In vitro characterization of calcium transport along the gastrointestinal tract of freshwater rainbow trout Oncorhynchus mykiss

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(Received 7 February 2011, Accepted 15 February 2012)

Using an in vitro gut-sac technique, this study examined the mechanisms of calcium (Ca) uptake along the gastrointestinal tract (GIT) of rainbow trout Oncorhynchus mykiss. Ca uptake into three different compartments (mucous-bound, mucosal epithelium and blood space) of four distinct GIT segments (stomach, anterior intestine, mid intestine and posterior intestine) was monitored after luminal exposure to 10 mM Ca saline (radiolabelled with $^{45}$Ca). Ca transport was determined to be both time-dependent and concentration-dependent. The concentration-dependent kinetics of Ca uptake was investigated using varying luminal concentrations of Ca (1, 10, 30, 60 and 100 mM). In the blood-space compartment, Ca uptake was saturable at high Ca concentrations in the mid intestine (suggesting mediated transport), while linear uptake was found in the other gut segments. In the mucous-bound and mucosal epithelium compartments, however, saturation kinetics were found for most GIT segments, also suggesting mediated transport. Manipulation of serosal saline osmotic pressure with mannitol demonstrated that Ca uptake was not greatly affected by solvent drag. Elevated mucosal cadmium (Cd) did not appear to inhibit Ca uptake into the blood space in any of the GIT sections, and Ca uptake did not appear to be sodium dependent. Maximum transport capacities for Ca and Cd were found to be comparable between the gills and gut, but affinities were much higher at the gills (up to 3000 times).

Key words: cadmium; gut sac; sodium dependence; solvent drag.

INTRODUCTION

Calcium (Ca) is an essential metal for aquatic species, serving many functions in fishes such as maintaining homeostasis, growth, bone formation, signal transduction in the nervous system and regulation of muscle contractions. Fishes have two main routes of Ca uptake: via their gills by waterborne exposure (primary route under most natural conditions, estimated to be c. 50–80% of total intake) or across their gastrointestinal tract (GIT) by dietborne exposure (Lovelace & Podoliak, 1952; Berg, 1968; Simkiss, 1974; Perry & Wood, 1985; Handy, 1993). Under some conditions, Ca transport across the gut epithelium is the more important route, particularly in marine
fishes [20–70% of total uptake (Schoenmakers et al., 1993; Guerreiro et al., 2002)] and for freshwater fishes when waterborne Ca levels are low and in times of greater need (such as during vitellogenesis) (Rodgers, 1984; Wood & Bucking, 2011).

Branchial transepithelial Ca transport is believed to occur via passive entry into epithelial cells by means of non-voltage-gated Ca channels on the apical membranes (Perry & Flik, 1988). Transport across the basolateral membranes is thought to occur by direct (Ca-ATPase; Flik et al., 1983, 1985) or indirect active transport (Na$^+$–Ca exchange; Verbost et al., 1994).

Less is known about GIT Ca uptake. Klaren et al. (1993) suggested that Ca transport across the brush-border membrane of the intestine of tilapia Oreochromis mossambicus (Peters 1852) had both saturable and non-saturable components. Klaren et al. (1997) later showed that Ca transport across the brush-border membrane increased when ATP was supplied, and suggested that Ca transport is mediated by a P$_2$ purinoceptor. Flik et al. (1990) found that net Ca uptake in freshwater O. mossambicus was dependent on the presence of a Na$^+$ gradient for basolateral transport (as has been shown for gill transport explained above), and expression of an Na$^+$–Ca$^{2+}$ exchanger has been found in the intestine of zebrafish Danio rerio (Hamilton 1822) (On et al., 2009). Only low or negligible expression of epithelial Ca$^{2+}$ channel (ECaC) along the GIT has been found in rainbow trout Oncorhynchus mykiss intestines (Shahsavarani et al., 2006), implying the existence of a different type of apical entry mechanism compared to the gills. In Atlantic cod Gadus morhua L. 1758 (a seawater fish), Larsson et al. (1998) reported that Ca uptake along the intestine was by means of L-type voltage-gated Ca channels, which are not found in the gills.

Cadmium (Cd) is a non-essential metal [except for some diatoms; Lane & Morel (2000)], which is toxic to fishes and other aquatic organisms at low concentrations. In addition to sharing divalent charge, Cd and Ca have similar atomic sizes and radii. At low concentrations, waterborne Cd is a powerful inhibitor of branchial Ca uptake, entering through the same Ca channels in a competitive manner and blocking the basolateral transporters of the gill ionocytes (Verbost et al., 1987, 1988, 1989; Niyogi & Wood, 2004). There is considerable circumstantial evidence that Cd and Ca share common uptake pathways in the GIT of O. mykiss at least in part, though probably different from that in the gills. For example, elevated dietary Ca protects against the uptake of Cd from the food (Baldisserotto et al., 2005; Franklin et al., 2005; Ng et al., 2009), and the protective effect appears to be particularly marked in the stomach (Wood et al., 2006). In vitro experiments using isolated gut sacs demonstrated that elevated mucosal Ca inhibits Cd uptake by the stomach (Ojo & Wood, 2008) and Ca v. Cd uptake rates were closely correlated in all sections of the GIT (Klinck et al., 2009). In a recent study (Klinck & Wood, 2011), the inhibitory effects of 10 mM Ca in the mucosal saline on the concentration-dependent kinetics of Cd uptake suggested the presence of a common uptake pathway for Cd and Ca in the stomach, anterior intestine and mid intestine. Additional pharmacological and competition experiments with gut-sac preparations indicated the participation of both L-type Ca channels and lanthanum-sensitive Ca channels, and to a lesser extent, the divalent metal transporter I and a zinc transporter (Klinck & Wood, 2011). There are no studies on basolateral transport in O. mykiss, but in O. mossambicus, basolateral transport of Cd has been linked to Na$^+$–Ca$^{2+}$ exchanger, the Ca$^{2+}$-ATPase and Na$^+$, K$^+$-ATPase (Schoenmakers et al., 1992).
The objectives of the current study were to use the same gut-sac technique to characterize the concentration-dependent kinetics of Ca uptake in the four GIT segments of *O. mykiss*, to investigate several possible mechanisms of Ca transport (solvent drag and Na dependence) and to look at the possible inhibitory effects of Cd on Ca transport. The present results on the gut facilitated quantitative comparison with previous data on Ca and Cd transport in the gills of freshwater *O. mykiss*.

**MATERIALS AND METHODS**

**EXPERIMENTAL ANIMALS**

Adult *O. mykiss* with a mass of c. 250 g (Humber Springs Hatchery; www.humbersprings.com) were housed in 500 l tanks containing constantly aerated Hamilton city dechlorinated tap water (temperature 11–13°C). Water had an approximate ion composition of (in mM): 0·5 [Na⁺], 0·7 [Cl⁻], 1·0 [Ca], 0·2 [Mg²⁺] and 0·05 [K⁺], pH 7·8–8·0, dissolved organic carbon (DOC) c. 3 mg Cl⁻¹, hardness c. 140 mg l⁻¹ as CaCO₃. Fish were held at these conditions for at least 2 weeks before experimentation. Fish were fed a maintenance ration of commercial trout dried pellet food [composition: crude protein 41%, crude fat 11%, crude fibre 3·5%, calcium 1%, phosphorus 0·85%, sodium 0·45%, vitamin A 6800 IU kg⁻¹, vitamin D₂ 100 IU kg⁻¹, vitamin E 80 IU kg⁻¹ (Martins Mills Inc.; www.martinmills.com)] on alternate days, but were left unfed for c. 48 h before commencement of all experiments.

**IN VITRO GUT-SAC TECHNIQUE**

Experiments were conducted using an in vitro gut-sac technique, closely following methods described by Klinck & Wood (2011). Fish were euthanized with an overdose of tricaine methanesulphonate (MS-222) and their entire GIT was removed and immediately transferred into ice-cold Cortland saline. Visceral fat surrounding the tissue was carefully pulled away. The bile duct was tied off using surgical silk and the liver and gall bladder were excised. The GIT was subsequently divided into four distinct sections: the whole stomach, anterior intestine, mid intestine and posterior intestine. The anterior intestine represented the intestinal portion directly following the stomach up to the distal most pyloric caecum. The posterior intestine was discerned from the mid intestine by its darker colour and annular rings. Each segment of the gut was individually flushed with Cortland saline to remove any remnant chyme, food or faecal matter. The anterior end of each gut section was tied closed using surgical silk, while the posterior end was fitted with a short flared catheter made of polyethylene tubing and secured in place with more surgical silk. The resulting sac was blotted dry and weighed empty. Gut sacs were then filled via the catheter with the appropriate saline solutions in a consistent manner. Based on earlier experiments (Klinck & Wood, 2011) in which pressure was measured by a pressure transducer (Statham P23BB, Statham Instruments; www.harvardapparatus.com) attached to a transducer amplifier (Harvard Apparatus; www.harvardapparatus.com) and an oscillograph (Harvard Apparatus), a filling pressure of c. 2·0 kPa was employed. The preparations were then sealed, reweighed and transferred to Falcon tubes containing serosal saline (35 ml for stomach and anterior intestine and 11 ml for mid and posterior intestines) for either 2 or 4 h depending on the experiment. Serosal baths were aerated with a mixture of 99·7% O₂ and 0·3% CO₂, resulting in pCO₂ levels mimicking natural partial pressure in fishes blood (c. 2·3 torr). Experimental temperature was c. 16°C.

After the appropriate incubation time, gut sacs were blotted dry with paper towel and weighed a final time. A 5 ml sample of the serosal saline was collected. Remaining luminal content was drained via the catheter and gut sacs were then cut open longitudinally. Tissues were individually rinsed with 5 ml of Cortland saline followed by second rinse with 5 ml of Cortland saline containing 1 mM of EDTA. The resulting rinse solutions were saved for analysis. Gut sacs were then blotted dry with paper towel (also collected). The surface area of each GIT section was determined using methods described by Grosell & Jensen (1999).
The mucosal epithelial layer was collected by gently scraping the luminal membrane with a microscope slide, and transferred with 2 ml of distilled water into individual containers. The remaining muscle layer was also collected. A set of seven samples was collected for each gut sac: final serosal saline, final mucosal saline, saline rinse, EDTA rinse, blotting paper, muscle layer and epithelial scraping, all of which were measured separately for $^{45}\text{Ca}$ activity.

Once samples were measured for radioactivity, some activity values were added together to calculate the amount of Ca present in three different compartments of the GIT: the mucous-binding fraction (rinse solutions + blotting paper), the mucosal epithelial layer (epithelial scrapings) and blood space (serosal saline + muscle layer). The mucous-binding fraction represents the Ca ions loosely bound to the luminal mucosal surface of the gut sac. The mucosal epithelial layer determines the amount of Ca that passed across the apical surfaces of enterocytes but not through the basolateral surface. Finally, the blood-space values represent the amount of Ca that had passed through the enterocytes, and was considered a conservative estimate of the Ca that would have been absorbed by the fish (Nadella et al., 2007; Ojo & Wood, 2008; Klinck & Wood, 2011).

SALINE AND TREATMENT SOLUTIONS

Saline rinse solutions used in experiments followed the basic recipe for Cortland saline described by Wolf (1963) [in mM: NaCl 122, KCl 5, CaCl$_2$·2H$_2$O 1, MgSO$_4$·7H$_2$O 1.9, NaHCO$_3$ 11.9, NaH$_2$PO$_4$·H$_2$O 2.9, glucose 5.5 (+1 mM of EDTA for 'EDTA rinse')]. Mucosal and serosal salines were modified Cortland salines with NaHCO$_3$ and NaH$_2$PO$_4$·H$_2$O eliminated to avoid Ca precipitation (in mM: NaCl 133, KCl 5, Ca(NO$_3$)$_2$·4H$_2$O 1, MgSO$_4$·7H$_2$O 1.9, glucose 5:5) (c. 270 mOsm). All saline solutions were brought to a pH of 7-4 by adding NaOH (except in series 4 where KOH was used). Five series of experiments were conducted: a time course and spatial distribution analysis of Ca uptake (series 1), a concentration-dependent kinetic analysis of Ca uptake (series 2), the effects of solvent drag on Ca uptake (series 3), the dependency of Ca uptake on Na (series 4) and the inhibition of Ca transport by Cd (series 5). Specific experimental treatment salines are described in their corresponding figure or table captions.

For Ca treatment solutions (nominally: 1, 10, 25, 30, 60 and 100 mM), additional Ca was added as Ca(NO$_3$)$_2$·4H$_2$O (Fisher Scientific; www.fishersci.ca). Each treatment solution contained 0.5 $\mu$Ci ml$^{-1}$ of radioactive $^{45}\text{Ca}$ (as CaCl$_2$, PerkinElmer; www.perkinelmer.com). For treatments containing Cd (82 and 500 $\mu$M), Cd was added as Cd(NO$_3$)$_2$·4H$_2$O (Fisher Scientific). Concentrations of Ca and Cd in the solutions were measured using flame atomic absorption spectrophotometry (FAAS; Varian SpectraAA-220FS; www.varianinc.com). Values obtained were verified against the analytical standards (TM-15), certified by the National Research Council of Canada (www.nrc-cnrc.gc.ca). Mannitol was used to manipulate osmotic pressure gradients in series 4, and to match osmolalities in series 5, as measured by a Wescor 5100C vapour pressure osmometer (Wescor; www.wescor.com). Depletion of Ca in the mucosal saline due to transport across the GIT was not significant.

ANALYTICAL TECHNIQUES AND CALCULATIONS

The seven collected samples described above were counted for $^{45}\text{Ca}$ $\beta$-radioactivity using a scintillation counter (PerkinElmer liquid scintillation analyser, Tri-carb 2900TR). Aliquots of 1 ml of all liquid samples (final serosal saline, saline rinse and EDTA rinse) were mixed with 10 ml of Optiphase scintillation fluid (PerkinElmer) and 4 ml of water. Muscle layers, epithelial scrapings and blotting papers were all digested separately in 5 ml (or 15 ml for stomach and anterior intestine muscle layers) of 1 N HNO$_3$ for 48 h at 60$^\circ$ C. One ml of each digest was then mixed with 5 ml of Ultima Gold scintillation liquid (PerkinElmer). Samples in scintillation fluid were first kept in the dark for at least 2 h to reduce any possible effects from chemiluminescence on $\beta$-radioactivity measurement. After counting and background subtraction, sample radioactivities measured were quench-corrected using the external standard ratio method to ensure the same counting efficiency as in fluid samples.

Fluid transport rates ($R_{FT}$) were estimated gravimetrically using the formula: $R_{FT} = (M_1 - M_T)t^{-1}A_{GS}$, where $M_1$ = the initial mass of gut bags [in $\mu$l (assuming 1 mg $\approx$ 1 $\mu$l)],
$M_F$ = the final mass of gut bags after flux (in μl), $A_{GS}$ = the gut surface area (in cm$^2$) and $t$ = the duration of flux (in h). Therefore, final FTRs are expressed as μl h$^{-1}$ cm$^{-2}$.

The term specific transport rate is used to indicate the uptake rate in a particular segment or compartment. The specific transport rate of Ca (absorption rate, $R_A$) was calculated using the following equation: $R_A = x (S_A t A_{GS})^{-1}$, where $x$ = counts per minute (cpm) (the total $^{45}$Ca activity found in samples) and $S_A$ = specific activity of treatment mucosal saline (in cpm nmol$^{-1}$). Final specific transport rates are expressed as nmol Ca h$^{-1}$ cm$^{-2}$.

**STATISTICAL ANALYSES**

In series 3, either a linear or hyperbolic curve was fitted to data (using SigmaPlot software, Windows version 10.0; SigmaPlot; www.sigmaplot.com) for each gut section and compartment. The best fit was determined on the basis of $r^2$ values. Hyperbolic curves were fitted by using a single rectangular two-parameter equation: $y = ax(x + b)^{-1}$, fitted by SigmaPlot (SigmaPlot) by non-linear regression so as to estimate parameters in the Michaelis–Menten equation: $J_{in} = J_{max} [X] ([X] + K_m)^{-1}$, where $J_{in}$ is the unidirectional influx rate, $[X]$ is the substrate (Ca) concentration (in mM), $J_{max}$ is the maximal unidirectional flux rate at an infinitely high substrate concentration and the $K_m$ value is the Ca concentration providing an uptake rate equal to half $J_{max}$. The $J_{max}$ values are reported in nmol h$^{-1}$ g$^{-1}$ and $K_m$ values in mM.

Statistical differences between groups of data were assessed using the SigmaPlot programme with SigmaStat integration (10.0) (SigmaPlot). Unpaired $t$-tests (two-tailed) or one-way ANOVA followed by Tukey’s multiple comparison post hoc tests were used. All data are presented as means ± s.e. ($n = 5$), and differences between two groups were considered significant at $P < 0.05$.

**RESULTS**

**SERIES 1: TIME COURSE AND SPATIAL DISTRIBUTION OF CA UPTAKE**

The specific rates of Ca binding to the mucus for all gut sections were quantitatively lower after the 4 h incubation time compared to 2 h incubation time (Fig. 1). The largest reductions in this compartment were observed in the mid intestine (by 57%) and posterior intestine (by 55%) ($P < 0.05$ in both cases). In the stomach and anterior intestine, there were also large decreases in mucus binding by 45% ($P < 0.05$) and 38% ($P > 0.05$), respectively.

Using the average total Ca uptake rates (calculated by adding average values of each fraction together), it was determined that after 2 h only c. 0.5% of the total initial amount of luminal Ca had been transported from the anterior mucosal saline, 1.5% from the posterior intestine mucosal saline, 0.8% from the mid intestine mucosal saline and only 0.4% from the stomach saline, and therefore a loss of luminal Ca after 2 h is negligible.

The effect of longer incubation time on Ca accumulation in the mucosal epithelium was similar to the mucus-binding results, showing a significant 56% reduction in the stomach, which also had the highest specific Ca transport rate (32 nmol h$^{-1}$ cm$^{-2}$ after 2 h). In the posterior intestine, there was a 58% reduction in the specific transport rate after 4 h compared to 2 h, and in the posterior intestine, there was a 61% reduction at 4 h compared to 2 h. The length of incubation did not affect specific uptake rates into the mucosal epithelium compartment for either the anterior intestine or for the mid intestine.
Fig. 1. (a) Fluid transport rates; positive values represent net fluxes from mucosal to serosal sides, whereas negative values represent net fluxes from serosal to mucosal sides and specific uptake rates of Ca into three compartments: (b) mucus binding, (c) mucosal epithelium and (d) blood space of four gastrointestinal segments (stomach, anterior intestine, mid intestine and posterior intestine) of Oncorhynchus mykiss after either 2 h (○) or 4 h flux (■) exposure to a 9.8 mM Ca luminal treatment (series 1). *, represent significant differences ($P < 0.05$). Values are means ± s.e. ($n = 5$).

A similar trend was also seen in the blood-space compartment. The greatest reduction of uptake was seen in the mid intestine (by 56%), followed by the posterior intestine (41%) ($P < 0.05$ in both cases). The 30% reduction in Ca blood-space absorption rates in the stomach and the 25% decline in the anterior intestine were not statistically significant.

Therefore, there was an overall general reduction in the specific transport rates of Ca in most compartments and gut sections between the 2 and 4 h incubation time. FTRs for all gut sections remained consistent between the two time periods (Fig. 1). Based on these results, subsequent experiments were conducted using a 2 h incubation time.

To analyse the spatial distribution of Ca uptake along the GIT on both an absolute basis [Fig. 2(a)] and a relative basis [Fig. 2(b)], data were compiled from control preparations of series 1, 2 and 4 (all having a 10 mM Ca mucosal saline and a 2 h incubation time).

The anterior intestine had the highest total-specific Ca uptake rate per cm$^2$ (77.6 ± 6.6 nmol h$^{-1}$ cm$^{-2}$), which was calculated by adding together the three compartments (mucus-binding, mucosal epithelium and blood space). The posterior intestine had nearly an identical total-specific uptake rate of 75.9 ± 7.9 nmol h$^{-1}$ cm$^{-2}$. The stomach and mid intestine had similar total-specific transport rates to each other (42.2 ± 6.0 and 52.8 ± 5.1 nmol h$^{-1}$ cm$^{-2}$, respectively) and were both significantly lower than the anterior and posterior intestines [Fig. 2(a)].
In the stomach, Ca transported into the blood-space compartment (c. 19%) contributed the least to the total Ca flux, compared to the other compartments of this segment. On the other hand, the blood-space compartment contributed the greatest percentage of the total for the intestinal segments [c. 78% in the anterior intestine, c. 59% in the mid intestine and c. 65% in the posterior intestine; Fig. 2(b)]. Much of the Ca taken up by the stomach portion of the GIT remained loosely bound to the mucus layer (c. 64%). Lesser amounts were mucous-bound in the three intestinal segments (between 17 and 38%). Ca accumulation in the mucosal epithelium
UPTAKE SERIES 2: CONCENTRATION-DEPENDENT KINETICS OF Ca UPTAKE

A saturating curve for the specific Ca uptake rate into the blood space was only found for the mid intestine \( (r^2 = 0.62; J_{\text{max}} = 347.3 \text{ nmol h}^{-1} \text{ cm}^{-2}; K_m = 78.9 \text{ mM}) \), whereas the specific uptake rates in all other sections had a better fit to a linear curve (Fig. 3). Similar to the series 1 data, the stomach was found to have the lowest uptake rates into the blood space \( (r^2 = 0.71; \text{slope} = 1.17 \text{ nmol h}^{-1} \text{ cm}^{-2} \text{ per mM}) \) across all concentrations compared to intestinal portions of the GIT. The anterior intestine had a slope of 5.59 \text{ nmol h}^{-1} \text{ cm}^{-2} \text{ per mM} \( (r^2 = 0.86) \) and the posterior intestine had a slope of 7.88 \text{ nmol h}^{-1} \text{ cm}^{-2} \text{ per mM} \( (r^2 = 0.77) \), both of which were greater than those in the mid intestine (Fig. 3).

For the mucous-binding compartment, saturable kinetics were seen in the stomach segment \( (r^2 = 0.75; J_{\text{max}} = 255.9 \text{ nmol h}^{-1} \text{ cm}^{-2}; K_m = 74.7 \text{ mM}) \), anterior compartment in the stomach was nearly equivalent to the absorption rate into the blood space \( (c. 19\%) \) [Fig. 2(a)]. This compartment, however, made up only a small percentage of the total Ca taken up in the intestinal segments \( (c. 5, 3 \text{ and } 4\% \text{ of the total for the anterior intestine, mid intestine and posterior intestine, respectively}) \).

Because each gut segment varies in surface area, it is important to take into account these differences when ranking gut section in terms of their contributions towards total body uptake (Table I). For example, the mid intestine on area-specific basis \( (\text{nmol h}^{-1} \text{ cm}^{-2}) \) had a total-specific Ca uptake rate 25% higher than that found in the stomach, but when surface area is accounted for (so as to calculate a total transport rate for the entire GIT in \text{nmol h}^{-1}) the order of importance changes, with the stomach having a two-fold higher total transport rate than the mid intestine because of its much larger surface area \( (\text{average of 11.8 cm}^2 \text{ compared to 4.7 cm}^2 \text{ of the mid intestine}) \). The combined average total Ca uptake rate for the whole GIT was found to be 2578 \text{ nmol h}^{-1} \text{ (for a c. 250 g fish)} \text{. The anterior intestine had both the highest specific rate per cm}^2 \text{, as well as the highest absolute total transport rate (1346 nmol h}^{-1} \text{ or 52\% of the total), and the highest specific transport rate into the blood-space compartment (1057 nmol h}^{-1} \text{ or 41\% of the total}) \text{ (Table I)\text{.}}

<table>
<thead>
<tr>
<th>Surface area (cm$^2$)</th>
<th>Total area-specific Ca uptake rate (nmol h$^{-1}$ cm$^{-2}$)</th>
<th>Total Ca uptake rate (nmol h$^{-1}$) into the blood-space compartment (nmol h$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stomach 11.8 ± 0.7</td>
<td>42.2 ± 6.0</td>
<td>82.6 ± 9.7</td>
</tr>
<tr>
<td>Anterior intestine 17.2 ± 0.8</td>
<td>77.6 ± 6.6</td>
<td>1056.9 ± 113.8</td>
</tr>
<tr>
<td>Mid intestine 4.7 ± 0.2</td>
<td>52.8 ± 5.1</td>
<td>149.1 ± 16.0</td>
</tr>
<tr>
<td>Posterior intestine 6.7 ± 0.2</td>
<td>75.9 ± 7.9</td>
<td>328.8 ± 33.9</td>
</tr>
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**SERIES 2: CONCENTRATION-DEPENDENT KINETICS OF Ca UPTAKE**

Table I. Average surface area and total Ca uptake rates \( (\text{mean ± s.e.}) \text{ (}\text{n} = 14–15\text{)} \text{ for each gastrointestinal tract segment of Oncorhynchus mykiss from series 1 \text{ (exposed luminally to 10 mM Ca for 2 h)}) \text{, given in three different units: total area-specific Ca uptake rate, \text{i.e. all fractions combined on a per unit surface area per hour basis, total Ca uptake rate of all fractions combined on an absolute basis per hour and total Ca transport rate into the blood space only on an absolute basis per hour}) \text{. For example, the mid intestine had both the highest specific rate per cm}^2 \text{, as well as the highest absolute total transport rate (1346 nmol h}^{-1} \text{ or 52\% of the total), and the highest specific transport rate into the blood-space compartment (1057 nmol h}^{-1} \text{ or 41\% of the total}) \text{ (Table I)\text{.}}
Fig. 3. Differences in fluid transport rates in four segments (stomach, anterior intestine, mid intestine and posterior intestine) of the gastrointestinal tract (GIT) of *Oncorhynchus mykiss* and Ca uptake rates into three compartments: (a) mucus binding, (b) mucosal epithelium and (c) blood space of the four GIT segments when exposed to mucosal saline of varying concentrations of Ca (in mM: 1.2, 8.0, 24.4, 45.4 and 91.4) (in series 2). Kinetic relationships were either linear or could be defined by a Michaelis–Menten equation $J = J_{\text{max}} [X] ([X] + K_m)^{-1}$. Values are means ± s.e. ($n = 5$).

intestine ($r^2 = 0.67; J_{\text{max}} = 521.0$ nmol h$^{-1}$ cm$^{-2}; K_m = 309.1$ mM) and posterior intestine ($r^2 = 0.67; J_{\text{max}} = 362.0$ nmol h$^{-1}$ cm$^{-2}; K_m = 168.7$ mM). Saturation was also found for the mucosal epithelium compartment of the stomach ($r^2 = 0.74; J_{\text{max}} = 90.7$ nmol h$^{-1}$ cm$^{-2}; K_m = 262.2$ mM), mid intestine ($r^2 = 0.47; J_{\text{max}} = 23.6$ nmol h$^{-1}$ cm$^{-2}; K_m = 182.0$ mM) and posterior intestine ($r^2 = 0.46; J_{\text{max}} = 12.2$ nmol h$^{-1}$ cm$^{-2}; K_m = 62.8$ mM). A linear curve fitted the data better for the mucous-binding and blood-space compartments of the anterior intestine (Fig. 3).

The FTRs in all gut sections and for all Ca treatment groups from this series are presented in Table II. The stomach consistently had a negative net flux (*i.e.* secretion into the mucosal compartment) regardless of the Ca concentration, with an average FTR of 4.71 μl h$^{-1}$cm$^{-2}$. For the three intestinal segments, there was a general trend for positive net fluid uptake rates from mucosal to serosal compartments at lower
mucosal Ca concentrations (1, 10 and 30 mM), with a reversal to negative fluid fluxes at higher mucosal Ca concentrations (60 and 100 mM). Significant differences \((P < 0.05)\) in FTRs caused by Ca levels were noted only in the mid intestine.

**SERIES 3: SOLVENT-DRAG EFFECT**

When the osmolality of the serosal saline was increased from 290 to 492 mOsm kg\(^{-1}\) by addition of mannitol, fluid transport was reversed from a small negative flux to high positive rates of transport from mucosal to serosal compartments (Fig. 4). Rather than the suspected possible increases due to solvent drag, the elevated serosal osmolality resulted in significant reductions in specific Ca uptake rates into the mucosal epithelium of the stomach \((c. 45\%)\) and anterior intestine \((c. 47\%)\) and into the blood space of the stomach \((c. 58\%)\). There was, however, a significant increase \((37\%)\) in specific Ca uptake rate into the blood space of the anterior intestine \((P < 0.05)\).

**SERIES 4: NA-FREE SALINE TREATMENT**

Removal of Na from the luminal and serosal salines resulted in a significant increase in FTR in the anterior and mid intestines \((by \ c. 3 \text{ fold and } c. 2 \text{ fold, respectively})\) (Table III). The absence of Na had no effect on specific Ca transport in either the mucosal epithelium or the blood-space compartments. The only effect seen in the mucous-binding fraction was in the anterior intestine where the treatment saline caused a significant increase \((by 32\%)\).

**SERIES 5: INHIBITION OF CA TRANSPORT BY Cd**

Overall, the concentration of Cd in the mucosal saline had little effect on specific Ca uptake rates among the two different experimental groups: 25 mM Ca + 82 \(\mu\)M Cd and 25 mM Ca + 500 \(\mu\)M Cd, compared with the control group (25 mM Ca) (Fig. 5). There were a few notable differences, however, one being the large reduction in specific Ca uptake found in the mucous-binding fraction of the anterior intestine.
CA TRANSPORT ALONG GUT OF O. MYKISS

Fig. 4. Differences in uptake rates of Ca into four gastrointestinal segments (stomach, anterior intestine, mid intestine and posterior intestine) (a) fluid transport rates and three different compartments: (b) mucus binding, (c) mucosal epithelium and (d) blood space of Oncorhynchus mykiss, when exposed to serosal salines of varying osmolality (series 3 experiment). All gut sacs were exposed to 83.9 mM Ca. *, significant differences between the two treatment groups ($P < 0.05$). Control gut-sac (□) serosal saline was not adjusted (290 mOsm kg$^{-1}$), while the osmolality of the treatment saline (■) was raised to 492 mOsm kg$^{-1}$ by the addition of mannitol. Osmolality was verified using an osmometer (Wescor 5100C Vapor Pressure Osmometer; www.wescor.com). For fluid transport rates, positive values represent net fluxes from mucosal to serosal sides; negative values represent net fluxes from serosal to mucosal sides. Values are presented as means ± s.e. ($n = 5$).

(by between c. 65 and 75% compared to controls) (Fig. 5). In the mid intestine, there was also a significant difference between the two Cd treatments, with the lower Cd concentration causing a higher Ca uptake rate into the blood space, but there was no difference between either of the Cd treatments and the control group. No changes in Ca accumulation were observed in the mucosal epithelium compartment and the only significant difference observed in FTR was the higher value in the posterior intestine for the 25 mM Ca + 82 μM Cd treatment relative to the 25 mM Ca control treatment (Table IV).

DISCUSSION

GASTROINTESTINAL CA TRANSPORT

The decrease of Ca uptake rates over time was somewhat unexpected as it had been previously reported by Nadella et al. (2006) that for another essential metal (Cu), uptake rates remain relatively constant over 4 h. Based on these Cu results and the consistent FTRs over time in their and the present preparations, gut cell
death is probably not an explanation. The reduced rate of Ca uptake over the longer time period was not due to a decrease in mucosal saline Ca concentration as this loss proved to be negligible. A more probable explanation is that in these in vitro preparations there was a lack of continual delivery of essential molecules normally supplied by the circulatory system in vivo. For instance, Ca transport may have slowed as the GIT tissues became depleted of ATP, as the presence of ATP has been linked with basolateral Ca transport (Flik et al., 1997). Regardless, to avoid this complication of rate change with time, all subsequent experiments used the same incubation time of 2 h.

When the specific Ca uptake rates (on an area-specific basis) of the three measured compartments of fish from the 2 h exposure in series 1 were added together, all gut section totals fell within a very narrow range (<2-fold variation amongst segments). As has been noted by Ojo & Wood (2007) and Klinck & Wood (2011), however, using an area-specific unit of measurement probably leads to an underestimation of the importance of the stomach and anterior intestine due to their relatively large surface areas, and an overestimation of the importance of the mid and posterior intestines, which have much less surface area. To compensate for this, the total measured surface areas were taken into account and total transport rates were expressed based as nmol h\(^{-1}\) cm\(^{-2}\) for a c. 250 g fish. The order of relative importance expressed in these units for total Ca transport rate was found to be anterior intestine > posterior intestine > stomach > mid intestine. The rank of importance for Cd using the same variables is similar; however, the order of the posterior intestine and anterior intestine was reversed. These similarities may further link the uptake mechanisms between Ca and Cd (Klinck et al., 2009). Traditionally, the intestinal portion of the GIT has been considered to be the most important site of nutrient and ion absorption, whereas the stomach’s primary role has been limited to mechanical and acidic digestion. This theory is supported by the rank order of the GIT segments. It therefore would not be surprising if the intestine has a greater number of Ca uptake sites [as has been suggested for Cu (Nadella et al., 2006) and Cd (Klinck & Wood,

<table>
<thead>
<tr>
<th>Gut section</th>
<th>Treatment</th>
<th>FTR (μl h(^{-1}) cm(^{-2}))</th>
<th>Mucus binding (nmol h(^{-1}) cm(^{-2}))</th>
<th>Mucosal epithelium (nmol h(^{-1}) cm(^{-2}))</th>
<th>Blood space (nmol h(^{-1}) cm(^{-2}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stomach</td>
<td>Control</td>
<td>-2.17 ± 0.89</td>
<td>17.48 ± 1.70</td>
<td>1.76 ± 0.37</td>
<td>4.57 ± 0.86</td>
</tr>
<tr>
<td></td>
<td>Na-free</td>
<td>-1.57 ± 0.24</td>
<td>16.96 ± 2.64</td>
<td>1.17 ± 0.06</td>
<td>6.54 ± 0.96</td>
</tr>
<tr>
<td>Anterior intestine</td>
<td>Control</td>
<td>5.24 ± 2.57</td>
<td>11.52 ± 0.63</td>
<td>4.01 ± 0.16</td>
<td>49.96 ± 12.40</td>
</tr>
<tr>
<td></td>
<td>Na-free</td>
<td>15.25 ± 2.32*</td>
<td>16.85 ± 1.72*</td>
<td>3.09 ± 0.36</td>
<td>61.34 ± 6.29</td>
</tr>
<tr>
<td>Mid intestine</td>
<td>Control</td>
<td>4.55 ± 1.21</td>
<td>19.65 ± 2.78</td>
<td>1.06 ± 0.03</td>
<td>31.12 ± 6.28</td>
</tr>
<tr>
<td></td>
<td>Na-free</td>
<td>10.02 ± 1.40*</td>
<td>23.21 ± 4.35</td>
<td>1.84 ± 0.11</td>
<td>43.22 ± 14.99</td>
</tr>
<tr>
<td>Posterior intestine</td>
<td>Control</td>
<td>4.60 ± 1.11</td>
<td>19.20 ± 3.88</td>
<td>1.51 ± 0.32</td>
<td>47.20 ± 3.75</td>
</tr>
<tr>
<td></td>
<td>Na-free</td>
<td>8.97 ± 2.32</td>
<td>16.44 ± 2.69</td>
<td>2.81 ± 0.67</td>
<td>47.38 ± 6.44</td>
</tr>
</tbody>
</table>

*aSignificant differences compared to respective controls (P < 0.05).
Fig. 5. Differences in uptake rates of Ca in four distinct segments (stomach, anterior intestine, mid intestine and posterior intestine) of the gastrointestinal tract and in three different compartments: (a) mucous-binding, (b) mucosal epithelium and (c) blood space of *Oncorhynchus mykiss* in response to additions of Cd to the mucosal saline (series 5 experiment). Gut sacs were luminaly exposed to either 25·4 mM Ca (○) or 25·6 mM Ca plus either 81·8 μM Cd (□) or 27·2 mM Ca plus 499·9 μM Cd (■). An appropriate amount of mannitol was added to the serosal saline to ensure similar osmolality levels between treatment solutions and serosal saline (c. 315 mOsm). Where lowercase letters are shown, means not sharing the same letter are significantly different from one another (P < 0·05). Values are means ± s.e. (n = 5).

A recent *in vivo* feeding experiment by Bucking & Wood (2007), however, found that the stomach of *O. mykiss* contributed the greatest Ca (as well as Na⁺, Mg⁺²) uptake of all GIT segments. Using the gut-sac technique in this study, the stomach actually had the lowest rates of absorption into the blood space (on a per area-specific basis). Perhaps, the differences can be explained in that *in vivo* the stomach has by far the highest fluid phase Ca concentration (up to 50 mM), and greatest latency of chyme movement (after ingestion of a single meal) (Bucking & Wood, 2007).

In comparison to marine fishes, the Ca uptake rates from freshwater *O. mykiss* in series 1 are about half as great. Sundell & Björnsson (1988) estimated that the
Table IV. Average fluid transport rates (means ± s.e.) (n = 5) along the gastrointestinal tract of *Oncorhynchus mykiss* from series 5 (inhibition of Ca transport by Cd). Positive values represent net fluxes from mucosal to serosal sides; negative values represent net fluxes from serosal to mucosal sides. Where lowercase superscript letters are shown, means not sharing the same letter are significantly different from one another within a segment (P < 0.05).

<table>
<thead>
<tr>
<th>Gut section</th>
<th>Treatment</th>
<th>Fluid transport rates (μl h⁻¹cm⁻²)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>25 mM Ca</td>
<td>25 mM Ca + 82 μM Cd</td>
</tr>
<tr>
<td>Stomach</td>
<td>–2.4 ± 0.5</td>
<td>–2.7 ± 0.8</td>
</tr>
<tr>
<td>Anterior intestine</td>
<td>23.6 ± 3.4</td>
<td>20.7 ± 2.6</td>
</tr>
<tr>
<td>Mid intestine</td>
<td>9.2 ± 1.8</td>
<td>7.7 ± 1.8</td>
</tr>
<tr>
<td>Posterior intestine</td>
<td>6.7 ± 1.7a</td>
<td>16.9 ± 2.7b</td>
</tr>
</tbody>
</table>

Ca influx rate across a portion of the intestine (approximately equivalent to the mid intestine in this present study) of *G. morhua* (marine fish) was c. 2.6 μmol Ca h⁻¹ kg⁻¹, whereas the mid intestine of fish from series 1 had rates of c. 1 μmol Ca h⁻¹ kg⁻¹ (based on an approximate average fish mass of 250 g). Björnsson & Nilsson (1985) calculated an approximate *in vivo* Ca uptake rate for the entire *G. morhua* GIT to be 20 μmol Ca h⁻¹ kg⁻¹, while in this study it is estimated that *O. mykiss* have a rate of 10.3 μmol Ca h⁻¹ kg⁻¹. These results highlight the differences in GIT Ca uptake between freshwater and seawater fishes.

In terms of concentration-dependent kinetics, the results from series 2 suggest that there are both saturable (facilitated transport) and non-saturable (passive) uptake sites along the GIT, indicating different types of Ca transporters and different routes of absorption. Similarly, both a channel-mediated mechanism and diffusive Ca uptake have been suggested for Ca absorption in intestines of rats (Miller & Bronner, 1981; Miller et al., 1982; Takito et al., 1990; Bronner, 1991). In *O. mykiss* GIT, biphasic relationships (uptake kinetics containing both a saturable and a linear component) have been shown for Ni (Leonard et al., 2009) and for Cu (Nadella et al., 2006) using the same *in vitro* gut-sac technique. The Ca transport was also found to be biphasic in brush-border membrane vesicles from the intestine of *O. mossambicus* at concentrations <5 mM (Klaren et al., 1993). Sundell & Björnsson (1988) found saturable (accounting for c. 60% of total uptake) and non-saturable components of Ca uptake (and efflux) in *G. morhua*. Larsson et al. (1998) gave further support for this using isolated intestinal cells, and suggested the saturable component was from transport via L-type Ca channels. It is possible that the saturable component found in the experiment described above by Klaren et al. (1993) may have been missed in the blood-space results of this present study due to the comparably higher Ca concentrations used. On the other hand, it could be argued that the range of Ca concentrations (1, 10, 25, 30, 60 and 100 mM) was not high enough to observe saturation, although this is doubtful since concentrations >100 mM are unlikely seen in diets of wild freshwater fishes. The results of the present study in part support the findings of a transport-mediated pathway reported in previous literature, showing saturation kinetics in the mucosal epithelium of all the gut segments (with the exception of the anterior intestine). It was only in the mid intestine, however, that specific Ca transport into the blood space fits a Michaelis–Menten equation.
(\(J_{\text{max}} = 347.3\ \text{nmol h}^{-1}\text{cm}^{-2};\ K_m = 78.9\ \text{mM}\)), thereby indicating that differences in transport mechanisms exist between different segments of the GIT.

With increasing luminal Ca concentration levels in series 2, FTRs decreased or reversed due to osmolality differences across the gut tissue. These changes in fluid transport could have potentially affected Ca uptake rates due to an opposing force created by the direction of fluid flow (i.e. a solvent drag effect). Tanrattana et al. (2004) reported that solvent drag of Ca occurred in the rat duodenum (first section of small intestine), thereby favouring Ca absorption. In contrast, changes in FTR by osmotic manipulation had only small effects on Ca transport rates in the present study. There was, however, an increase in Ca uptake into the blood space of the anterior intestine, which could indicate some influence of solvent drag effect in this compartment of this specific GIT segment. Overall, a solvent drag effect is probably not a major player in Ca transport in most sections of the GIT of *O. mykiss*.

Using an Na-free saline solution, the Na dependence of Ca absorption was studied. Initial trials demonstrated that unilateral Na\(\text{\textsuperscript{+}}\)-free conditions could not be maintained for more than a few minutes, because of rapid Na\(\text{\textsuperscript{+}}\) entry from the contralateral solutions. Exposure of gut sacs to an Na-free saline had no effect on Ca transport in the stomach, mid intestine and posterior intestine. In the anterior intestine, the absence of Na increased Ca mucus-binding. These findings were surprising given that Ca transport across basolateral epithelia of enterocytes has been reported to be strongly associated with Na\(\text{\textsuperscript{+}}\)–Ca\(\text{\textsuperscript{2+}}\) exchange in many studies (Taylor, 1989; Flik et al., 1990; Schoenmakers et al., 1992, 1993), and therefore Na\(\text{\textsuperscript{+}}\)-dependent. The organic cation N-methyl-D-glucamine (NMDG) has been commonly used to replace extracellular Na in experimental studies. Mroz & Lechene (1993) found that replacing Na with NMDG\(\text{\textsuperscript{+}}\) in a fluid bath containing goldfish *Carassius auratus* (L. 1758) hair cells caused cells to lose Na, K and Cl and caused pH to decrease. If enterocytes are losing Na and Cl, this would explain the significant increase in FTR in the anterior intestine and mid intestine (and the trend seen in the posterior intestine) due to osmotic pressure changes. Perhaps, clearer results would have been seen if a serosal saline lacking Na and a luminal saline containing Na, and *vice versa*, had been used, but this proved impractical.

Spatial distribution for Ca uptake was very similar to that found by Klinck & Wood (2011) for Cd uptake, adding to mounting evidence that Ca and Cd are taken up by a similar pathway (Schoenmakers et al., 1992; Baldisserotto et al., 2004, 2005; Franklin et al., 2005; Klinck & Wood, 2011). For example, in the stomach the greatest percentage of both metals was found loosely bound to the mucous layer (c. 64% for Ca and c. 50% for Cd), and the remaining Ca and Cd was equally distributed between the mucosal epithelium and blood-space compartments (c. 19% for Ca and c. 25% for Cd). In the intestine, for both Ca and Cd, <6% of the total accumulation was found in the mucosal epithelium compartment, while the majority was found in the blood-space fraction. Compared to Cd, Ca had a higher percentage (c. 2–3 times as much) in the mucous-binding compartment for the mid and posterior intestines. This may be because the affinity for Cd is much higher (i.e. lower \(K_m\) value) compared to Ca.
INHIBITION OF CA TRANSPORT BY Cd

Many studies have documented the inhibitory effect of elevated mucosal Ca on Cd uptake in the GIT both in vitro and in vivo, but the possible reciprocal influence of mucosal Cd on Ca uptake has been largely overlooked. The concentrations of Ca and Cd used here were based on measurements of $K_m$ for the transport of these two metals. At 25 mM, Ca concentration was at or below $K_m$ values measured in series 2 of the present study, and at 82 and 500 μM, Cd concentrations were at or above $K_m$ values measured by Klinck & Wood (2011). Thus, if Cd and Ca compete for the same transporter, inhibition of Ca uptake by Cd would have been expected. Uptake rates of Ca in the mucous-binding fraction of the anterior intestine decreased when treated with both Cd concentrations (82 and 500 μM), providing some evidence of competitive inhibition. It was surprising therefore to find that Ca uptake rates in all compartments of the stomach and posterior intestine, and most of the compartments of the anterior and mid intestine, were unaffected by either of the Cd treatments. Ostensibly, this indicates that Cd is not transported via Ca transporters on the apical membrane. It remains possible, however, that Cd could exert toxic effects on basolateral Ca transport via Ca-ATPase (Flik et al., 1983, 1985) and Na$^+-$Ca exchanger (Verbost et al., 1994), and that such effects were not seen in the present experiments because of access limitation. If true, this might mean that bloodborne Cd may be more toxic to the gut compared to dietborne Cd. It is also possible that the ratios between Cd and Ca may not have been high enough (Cd concentrations were 50–300 times lower than Ca), although they were certainly within the ranges of the ratios of the measured $K_m$ values. Breitwieser et al. (2004) also list similar ratios for the $K_m$ of Cd and Ca at the Ca-sensing receptors. Overall, these data suggest that the interactions between Ca and Cd and their transport along the GIT of O. mykiss are more complicated than simple competitive inhibition, and therefore this is another difference from transport mechanisms at the gills (Niyogi & Wood, 2004). Clearly, there is a need for further investigation into dietary Ca transport in fishes and the effects of Cd on its uptake.

QUANTITATIVE COMPARISON OF CA AND Cd TRANSPORT AT THE GIT V. THE GILLS

If the blood-space data of the mid intestine from this study are extrapolated to represent the total amount of dietary Ca uptake (by multiplying the $J_{max}$ value by the total surface area of the GIT and then dividing by the mass of the whole fish), it is possible to make a rough comparison with gill Ca uptake kinetics which have been reported by Niyogi & Wood (2004) [Fig. 6(a)]. This can also be done for Cd using gut data presented by Klinck & Wood (2011) and gill data from Niyogi & Wood (2004) [Fig. 6(b)]. Interestingly, the $J_{max – gut}$ and $J_{max – gill}$ values were similar for both Ca and Cd, with Ca having much higher values (for Ca: $J_{max – gut} = 54.2$ nmol h$^{-1}$ g$^{-1}$ and $J_{max – gill} = 188.7$ nmol h$^{-1}$ g$^{-1}$; for Cd: $J_{max – gut} = 0.27$ nmol h$^{-1}$ g$^{-1}$ and $J_{max – gill} = 0.40$ nmol h$^{-1}$g$^{-1}$). Therefore, the overall transport capacities for Ca and Cd are fairly similar in the gut and gills of O. mykiss. When the $K_m$ values (concentration of substrate at which half the transporters are saturated, which is a measure of substrate-binding affinity) are compared, however, there is about a 3000 times higher affinity for Cd at the gills compared to that at the gut ($K_m – gill = 31$ nM, $K_m – gut$ c. 102 000 nM) and about a 315-fold higher affinity for Ca at the gills.
Fig. 6. (a) Ca and (b) Cd uptake kinetics in *Oncorhynchus mykiss* at the gill (△, ...) (adapted from Niyogi & Wood, 2004) and for the gastrointestinal tract (○, ...) using extrapolated data from the mid intestine data [for Ca using data presented in Fig. 3, and for Cd using data from Klinck & Wood (2011)]. The kinetic relationships are defined by a Michaelis–Menten equation $J_{in} = J_{max} [X] ([X] + K_m)^{-1}$, where $J_{in}$ is the unidirectional influx rate (in nmol h$^{-1}$ g$^{-1}$), [X] is the substrate (Ca or Cd) concentration, $J_{max}$ is the maximum transport rate when the system is saturated with substrate and the $K_m$ value (...) is the concentration providing an uptake rate equal to half $J_{max}$. ...
(\(K_m\text{–gill} = c.\ 244\ \mu M\)) than at the gut (\(K_m\text{–gut} = c.\ 77\ 000\ \mu M\)). It is interesting to note that these different \(K_m\) values roughly correspond to differences in metal concentrations normally present in water (Cd = nM range, Ca = \(\mu M\) range) vs. those normally present in chyme (Cd = \(\mu M\) range, Ca = mM range) (Bucking & Wood, 2007). Transport affinities appear to be set at appropriate values for the concentrations normally encountered (Perry & Wood, 1985; Niyogi & Wood, 2004; Baldisserotto et al., 2005; Bucking & Wood, 2007) at the two uptake surfaces.

This research was supported by an NSERC Discovery Grant to C.M.W. J.S.K. is supported by an NSERC postgraduate scholarship, and C.M.W. is supported by the Canada Research Chair Program. We thank two anonymous reviewers, and also S. Klinck and P. Gillis for their constructive comments, and S. Nadella and L. Diao for their technical help. All experiments were in compliance with regulations set by the Canadian Council on Animal Care.

References


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