RESEARCH ARTICLE

An *in vitro* study of urea, water, ion and CO_2/HCO_3^- transport in the gastrointestinal tract of the dogfish shark (*Squalus acanthias*): the influence of feeding

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SUMMARY

In vitro gut sac preparations made from the cardiac stomach (stomach 1), pyloric stomach (stomach 2), intestine (spiral valve) and colon were used to examine the impact of feeding on transport processes in the gastrointestinal tract of the dogfish shark. Preparations were made from animals that were euthanized after 1–2 weeks of fasting, or at 24–48 h after voluntary feeding on a 3% ration of teleost fish (hake). Sacs were incubated under initially symmetrical conditions with dogfish saline on both surfaces. In comparison to an earlier *in vivo* study, the results confirmed that feeding caused increases in H^+ secretion in both stomach sections, but an increase in Cl^- secretion only in stomach 2. Na^+ absorption, rather than Na^+ secretion, occurred in both stomach sections after feeding. All sections of the tract absorbed water and the intestine strongly absorbed Na^+ and Cl^-, regardless of feeding condition. The results also confirmed that feeding increased water absorption in the intestine (but not in the colon), and had little influence on the handling of Ca^{2+} and Mg^{2+}, which exhibited negligible absorption across the tract. However, K^+ was secreted in the intestine in both fasted and fed preparations. Increased intestinal water absorption occurred despite net osmolyte secretion into the mucosal saline. The largest changes occurred in urea and CO_2/HCO_3^- fluxes. In fasted preparations, urea was absorbed at a low rate in all sections except the intestine, where it was secreted. Instead of an increase in intestinal urea secretion predicted from *in vivo* data, feeding caused a marked switch to net urea absorption. This intestinal urea transport occurred at a rate comparable to urea reabsorption rates reported at gills and kidney, and was apparently active, establishing a large serosal-to-mucosal concentration gradient. Feeding also greatly increased intestinal CO_2/HCO_3^- secretion; if interpreted as HCO_3^- transport, the rates were in the upper range of those reported in marine teleosts. Phloretin (0.25 mmol l^{-1}, applied mucosally) completely blocked the increases in intestinal urea absorption and CO_2/HCO_3^- secretion caused by feeding, but had no effect on Na^+, Cl^- or water absorption.

Key words: cardiac stomach, pyloric stomach, intestine, spiral valve, colon, gut sac.

INTRODUCTION

Prior to the last decade, there was relatively little research on the physiological events associated with feeding and fasting in elasmobranchs (reviewed by Holmgren and Nilsson, 1999). However, since 2005, we have initiated a research program in this area (reviewed by Wood et al., 2010) on the spiny dogfish shark (*Squalus acanthias*), chosen because it is an opportunistic predator that is relatively abundant, and because its feeding ecology has been well studied (Bonham, 1954; Holden, 1966; Jones and Geen, 1977; Tanasichuk et al., 1991; Hanchet, 1991; Laptikhovsky et al., 2001; Alonso et al., 2002). In nature, the dogfish consumes large meals at irregular intervals; many dogfish are caught with nothing in their stomach, but a single meal may be very large (1–10% of body mass). Digestive processing is slow, taking more than 5 days at 11°C (Wood et al., 2007b). During this period there are profound changes in acid-base balance (Wood et al., 2005; Wood et al., 2007a; Wood et al., 2009), metabolism (Walsh et al., 2006; Kajimura et al., 2006; Wood et al., 2010) and ionoregulation (Kajimura et al., 2006; Matey et al., 2009). However, studies on the gastrointestinal tract itself in relation to feeding are limited to one descriptive investigation (Wood

et al., 2007b) of changes in chyme volume and composition in various segments over this 5+day digestive period.

In that study (Wood et al., 2007b), the animals were fed with dead hake (*Merluccius productus*), a teleost fish that is one of their most common natural prey items (Jones and Geen, 1977), with an internal osmolality only ~40% that of *S. acanthias*. It was concluded that the seawater drinking rate, which is very low, probably remained unchanged after feeding, but that nevertheless, the osmolality of the chyme was quickly raised to that of the blood plasma of the shark. This appeared to occur as a consequence of large additions of urea, Na^+, Cl^- and unmeasured osmolytes to the chyme, though removal of water by osmosis may have also played a role. HCl in particular appeared to be secreted into the chyme by the two sections of the stomach, creating the systemic 'alkaline tide' (Wood et al., 2005). Na^+ and Cl^- ions appeared to be secreted mainly in the two sections of the stomach, and urea mainly in the intestine. However, it was unclear whether the urea secretion originated from the bile, the pancreas or intestinal wall secretions. Later in the digestive process, Na^+, Cl^-, K^+ and urea appeared to be reabsorbed by the intestine and perhaps also the colon, together with a

considerable amount of water. At the same time, the potentially toxic divalent cations Ca^{2+} and Mg^{2+} seemed to be largely excluded. There also appeared to be a massive addition of basic equivalents (presumably as HCO_3^-) to neutralize the very acidic chyme as it entered the intestine. Again, however, the source of this base addition to the intestinal chyme was uncertain. Furthermore, in the absence of fluid phase and solid phase markers (cf. Wood and Bucking, 2011), all these conclusions must be considered circumstantial (Wood et al., 2007b).

Isolated gut sac preparations have been used extensively to study ion and toxicant transport processes in the various sections of the gastrointestinal tract in teleost fish, but only a few studies have extended this approach to look at changes in gut physiology associated with fasting and feeding (reviewed by Wood and Bucking, 2011). Recently, Anderson et al. (Anderson et al., 2007; Anderson et al., 2010) have adopted this approach for studies on ion transport in two elasmobranchs, but the preparations were made only from fasted animals, and only two sections (intestine and colon) were investigated. In the present study, we have applied the *in vitro* gut sac approach to all four sections of the gastrointestinal tract (cardiac stomach, pyloric stomach, intestine and colon) in *S. acanthias*, comparing preparations from fasted animals with those taken at 24–48 h post-feeding.

Based on the earlier descriptive *in vivo* study (Wood et al., 2007b), we hypothesized that feeding would: (1) increase H^+, Cl^- and Na^+ secretion in the stomach sections; (2) increase urea secretion in the intestine; (3) increase HCO_3^- secretion in the intestine; (4) increase Na^+, Cl^-, K^+ and water absorption in the intestine and colon; and (5) have little or no effect on the handling of Ca^{2+} and Mg^{2+}, which would not be absorbed across the tract. Some of these hypotheses were confirmed, but there were several surprising contradictions, particularly with respect to urea transport in the intestine after feeding. An apparent active absorption of urea in the intestine of fed animals was further investigated using phloretin, a general non-competitive blocker of urea transporters in many systems (e.g. Levine et al., 1973; Kato and Sands, 1998; Walsh and Smith, 2001).

MATERIALS AND METHODS
Experimental animals

Male spiny dogfish sharks (*Squalus acanthias suckleyi*, Linnaeus; 1.41±0.06 kg, N=28) were trawled from Barkely Sound, British Columbia, by a commercial fisherman in early July 2011. [The systematics of the *Squalus acanthias* group has been controversial, and Ebert et al. (Ebert et al., 2010) have recently proposed that these northeast Pacific dogfish may be a separate species (*Squalus suckleyi*) rather than a subspecies of *S. acanthias*]. At Bamfield Marine Sciences Centre (BMSC), the fish were held under a seasonal photoperiod in a large 151,000 l tank served with running seawater at the experimental temperature (12±1°C), salinity (30±2 p.p.t.) and pH (7.95±0.10) until August, when experiments were performed. During this period they were entrained to a feeding regime in which a 3% ration of dead hake (*Merluccius productus*) was delivered every fourth day; all the food was consumed. For fed treatments, fish were sampled at 24–48 h after feeding, employing visual observation of the external surface of the abdomen to ensure that they had fed well. For fasting treatments, fish were removed to smaller 1000 l tanks where they were held under the same conditions for 1–2 weeks without feeding, prior to sampling. All procedures were in accord with Canada Council for Animal Care guidelines and were approved by BMSC and McMaster University Animal Care Committees.

Gut sac preparations

Preparations of all four gut sections (see below) were made from fasted fish (N=10) and fed fish (N=12). Additionally, preparations of intestinal sacs only were made from fed fish (N=6), in which the mucosal saline contained 0.25 mmol l^{-1} phloretin plus 0.1% DMSO (Sigma-Aldrich, St Louis, MO, USA).

Dogfish were terminally anaesthetized in MS-222 (0.2 g l^{-1}; Syndel Laboratories, Qualicum Beach, BC, Canada). The body cavity was opened by a long mid-ventral incision. The ducts of the rectal gland, pancreas and biliary system were tied off with silk suture. The cardiac stomach (stomach 1), the pyloric stomach (stomach 2), the intestine (spiral valve) and the colon were excised separately, and thoroughly flushed with saline to remove intestinal fluids and chyme. The saline used in all experiments was similar to that developed by Pärt et al. (Pärt et al., 1998) for *S. acanthias* except that the trimethyl amine oxide (TMAO) concentration was set to a higher concentration to better represent *in vivo* plasma values (Robertson, 1975; Kajimura et al., 2006), and the colloids (albumen and PVP-40) were omitted. The components (in mmol l^{-1}) were: 257 NaCl, 7 Na$_2$SO$_4$, 6 NaHCO$_3$, 0.1 Na$_2$HPO$_4$, 4 KCl, 3 MgSO$_4$·7H$_2$O, 2 CaCl$_2$·2H$_2$O, 5 glucose, 85 TMAO and 350 urea. The saline was pre-equilibrated with a precision gas mixture of 99.7% O$_2$ and 0.3% CO$_2$, to maximize O$_2$ supply, which set the partial pressure of CO$_2$ (P_{CO2}) to approximately 2.3 Torr (306.6 Pa) and pH to 8.1.

The posterior end of each section was cannulated with a short length (2 cm) of polyethylene tubing tipped with the lip cut off from a 0.4 ml 'hornet' centrifuge tube. This facilitated the making of a tight seal of tissue around the lip with silk suture. The anterior end of the section was ligated with silk suture, and the sac was filled with saline. A sample of this saline was taken for analysis of initial composition. Typical filling volumes for the four sections were approximately 12 ml (stomach 1), 15 ml (stomach 2), 20 ml (intestine) and 3 ml (colon). The filling cannula was plugged, and the sac (plus cannula) was then blotted dry, weighed to 0.0001 g, and immersed at time 0 h in a beaker containing 150 ml of the same saline. Thus gut sacs were set up under conditions that were initially symmetrical – i.e. identical saline on mucosal and serosal surfaces. The serosal saline was continuously bubbled with the 99.7% O$_2$/0.3% CO$_2$ gas mixture, and temperature was maintained at 12°C by standing the beakers in a flowing seawater bath. After 3 h of incubation, the gut sac was removed, blotted again and reweighed. The contents were then drained into a collection vial for analysis of final composition. The sac was then cut open, thoroughly blotted and weighed, together with its cannula and plug; this was designated as 'preparation mass' (see below). The tissue was then spread onto graph paper, and its surface area was measured.

Analytical techniques

All initial and final samples (and some serosal samples) were analyzed immediately for pH (Radiometer-Copenhagen GK401C combination glass pH electrode and pHm 82 meter, Brønshøj, Denmark). Subsamples were stored at 4°C for analysis of total CO$_2$ content (Corning 965 total CO$_2$ analyser, Midland, MI, USA), osmolality (Wescor Vapro 5520 vapour pressure osmometer, Logan, UT, USA) and Cl$^-$ (Labconco 4425120 digital chloridometer, Kansas City, MO, USA) within 24 h, or frozen at −20°C for later measurements of other parameters. The latter included urea by the colorimetric assay of Rahmatullah and Boyde (Rahmatullah and Boyde, 1980) and Na$^+$, K$^+$, Mg^{2+} and Ca^{2+} by atomic absorption spectroscopy (Varian SpectrAA-220FS, Mulgrave, Vic, Australia).

Calculation, display of data and statistics

Flux rates were calculated on a surface area (A) basis from measured changes in the mucosal solutions over time (T), taking into account measured changes in mucosal fluid volume (V_1–V_2). Concentration changes in the 7.5- to 50-fold higher volume serosal solutions were generally negligible. The initial volume (V_1; ml) was calculated at the initial sac mass minus preparation mass (see above) and the final volume (V_2; ml) was calculated at the final sac mass minus preparation mass. Thus the water flux rate was calculated as:

$$\frac{(V_1 - V_2)}{T \times A} \times 1000 \,, \tag{1}$$

where 1000 converts millilitres to microlitres, and the flux rates (μmol cm^{-2} h^{-1}) of various parameters (C; μmol ml^{-1}) were calculated as:

$$\frac{(V_1 \times C_1) - (V_2 \times C_2)}{T \times A} \,. \tag{2}$$

Thus negative flux values represent net secretion into the mucosal solution, and positive flux values represent net absorption out of the mucosal solution.

In most figures, final measured mucosal concentrations (mmol l^{-1}) have been displayed relative to starting (=serosal) concentrations in panel A, to indicate the concentration gradients achieved over 3 h by the gastrointestinal transport processes, whereas in panel B, the flux rates (μmol cm^{-2} h^{-1}) have been tabulated.

Surface areas are only coarse measurements that do not take into account the fine structure of villi and microvilli, or the architecture of the rugae in the two stomachs and the folds of the spiral valve. Therefore, to facilitate conversion of flux rates to a whole organism basis (i.e. per kilogram of original body mass), measured surface areas of the gut sac preparations have been reported in Table 1 in units of cm^{-2} kg^{-1}.

Data have been expressed as means ± 1 s.e.m., where N is the number of preparations (i.e. animals). Data were checked for normality and homogeneity of variance, and in a few cases were log or square root transformed to achieve this. Means were compared by Student's unpaired *t*-test (two-tailed) for fasted *versus* fed comparisons, with the Bonferroni correction for fasted *versus* fed *versus* phloretin comparisons. A significance level of $P<0.05$ was used throughout.

RESULTS

Gastrointestinal surface areas

Surface areas of the gut sac preparations per kilogram of original body mass were in the order stomach 1 (cardiac) > stomach 2 (pyloric) > intestine > colon (Table 1). There were no significant differences associated with fasted *versus* fed or fed + phloretin treatments. We estimate that in each case, the measured surface area is approximately 70% of the true area, 30% being lost to trimming and the ligatures at the two ends.

Urea transport

Under fasting conditions, stomach 1, stomach 2 and the colon all absorbed urea on a net basis, albeit at low rates that were not significantly different from zero in some cases, and this situation did not change with feeding (Fig. 1B). However, the intestines from fasted fish secreted urea into the lumen at approximately –2 μmol cm^{-2} h^{-1}, and with feeding, this changed to net absorption at a rate of approximately +8 μmol cm^{-2} h^{-1}. Urea absorption by fed preparations lowered measured mucosal urea levels from approximately 360 to 290 mmol l^{-1} after 3 h, thereby establishing a considerable gradient against the serosal concentration (Fig. 1A). This marked effect of feeding was entirely blocked by phloretin, which returned urea transport to approximately –4 μmol cm^{-2} h^{-1} and final mucosal urea concentration to above serosal values.

Water transport

All sections of the gut tended to absorb water on a net basis under both fasting and fed conditions (Fig. 2). Rates tended to be lower in stomach 2 than in the other sections. However, feeding significantly increased water absorption twofold to threefold in both stomach 2 and the intestine. A comparable increase in stomach 1 was not significant, whereas rates in the colon did not change. Phloretin did not affect the increase in water absorption associated with feeding.

Transport of ions

In preparations from fasted animals, Na$^+$ was secreted at very low rates in stomach 1, stomach 2 and the colon, but was absorbed at a rate of approximately +4 μmol cm^{-2} h^{-1} in the intestine (Fig. 3B). With feeding, both stomach 1 and stomach 2 (but not the colon) switched over to net Na$^+$ absorption at a low rate of approximately +0.5 μmol cm^{-2} h^{-1}. However, the net Na$^+$ absorption in the intestine remained unchanged, and this pattern was not affected by phloretin. In all three treatments, Na$^+$ absorption rates lowered mucosal Na$^+$ concentrations to 200–220 mmol l^{-1} over 3 h, substantially below the serosal concentration of approximately 270 mmol l^{-1} (Fig. 3A).

Patterns of Cl$^-$ transport were similar to those of Na$^+$ in the intestine, with net absorption of approximately +3 μmol cm^{-2} h^{-1} in both fasted and fed preparations establishing a substantial gradient against the serosal concentration; again there was no significant effect of phloretin (Fig. 4). However, stomach 1 and colon tended to absorb Cl$^-$ on a net basis in both fasted and fed preparations, whereas feeding activated net Cl$^-$ secretion in stomach 2, sufficient to raise mucosal Cl$^-$ concentration by approximately 15 mmol l^{-1}.

Net K$^+$ flux rates were negligible in stomach 1 and stomach 2, and there was a small but significant net K$^+$ secretion (approximately –0.2 μmol cm^{-2} h^{-1}) in the colon in both fasted and fed preparations (Fig. 5B). In contrast, the intestine secreted K$^+$ at a 10-fold higher rate (approximately –2 μmol cm^{-2} h^{-1}) in both fasted and fed preparations, and this rate was significantly doubled when phloretin was administered to fed preparations. Net K$^+$ secretion rates were

Table 1. Surface area per unit original body mass (cm^2 kg^{-1}) of the dogfish gastrointestinal sac preparations

	Fasted (N=10)	Fed (N=12)	Fed + phloretin (0.25 mmol l^{-1}; N=6)
Stomach 1 (cardiac)	47.55±4.21	55.12±3.36	–
Stomach 2 (pyloric)	37.07±2.65	45.98±3.82	–
Intestine	27.98±1.55	32.84±4.06	32.83±4.53
Colon	11.31±1.26	10.97±1.85	–

Data are means ± 1 s.e.m.
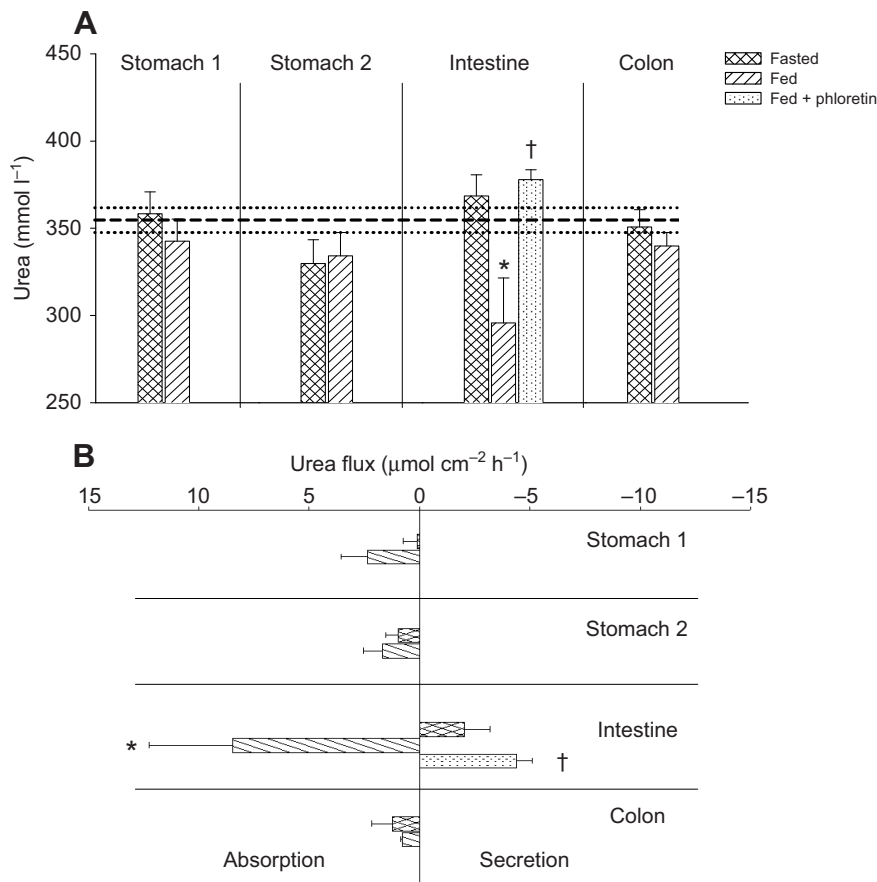There were no significant differences ($P<0.05$) among the three treatments for any section.

Fig. 1. Urea transport by *in vitro* gut sac preparations of the dogfish shark (means ± 1 s.e.m.) for fasted (N=10), fed (N=12) and fed preparations (intestine only, N=6) treated with 0.25 mmol l^{-1} phloretin in the mucosal saline. (A) Final measured mucosal concentrations (mmol l^{-1}) relative to the starting (=serosal) concentration (dashed horizontal line represents mean, dotted horizontal lines represent 1 s.e.m.), indicating the concentration gradients achieved over 3 h by the gastrointestinal transport processes. (B) Flux rates (µmol cm^{-2} h^{-1}) per unit surface area (means ± 1 s.e.m.). Positive flux rates represent net absorption out of the mucosal saline, negative values indicate net secretion into the mucosal saline. Asterisk indicates mean significantly different (P<0.05) from fasted mean; dagger indicates fed + phloretin mean significantly different (P<0.05) from fed mean.

sufficient to raise mucosal K$^+$ concentrations up to 25–40 mmol l^{-1}, almost 10-fold greater than the serosal concentrations (Fig. 5A).

Net Mg^{2+} and Ca^{2+} flux rates (Table 2) were much smaller than the fluxes of other ions and urea (cf. Fig. 1 and Figs 3–5) and were somewhat similar to one another, though Ca^{2+} was far more variable. In fasted preparations, slight net secretion of Mg^{2+} in stomach 1 and stomach 2 of fasted preparations changed significantly to slight net absorption with feeding, whereas slight net secretion in the intestine remained unchanged. Mg^{2+} flux rates in the colon were not significantly different from zero. Ca^{2+} transport rates were not significantly different from zero in any segment, in either fasted or fed preparations. Notably however, for both divalent cations, phloretin treatment of fed preparations led to significant increases in net secretion rates (Table 2).

Net osmolyte transport

Net osmolyte flux rates were generally small and variable, and were unaffected by feeding in stomach 1, stomach 2 and the colon (Fig. 6B). However, in the intestine, there was a strong net secretion of osmolytes at approximately –6 mOsmol cm^{-2} h^{-1} in fasted preparations. This rate doubled with feeding but the increase was not significant; phloretin had no effect. Intestinal net osmolyte secretion over 3 h resulted in 50 to 100 mOsmol l^{-1} elevations in the osmolality of the mucosal saline (Fig. 6A). This occurred despite the substantial net absorptions of urea, Na$^+$ and Cl$^-$ in the intestine, especially in preparations from fed dogfish (Figs 1, 3, 4).

H$^+$ and CO$_2$/HCO$_3^-$ transport

In all segments under all conditions, over the 3 h flux period, the pH of the mucosal saline fell substantially below the starting/serosal pH of approximately 8.1 (Fig. 7), though it was highest in the colon

(~7.5). In both sections of the stomach, the decrease in mucosal pH was greater with feeding, especially in stomach 2, where the final pH fell to ~5.7 *versus* 6.7 in fasted preparations. In stomach 1, the decrease with feeding was less, from ~7.0 to 6.5, and in the colon, pH did not change. Neither feeding nor phloretin had any effect on final intestinal pH, which averaged approximately 6.4.

As explained in the Discussion, the total CO$_2$ measurements cannot absolutely distinguish between movements in the form of CO$_2$ *versus* HCO$_3^-$, so we refer to CO$_2$/HCO$_3^-$ transport. In fasted preparations, flux rates were small, not significantly different from zero in all segments (Fig. 8B). Feeding clearly stimulated CO$_2$/HCO$_3^-$ transport, with net secretion in all sections, but with high variability. Mean flux rates in stomach 1 and colon reached –1.5 to –2.0 µmol cm^{-2} h^{-1}, and the largest increase was in the intestine, to approximately –5.5 µmol cm^{-2} h^{-1}. These fluxes brought final mucosal mean total CO$_2$ concentrations up to between 20 mmol l^{-1} (in stomach 1 and colon) and 60 mmol l^{-1} (in intestine) relative to starting/serosal concentrations of approximately 6 mmol l^{-1}. Exposure to phloretin completely blocked the net secretion of CO$_2$/HCO$_3^-$ by the intestine.

DISCUSSION
Answers to hypotheses

With respect to our initial hypotheses (see Introduction), the results with *in vitro* gut sac preparations: (1) confirmed an increase in H$^+$ secretion in both stomach sections (Fig. 7) and an increase in Cl$^-$ secretion only in stomach 2 (Fig. 4), but found that Na$^+$ absorption, rather than Na$^+$ secretion, occurred in both stomach sections after feeding (Fig. 3); (2) contradicted the predicted increase in urea secretion by the intestine after feeding, showing instead a marked switch to urea absorption (Fig. 1); (3) showed a substantial increase

Fig. 2. Water transport by *in vitro* gut sac preparations of the dogfish shark (means ± 1 s.e.m.) for fasted (*N*=10), fed (*N*=12) and fed preparations (intestine only, *N*=6) treated with 0.25 mmol l⁻¹ phloretin in the mucosal saline. Flux rates (µl cm⁻² h⁻¹) per unit surface area (means ± 1 s.e.m.) are shown. Positive flux rates represent net absorption out of the mucosal saline, negative values indicate net secretion into the mucosal saline. Other details as in legend of Fig. 1.

Fig. 3. Na⁺ transport by *in vitro* gut sac preparations of the dogfish shark (means ± 1 s.e.m.) for fasted (*N*=10), fed (*N*=12) and fed preparations (intestine only, *N*=6) treated with 0.25 mmol l⁻¹ phloretin in the mucosal saline. Other details as in legend of Fig. 1.

in CO_2/HCO_3^- secretion in the intestine after feeding (Fig. 8) which probably reflects the predicted increase in HCO_3^- secretion, as discussed below; (4) confirmed increased water absorption (Fig. 2) whereas Na⁺ (Fig. 3) and Cl⁻ (Fig. 4) absorption remained unchanged, and K⁺ was secreted regardless of feeding condition (Fig. 5), with negligible changes in colonic fluxes; and (5) confirmed that feeding had little influence on the handling of Ca^{2+} and Mg^{2+}, which were not absorbed across the tract to any appreciable extent (Table 2). The many marked differences in transport seen between fed and fasted preparations presumably result from the persistent effects (i.e. post-excision) of neural, hormonal, chemical and/or mechanical stimuli associated with feeding (Holmgren and Nilsson, 1999). These were beyond the scope of the present study but constitute a rich area for future investigation.

Urea transport
Although contrary to our initial hypothesis, the finding of substantial urea absorption by the intestine after feeding (Fig. 1) is an exciting discovery that may help clarify the mystery of how elasmobranchs retain urea against such immense gradients (Smith, 1929; Smith, 1936). In the present study, this intestinal net absorption was completely blocked by phloretin, resulting in a return to net secretion (Fig. 1B). Phloretin is a non-competitive inhibitor of both facilitated diffusion and active transport systems for urea (e.g. Levine et al., 1973; Kato and Sands, 1998; Walsh and Smith, 2001), and in an oocyte expression system has been shown to block the only urea transporter (ShUT) that has ever been identified at the molecular level in *S. acanthias* (Smith and Wright, 1999). Although it was not found in the intestine, mRNA for a larger homologue was detected. However, ShUT is thought to be a facilitated diffusion
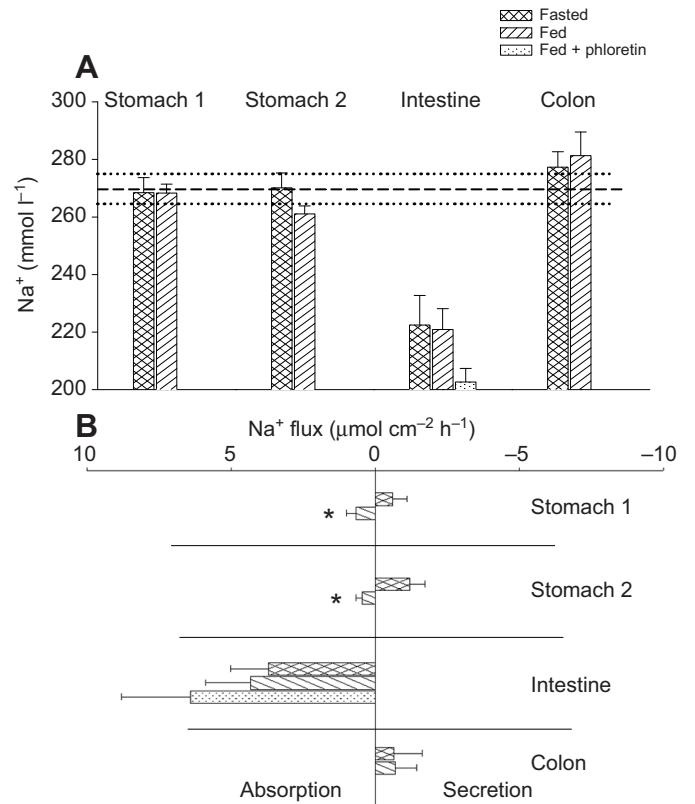
transporter. In the present study, the additional fact that the net urea absorption established a pronounced urea concentration difference between mucosal and serosal solutions suggests that the transport is both carrier-mediated and active. Previous studies have shown the presence of apparent active urea reabsorption sensitive to a range of inhibitors at both the gills (Wood et al., 1995; Wood et al., 2013; Pärt et al., 1998; Fines et al., 2001) and the kidneys (Kempton, 1953; Schmidt-Nielsen and Rabinowitz, 1964; Schmidt-Nielsen et al., 1972; Hays et al., 1977; Wood et al., 1995; Morgan et al., 2003) of elasmobranchs, but the exact mechanisms have remained controversial for decades (Boylan, 1967; Boylan, 1972; Friedman and Hebert, 1990; Walsh and Smith, 2001; Hill et al., 2004; Wood et al., 2013). Much of this uncertainty has originated from the complex anatomy of both gills and kidney, whereas the intestine offers a relatively simple, uniform, flat epithelium that should be amenable to future gut sac, Ussing chamber or isolated membrane studies.

The magnitude of the net transport of urea by the intestine is impressive. Adjusting the intestinal surface area data of Table 1 by the fraction 1/0.7 (to account for the area lost in making the sac preparation), a net absorption rate of +8 µmol cm⁻² h⁻¹ translates to a net urea transport rate of approximately +375 µmol kg⁻¹ h⁻¹, which appears to be close to those at gills and kidneys in *S. acanthias*. Reabsorption rates of urea at the gills have never been measured directly, but based on vesicle studies, Fines et al. (Fines et al., 2001) estimated a unidirectional reabsorption rate of +535 µmol kg⁻¹ h⁻¹ against an overall branchial net loss rate of –250 to –400 µmol kg⁻¹ h⁻¹ typically measured *in vivo* (Wood et al., 1995;
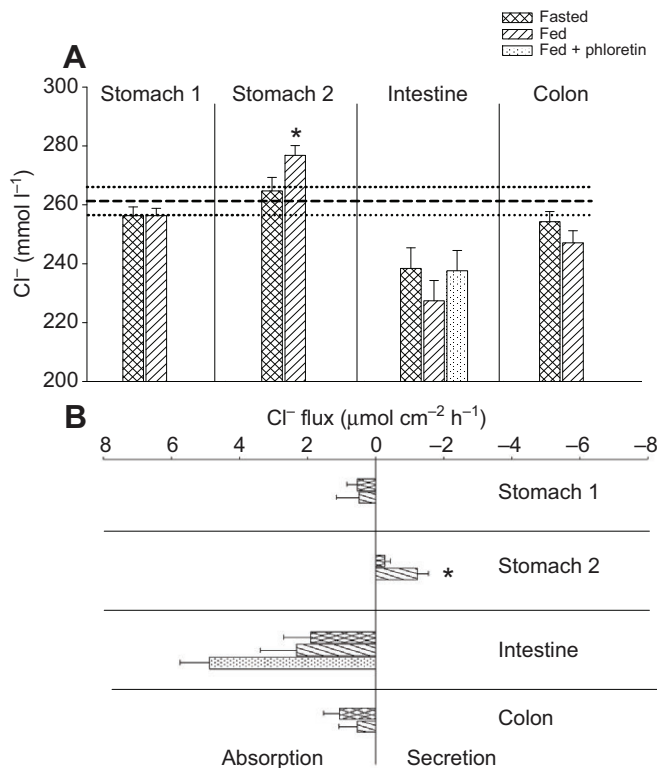
Fig. 4. Cl⁻ transport by *in vitro* gut sac preparations of the dogfish shark (means ± 1 s.e.m.) for fasted (N=10), fed (N=12) and fed preparations (intestine only, N=6) treated with $0.25\,mmol\,l^{-1}$ phloretin in the mucosal saline. Other details as in legend of Fig. 1.

Fig. 5. K⁺ transport by *in vitro* gut sac preparations of the dogfish shark (means ± 1 s.e.m.) for fasted (N=10), fed (N=12) and fed preparations (intestine only, N=6) treated with $0.25\,mmol\,l^{-1}$ phloretin in the mucosal saline. Other details as in legend of Fig. 1.

Wood et al., 2005; Kajimura et al., 2006). In the kidney, a net reabsorption rate of approximately $+480\,\mu mol\,kg^{-1}\,h^{-1}$ may be calculated from the data of Schmidt-Nielsen et al. (Schmidt-Nielsen et al., 1972).

It is possible that the net secretion of urea (at a lower rate of approximately $-2\,\mu mol\,cm^{-2}\,h^{-1}$ or $-60\,\mu mol\,kg^{-1}\,h^{-1}$) seen in intestinal preparations from fasting fish (Fig. 1B) is also active and carrier-mediated, because it did establish a small excess concentration of urea in the mucosal fluid relative to the serosal compartment (Fig. 1A). In isolated gut sac preparations from starved animals, the intestine secreted urea at a comparable rate in the little skate, *Leucoraja erinacea* [$-110\,\mu mol\,kg^{-1}\,h^{-1}$ (Anderson et al., 2010)], and at a higher rate ($-240\,\mu mol\,kg^{-1}\,h^{-1}$) in the white-spotted bamboo shark, *Chiloscyllium plagiosum* (Anderson et al., 2007). However, the fact that fed preparations treated with phloretin did not exhibit zero urea flux but rather an even greater net secretion rate (Fig. 1B) could be interpreted as evidence against this. Indeed we favour the idea that the net secretion seen in fasted and phloretin-treated preparations is mainly due to endogenous synthesis of urea, as a full complement of ornithine-urea cycle enzymes has been found in the intestinal tissue of *S. acanthias* (Kajimura et al., 2006). The net absorptive flux of urea seen in fed preparations would be running against this background of secretion due to endogenous production, so the unidirectional absorptive flux would be substantially greater than the net absorptive flux. Further research is needed to clarify this point.

What is the explanation for the discrepancy between the previous *in vivo* study (Wood et al., 2007b), indicating net addition of urea into the intestine after feeding, and the present *in vitro* data
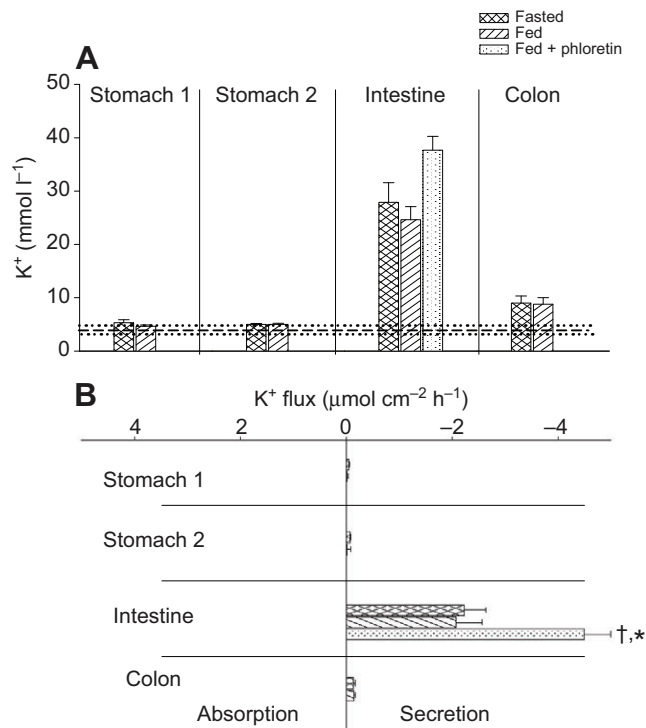
(Fig. 1B), showing the opposite – i.e. net urea reabsorption? While endogenous production by the intestinal ornithine-urea cycle (Kajimura et al., 2006) may play a role *in vivo*, other sources should also be investigated. Although the stomach is reported to be capable of urea secretion (MacIntosh, 1936), both sections appeared to perform net urea reabsorption in the present study (Fig. 1B). Bile is quite high in urea ($\sim315\,mmol\,l^{-1}$) in *S. acanthias* (Wood et al., 2007b; Anderson et al., 2012) and the rate of bile discharge appears to increase after feeding (Wood et al., 2007b), so hepatobiliary input, perhaps together with pancreatic secretions, could well be an important source. Presumably urea reabsorption by the intestinal epithelium is activated after feeding in order to balance and recover urea secreted into the chyme from these sources. Urea is too valuable an osmolyte to discharge through the colon. The colon itself also appears to play at least a small role in this reabsorption (Fig. 1B), in agreement with recent Ussing chamber experiments on this segment (Anderson et al., 2012).

Water and net osmolyte transport

All sections of the tract absorbed water on a net basis in fasted preparations, and water absorption increased with feeding in stomach 2 and especially in the intestine (Fig. 2), in accord with original predictions. The finding of water absorption even in fasting preparations agrees with data from intestinal sacs of *C. plagiosum* (Anderson et al., 2007) and from both intestinal and colonic sacs of *L. erinacea* (Anderson et al., 2010). This is in accord with the view that elasmobranchs, including *S. acanthias* (DeBoeck et al., 2001), drink at very low levels even in 100% seawater and attempt to conserve water from the chyme, drying the faeces prior to

Table 2. Net flux rates of Mg^{2+} and Ca^{2+} in gut sac preparations of four segments of the gastrointestinal tract in dogfish

	Mg^{2+} ($\mu mol\,cm^{-2}\,h^{-1}$)			Ca^{2+} ($\mu mol\,cm^{-2}\,h^{-1}$)		
	Fasted (N=10)	Fed (N=12)	Fed + phloretin (0.25 mmol l^{-1}; N=6)	Fasted (N=10)	Fed (N=12)	Fed + phloretin (0.25 mmol l^{-1}; N=6)
Stomach 1	−0.07±0.05	+0.05±0.02*		−0.30±0.20	+0.11±0.08	
Stomach 2	−0.05±0.01	+0.03±0.04*		+0.07±0.11	−0.28±0.33	
Intestine	−0.18±0.11	−0.08±0.17	−0.63±0.13†	+0.04±0.14	0.00±0.27	−1.02±0.22*,†
Colon	+0.01±0.01	−0.05±0.04		+0.08±0.09	−0.22±0.28	

Data are means ± 1 s.e.m. Note: positive values indicate net absorption out of the mucosal compartment, negative values indicate net secretion into the mucosal compartment.
*Significantly different (P<0.05) from corresponding fasted value.
†Significantly different (P<0.05) from corresponding fed value.

discharge (reviewed by Anderson et al., 2007). Integrated over the whole intestinal tract, the water absorption rate amounted to approximately $0.30\,ml\,kg^{-1}\,h^{-1}$, reasonably close to the measured drinking rate in this species of $0.16\,ml\,kg^{-1}\,h^{-1}$ (DeBoeck et al., 2001). At least in *S. acanthias*, net intestinal water absorption in fed preparations correlated with net absorptive transport of urea (Fig. 1B), Na^+ (Fig. 3B) and Cl^- (Fig. 4B). The calculated 'concentrations' of these moieties in the absorbed fluid would be massive (~$2700\,mmol\,l^{-1}$ for urea, ~$1200\,mmol\,l^{-1}$ for Na^+ and Cl^-). However, it is curious that in the present investigation, as well as in some of the intestinal and colonic sac studies on *Chiloscyllium plagiosum* (Anderson et al., 2007) and *Leucoraja erinacea* (Anderson et al., 2010), the net absorption of water occurred in the face of overall net osmolyte secretion (e.g. Fig. 6). It is also curious that water absorption was not impeded (Fig. 2) when urea absorption

was completely blocked by phloretin (Fig. 1). In the present study, the net osmolyte appearance in the intestine (Fig. 6) could not be quantitatively explained by the measured ions, though K^+ (Fig. 5) and CO_2/HCO_3^- secretion (Fig. 8) were significant contributors. Presumably secretion of organic molecules (e.g. amino acids, enzymes, metabolites) by the intestinal epithelium also played a role.

Water transport in the opposite direction from net osmolyte transport, as clearly seen in the intestine (cf. Figs 2, 6), is surprising. The transport of fluid across biological membranes is a fundamental process in physiology, but as Hill (Hill, 2008) has noted, despite a voluminous literature, we still have no clear idea how it occurs. Three explanations for the current observation may be discussed. Firstly, it is tempting to attribute the absorption of water against the osmotic gradient to hydrostatic pressure, because the preparations exhibited obvious motility and contractility during the flux period, and fed preparations (in which fluid absorption was higher) seemed to be more active in this regard. However, the hydrostatic pressure needed to sustain an opposing osmotic gradient of up to $100\,mOsmol\,l^{-1}$, as observed at the end of the 3 h flux period (Fig. 6A), would be 2.24 atm or ~1700 mmHg, which is clearly improbable, so hydrostatic flux likely would make only a small contribution. Secondly, the water flux measurement is an artefact
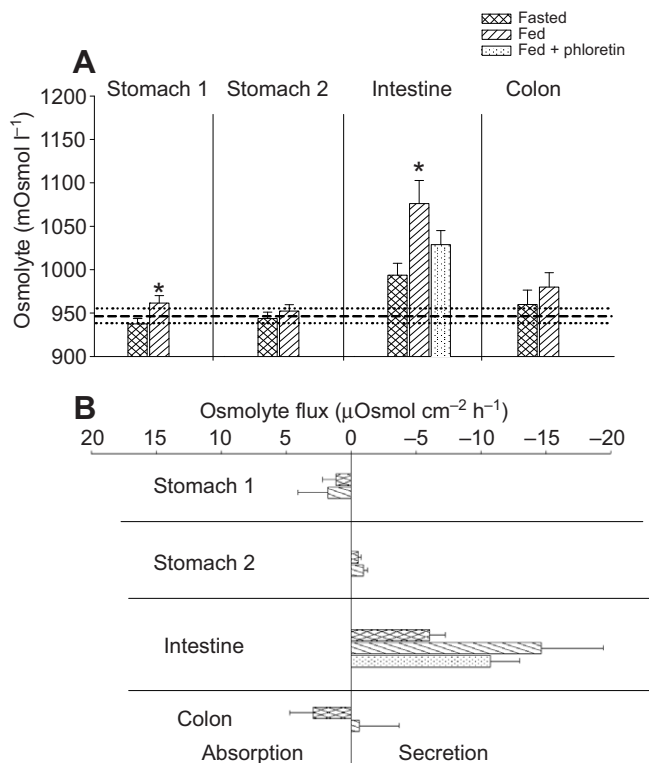

Fig. 6. Osmolyte transport by *in vitro* gut sac preparations of the dogfish shark (means ± 1 s.e.m.) for fasted (N=10), fed (N=12) and fed preparations (intestine only, N=6) treated with 0.25 mmol l^{-1} phloretin in the mucosal saline. Other details as in legend of Fig. 1.
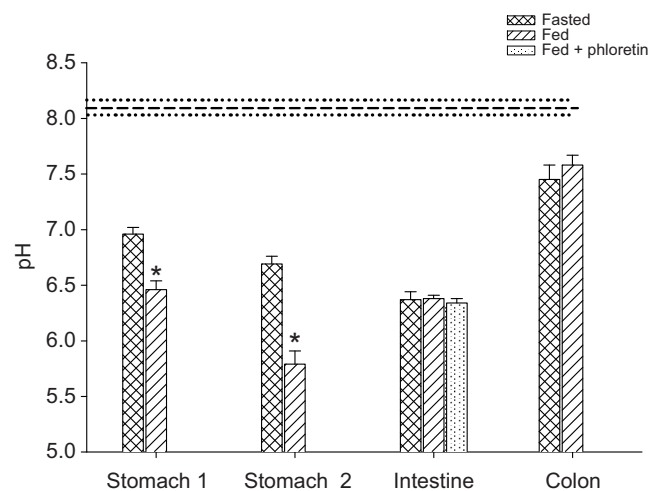

Fig. 7. Final pH values measured in the mucosal saline of *in vitro* gut sac preparations of the dogfish shark (means ± 1 s.e.m.) for fasted (N=10), fed (N=12) and fed preparations (intestine only, N=6) treated with 0.25 mmol l^{-1} phloretin in the mucosal saline. The starting (=serosal) pH (dashed horizontal line represents mean, dotted horizontal lines represent 1 s.e.m.) is also shown to indicate the changes occurring over 3 h. Other details as in legend of Fig. 1.
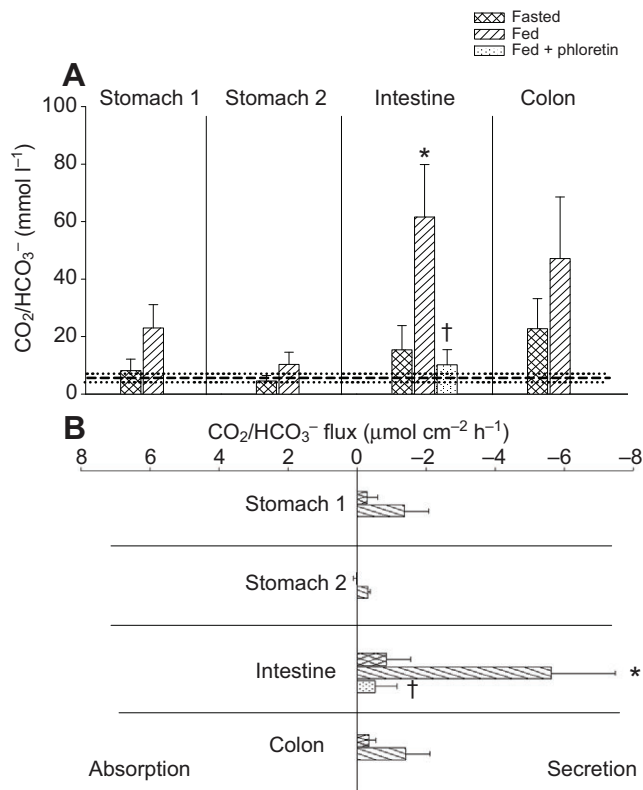
Fig. 8. CO_2/HCO_3^- transport by *in vitro* gut sac preparations of the dogfish shark (means ± 1 s.e.m.) for fasted (N=10), fed (N=12) and fed preparations (intestine only, N=6) treated with 0.25 mmol l^{-1} phloretin in the mucosal saline. Other details as in legend of Fig. 1.

of the gravimetric technique. The tissue is spongy; if at the start, fluid were trapped in the interstitial fluid spaces of the gut tissue, and by the end some of this trapped fluid had been mechanically extruded through the serosal surface by the contractility, then an 'apparent' water absorption would be calculated without any real change in the mucosal fluid volume. The way around this problem would be to measure water flux by assaying changes in the concentration of an inert non-absorbed marker in the mucosal fluid. The difficulty comes in choosing and validating such a marker. For example, polyethylene glycol-4000 (PEG-4000) is often employed as an inert marker for drinking rate estimates in teleost fish, and it was used by Anderson et al. (Anderson et al., 2010) for the calculation of fluid fluxes in elasmobranch gut sac preparations, some of which exhibited water absorption in the face of apparent net osmolyte secretion, as in the present study. However, a recent study has shown that PEG-4000 permeates teleost gut tissue by a paracellular route, albeit at a low rate (Wood and Grosell, 2012). A final possibility is that the transport of water in the opposite direction from net osmolyte transport is a real phenomenon mediated by epithelial transport processes. Of the five current theories of trans-epithelial water transport reviewed by Hill (Hill, 2008), only one, the 'osmosensor feedback theory', does not depend on osmosis, and therefore could reasonably fit the present observations. This is decribed as a 'radical departure' from past ideas, and paracellular fluid flow is a critical component of this model. In this context, future experiments using radiolabelled PEG-4000 as a marker of paracellular fluid flow across the dogfish intestine (rather than as an inert marker) might actually prove quite instructive.

Transport of ions

The substantial net absorptive fluxes of Na^+ (Fig. 3B) and Cl^- (Fig. 4B) in the intestine did not increase with feeding and were not seen in the colon. At least at a macroscopic level, these intestinal fluxes appear to be due to active transport, because substantial concentration gradients against the serosal fluid were achieved (Fig. 3A, Fig. 4A). In this respect, the marine elasmobranch is similar to the marine teleost (reviewed by Grosell, 2011; Wood and Bucking, 2011). Net absorptive fluxes of Na^+ and Cl^- of comparable magnitude were also reported in isolated intestinal sacs from fasted *C. plagiosum* (Anderson et al., 2007) and in intestinal, but not colonic, sacs of fasted *L. erinacea* (Anderson et al., 2010), in agreement with the present observations. Na^+ was also absorbed to a small extent in both stomach sections in fed preparations (Fig. 3), in accord with increasing evidence that the stomach is also important in Na^+ uptake in teleost fish (Wood and Bucking, 2011). Note that Na^+ transport appeared to be independent of Cl^- transport in the stomach (Fig. 3 *versus* Fig. 4), which is not surprising in light of the role of these sections in HCl secretion, as discussed below.

In all three species, there were substantial secretory fluxes of K^+ in the intestine, and in *S. acanthias*, these did not change with feeding and actually increased with phloretin treatment (Fig. 5). These observations do not agree with predictions of net intestinal K^+ absorption from the *in vivo* study (Wood et al., 2007b), or with the situation in marine teleosts where K^+ is efficiently absorbed (Grosell, 2011; Wood and Bucking, 2011). Possibly, the result is an artefact of K^+ leakage from intestinal muscle or neural tissue during incubation, especially because phloretin is well known to affect K^+ channels, increasing K^+ conductance (e.g. Owen, 1974). Phloretin also blocks glucose uptake in many systems (e.g. Kletzien et al., 1975; Levine et al., 1973; Forman et al., 1982), and these actions may also explain the significant Ca^{2+} and Mg^{2+} effluxes that occurred into the mucosal saline in the presence of phloretin (Table 2). Regardless, the more important finding is that Mg^{2+} and Ca^{2+} fluxes were negligible in both fasted and fed preparations (Table 2), so the gastrointestinal tract of the elasmobranch (Smith, 1931), like that of the marine teleost (Grosell, 2011; Wood and Bucking, 2011), effectively excludes these toxic divalent cations.

H^+ and CO_2/HCO_3^- transport

All four gut sections acidified the mucosal saline (Fig. 7). The pH inside the intestinal sacs was brought to 6.4, regardless of feeding or phloretin treatment, which is almost identical to the invariant value of 6.5 recorded *in vivo* for intestinal contents, regardless of feeding status (Wood et al., 2007b). The mucosal saline pH of 7.5 seen in the colon *in vitro* was also close to the constant *in vivo* value of 7.2. This suggests that there are homeostatic mechanisms that carefully regulate the pH of the intestinal chyme, which could be very important for the optimal functioning of digestive enzymes and transporters.

In contrast, the pH values seen *in vitro* in the two stomach segments (5.8–7.0; Fig. 7), were far above those recorded *in vivo* in either fasted (1.8–2.1) or fed animals (3.2–4.0, higher because of buffering by ingested food) (Wood et al., 2007b). The explanation is probably twofold. Firstly, HCl secretion was probably far lower *in vitro* than *in vivo* in the absence of the normal neural, hormonal, chemical and/or mechanical stimuli associated with food ingestion (Holmgren and Nilsson, 1999). This conclusion is supported by the finding of no net secretion of Cl^- in stomach 1 in either fasted or fed preparations, though a modest net Cl^- secretion (approximately $-1.5\,\mu mol\,cm^{-2}\,h^{-1}$) was stimulated by feeding in stomach 2 (Fig. 4). This secretion rate is typical of many *in vitro* studies on isolated

elasmobranch stomach epithelia stimulated by various treatments (e.g. Rehm, 1962; Hogben, 1959; Hogben 1967; Kidder, 1976; Kidder, 1991), and appears to be much less than that recorded in some early informative but crude experiments *in vivo* (Babkin et al., 1935; MacIntosh, 1936). Secondly, the initial $6\,\mathrm{mmol\,l^{-1}}$ HCO_3^- present in the mucosal saline in the present study would have acted as a buffer, so the pH values resulting from a given rate of HCl secretion would be higher than those recorded *in vivo* or with HCO_3^--free salines *in vitro* in the aforementioned studies.

Indeed there was a net secretion of CO_2/HCO_3^- into the mucosal saline in these stomach preparations (Fig. 8A). Most likely this resulted from the buildup of CO_2 in a closed system, because HCO_3^- secretion by the stomach is most unlikely, and CO_2/HCO_3^- is undetectable in gastric chyme *in vivo* (Wood et al., 2007b). Indeed CO_2/HCO_3^- flux tended to be higher in fed preparations (Fig. 8A) where a higher tissue metabolic rate would be expected. This highlights a problem in data interpretation: while one can accurately measure the total CO_2/HCO_3^- flux, and partition the concentration into the CO_2 and HCO_3^- components using the Henderson–Hasselbalch equation and known constants for elasmobranchs (Boutilier et al., 1984), it is technically not possible to determine the actual molecular species (CO_2, HCO_3^-, CO_3^{2-}) that were secreted using the present preparation. However, the contributions of CO_2 *versus* basic equivalents (HCO_3^-, CO_3^{2-}) could be separated in future studies by applying the pH-stat technique of Grosell and Genz (Grosell and Genz, 2006) to isolated epithelial membranes.

In the intestine, there was a very marked (but variable) secretion of CO_2/HCO_3^- ($-5.6\,\mathrm{\mu mol\,cm^{-2}\,h^{-1}}$) stimulated by feeding (Fig. 8B) sufficient to raise the mucosal concentration to $\sim60\,\mathrm{mmol\,l^{-1}}$ (Fig. 8A). CO_2/HCO_3^- secretion was also observed in intestinal sacs of fasted *C. plagiosum* (Anderson et al., 2007) and *L. erinacea* (Anderson et al., 2010), though the rates were only approximately 1–3% of those observed in the present study. *In vivo*, intestinal fluid CO_2/HCO_3^- concentrations averaging $\sim5\,\mathrm{mmol\,l^{-1}}$ in *Squalus acanthias* regardless of feeding status (Wood et al., 2007b) or $15\,\mathrm{mmol\,l^{-1}}$ in fasted animals (Anderson et al., 2012) have been reported. Comparable values of $9\,\mathrm{mmol\,l^{-1}}$ in the clear-nosed skate (*Raja eglanteria*) and $13\,\mathrm{mmol\,l^{-1}}$ in *L. erinacea* were recorded by Anderson et al. (Anderson et al., 2010), but $73\,\mathrm{mmol\,l^{-1}}$ in *C. plagiosum* (Anderson et al., 2007). In the present experiments, at final intestinal pH (6.4), approximately 60% of this secreted CO_2/HCO_3^- would be in the form of HCO_3^- and 40% as dissolved CO_2, but again the form secreted cannot be proven. Very likely, both CO_2 (from increased post-prandial metabolism of the tissue associated with increased transport and digestive activity) and HCO_3^- were secreted. The masses of the gut sac tissues were measured in each preparation, and the intestinal CO_2/HCO_3^- secretion rate of $5.6\,\mathrm{\mu mol\,cm^{-2}\,h^{-1}}$ translates to $34{,}000\,\mathrm{\mu mol\,kg^{-1}\,intestinal\,tissue\,h^{-1}}$. By contrast, rates for the stomach 1 and 2 translated to 6300 and $2100\,\mathrm{\mu mol\,kg^{-1}\,stomach\,tissue\,h^{-1}}$, respectively. The metabolic rate of an intact dogfish during the period of specific dynamic action at 24–48 h post-feeding is only $\sim2200\,\mathrm{\mu mol\,O_2\,kg^{-1}\,h^{-1}}$ (Wood et al., 2007a), or 6% of the intestinal CO_2/HCO_3^- secretion rate. This argues in favour of the notion that a substantial portion of the intestinal CO_2/HCO_3^- secretion in fed sharks occurs as HCO_3^- flux. Indeed, on a whole-animal basis, this would amount to an intestinal HCO_3^- flux of $\sim250\,\mathrm{\mu mol\,kg^{-1}\,h^{-1}}$, in the upper range of marine teleosts (reviewed by Grosell, 2006; Grosell, 2011).

In marine teleosts, intestinal HCO_3^- secretion in exchange for Cl^- plays an integral role in the strategy of osmoregulation so as to facilitate water absorption (reviewed by Grosell, 2006; Grosell, 2011), and this process appears to be stimulated by feeding (reviewed by Wood and Bucking, 2011). Because elasmobranchs are approximately isosmotic to seawater, they are thought not to need such a mechanism to help water uptake. However, Taylor and Grosell (Taylor and Grosell, 2006) reported high intestinal CO_2/HCO_3^- concentrations in live specimens of *C. plagiosum* that had been experimentally dehydrated. They suggested that Cl^-/HCO_3^- exchange was activated to enhance water absorption, but this was not supported by subsequent intestinal sac experiments (Anderson et al., 2007). In the present study, phloretin completely blocked intestinal CO_2/HCO_3^- secretion (Fig. 8). Phloretin is well known to inhibit Cl^-/HCO_3^- exchange and other anion exchangers (Jennings and Solomon, 1976; Forman et al., 1982), so the result is not surprising. However, phloretin had absolutely no effect on net Cl^- absorption (Fig. 4) or net water absorption (Fig. 2), so perhaps the elevated CO_2/HCO_3^- secretion stimulated by feeding is entirely for digestive purposes. Phloretin also has many other potential actions, including blockade of glucose uptake (Levine et al., 1973; Forman et al., 1982), which confound interpretation.

Concluding remarks

The present study, while clarifying some aspects of gastrointestinal physiology of *S. acanthias* during feeding, has raised far more questions, especially with respect to urea, water and CO_2/HCO_3^- transport in the intestine. In future studies, it will be useful to further characterize the present preparations as to the time course of responses (i.e. viability), transport kinetics and fuel substrate preferences, and to address the methodological and theoretical issues associated with the paradox of water transport against the osmotic gradient, as discussed earlier. A recent comprehensive synthesis of the multiple functions of the gut in fish (Grosell et al., 2011) summarizes a great deal of knowledge about these processes in teleosts, but reveals very little information in elasmobranchs. The time is ripe for comparable focus on this ancient group, many of which are currently endangered, using integrated molecular, cellular, physiological and behavioural approaches. Because of its relative abundance, and the information available to date, *S. acanthias* is an ideal model for future investigations. In particular, it will be useful to compare *S. acanthias* with other elasmobranchs to validate that it can serve as a model for the many tropical and subtropical species that are threatened or endangered.

AUTHOR CONTRIBUTIONS

H.J.L. performed the experiments, analyzed the data and edited the manuscript. G.D.B. performed the experiments, contributed reagents/materials/analysis tools and edited the manuscript. C.M.W. conceived and designed the experiments, performed the experiments, analyzed the data, contributed reagents/materials/analysis tools and wrote the paper.

COMPETING INTERESTS

No competing interests declared.

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