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Examining urea flux across the intestine of the spiny dogfish, *Squalus acanthias*

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ABSTRACT

Recent examination of urea flux in the intestine of the spiny dogfish shark, *Squalus acanthias*, has shown that feeding significantly enhances urea uptake across the intestine, and this was significantly inhibited following mucosal addition of phloretin. The present study examined potential mechanisms of urea uptake across the dogfish intestine in starved and fed dogfish. Unidirectional flux chambers were used to examine the kinetics of urea uptake, and to determine the influence of sodium, ouabain, competitive urea analogues, and phloretin on urea uptake across the gut of fed dogfish. Intestinal epithelial preparations from starved and fed dogfish were mounted in Ussing chambers to examine the effect of phloretin on bidirectional solute transport across the intestine. In the unidirectional studies, the maximum uptake rate of urea was found to be 35.3 ± 6.9 μmol.cm^{-2}.h^{-1} and K_m was found to be 291.8 ± 9.6 mM in fed fish, and there was a mild inhibition of urea uptake following mucosal addition of competitive agonists. Addition of phloretin, Na-free Ringers and ouabain to the mucosal side of intestinal epithelia also led to a significant reduction in urea uptake in fed fish. In the Ussing chamber studies there was a net influx of urea in fed fish and a small insignificant efflux in starved fish. Addition of phloretin blocked urea uptake in fed fish when added to the mucosal side. Furthermore, phloretin had no effect on ion transport across the intestinal epithelia with the exception of the divalent cations, magnesium and calcium.

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1. Introduction

Homer Smith was the first to examine the physiological consequences of the ureosmotic strategy employed by marine elasmobranch fish (Smith, 1936) where the internal osmolality is maintained similar to or slightly higher than that of the marine environment largely through the retention of high concentrations of urea. Despite the significant molecular and structural modifications to gill epithelia cells in elasmobranch fish, thought to be related to urea retention (Fines et al., 2001), it is recognised following extensive studies on the dogfish shark, *Squalus acanthias*, that not only are these fish ureosmotic but they are also ureotelic (Wood et al., 1995), with most urea being lost across the gill epithelia (Pärt et al., 1998). This incipient urea loss has been demonstrated in long term starvation studies (Cohen et al., 1958; Kajimura et al., 2008) such that it was estimated that *S. acanthias* would need to feed every 5–6 days to maintain nitrogen balance (Kajimura et al., 2006). Thus, despite the high concentration of urea in the plasma of the dogfish shark they are in fact severely nitrogen limited (Wood et al., 2005). These findings are part of a significant research effort into understanding the role of the fish intestine and how feeding results in profound changes in homeostatic regulation of acid–base balance, nitrogen balance, ion regulation and metabolism (Wood and Bucking., 2011). However, despite the obvious potential for dramatic changes in the dogfish shark intestinal tract following a single meal that may be as much as 10% body mass (Wood et al., 2007), examination of intestinal physiology in fed and starved elasmobranch fish is limited to much fewer studies (Anderson et al., 2012; Liew et al., 2013; Wood et al., 2007).

In 2007 Wood et al. reported a rapid increase in the osmolality of chyme entering the intestine from the stomach following a single meal. This was found to be largely the result of increases in urea, Na^+ and Cl^-. In more distal regions of the intestine and/or the colon the same solutes were thought to be reabsorbed. Indeed accumulation of ^{14}C-urea in the mucosa of colonic tissue from starved dogfish provided some support for this hypothesis (Anderson et al., 2012). Most recently, Liew et al. (2013) using intestinal sac preparations reported a net loss of

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urea to the intestinal lumen in starved fish similar to the result previously shown in the little skate, *Leucoraja erinacea* (Anderson et al., 2010). However, this was significantly reversed to a net urea uptake within 24–48 h following a feeding event. Furthermore, the intestine (or spiral valve) was the only region of the GI tract of the four examined (cardiac stomach, pyloric stomach, intestine, and colon) where this occurred, and the effect was significantly blocked through the addition of the general urea transport blocker, phloretin, to the mucosal side of the preparation (Liew et al., 2013). The significant uptake of urea from the isolated intestinal sacs reported by Liew et al. (2013) was in direct contrast to the assumed secretion, at least in the anterior part of the intestine in vivo, reported by Wood et al. (2007). Liew et al. (2013) suggest that the contrasting results may be due to addition of urea by elevated biliary and pancreatic secretions that would resemble a net intestinal secretion of urea post-feeding (Wood et al., 2007).

In the present study two preparations were employed, both using ^{14}C-urea to examine the effect of feeding and various known inhibitors and competitors on urea transport in isolated intestinal epithelia from the dogfish shark. *Series 1* used only tissue from fed fish and was similar to the protocol developed for examination of nutrient uptake across the skin in the Pacific Hagfish, *Eptatretus stoutii* (Glover et al., 2011). Only fed fish were examined in this first series because Liew et al. (2013) had reported that net uptake of urea meeting a key criterion of active transport (transport against the concentration gradient) occurred only under fed conditions, and not under starved conditions. Therefore, kinetic and pharmacological investigation of unidirectional urea transport in the fed preparation would likely yield the most informative results. Urea uptake was determined based on the disappearance of ^{14}C-urea from the mucosal side of the preparation. Various experiments examined the concentration-dependent kinetics of urea uptake, the influence of sodium removal and ouabain on urea uptake, as well as the effects of phloretin as an antagonist and thiourea, methylurea and acetamide as competitive agonists. In *Series 2*, we then followed up using Ussing chambers to examine the differences in bi-directional and net flux rates between fed and starved preparations. Bidirectional flux of ^{14}C-urea was examined in both fed and starved individuals under control conditions and following addition of phloretin to the mucosal and serosal sides of the preparation. These latter experiments allowed for comparison of bidirectional flux, and therefore the two components of net flux, whereas previous work with gut sac preparations measured only net flux (Liew et al., 2013).

2. Materials and methods

Male spiny dogfish, *S. acanthias*, were caught by rod and line or trawl by commercial fishermen in Barkley Sound British Columbia in July of 2012 ($n = 18$; mean body mass 1.42 ± 0.12 kg). Following capture, fish were transferred to a 151,000-L indoor flow-through aquarium at Bamfield Marine Sciences Centre, where water was held at ambient temperature (12 ± 0.1 °C), salinity (30 ± 2 ppt) and photoperiod. Both fed and starved free-swimming fish were used in the present study where feeding and fasting regimens followed those previously described (Liew et al., 2013). Briefly, for the fed fish the diet consisted of approximately 3% ration of frozen hake, *Merluccius productus*, delivered every 4 days. Fed fish were sacrificed between 24 and 48 h post-feeding and food was with-held from starved fish for a minimum of 7 days prior to sacrifice. 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.

Following immersion in a terminal dose of tricaine methanesulfonate (250 ppm MS-222), the intestine was removed, and a longitudinal incision was made to open the spiral valve. The anterior two intestinal folds were dissected out and set aside for mounting in the Ussing chambers and the remaining folds were dissected out and mounted in modified flux chambers as previously described (Glover et al., 2011).

2.1. Series 1: unidirectional flux measurement

As described, intestinal folds from the spiral valve were carefully removed between 24 and 48 h after the feeding event. Both sides of an intestinal fold are covered with mucosal epithelium, so in this series, the fold was kept intact, and the surface exposed to the experimental solution was considered the mucosal surface. Unidirectional uptake was measured by disappearance of ^{14}C-urea from this experimental (mucosal) solution.

Each fold was rinsed with Elasmobranch Ringers (in mM; 257 NaCl, 7 Na_2SO_4, 6 $NaHCO_3$, 0.1 Na_2HPO_4, 4 KCl, 3 $MgSO_4 \cdot 7H_2O$, 2 $CaCl_2 \cdot 2H_2O$, 5 glucose, 100 TMAO and 350 urea, pH 7.8) to remove any chyme or undigested material. The saline composition was identical to that of Pärt et al. (1998), except for the removal of colloids and the elevation of TMAO from 15 mM to 100 mM. A section was then cut and fitted over the opening of a 7-ml scintillation vial. An aperture of known surface area (1.13 cm^2) was present in the lid of the scintillation vial. This lid was then screwed onto the vial to seal the tissue in place. The vial contained 2 ml of the experimental (mucosal) solution (modified Elasmobranch Ringers) which had been pre-equilibrated with a specialty gas mixture of 99.7% O_2:0.3% CO_2 and labelled with 3.7 $kBq \cdot ml^{-1}$ (0.1 $\mu Ci \cdot ml^{-1}$) of ^{14}C-urea (original specific activity 2.04 $MBq \cdot mmol^{-1}$; Perkin Elmer, Waltham, MA, USA). A sample of the experimental solution was taken for measurements of initial radioactivity. The whole preparation was then blotted gently, weighed to 1 mg accuracy, inverted, and placed into a bathing solution (serosal) containing 5 ml of Elasmobranch Ringers which was continuously bubbled with the same gas mixture. Isosmotic conditions on the two sides of the fold were maintained in all treatments, as explained below. The preparation was then allowed to incubate in a water jacketed chamber at 12 °C for the following 3 h. After the 3-h incubation period, the preparation was removed, blotted, and reweighed, and a mucosal sample was taken for measurement of final radioactivity, so as to allow calculation of uptake rates. Data from preparations showing weight changes of more than 50 mg (indicative of potential leakage) were discarded.

The concentration-dependent kinetics of urea uptake were determined based on the disappearance of ^{14}C-urea from the mucosal side. Mucosal urea concentration was adjusted to between 70 and 700 mM and the serosal urea concentration was maintained at 350 mM. The osmotic pressure of both the mucosal and serosal solutions in this series was balanced with the addition of mannitol to the Ringers solutions, as verified with a Vapro 5520 vapour pressure osmometer (Wescor, Logan, UT, USA). The effect of sodium on urea uptake was examined through; a) the removal of NaCl from the mucosal Ringers with NaCl being replaced by equimolar amounts of N-methyl-D-glucamine (NMDG) and; b) addition of the Na,K,ATPase inhibitor, ouabain (1 mM), to the mucosal Ringers solution. The effects of urea agonists on urea uptake were determined following the addition to the mucosal Ringers solution of 350 mM of one of thiourea, acetamide or N-methylurea (i.e. equimolar to urea). Again the serosal Ringers solution had 350 mM urea and any difference in osmotic pressure between the serosal and mucosal Ringers solutions was resolved with the addition of mannitol. Finally the potential involvement of specific urea transporters in urea uptake across the intestinal epithelia in fed dogfish was examined following the addition of phloretin (0.25 mM in 0.1% dimethyl sulfoxide, DMSO), or 0.1% DMSO alone, to the mucosal Ringers solution. In all experiments, a minimum of 3 preparations (i.e. 3 folds of intestinal tissue) were examined from one animal for each treatment, including the control, and the results of the replicates were averaged as $N = 1$. Actual N numbers (number of animals) are reported in the figure legends.

2.2. Series 2: bidirectional flux measurement — Ussing chambers

Intestinal tissue was removed as described in both fed and starved dogfish. The anterior one to two folds were carefully rinsed in dogfish

Ringers to remove any chyme or particulates. One side was then carefully scraped to remove any intestinal epithelia while leaving the opposing side intact. The intact side was henceforth labelled as the mucosal side and the scraped side was labelled as the serosal side of the preparation. A small section was cut and mounted onto tissue holders (Physiologic Instruments, San Diego, CA, USA) with a 0.2 cm^2 aperture. The tissue holders were then mounted into the Ussing chamber and 4 ml of Elasmobranch Ringers (in mM; 250 NaCl, 7 Na_2SO_4, 5 $NaHCO_3$, 0.1 Na_2HPO_4, 4 KCl, 3 $MgSO_4 \cdot 7H_2O$, 2 $CaCl_2 \cdot 2H_2O$, 5 glucose, 100 TMAO and 450 urea, pH 7.8) was added to each side of the chamber. Each chamber was supplied with a specialty gas mix of 99.7% O_2:0.3% CO_2 and cooled to 12 ± 1 °C by a re-circulating chiller (Haake, Fisher Scientific, Mississauga, ON, Canada). It is worth noting that the recipe for Ringers between this and the unidirectional series of experiments was similar with the exception of a large (100 mM) increase in urea concentration. The large increase in urea was implemented in light of the V_{max} for urea as determined in *Series 1*, thus maximising urea transport across the tissue in the Ussing chamber experiments.

^{14}C-urea (original specific activity 2.04 $MBq \cdot mmol^{-1}$) was then added to the mucosal or serosal side of a preparation to a final concentration of 0.925 $KBq \cdot ml^{-1}$ (0.025 $\mu Ci \cdot ml^{-1}$) and the first 20 μl sample representing time zero was taken from both sides of each preparation. Subsequent 20 μl samples were then taken at 1, 2 and 3 h from each side of the preparation for ^{14}C-urea analysis. The effect of phloretin was also examined to determine if there was any contribution of specific urea transporters to urea flux in both fed and starved fish. Phloretin (0.25 mM in 0.1% DMSO) was added approximately 5 min prior to the addition of the ^{14}C-urea to either the mucosal or serosal side of the preparation. 0.1% DMSO alone was also added to determine if this chemical influenced urea transport independent of phloretin. A 1-ml sample of Elasmobranch Ringers solution was taken at the start of each experiment and a 1-ml sample of both the mucosal and serosal fluids was taken at the end of each incubation. These samples were used to determine ion concentrations over the course of the 3-h incubation period.

2.3. Fluid analysis

1-ml samples from *Series 1* experiments and 20 μl samples (plus 1 ml of water) from *Series 2* experiments respectively were added to 4 ml of scintillation cocktail (Ultima Gold, Perkin Elmer) in a 7-ml scintillation vial, and the radioactivity was counted using a liquid scintillation counter (Beckman LS6000, Beckman Coulter, Brea, CA, USA) or due to unforeseen machine failure a Triathler portable counter (Hidex, Helsinki, Finland) or a Tricarb Wizard scintillation counter (Perkin Elmer). Tests showed that quench was constant. Ion concentrations in *Series 2* were assessed 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 as previously described (Anderson et al., 2012).

2.4. Calculations

Urea uptake flux in the unidirectional studies (*Series 1*) was assessed based on the disappearance of ^{14}C-urea from the mucosal Ringers solution and was calculated using the following equation:

$$\text{Uptake} = V \times (R_1 - R_2)/(\text{specific activity} \times SA \times T)$$

where V is 2 ml (mucosal solution volume), R_1 and R_2 are the measured radioactivities of the samples on the labelled mucosal side taken at the beginning and end of the incubation respectively ($cpm \cdot ml^{-1}$), specific activity is the initial actual specific activity ($cpm \cdot \mu mol^{-1}$) of the labelled experimental Ringers used on the mucosal side in the experiment ($cpm \cdot \mu mol^{-1}$), SA is the surface area of the aperture (1.13 cm^2), and T is 3 h.

Urea flux in the Ussing chamber experiments (*Series 2*) was calculated using the following equation (adapted from Clarke, 2009):

$$J_{ms} (\text{or } J_{sm}) = V \times (S_2 - S_1)/(\text{specific activity} \times SA \times T)$$

where J_{ms} is the mucosal to serosal flux and J_{sm} is the reciprocal; V is the volume of Ringers (ml); S_1 and S_2 are the radioactivities of the samples on the non-labelled side taken at the beginning and end of the incubation respectively ($cpm \cdot ml^{-1}$); specific activity is the actual specific activity ($cpm \cdot \mu mol^{-1}$) on the labelled side used in the experiment ($cpm \cdot \mu mol^{-1}$); SA is the surface area of the aperture (0.2 cm^2) in the tissue holders, and T is the time of the flux period (h).

Ion flux was calculated based on the change in ion concentration over the 3-h incubation period in both the serosal and mucosal bath using the following equation:

$$J_{ms} (\text{or } J_{sm}) = (V_1 \times C_1) - (V_2 \times C_2)/T \times SA$$

where V_1 and C_1 are the volume (ml) and concentration ($mmol \cdot ml^{-1}$) of the flux chamber and solute respectively at the start and at the end (V_2 and C_2) of the incubation period (removal of 20 μl for ^{14}C-urea flux determinations throughout the experiment was accounted for in V_2) and T is the time (h) and SA is the surface area (cm^2). Net flux was calculated as:

$$\text{Net flux} = J_{ms} - J_{sm}.$$

Consequently a negative value depicted a net efflux and a positive value depicted a net influx or uptake of the labelled urea or ion from the mucosal to the serosal solution.

All data have been expressed as means ± 1 SEM (N), and significance was taken at $P < 0.05$. In *Series 1*, for all drug tests there was a paired design, with a control treatment for the same animal, so either a paired Student's t-test (for single treatments), or a repeated measures analysis of variance (ANOVA) followed by a Bonferroni's test (for multiple treatments) was used. In *Series 2*, statistically significant differences were determined using one way analysis of variance (ANOVA) followed by a Bonferroni's post hoc test for multiple comparisons, or an unpaired Student's t-test for single comparisons.

3. Results

3.1. Series 1 — unidirectional flux measurements

^{14}C-urea uptake in the isolated intestinal tissue from fed fish demonstrated a concentration dependent rate that was best fitted to a sigmoidal relationship with a maximum uptake rate or J_{max} of 35.3 ± 6.9 $\mu mol \cdot cm_2^{-1} \cdot h^{-1}$ and an affinity constant (K_m) of 291.8 ± 9.6 mM (Fig. 1). Addition of phloretin (0.25 mM in 0.1% DMSO) to the mucosal side of the preparation significantly inhibited unidirectional urea uptake compared to control rates, and addition of 0.1% DMSO alone as the vehicle for the dissolution of phloretin had no significant effect (Fig. 2). Relative to the 0.1% DMSO rate, the inhibition by phloretin was approximately 40%. Further, both sodium-free Ringers and addition of ouabain (1 mM) to the mucosal side of the preparation significantly inhibited unidirectional urea uptake (Fig. 3). Finally, addition of either acetamide, thiourea or N-methylurea at 350 mM (equimolar to urea) to the mucosal side of the preparation caused small but significant reductions in unidirectional urea uptake rate compared to the control treatment (Fig. 4).

3.2. Series 2 — Ussing chamber experiments

There was a net influx of ^{14}C-urea across the intestine in fed fish intestinal preparations and if anything a small net efflux of urea in starved fish (Fig. 5), which is in agreement with previous reports on urea transport in intestinal sacs of spiny dogfish intestine (Liew et al.,

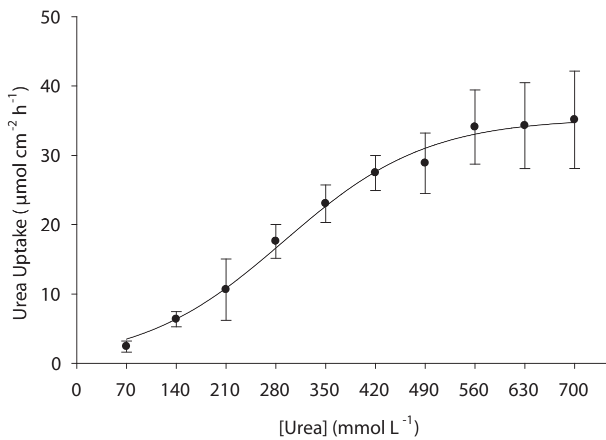

Fig. 1. Concentration-dependent kinetics of unidirectional uptake rates of ^{14}C-urea in isolated spiny dogfish intestinal preparations from fed fish (*Series 1*). The uptake kinetics were best fitted by a sigmoidal relationship. Osmolality was compensated by addition of mannitol to the mucosal solution (for mucosal urea concentrations less than 350 mM), and to the serosal solution (for mucosal urea concentrations greater than 350 mM). Maximal transport rate (J_{max}) was calculated as 35.3 ± 6.9 μmol·h^{-1}·cm^2 and the affinity constant (K_m) was calculated as 291.8 ± 9.6 mM urea. Means ± 1 SEM. N = 3 at each point with the exception of 350 mM urea where N = 6.

2013) and experiments conducted in *Series 1* of this study. Addition of phloretin to the mucosal side of intestinal preparations taken from fed fish reduced J_{ms} of ^{14}C-urea within the first hour and significantly so by the end of the flux period (Fig. 6). When phloretin was added to the serosal side of intestinal preparations from fed fish mounted in the Ussing chamber, J_{sm} of ^{14}C-urea had a tendency to increase at all three time-points during the flux period although the increase was not significant compared to controls. In starved fish the addition of phloretin to the mucosal side of the preparation had no effect on J_{ms} of ^{14}C-urea and similarly when phloretin was added to the serosal side of the preparation in starved fish, J_{sm} of ^{14}C-urea was no different from controls (Fig. 6).

Net flux rates of sodium, potassium, chloride and sulphate were highly variable in control preparations and following phloretin treatment. While there were no statistical differences between preparations from fed and starved fish or following the addition of phloretin, the pattern of flux rates appeared quite different between fed and starved fish, particularly following the addition of phloretin (Table 1). However, addition of phloretin significantly influenced the net flux of the divalent cations, magnesium and calcium. In fed and starved fish, addition of phloretin to the serosal side of the preparation resulted in a significantly greater influx of both divalent cations compared to preparations in fed

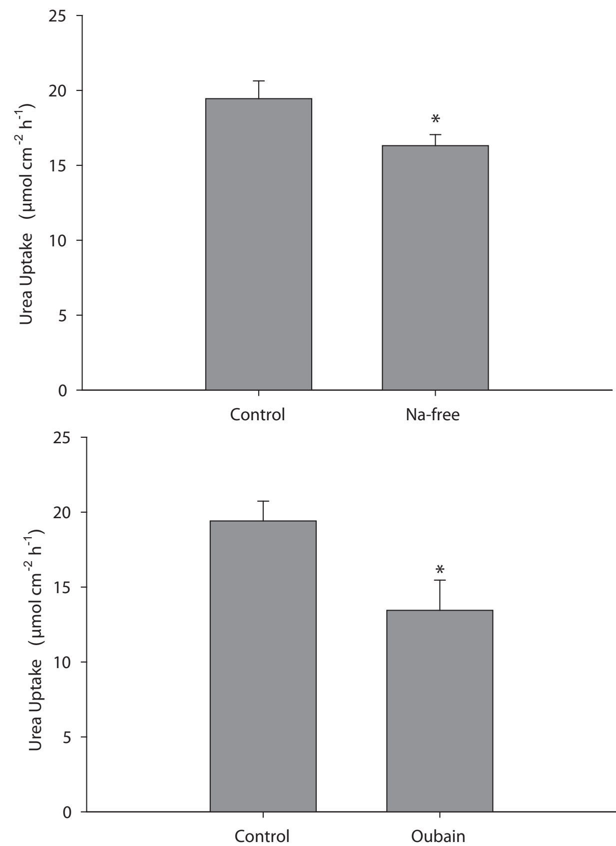

Fig. 3. Unidirectional uptake rate of ^{14}C-urea in control, ouabain (1 mM) and sodium-free treated isolated spiny dogfish intestinal preparations from fed fish (*Series 1*). Ouabain and sodium-free Ringers were added to the mucosal side of the preparation, and osmolality in sodium free Ringers was balanced with the addition of N-methyl-D-glucamine (NMDG). Means ± 1 SEM (N = 6). * represents significant differences (P < 0.05) between control and treated preparations, using paired Student's t-tests.

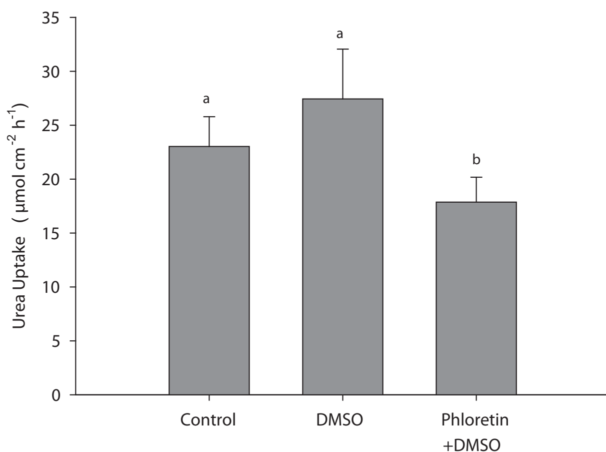

Fig. 2. Unidirectional uptake rate of ^{14}C-urea in control, phloretin treated (0.25 mM in 0.1% DMSO) and DMSO (0.1%) treated isolated spiny dogfish intestinal preparations from fed fish (*Series 1*). Phloretin and DMSO were added to the mucosal side of the preparation. Means ± 1 SEM (N = 6). Different letters represent significant differences (P < 0.05) using repeated measures ANOVA followed by Bonferroni's test.

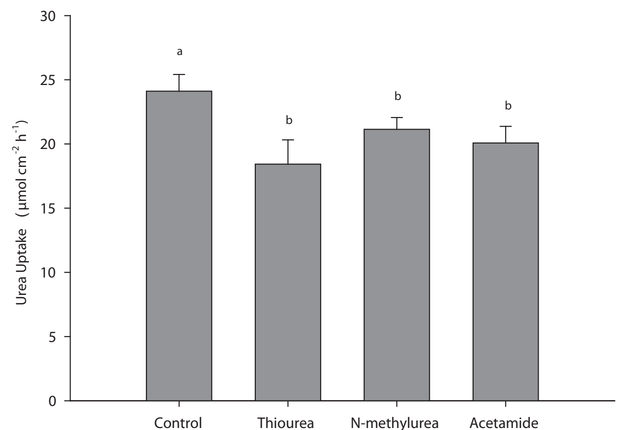

Fig. 4. Unidirectional uptake rate of ^{14}C-urea in control, thiourea, N-methylurea, and acetamide treated isolated spiny dogfish intestinal preparations from fed fish (*Series 1*). Thiourea, N-methylurea and acetamide were added to the mucosal side of the preparation at equimolar concentrations to urea (350 mM). The increase in mucosal Ringers osmolality was compensated by the addition of mannitol to the serosal side. Means ± 1 SEM (N = 6). Different letters represent significant differences between treatments (P < 0.05 using repeated measures ANOVA followed by Bonferroni's test).

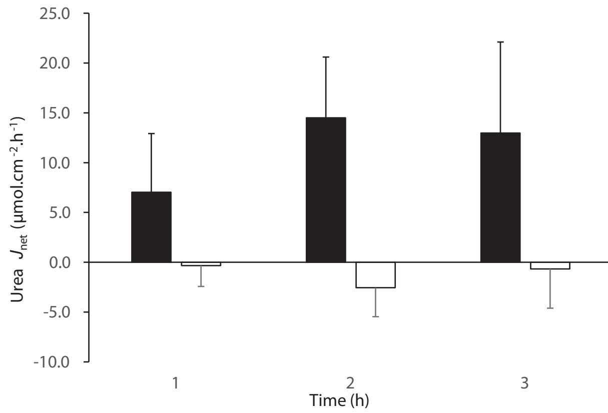

Fig. 5. Net flux rates of ^{14}C-urea in isolated spiny dogfish intestinal preparations from fed (filled bars) and starved (open bars) dogfish (*Series 2*). Positive values represent a net influx of ^{14}C-urea and negative values represent a net efflux of ^{14}C-urea. Means ± 1 SEM (N ≥ 5). * symbols represent significant differences between fed and starved fish at the same time-point (P < 0.05 using Student's t-test).

and starved fish where the addition of phloretin to the mucosal side resulted in a net efflux of Ca^{2+} and Mg^{2+} (Fig. 7).

4. Discussion

In the present study we have firmly established the intestinal epithelia as an ideal tissue for the study of urea transport mechanisms in elasmobranch fish. While the gills represent the biggest site of urea loss (Wood et al., 1995; Pärt et al., 1998) and therefore an obvious site of urea transport (Fines et al., 2001; Pärt et al., 1998; Wood et al., 2013), they are a heterogeneous mix of cells that are technically challenging to establish in cell culture. The kidneys are considered the major site of urea regulation in elasmobranchs (Goldstein and Forster, 1971), however, the complex architecture of the nephron (Hentschel, 1987; Lacy and Reale, 1995) has led to uncertainties regarding the mechanism of urea reabsorption (Boylan, 1972; Hyodo et al., 2004; Kempton, 1953; Morgan et al., 2003b; Schmidt-Nielsen et al., 1972) that can only be inferred from molecular mapping and whole kidney perfusion studies given that microdissection would disrupt the functional integrity of the renal tubular epithelia. Only recently has attention turned to intestinal epithelia as a viable alternative for the study of urea transport in elasmobranch fish (Anderson et al., 2010, 2012; Liew et al.,

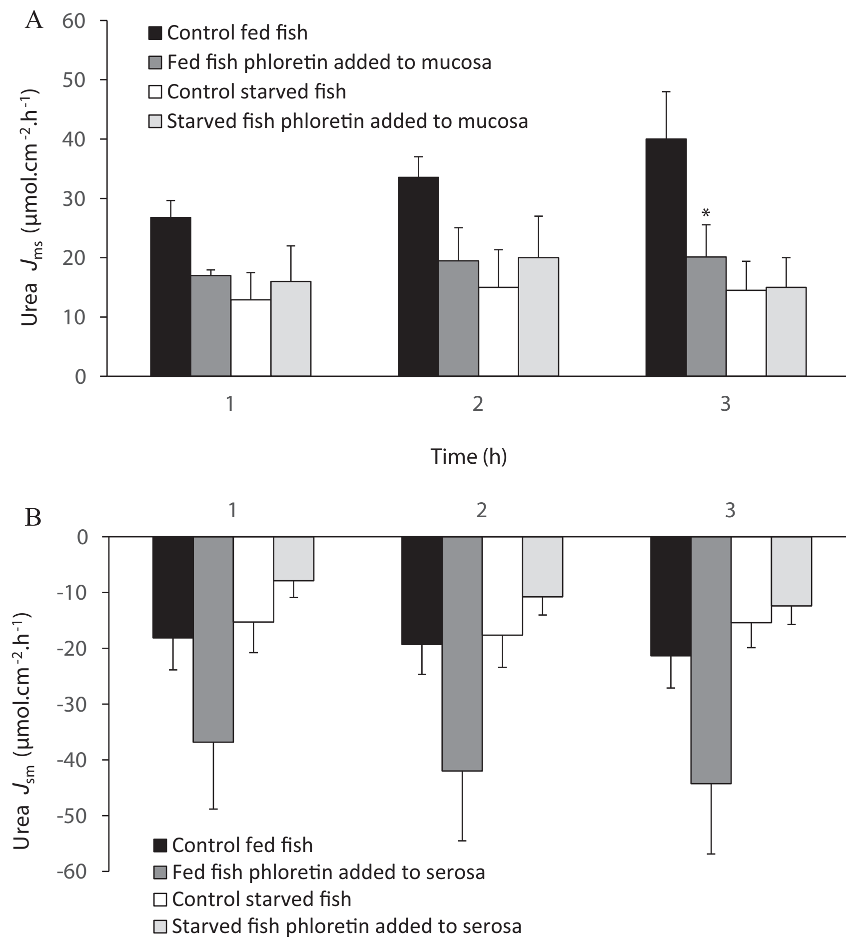

Fig. 6. Unidirectional flux rates of ^{14}C-urea in isolated spiny dogfish intestinal preparations from starved and fed dogfish treated with phloretin on either: the mucosal (panel A) or the serosal (panel B) side of the preparation (*Series 2*). Means ± 1 SEM (N ≥ 5). Top panel: J_{ms} (mucosal to serosal flux) in control starved (open bars) and fed (black bars) dogfish. Phloretin (0.25 mM in 0.1% DMSO) addition to the mucosal side of the preparation in starved (light grey bars) and fed (dark grey bars) dogfish. Bottom panel: J_{sm} (serosal to mucosal flux) in control starved (open bars) and fed (black bars) dogfish. Phloretin (0.25 mM in 0.1% DMSO) addition to the serosal side of the preparation in starved (light grey bars) and fed (dark grey bars) fish. Tests examining the influence of 0.1% DMSO alone were conducted and found no effects (data not shown). Asterisk represents significant difference between control and phloretin treated (mucosa) preparations in fed fish (P < 0.05 using ANOVA followed by Bonferroni's test).

Table 1
Net ion flux rates in isolated spiny dogfish intestinal preparations from fed and starved dogfish mounted in Ussing chambers (*Series 2*). Means ± 1 SEM (N ≥ 5). Control and phloretin treated preparations (0.25 mM in 0.1% DMSO) are shown. Phloretin was added to either the mucosal or serosal side in each preparation. Tests examining the influence of 0.1% DMSO alone were conducted and found no effects (data not shown). Negative values denote a net efflux and positive values denote a net influx. No significant differences within or between treatments were detected.

	Starved fish			Fed fish		
	Control	Mucosal treated phloretin	Serosal treated phloretin	Control	Mucosal treated phloretin	Serosal treated phloretin
Na^+	0.17 ± 0.68	−2.29 ± 1.31	−1.03 ± 0.83	1.38 ± 0.62	0.70 ± 0.65	0.66 ± 1.67
K^+	0.00 ± 0.01	−0.02 ± 0.03	−0.01 ± 0.02	0.02 ± 0.01	0.01 ± 0.01	0.04 ± 0.01
Cl^-	0.35 ± 0.46	−0.39 ± 0.71	−0.49 ± 1.49	−0.06 ± 0.60	0.44 ± 0.76	−1.08 ± 1.10
SO_4^{2-}	0.04 ± −0.04	−0.11 ± 0.05	−0.12 ± 0.09	0.03 ± 0.04	0.05 ± 0.03	−0.06 ± 0.06

2013; Wood et al., 2007). Data using gut sacs from fed and starved spiny dogfish (Liew et al., 2013) and gut sac preparations from starved little skate, *L. erinacea* (Anderson et al., 2010), support the unidirectional and bidirectional results in the present study, indicating that the intestinal epithelium represents a robust model for the study of urea transport in elasmobranchs. Its simple two-dimensional anatomy, ideal for sac and Ussing chamber techniques, is a particular advantage.

Liew et al. (2013) reported a net urea uptake rate of 8 $\mu mol \cdot h^{-1} \cdot cm^{-2}$ across intestinal gut sac preparations in fed dogfish when a concentration of 350 mM urea was used in the mucosal and serosal Ringers. This is approximately 4.4 fold less than the maximal uptake rate of 35.3 ± 6.9 $\mu mol \cdot h^{-1} \cdot cm^{-2}$ reported here using the unidirectional flux chambers (*Series 1*). It is important to note that these experiments provided only unidirectional flux rates, not net flux rates. Furthermore, Liew et al. (2013) measured whole urea net fluxes and therefore were not able to discriminate uptake from the mucosal Ringers from potential endogenous production of urea within the intestinal tissue (Kajimura et al., 2006). Based on the kinetic curve in the unidirectional experiments and previously reported urea values of 453.2 ± 10.6 mM in the intestinal chyme of starved spiny dogfish (Anderson et al., 2012) we used 450 mM urea in the Ussing chamber experiments (*Series 2*) to maximise the potential for net urea transport and achieved a net urea uptake rate of 14.5 ± 6.1 $\mu mol \cdot h^{-1} \cdot cm^{-2}$ in fed fish. As labelled urea was used in this study, the value would be reflective of uptake from the mucosal Ringers minus loss from serosal Ringers, without confounding effects of endogenous urea production by the intestinal tissue. This therefore demonstrates a tremendous capacity for urea uptake across the intestinal epithelia of fed spiny dogfish. Given an intestinal surface area of approximately 33 $cm^2 \cdot kg^{-1}$ in fed dogfish (Liew et al., 2013), which is undoubtedly an underestimate given the complexity of the spiral valve folds, this organ could return about 480 $\mu mol \cdot kg^{-1} \cdot h^{-1}$ of urea from chyme to blood, a net uptake rate somewhat higher than net urea loss rates at the gills (e.g. Wood et al., 1995, 2005; Kajimura et al., 2006). If this scavenging mechanism was not present, this amount of urea would presumably be excreted through the rectum.

Net uptake of urea in the absence of or against the concentration gradient (Liew et al., 2013, *Series 2* data), in combination with the concentration-dependent kinetics for unidirectional ^{14}C-urea uptake (*Series 1*), as well as inhibition by ouabain blockade of Na,K,ATPase, indicates that the uptake of urea is both active and saturable. Two principal transport mechanisms have been implicated in epithelial urea transport in elasmobranch fish. Reviewed by McDonald et al. (2006), facilitated transport is achieved largely through the presence of specific urea transport (UT) proteins which were first identified by molecular means in renal tissue of the spiny dogfish (Smith and Wright, 1999) and have since been identified in five additional species, the little

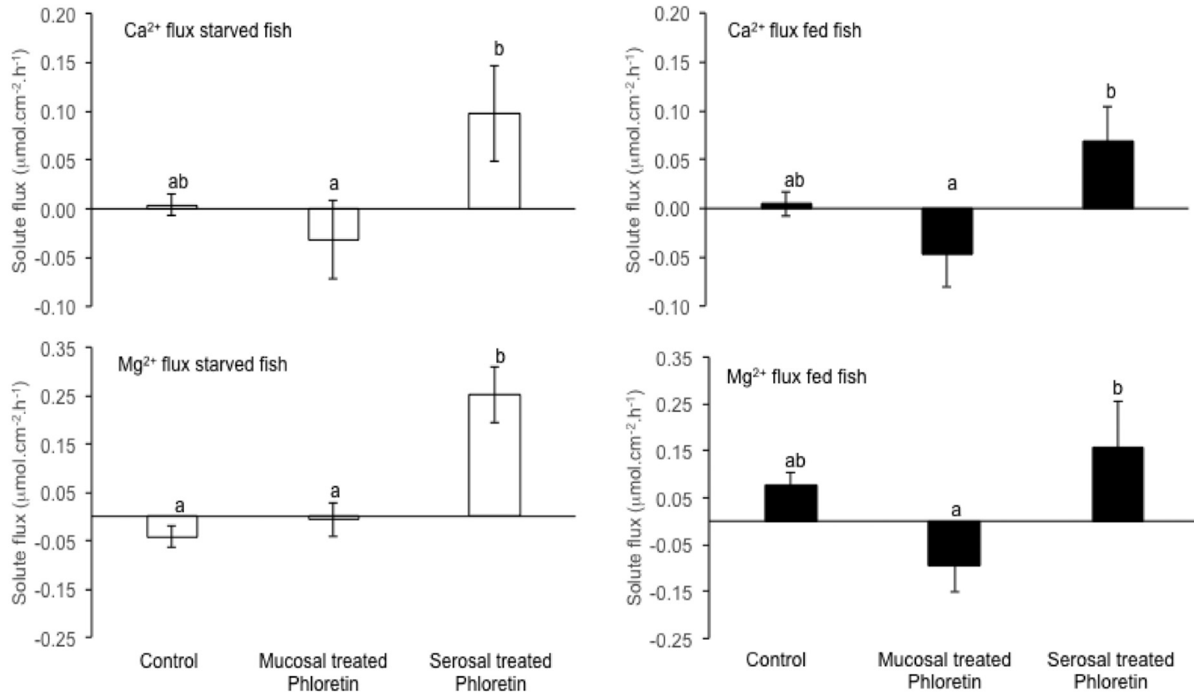

Fig. 7. Net calcium and magnesium flux rates in isolated spiny dogfish intestinal preparations from fed (filled bars) and starved (open bars) dogfish mounted in Ussing chambers (*Series 2*). Control and phloretin treated preparations (0.25 mM in 0.1% DMSO) are shown. Phloretin was added to either the mucosal or serosal side in each preparation. Tests examining the influence of 0.1% DMSO alone were conducted and found no effects (data not shown). Negative values denote a net efflux and positive values denote a net influx. Different letters represent significant differences ($P < 0.05$ using ANOVA followed by Bonferroni's test).

skate, *L. erinacea* (Morgan et al., 2003a), the Japanese dogfish, *Triakis scyllia* (Hyodo et al., 2004), the winter skate, *Leucoraja ocellata*, bluntnose stingray, *Dasyatis say* (Janech et al., 2008) and Atlantic stingray, *Dasyatis sabina* (Janech et al., 2003). Of note UT expression has been reported in the intestinal tissue of the spiny dogfish (Smith and Wright, 1999) likely as a different isoform to that expressed in the brain or kidney, and also in the little skate (Anderson et al., 2010), albeit at somewhat lower levels compared to renal tissue. A second transport mechanism, sodium-linked transport has also been implicated in the transport of urea in the renal (Schmidt-Nielsen et al., 1972; Morgan et al., 2003b) and gill epithelial cells (Fines et al., 2001) of elasmobranchs (reviewed by McDonald et al., 2006), however, to date there are no reports of the molecular identification of this transporter in any elasmobranch fish.

In the unidirectional experiments of *Series 1*, addition of the competitive agonists to facilitated urea transport, N-methylurea, thiourea and acetamide (all urea analogues) to the mucosal side of the preparation all significantly inhibited unidirectional ^{14}C-urea, albeit to a small extent (<25%), and somewhat less than the 40% blockade caused by phloretin. Likewise addition of sodium-free Ringers (15% inhibition) and ouabain (30% inhibition by this specific inhibitor of Na,K,ATPase) to the mucosal side of the preparation both significantly reduced urea uptake in fed fish (*Series 1*). Combined, these data suggest a role for both facilitated transport and sodium-linked transport. However, based on the kinetics of ^{14}C-urea uptake, the level of inhibition by all these pharmacological agents was somewhat lower than anticipated, suggesting additional routes for urea uptake across intestinal epithelia. The current working hypothesis for urea transport across red blood cell (RBC) membranes in elasmobranchs excludes facilitated transport and may simply be the result of passive diffusion (Carlson and Goldstein, 1997) as there was no measurable K_m or V_{max} across the RBCs from the lesser spotted dogfish *Scyliorhinus canicula* and urea uptake was not impeded following the addition of the facilitated urea transport blocker phloretin (Walsh et al., 1994). Given the saturable kinetics displayed in *Series 1* it is hard to justify urea uptake in the dogfish intestine as the result of diffusion alone. It would be interesting to co-administer phloretin and ouabain in Na^+-free Ringers and determine the combined inhibitory effect. If results were additive and urea uptake was eliminated this would provide substantial support for multiple pathways for urea uptake across the epithelia.

Addition of phloretin to the mucosal side of intestinal epithelia in the present study significantly inhibited the uptake of ^{14}C-urea (*Series 1 and 2*) in fed fish but had no effect on starved fish (*Series 2*). Furthermore, addition of phloretin to the serosal side of the intestinal epithelia resulted in a tendency for increased efflux of ^{14}C-urea in fed fish but again had no effect on starved fish (*Series 2*). These results are supported by previously published data examining net flux of whole urea using intestinal gut sac preparations (Liew et al., 2013) and underscore two important components of urea transport in the dogfish intestine: 1) feeding dramatically alters urea transport in the intestine and 2) alternative transport routes for this important osmolyte must be present in elasmobranch intestinal epithelial cells.

In reference to the first point, physiological and anatomical changes in the gastro-intestinal tract following a feeding event are recognised in all animals (Karasov and Diamond, 1987) including the fishes (for review see: Holmgren and Nilsson, 1999; Wilson and Castro, 2011; Wood and Bucking, 2011). These changes are most pronounced in animals that feed intermittently and are particularly dramatic when the temporal separation of feeding events is prolonged with many snake species being prime examples (Secor and Diamond, 1995). The intermittent and gorge feeding behaviour of many elasmobranch species (Wetherbee and Cortes, 2004) certainly suggests profound morphological and physiological changes in the intestine following feeding. Wood et al. (2007) reported increases in the relative masses of tissues of the gastrointestinal tract of *S. acanthias* following feeding, effects that appeared to be driven mainly by fluid engorgement, and the differential effect of phloretin between fed and starved animals in this study suggests the potential for molecular reshuffling in the elasmobranch intestine that is dependent on feeding. However, much remains to be determined, in particular potential morphological and molecular changes that may be involved in aiding assimilation of nutrients meanwhile maintaining ion, nitrogen and acid–base balance in the whole animal. For example, in future studies it would be of interest to compare the concentration-dependent kinetics of unidirectional urea fluxes in fed versus fasted animals.

Previously Liew et al. (2013) reported a net influx of sodium and chloride and a net efflux of potassium in gut sac preparations from starved spiny dogfish and this result did not change following feeding or following the addition of phloretin to the mucosal side of gut sacs taken from fed fish. In the present study there were no significant differences in net flux of sodium, chloride, potassium or sulphate between fed and starved fish (Table 1) nor did the addition of phloretin to the mucosal or serosal side of the preparation influence flux rates of these ions (Table 1). However, addition of phloretin to the mucosal side of the preparation resulted in a net efflux of calcium and magnesium whereas addition of phloretin to the serosal side of the preparation resulted in a net influx of calcium and magnesium regardless of whether the fish had recently fed or not. Furthermore, mucosal addition of phloretin to gut sac preparations caused a net efflux of Ca^{2+} and Mg^{2+} (Liew et al., 2013). Interestingly, in a number of mammalian cell types glucose transport is linked with Mg^{2+} transport (Torres et al., 2005). Furthermore, the sodium dependent glucose transporter is also known to transport urea (Leung et al., 2000). However, while glucose transporters have been identified in the kidney (Althoff et al., 2006; Kipp et al., 1997) and brain (Balmaceda-Aguilera et al., 2012) in elasmobranch fish they have yet to be identified in the intestine. Clearly further study is required to determine the relationship between divalent cation movement and urea transport across the intestinal epithelia.

In summary, results from the present study are supported by previous data using intestinal gut sac preparations (Liew et al., 2013) and provide a strong case for the use of intestinal epithelia for urea transport studies in elasmobranch fish. Further, while there is clearly a significant amount of research to be conducted, the data suggest that urea transport across the epithelia is not limited to specific UT transporters but more likely may make use of a number of alternative pathways such that the potential for urea uptake across the gut is maximised; a hypothesis that fits well with the nitrogen limited elasmobranch fish.

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