood, 2009). Two Rh proteins have thus far been identified in elasmobranch fish, Rhp2 in the kidney of the Japanese dogfish, *Triakis scyllia* (Nakada et al., 2010) and a Rhbg-like transporter in the intestine, kidney and rectal gland of the little skate, *L. erinacea* (Anderson et al., 2010). To the authors' knowledge the present paper is the first to identify expression of any Rh protein in the holocephalans. This putative transporter was Rhbg-like, and was highly expressed in the gills and colon of the ratfish but interestingly not the kidney. It is important to note that additional isoforms, such as primitive Rh proteins analogous to the Rhp identified in the kidney of the Japanese dogfish (Nakada et al., 2010) were not sought in the tissues of the ratfish and may well be expressed in these tissues.

In elasmobranch fish ureolytic bacteria have been identified in a number of tissues (Grimes et al., 1985; Knight et al., 1988), however, there are no reports to date suggesting any functional significance regarding a potential influence of these bacteria on nitrogen balance. In the intestine of the little skate, molecular expression of the Rhbg-like protein was orders of magnitude higher than that of the UT (Anderson et al., 2010). Furthermore glutamine synthetase, required for the conversion of ammonia to urea in the ornithine urea cycle of elasmobranch fish (Anderson, 2001) is highly active, and indeed all enzymes of the OUC are present in the intestine (Kajimura et al., 2006) suggesting the assimilation of nitrogen in the form of ammonia rather than urea. However, the present study does not support this hypothesis, as addition of 2 mM NH_4Cl to the mucosal side of the colon, for both species, did not result in a reduced concentration of ammonia throughout the experimental period. This concentration was chosen to be physiologically realistic, based on measured levels of 1–3 mM total ammonia in the chyme of *S. acanthias* (Wood et al., 2009). It remains to be determined if ureolytic bacteria do play a significant role in nitrogen balance in ureosmotic fish.

In summary, a role for the colon in the dogfish in regulating urea balance has been demonstrated, however, such a role for this tissue was not evident in the ratfish. Molecular analysis of the gills, kidney and intestinal epithelia in the ratfish has revealed 3 UT isoforms that are closely related to the UT-1, 2 and 3 isoforms identified in the elephantfish (Kakumura et al., 2009). However, comparison between the elephantfish (Hyodo et al., 2007) and the ratfish suggests that the ratfish may adopt a ureosmotic strategy that is different to the elephantfish. In support of this, recent phylogenetic analysis of

the extant holocephalans places the genus *Hydrolagus* in a monophyletic group to the exclusion of the genus *Callorhinchus* (Inoue et al., 2010). Regardless of the evolutionary position of these two sister species, it is clear that our understanding of the ureosmotic strategy in holocephalans lags far behind that of elasmobranch fish.

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