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SHORT COMMUNICATION

The teleost fish intestine is a major oxalate-secreting epithelium

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ABSTRACT

Oxalate is a common constituent of kidney stones, but the mechanism of its transport across epithelia is not well understood. With prior research on the role of the intestine focused on mammals, the present study considered oxalate handling by teleost fish. Given the osmotic challenge of seawater (SW), marine teleosts have limited scope for urinary oxalate excretion relative to freshwater (FW) taxa. The marine teleost intestine was hypothesized as the principal route for oxalate elimination, thus demanding epithelial secretion. To test this, intestinal ^{14}C-oxalate flux was compared between FW- and SW-acclimated sailfin molly (*Poecilia latipinna*). In SW, oxalate was secreted at remarkable rates (367.90±22.95 pmol cm^{-2} h^{-1}), which were similar following FW transfer (387.59±27.82 pmol cm^{-2} h^{-1}), implying no regulation by salinity. Nevertheless, this ability to secrete oxalate at rates 15–19 times higher than the mammalian small intestine supports this proposal of the teleost gut as a major, previously unrecognized excretory pathway.

KEY WORDS: Bicarbonate secretion, Slc26, Anion exchange, Calcium carbonate, Ussing chamber, DIDS

INTRODUCTION

The oxalate anion is a non-functional end-product of metabolism and also part of the diet. When permitted to accumulate, it is potentially damaging, readily binding with calcium to form calcium oxalate, the main constituent of the vast majority of kidney stones (Asplin, 2002). The excretion of oxalate is therefore imperative and the kidney typically expels 90–95% of oxalate in the urine (Osswald and Hautmann, 1979; Prenen et al., 1982; Costello et al., 1992). The intestine, while making a relatively minor contribution to excretion under normal circumstances, can also exert a significant influence on oxalate homeostasis (Whittamore and Hatch, 2017). For example, the systemic oxalate burden associated with metabolic disorders, such as primary hyperoxaluria, or from excessive consumption of oxalate (or its precursors), can be targeted by triggering its secretion into the gut (Hatch et al., 2006, 2011). Similarly, when kidney function is compromised by chronic renal failure, fecal oxalate excretion can increase by more than 4-fold (Costello et al., 1992), in association with elevated intestinal secretion (Hatch et al., 1994), while a chronic metabolic acidosis can also induce a similar secretory response (Whittamore and Hatch, 2015). For decades, these and related studies examining oxalate transport across the intestinal epithelium have been confined to mammalian models (Whittamore and Hatch, 2017). The following brief report therefore considers how the largest and most diverse vertebrate group, teleost fish, might handle oxalate.

To cope with the dehydrating effect of seawater (SW), teleost fish minimize water loss with reduced rates of urine production (Larsen et al., 2014). Because the teleost kidney is not the principal osmoregulatory organ that it has evolved to be in mammals, and with limited scope for eliminating oxalate in the urine, one strategy would be for the intestine to assume a primary role in excretion. The teleost intestine efficiently absorbs water from SW, which is being continuously consumed to replenish lost body water (Larsen et al., 2014). However, the surfeit of calcium in imbibed SW (~10 mmol l^{-1}) does not disproportionately burden the kidney. A large fraction (>80%) of calcium remains within the gut and is excreted, with 40–60% of this sequestered as solid calcium carbonate ($CaCO_3$) (Shehadeh and Gordon, 1969; Wilson and Grosell, 2003). This process of enteric biomineralization, unique to SW teleosts, was suggested to reduce the risk of kidney stone formation (Wilson and Grosell, 2003) and, as proposed here, will also capture oxalate (Fig. 1), but this critically depends on the ability of the intestine to secrete it from the blood into the lumen.

In an initial pilot study, excreted $CaCO_3$ was collected from fasted sea bass (*Dicentrarchus labrax*) and turbot (*Scophthalmus maximus*), dissolved in 3 mol l^{-1} HCl (14 µl per mg dry $CaCO_3$) and the oxalate liberated was determined by enzymatic assay (Trinity Biotech, Ireland). Oxalate excretion was calculated to be 4.6 nmol kg^{-1} h^{-1} (sea bass) and 5.5 nmol kg^{-1} h^{-1} (turbot). Because $CaCO_3$ was collected from starved fish, this implied the oxalate detected must be derived from endogenous sources and is therefore being secreted across the intestine. Furthermore, the alkaline luminal environment (pH 8–9) characteristic of the marine teleost intestine, favoring $CaCO_3$ formation, is accomplished by chloride/bicarbonate (Cl^-/HCO_3^-) anion exchangers belonging to the Slc26 gene family, specifically Slc26a3 and Slc26a6 (Kurita et al., 2008; Grosell et al., 2009; Gregorio et al., 2013). Mammalian homologs of these proteins already possess established roles in oxalate transport based on studies with knockout mice (Freel et al., 2006, 2013) and cultured human cells (Freel et al., 2009). The intestinal secretion of oxalate anticipated for marine teleosts may therefore involve these same molecular pathways (Fig. 1).

In contrast to SW species, freshwater (FW) teleosts have the opposite osmoregulatory problem of passive water gain. To cope with this, they do not actively drink or produce $CaCO_3$, but excrete copious amounts of very dilute urine (Larsen et al., 2014); hence the kidney, rather than the gut, would be the projected path for oxalate disposal in this circumstance. Euryhaline species, with the ability to occupy either FW or SW, therefore offer a simple test of the hypothesis that the osmotic challenge of SW demands oxalate secretion by the intestine. The goal of this study was to measure and compare oxalate transport by the intestine from FW- and SW-acclimated forms of the sailfin molly [*Poecilia latipinna* (Lesueur 1821)] to address whether oxalate is being actively secreted and whether this process is regulated by salinity.

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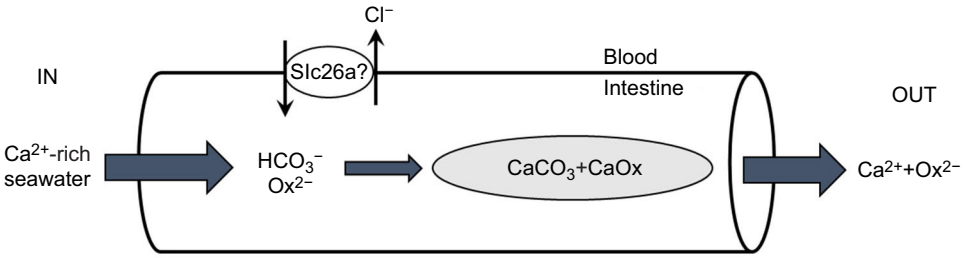

Fig. 1. The intestine of marine teleost fish is proposed to be a major pathway for eliminating oxalate (Ox^{2-}). This occurs in association with epithelial bicarbonate (HCO_3^-) secretion, possibly involving Slc26-mediated anion exchange, and intraluminal calcium carbonate ($CaCO_3$) formation which can sequester oxalate as calcium oxalate (CaOx), thus mitigating the risk for kidney stone formation.

MATERIALS AND METHODS

A total of 30 *P. latipinna* of either sex (mean±s.e.m. body mass 2.7±0.1 g) were maintained at 25±1°C in one-third-strength artificial SW (Instant Ocean®) and fed TetraMin® flake food twice daily. Over the course of 7 days, a randomly allocated cohort was gradually acclimated to either full-strength SW by the addition of artificial sea salts or FW (50 mOsm kg^{-1}) by dilution with deionized water and held there for at least 14 days prior to entering flux experiments. All experimentation was conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. For measurements of oxalate transport, fish were euthanized with an overdose of buffered MS-222 (0.5 g l^{-1}; Sigma-Aldrich), and the intestine was removed and placed in ice-cold buffer. The proximal intestine was selected for study because this portion has been reported to possess the highest rates of HCO_3^- secretion and Slc26 transporter expression (Grosell et al., 2009; Gregorio et al., 2013; Alves et al., 2019). Unidirectional oxalate flux was measured in modified Ussing chambers (P2300; Physiologic Instruments) under symmetrical, short-circuit conditions as described previously (Hatch et al., 2011; Freel et al., 2013; Whittamore and Hatch, 2017), but with the following modifications. The first 10 mm of the proximal intestine was mounted on a slider (P2303A) exposing 0.1 cm^2, bathed with 4 ml of buffer either side and maintained at 25°C while gassed with a humidified gas mixture of 0.3% CO_2/99.7 O_2. The buffer contained (in mmol l^{-1}): 156.0 Na^+, 148.4 Cl^-, 10 HCO_3^-, 5.4 K^+, 2.4 HPO_4^{2-}, 0.6 $H_2PO_4^-$, 1.2 Ca^{2+}, 0.9 Mg^{2+}, 0.9 SO_4^{2-}, 10 D-glucose (serosal) and 10 D-mannitol (mucosal), pH 7.8. To trace oxalate fluxes, 0.36 µCi ^{14}C-oxalate (specific activity 115 mCi $mmol^{-1}$; Vitrax Radiochemicals) was added to either the mucosal or serosal side, with 1 mmol l^{-1} Na_2-oxalate used to achieve the desired final concentration of 1.5 µmol l^{-1} oxalate in each chamber. Net oxalate flux (J_{net}^{Ox}) was calculated by $J_{net}^{Ox}=J_{ms}^{Ox}-J_{sm}^{Ox}$ (where J_{ms}^{Ox} and J_{sm}^{Ox} are oxalate fluxes from mucosal to serosal, and vice versa, respectively) from tissues matched on the basis of transepithelial conductance G_T (with no greater than a ±15% difference between tissue pairs). To probe the possible involvement of an anion exchange mechanism, some experiments utilized the prototypical inhibitor 4,4′-diisothiocyano-2,2′-stilbene disulfonic acid (DIDS). Adopting a paired design, these flux experiments were divided into two time periods consisting of an initial 'control' period (period I; 0–45 min) followed by a second 'test' period (period II; 60–105 min), following the addition of DIDS at 45 min. Data are presented as means±s.e.m. Significant differences ($P<0.05$) between FW- and SW-adapted fish were determined by independent *t*-test. For two-period experiments, significant changes to oxalate flux in the presence of 200 µmol l^{-1} DIDS were determined by repeated-measures one-way ANOVA and, where appropriate, Holm–Šidák *post hoc* tests (SigmaPlot v14.0, Systat Software Inc.).

RESULTS AND DISCUSSION

To assist the conservation of body water, marine teleost fish possess low rates of urine production (Larsen et al., 2014), limiting their ability to excrete oxalate (and calcium) at the risk of calcium oxalate kidney stone formation. For SW teleosts, the intestine, rather than the kidney, is hereby proposed to be the main excretory pathway for oxalate and reliant on epithelial secretion (Fig. 1). The present study set out to explore this hypothesis by comparing oxalate flux across the proximal intestine from *P. latipinna* acclimated to either SW or FW. Fig. 2 shows this epithelium supported considerable net oxalate secretion *in vitro*, but these impressive transport rates were unaffected by salinity (P=0.595) contrary to expectation. This does not, however, invalidate the proposed concept. The most compelling finding was the sheer magnitude of J_{sm}^{Ox} conferring a remarkable and unprecedented net oxalate secretion that was acutely sensitive to serosal DIDS. For marine teleosts, such high transport rates into the lumen would make oxalate available to bind with calcium from ingested SW, becoming incorporated into $CaCO_3$, which may account for its detection in carbonates collected from *D. labrax* and *S. maximus*. The excretion of oxalate may thus highlight another unique contribution of intestinal HCO_3^- secretion and $CaCO_3$ formation to the physiology of marine teleost fish alongside water absorption (Wilson et al., 2002), blood–gas transport and acid–base regulation (Cooper et al., 2010), calcium homeostasis (Wilson and Grosell, 2003) and, on a global environmental scale, the marine inorganic carbon cycle (Wilson et al., 2009).

Comparisons with conventional mammalian models emphasize this remarkable capability of the teleost intestine where oxalate is being secreted on a net basis at rates 15 and 19 times higher than mouse duodenum (Whittamore and Hatch, 2017) and rat proximal jejunum (Hatch et al., 1994), respectively, under symmetrical, short-circuit conditions in the presence of 1.5 µmol l^{-1} oxalate. Beyond serving enteric oxalate excretion, these striking findings have led to consideration of why else J_{sm}^{Ox} might be so exaggerated. One was the presence of the non-pathogenic, oxalate-degrading gut bacterium *Oxalobacter formigenes*, which has been shown to stimulate intestinal oxalate secretion in rodent models (Hatch et al., 2006, 2011). Whether the teleost intestine can also host this bacterium has not been determined, but it was ruled an unlikely factor here because the gut contents of *P. latipinna* tested negative for *O. formigenes* colonization, using the method previously described (Allison et al., 1985). Another consideration was a dependence on body mass, given the small size of *P. latipinna* (<3 g) and recent data showing HCO_3^- secretion by the teleost intestine negatively correlates with body mass (Alves et al., 2019). If oxalate were to follow a similar allometry, a reasonable assumption given that oxalate and HCO_3^- can share transporters, projected secretion rates decline with increasing body mass but remain extraordinarily high. For example, if *P. latipinna* had a body mass of 25 g, then net oxalate secretion is calculated to be 250 pmol cm^{-2} h^{-1} (~10 times greater

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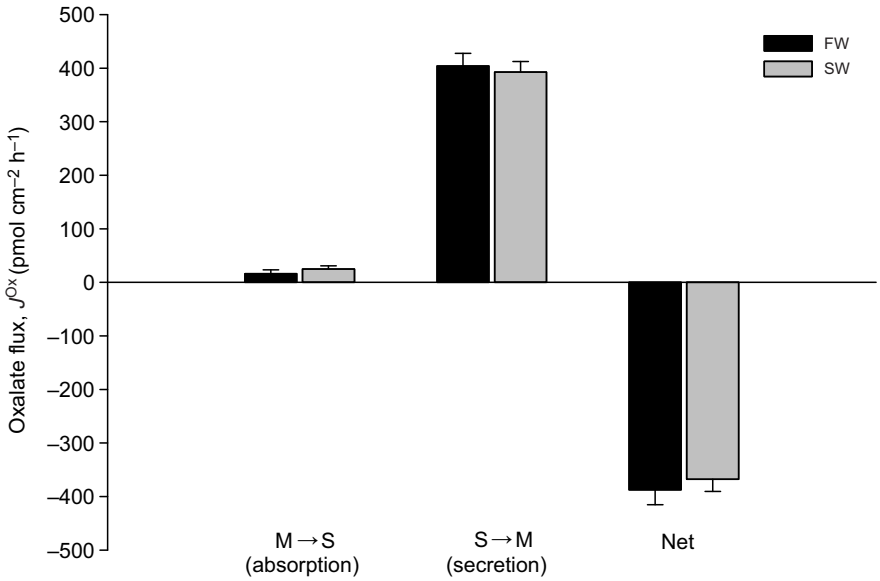

Fig. 2. Salinity does not impact oxalate flux across the euryhaline teleost intestine. A comparison of unidirectional and net oxalate flux (pmol cm^{-2} h^{-1}) under symmetrical, short-circuit conditions across isolated segments of the proximal intestine from *Poecilia latipinna* acclimated to either freshwater (FW) or seawater (SW) (*n*=6 and *n*=9 tissue pairs, respectively). Short-circuit current and transepithelial conductance were –0.03±0.07 µeq cm^{-2} h^{-1} and 25.64 ±2.58 mS cm^{-2} (FW) and –0.23±0.06 µeq cm^{-2} h^{-1} and 26.90±1.67 mS cm^{-2} (SW). M, mucosal; S, serosal.

than mouse duodenum) and 133 pmol cm^{-2} h^{-1} if body mass were 250 g (almost seven times greater than rat jejunum).

Transcellular secretion requires the coordinated efforts of transport proteins residing in the apical and basolateral membranes of enterocytes. Oxalate has been shown to be a substrate of apical anion exchangers encoded by the genes Slc26a3 and Slc26a6, which are implicated in Cl^- absorption and HCO_3^- secretion along the teleost (Kurita et al., 2008; Grosell et al., 2009; Gregorio et al., 2013) as well as the mammalian (Alper and Sharma, 2013; Seidler and Nikolovska, 2019) intestine. From studies of knockout mouse models, Slc26a3 also contributes to transcellular oxalate absorption (Freel et al., 2013) and Slc26a6 is crucial for intestinal oxalate secretion (Freel et al., 2006). Although teleost Slc26a3 has yet to be characterized, Slc26a6 cloned from mefugu (*Takifugu obscurus*) was shown to mediate Cl^-/oxalate exchange at rates comparable to the mouse homolog when expressed in *Xenopus* oocytes *in vitro* (Kato et al., 2009), but whether it might be participating in epithelial oxalate transport by the teleost intestine is not known. In the present study (Fig. 3A), DIDS was added to the mucosal bath to probe whether oxalate secretion might indeed involve a stilbene-sensitive apical anion exchanger such as Slc26a6. While J_{sm}^{Ox} displayed a significant downward trend in response to

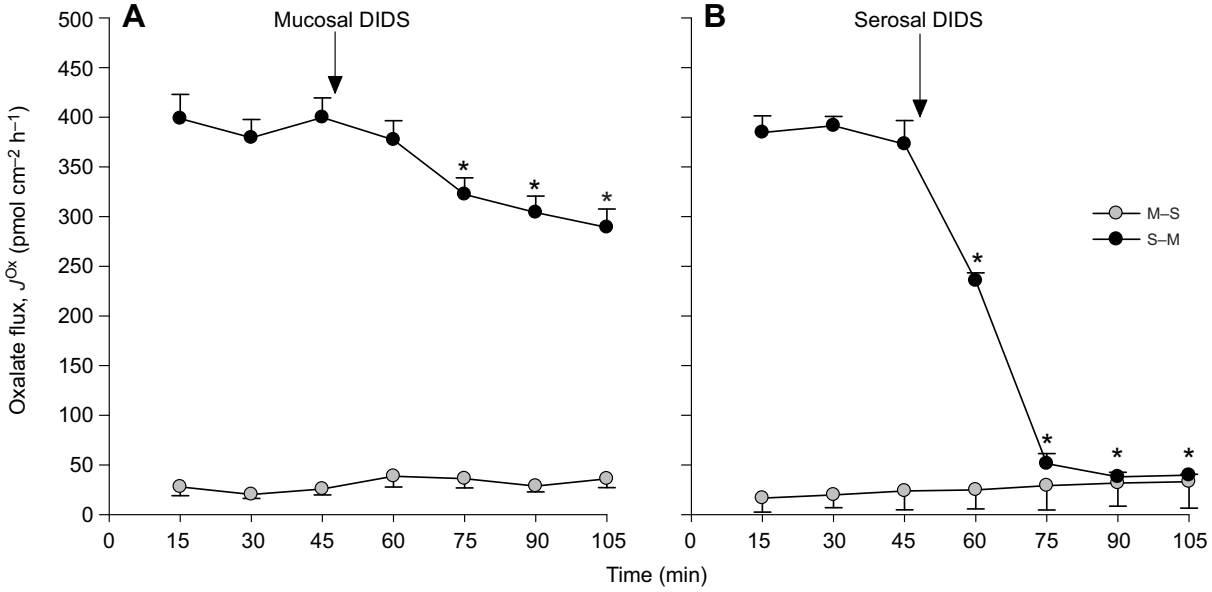

Fig. 3. Oxalate secretion across the euryhaline teleost intestine is DIDS-sensitive. The effect of 200 µmol l^{-1} DIDS on unidirectional oxalate flux (pmol cm^{-2} h^{-1}) under symmetrical, short-circuit conditions across isolated segments of the proximal intestine from *Poecilia latipinna*. Each data point represents the mean±s.e.m. from *n*=9 (mucosal DIDS) and *n*=3 (serosal DIDS) tissue pairs. An asterisk indicates a significant difference ($P \leq 0.05$) from the preceding control period (0–45 min), as determined by one-way repeated-measures ANOVA followed by Holm–Šidák multiple pairwise comparisons. (A) DIDS applied to the mucosal side of the intestinal epithelium from SW-acclimated *P. latipinna*. Short-circuit current (I_{sc}): –0.23±0.06 µeq cm^{-2} h^{-1} (control) versus –0.16±0.07 µeq cm^{-2} h^{-1} (+DIDS; 60–105 min) (paired *t*-test, *P*=0.019). Transepithelial conductance (G_T): 26.90±1.67 mS cm^{-2} (control) versus 26.12±2.17 mS cm^{-2} (+DIDS; 60–105 min) (paired *t*-test, *P*=0.605). (B) DIDS applied to the serosal side of the intestinal epithelium from FW-acclimated *P. latipinna*. I_{sc}: –0.12±0.07 µeq cm^{-2} h^{-1} (control) versus –0.09±0.09 µeq cm^{-2} h^{-1} (+DIDS) (paired *t*-test, *P*=0.524). G_T: 29.24±4.67 mS cm^{-2} (control) versus 30.54±1.10 mS cm^{-2} (+DIDS) (paired *t*-test, *P*=0.761).

DIDS the overall decrease after almost 1 h was relatively modest (22%). Conversely, when applied to the serosal bath, DIDS effectively eliminated net oxalate secretion within 20 min through inhibition of J_{sm}^{Ox} (Fig. 3B). This suggests the transport pathway at the basolateral membrane, which forms the critical rate-limiting step for transepithelial secretion and ultimately determines the magnitude of J_{sm}^{Ox}, is acutely DIDS-sensitive. The identity of the basolateral transporter(s) responsible, however, remain elusive. One candidate is the sulfate/anion exchanger, Slc26a1 (Alper and Sharma, 2013; Whittamore and Hatch, 2017). In mice, basolateral Slc26a1 was proposed to mediate intestinal oxalate secretion (Dawson et al., 2010), although subsequent work failed to confirm this (Ko et al., 2012; Whittamore et al., 2019). Another possibility is the electrogenic Na^+-HCO_3^- cotransporter (NBCe1; Slc4a4), identified as the DIDS-sensitive driver of transcellular HCO_3^- secretion (Kurita et al., 2008; Taylor et al., 2010). While oxalate is not considered a substrate of mammalian Slc4a4 (Lee et al., 2013), human and teleost homologs have shown clear differences in their transport characteristics (Chang et al., 2012), but whether this extends to selectivity for oxalate is unknown.

In summary, this is the first report of epithelial oxalate transport by a non-mammalian vertebrate, revealing extremely high rates of net secretion across the intestine of *P. latipinna* under symmetrical, short-circuit conditions *in vitro*, supporting the concept of the teleost gut serving as an important pathway for excretion of this waste metabolite. The initial hypothesis reasoned that this role was connected to the osmoregulatory demands associated with living in SW and low rates of urine production, where $CaCO_3$ formation within the gut would provide a convenient route for capturing and excreting oxalate. However, after transfer of *P. latipinna* to FW, the rate of oxalate secretion was unchanged, suggesting that the responsible transport process is not directly regulated by salinity, at least for this species. Nevertheless, such prominent secretion rates highlight a unique and previously unrecognized capability, and the key basolateral transport pathway could be abruptly blocked by DIDS. In terms of human health, characterizing the mechanisms of oxalate transport are important for understanding the various etiologies behind hyperoxaluria (elevated urine oxalate), a major risk factor for calcium oxalate kidney stone formation (Asplin, 2002). Identifying the teleost transporters responsible and what makes them so effective, relative to their mammalian forms, may offer potential insights into ongoing work investigating enteric oxalate excretion (Whittamore and Hatch, 2017).

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Competing interests
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