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RESEARCH ARTICLE

The influence of environmental calcium concentrations on calcium flux, compensatory drinking and epithelial calcium channel expression in a freshwater cartilaginous fish

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SUMMARY

Calcium metabolism and mRNA levels of the epithelial calcium channel (ECaC) were examined in a freshwater cartilaginous fish, the lake sturgeon *Acipenser fulvescens*. Lake sturgeon were acclimated for ≥2 weeks to 0.1 (low), 0.4 (normal) or 3.3 (high) mmol l^{-1} environmental calcium. Whole-body calcium flux was examined using ^{45}Ca as a radioactive marker. Net calcium flux was inward in all treatment groups; however, calcium influx was greatest in the low calcium environment and lowest in the high calcium environment, whereas efflux had the opposite relationship. A significant difference in the concentration of ^{45}Ca in the gastrointestinal tract (GIT) of fish in the low calcium environment led to the examination of drinking rate and calcium flux across the anterior-middle (mid) intestine. Drinking rate was not different between treatments; however, calcium influx across the mid-intestine in the low calcium treatment was significantly greater than that in both the normal and high calcium treatments. The lake sturgeon ECaC was 2831 bp in length, with a predicted protein sequence of 683 amino acids that shared a 66% identity with the closest sequenced ECaCs from the vertebrate phyla. ECaC mRNA levels were examined in the gills, kidney, pyloric caeca, mid-intestine and spiral intestine. Expression levels were highest in the gills, then the kidneys, and were orders of magnitude lower in the GIT. Contrary to existing models for calcium uptake in the teleost gill, ECaC expression was greatest in high calcium conditions and kidney ECaC expression was lowest in low calcium conditions, suggesting that cellular transport mechanisms for calcium may be distinctly different in these freshwater cartilaginous fishes.

Key words: calcium, epithelial calcium channel (ECaC), flux, intestine, sturgeon, calcium metabolism.

INTRODUCTION

The regulation and balance of ionic calcium (Ca^{2+}) is vital to all organisms. Ca^{2+} is necessary for a multitude of physiological processes and is important as a structural element in both invertebrates and vertebrates. Aquatic vertebrates have a source of ambient Ca^{2+} in water, unlike terrestrial vertebrates, which can only uptake Ca^{2+} *via* ingestion (Flik et al., 1995). Fishes, as aquatic vertebrates, have either a bony skeleton or a cartilaginous skeleton. While bony fishes are abundant in both freshwater and seawater environments, cartilaginous fishes generally occupy high-calcium marine environments, and examples of cartilaginous fishes inhabiting freshwater environments are comparatively rare (Ballantyne and Robinson, 2010; Compagno, 1990). Interestingly, many of the fishes with this body design and life history are primitive fishes, which are also known to maintain very low circulating concentrations of Ca^{2+} (Allen et al., 2009c).

Fishes are able to maintain plasma Ca^{2+} concentrations within fairly narrow ranges, although the ranges are generally not as narrow as in terrestrial vertebrates (Dacke, 1979; Flik and Verbost, 1993). Internal Ca^{2+} concentrations are maintained by a balance between influx and efflux. However, the influence of environmental calcium concentration on Ca^{2+} regulation has not been studied extensively in the primitive fishes, and may pose particular challenges to primitive cartilaginous fishes inhabiting fresh water, such as the acipenserids or sturgeons.

In fishes studied to date, the uptake of Ca^{2+} is achieved through the gills and the gastrointestinal tract (GIT) (Flik and Verbost, 1993), although the skin may play a role as well (Perry and Wood, 1985). The predominant uptake site is at the gills (Flik and Verbost, 1993; Flik et al., 1995), through mitochondria-rich cells and pavement cells (Shahsavarani and Perry, 2006), with the GIT playing a much smaller role (Bucking and Wood, 2007; Flik and Verbost, 1993; Sundell and Björnsson, 1988). In the gills, Ca^{2+} entry is a passive process, whereby Ca^{2+} enters epithelial cells *via* apical channels (Hwang and Lee, 2007), driven by very low intracellular concentrations (Flik, 1997). Once in the cell, Ca^{2+} is bound to proteins and actively pumped across the basolateral membrane into the blood by Ca^{2+}-specific ATPases or exchanged for Na^+ in Na^+/Ca^{2+} exchangers facilitated by the Na^+ gradient created by Na^+,K^+-ATPase (Hwang and Lee, 2007). In the GIT, Ca^{2+} uptake occurs following dietary ingestion (Bucking and Wood, 2007) and drinking (Flik et al., 1995; Guerreiro et al., 2004). Drinking may play a larger role in marine fishes, whereby the drinking of seawater offsets dehydrative water loss, and is also a potential source of Ca^{2+} uptake, particularly in juvenile fish (Flik et al., 1995; Guerreiro et al., 2002; Guerreiro et al., 2004). However, in hyperosmotic

environments, GIT Ca^{2+} uptake may be minimized through reduced transcellular transport and increased excretion *via* paracellular pathways (Schoenmakers et al., 1993), and luminal Ca^{2+} obtained through drinking is largely precipitated by the bicarbonate base (Wilson et al., 2002). The uptake of Ca^{2+} in the GIT across the basolateral membrane of enterocytes relies more on Na^+/Ca^{2+} exchangers than on Ca^{2+}-ATPase (Flik et al., 1990; Schoenmakers et al., 1993).

To date, examination of calcium metabolism in fishes has almost been entirely devoted to teleost fishes. In freshwater cartilaginous fishes, because internal calcium stores are more limited than in teleosts, the GIT may play a greater role in Ca^{2+} uptake, supplementing gill uptake particularly during times of calcium need [i.e. juvenile growth, female vitellogenesis, low ambient calcium (Berg, 1968; Ichii and Mugiya, 1983)]. In addition, certain groups of freshwater cartilaginous fishes, such as the sturgeons, are known to consume calcium-rich invertebrate prey (Miller, 2004). Furthermore, because primitive fishes are closer phylogenetically to higher vertebrates, which rely on GIT uptake of Ca^{2+}, mechanisms for Ca^{2+} uptake in primitive fishes may be more comparable to those used by higher vertebrates than to those of the more derived teleost fishes commonly studied.

In teleost fishes, increased calcium demands appear to be met through an upregulation of the epithelial Ca^{2+} channel (ECaC) in the gills (Liao et al., 2007; Shahsavarani and Perry, 2006). Interestingly, the active processes moving Ca^{2+} out of the cell at the basolateral membrane (i.e. Na^+/Ca^{2+} exchangers and plasma membrane Ca^{2+}-ATPases) do not appear to be upregulated (Liao et al., 2007). Endocrine control by the anti-hypercalcemic hormone stanniocalcin supports this, as it downregulates ECaC mRNA expression (Tseng et al., 2009). However, in some primitive fishes, such as sturgeons, the corpuscles of Stannius, the recognized source of stanniocalcin, are not present (Sasayama, 1999), indicating that the endocrine regulation of calcium may be somewhat different in primitive fishes. However, because studies in mammals have found that stanniocalcin is synthesized in a number of tissues rather than being released from an endocrine gland such as the corpuscles of Stannius, with similar regulatory effects in mammals as in teleosts (Gerritsen and Wagner, 2005; Hoenderop et al., 2005), the possibility does exist that stanniocalcin may be present in primitive fishes that lack corpuscles of Stannius.

ECaC has been characterized from a limited number of fishes (Pan et al., 2005; Qiu and Hogstrand, 2004; Shahsavarani et al., 2006), all of which are more derived bony fishes. ECaC has not been characterized in primitive fishes, nor have changes in mRNA levels been described, particularly in response to varying calcium concentrations typical of the aquatic environment. Furthermore, the role of ECaC has not been examined in the fish kidney in regard to environmental calcium concentrations. Although little is known in terms of kidney Ca^{2+}-related function in primitive fishes (Wright, 2007), the kidney may play a large role in Ca^{2+} regulation owing to its importance for Ca^{2+} regulation in fishes (Björnsson and Nilsson, 1985) and higher vertebrates (Friedman and Gesek, 1995). Therefore, the objectives of this study were to characterize the function of ECaC in lake sturgeon, and to examine whole-body and intestinal Ca^{2+} flux as they relate to environmental calcium concentrations. Furthermore, the hypotheses that ECaC is upregulated in low calcium environments and that intestinal Ca^{2+} uptake plays a significant role in overall Ca^{2+} balance in this species were tested.

MATERIALS AND METHODS

Acclimation to calcium treatments

Juvenile lake sturgeon were derived from wild broodstock from the Winnipeg River, Manitoba, Canada, that were artificially induced to spawn. These juvenile fish were reared in 1701 tanks in dechlorinated tap water, and, for experimental purposes, were randomly divided into one of three calcium treatment groups, transferred to 1201 recirculating systems, and acclimated for at least 2 weeks in their respective treatments. The initial mean body mass of fish was not different between treatments (mean initial wet mass: 22.0±0.6, 23.0±0.7 or 23.7±0.5 g). $CaCl_2$ was used to adjust treatment calcium concentrations in recirculating systems to 0.1 (low), 0.4 (normal) or 3.3 (high) mmol l^{-1} environmental calcium. The low concentration was arrived at following a pilot experiment in which fish health appeared compromised at a calcium concentration of 0.03 mmol l^{-1} (i.e. red fin membranes, loss of equilibrium and death). Normal calcium water conditions were based on a 45 year dataset for water concentrations in the Winnipeg River system from sampling locations up to 50 km apart (Bing Chu, Environment Canada, personal communication), which showed relatively little variance (0.1 mmol l^{-1}) over time or location. The high calcium concentrations were higher than typical circulating concentrations in lake sturgeon (Allen et al., 2009c). The chemistry of treatment water was otherwise made to mimic natural conditions of the Winnipeg River through the addition of NaCl, KCl, $MgSO_4$ and Na_2HPO_4, and $NaHCO_3$ was used to adjust pH within a range of 7.3–7.6 (Table 1). Only Cl^- was unlike natural conditions owing to the unavoidable increase through addition of $CaCl_2$, but the use of $CaCl_2$ was preferable to alternatives, such as $CaCO_3$ (low solubility) or $Ca(NO_3)_2$ (potential toxic effects in a recirculating system). The use of $CaCl_2$ is also supported by previous studies examining environmental effects of calcium in teleost fishes (Flik et al., 1986; Hwang et al., 1996).

Fish were fed bloodworms twice daily at a total of 1.5% body weight/day based on dry mass of bloodworms. Calcium content of bloodworms was low (0.0079±0.0005 mmol l^{-1} calcium g^{-1} wet bloodworms, N=6) based on 2 mol l^{-1} HNO_3 digestion. Fecal matter and uneaten food were removed 30 min after the addition of food. To maintain water quality, water was continually flushed through aquarium canister filters, and a 50% water change was conducted daily. Fish were maintained at 16°C in a temperature-

Table 1. Ion concentrations (mmol l^{-1}) of acclimation and treatment waters

Treatment	Ca	Na^+	K^+	Mg^{2+}	Cl^-	PO_4^{3-}	SO_4^{2-}
Low Ca	0.1±0.03	0.54±0.09	0.060±0.003	0.16±0.005[a]	0.33±0.02[a]	0.007±0.0020[a]	0.13±0.01
Normal Ca	0.4±0.01	0.53±0.03	0.060±0.005	0.18±0.005[b]	0.71±0.02[b]	0.005±0.0005[a]	0.14±0.01
High Ca	3.3±0.04	0.54±0.02	0.048±0.004	0.15±0.005[a]	5.18±0.09[c]	0.016±0.0004[b]	0.15±0.01

Values are means ± s.e.m. (N=8–9). Each water sample is from a different day analyzed by ion chromatography except for Ca (calcium), which was analyzed by atomic absorption spectrometry.

Values with different superscript letters indicate significant differences between treatments (ANOVA with Tukey's *post hoc* test, P<0.05).

controlled room, and kept on a 12 h light:12 h dark photoperiod during the experiment. Daily water samples were collected from each treatment throughout the acclimation period and analyzed by ion chromatography (Metrohm-Peak, Herisau, Switzerland) or atomic absorption spectrometry (AA240FS, Varian Inc., Palo Alto, CA, USA) for ion content. A different group of fish was used for each of the described experiments. All experiments were conducted under animal protocol F05-021, approved by the animal protocol management review committee of the University of Manitoba.

Whole-body Ca^{2+} flux

Following acclimation, fish were quickly transferred to individual flux chambers with a minimum body mass:volume ratio of 1 g:10 ml (Wood, 1992) covered with an opaque lid to reduce light intensity and potential effects of stress. The chambers contained 300 ml of water at the same ion concentrations and temperature as the treatment water. An airstone was placed in each chamber to maintain oxygen concentration and assist in mixing. All water, tissue digest and plasma samples were run in duplicate on a scintillation counter and atomic absorption unit. All experiments were conducted at the same time of day.

Influx

Ca^{2+} influx was determined through the uptake of ^{45}Ca over a 4 h period in a total of eight fish per treatment. Fifteen minutes prior to adding fish to the flux chamber, $^{45}CaCl_2$ was added at a final chamber concentration of 75 kBq l^{-1}. Two 1.5 ml water samples were collected prior to adding a fish (time 0), and at 2 and 4 h during the incubation period. The 2 h sample was not used in calculations, but was used to confirm the influx pattern. At 4 h, fish were quickly euthanized with an overdose of anesthetic (500 mg l^{-1} tricaine methanesulfonate), briefly immersed in tracer-free treatment water to remove surface contamination of ^{45}Ca, blotted dry with paper towels and immediately frozen at –20°C. After fish were frozen, the GIT was carefully removed by a scalpel and forceps. Fish and the GIT were weighed to the nearest 0.001 g and then digested separately in 2.5× *v:w* of 2 mol l^{-1} HNO_3 at 60°C for 1 week. Samples were diluted (1 ml:4 ml) in an acid-tolerant scintillation fluid (Ultima Gold AB, Perkin Elmer, Waltham, MA, USA) and read for 5 min on a β-scintillation counter (LS 6500, Beckman Coulter, Mississauga, ON, Canada). Influx (in nmol h^{-1} g^{-1}) was determined from the equation:

$$\text{Influx} = \frac{\text{Total fish CPM without GIT}}{\text{Water } ^{45}\text{Ca specific activity} \times \text{CE} \times \text{Time} \times \text{Fish mass}} , \quad (1)$$

where total fish CPM without GIT is the total c.p.m. for the entire fish excluding the GIT, water ^{45}Ca specific activity is in c.p.m. $nmol^{-1}$ and is the water ^{45}Ca (c.p.m. ml^{-1}) divided by the total water calcium (nmol ml^{-1}), counting efficiency (CE) is the mean ($CPM_{fish}\,CPM_{water}^{-1}$), which was determined by the recovery of spiked samples over the range of values found in tissue digest and water samples (5000 c.p.m. to 75 c.p.m.), time is 4 h, and fish mass is the mass of fish in g without the GIT.

Efflux

Ca^{2+} efflux was determined through the efflux of ^{45}Ca over a 7 h period in a total of eight fish per treatment. A lake sturgeon Ringer's solution without Ca^{2+} was made based on previous experiments (Allen et al., 2009a; Allen et al., 2009c), which consisted of: 128 mmol l^{-1} Na^+, 1.8 mmol l^{-1} K^+, 0.8 mmol l^{-1} Mg^{2+}, 102 mmol l^{-1} Cl^-, 2.3 mmol l^{-1} PO_4^{3-} and 0.8 mmol l^{-1} SO_4^{2-}, pH 7.6. ^{45}Ca was added to the Ringer's solution to give a final concentration of 746 kBq ml^{-1}. Fish were removed from acclimation tanks and a 150–200 µl (112–150 kBq or 5.5 kBq g^{-1}) intraperitoneal injection of the solution was administered; fish were then placed into new tracer-free 80 l recirculating systems in their respective treatment water conditions. Fish were held in these tanks for 40 h to allow for equilibration of ^{45}Ca efflux (Flik et al., 1986). Fish were then placed into individual flux chambers. Two 1.5 ml water samples were collected prior to fish addition, and then every hour for 7 h. Because of the much lower rates of efflux than influx, a longer time interval was used to establish a constant rate of ^{45}Ca efflux. At the conclusion of the experiment, fish were euthanized by the addition of 500 mg l^{-1} anesthetic to the water, removed, blotted dry, quickly weighed to the nearest 0.001 g, and a blood sample collected using a syringe inserted into the caudal vasculature immediately posterior to the anal fin. Blood samples were centrifuged at 5000 ***g*** for 5 min, and plasma removed and measured for ^{45}Ca (40 µl plasma, 1:25 dilution in water, 4 ml scintillation cocktail) and total calcium (50 µl plasma, 1:21 dilution in 0.1% HNO_3). Efflux (in nmol h^{-1} g^{-1}) was determined from the equation:

$$\text{Efflux} = \frac{\text{Total water CPM}}{\text{Plasma } ^{45}\text{Ca specific activity} \times \text{Time} \times \text{Fish mass}} , \quad (2)$$

where total water CPM is the total c.p.m. in the water, plasma ^{45}Ca specific activity is in c.p.m. $nmol^{-1}$ and is the plasma ^{45}Ca (c.p.m. ml^{-1}) divided by the total plasma calcium (nmol ml^{-1}), time is 7 h, and fish mass is the total mass of the fish in g. A counting efficiency correction factor was not required as the measured samples were essentially water.

Net flux

Net Ca^{2+} flux (in nmol h^{-1} g^{-1}) was determined from the equation:

$$\text{Net flux} = \text{Influx} - \text{Efflux} . \quad (3)$$

Intestinal Ca^{2+} flux

Initially, the GIT of each fish used in the calcium influx experiment (digested in HNO_3 as described above) was quantified for total counts of ^{45}Ca using 7 ml scintillation vials and 4 ml of scintillation cocktail over a 5 min period. The relative contribution of the GIT to whole-body calcium uptake was calculated from GIT ^{45}Ca values and whole-body ^{45}Ca values. Following these measurements, a separate experiment was conducted to measure rates of Ca^{2+} flux across the anterior–middle (mid) intestine in response to environmental calcium. Juvenile lake sturgeon (*N*=7 per treatment) were acclimated for at least 2 weeks to the same treatment water conditions and feeding regime as described above. Following acclimation, fish were euthanized in 500 mg l^{-1} tricaine methanesulfonate, pithed to ensure death, and the mid-intestine quickly dissected out of each fish. At least two rectangular tissue sections (0.25 cm^2) from the mid-intestinal region of the GIT in each fish were mounted in modified Ussing chambers (models: P2300 and EM-CSYS-2, Physiological Instruments, San Diego, CA, USA), so that both influx and efflux could be monitored in the same individual. Each side of the epithelium was bathed in 4 ml of lake sturgeon Ringer's solution under symmetrical conditions (similar to the efflux experiment but with 1.9 mmol l^{-1} $CaSO_4$) and gently aerated. After a 30 min acclimation period, 1.7 ml of Ringer's solution was removed from one side, and immediately replaced by 1.7 ml of Ringer's solution containing 1.25 µl of ^{45}Ca (specific activity, 764.73 MBq mg^{-1}). Calcium concentration was equalized between Ussing chamber sides by removing 10 µl of Ringer's

Table 2. Primers used for real-time PCR, and 5′ and 3′ RACE of lake sturgeon epithelial Ca^{2+} channel (ECaC)

Primer	Nucleotide sequence (5′→3′)	Annealing temperature (°C)	Product size (bp)
Real-time PCR			
ECaC-RTF4-F	GTGCACCTGCTGATTGAAGA	64	233
ECaC-RTR4-R	AACATCACCAAGTTGCCCTC	64	
EF-1α-RTF1-F	TGGCATCACCATTGACATCT	64	237
EF-1α-RTR1-R	AGCTGCTTCACACCCAGAGT	64	
GAPDH-F2	TACATGGTYTAYATGTTCAAGTA	53	364
GAPDH-R1	CAGRGGRGCCAGGCAGTT	65	
5′ RACE			
ECaC-F	AATGAACACTGCGTTTGCTG		
ECaC-R-inner	TCCCCTTGGTAGAGGTCAGA		
ECaC-R-mid	GCAATGTGCAGAGCTGTCTC		
ECaC-R-outer	TATGACCCGGTTACTCTGGG		
3′ RACE			
ECaC-F-inner	CCCAGAGTAACCGGGTCATA		
ECaC-F2-inner	ATGGGTCTCATAGACGTGCC		
ECaC-F-mid	AGAATGTCAATCTGGTCCGC		
ECaC-F-outer	GCACATTGCTGTGGTCAATC		
ECaC-F2-outer	TGGTTGATGATCGTTGTGCT		
ECaC-R	GCGAGCACAGAATTAATACGA		

F, forward; R, reverse; bp, base pairs.

solution from the opposite side and immediately replacing it with 10 µl of 3.43×10^{-2} mmol l^{-1} $CaCl_2$. Both the apical and basolateral surfaces of the intestine were subjected to this same ^{45}Ca exposure in separate preparations. Ca^{2+} influx or efflux was determined by collecting 50 µl samples from each side every 30 min for 3 h, and samples were read on a scintillation counter as described above. The change in Ca^{2+} (in nmol cm^{-2} h^{-1}) for every 30 min interval was calculated using the following equation:

$$\text{Flux rate} = [(\text{CPM}_{T2} - \text{CPM}_{T1}) - 11.53]\,(1.6 \times 10^{-8})\,(1.3 \times 10^{-6}) \times 0.02 \times (V5.6 \times 10^{10})], \quad (4)$$

where CPM_{T1} was the c.p.m. in the sample at time one, CPM_{T2} was the c.p.m. in the sample at time two, 11.53 was the background c.p.m. for non-radioactive samples, 1.6×10^{-8} is the conversion factor for c.p.m. to mega-becquerels (MBq), 1.3×10^{-6} is the specific activity of the isotope in MBq mg^{-1} and $0.02(V5.6\times10^{10})$ is the conversion from specific activity in MBq to the final unit of nmol cm^{-2} h^{-1}, where V is the volume in µl for one half of the Ussing chamber.

Drinking rate

Drinking rate was determined by the uptake of tracer (^{51}Cr-EDTA) over a 6 h period in a total of eight fish per treatment. Tracer was added to 300 ml of treatment water at a final water concentration of 3.3 MBq l^{-1}. Fish were individually placed into experimental flux chambers as described above and triplicate 1 ml water samples were collected prior to the addition of fish, and at 6 h post-incubation. Fish were then euthanized as above, removed from the flux chamber, blotted dry, weighed to the nearest 0.001 g, and a blood sample removed from the caudal vasculature. A careful incision was made along the ventral surface to expose the GIT while the fish was in a supine position. The GIT was ligated at the esophagus and rectum with silk sutures and removed from the fish. The spleen and liver were then removed from the GIT with forceps and the GIT was then added to a test tube and the tracer counted in a gamma counter (Cobra, Packard, Waltham, MA, USA) for 1 min. Blood and liver samples were also added to separate test tubes and read on a gamma counter to confirm the ^{51}Cr was restricted to the GIT. Drinking rate (in ml h^{-1}) was calculated according to the following equation (Carrick and Balment, 1983; Hazon et al., 1989):

$$\text{Drinking rate} = C\,(Mt)^{-1}, \quad (5)$$

where C is the total counts from the GIT (cpm kg^{-1} fish), M is the counts per milliliter (cpm ml^{-1}) in the bathing medium, and t is the time in bathing medium (6 h).

Blood and liver samples did not contain tracer, indicating that there had been no degradation and subsequent uptake of the radiolabel.

ECaC sequencing

Degenerate primers were used to isolate fragments of the lake sturgeon ECaC sequence. An RNA ligase-mediated rapid amplification of cDNA ends (RLM RACE) commercial kit (Ambion, Inc., Austin, TX, USA) was used to obtain 5′ and 3′ ends of lake sturgeon ECaC. These were cloned using a commercial kit (Invitrogen, Carlsbad, CA, USA) inserted into a plasmid vector (Topoisomerase I from *Vaccinia* virus) and transformed by heat shock into chemically competent *Escherichia coli* (DH5α-T1), which were then plated onto LB agar plates treated with 50 µg ml^{-1} ampicillin following the manufacturer's directions. Positive transformant clones were determined through X-gal staining and polymerase chain reaction (PCR) amplification with provided primers, subsequently amplified in 5 ml of LB medium containing 100 µg ml^{-1} ampicillin, and then purified for DNA sequencing through centrifugation using a commercial kit (Miniprep, QIAGEN Inc., Mississauga, ON, Canada). Sequencing was conducted on a Hitachi 3130 Genetic Analyzer using ABDNA Sequencing Analyzing software (Applied Biosystems, Foster City, CA, USA).

Real-time PCR ECaC expression

Eight fish per treatment were euthanized and sampled for blood as described above, and 100 mg gill, kidney and GIT (pyloric caeca,

mucosal scraping from mid-intestine, and spiral intestine) tissue samples were immediately removed, placed into 1 ml of RNAlater (Ambion), snap-frozen in liquid N_2 and subsequently stored at –80°C. Afterwards, blood plasma ion concentrations were analyzed using ion chromatography and atomic absorption spectrometry. Tissue samples were thawed, removed from RNAlater, and total RNA extracted using TRIzol reagent (Invitrogen). Manufacturer's instructions were followed and samples were resuspended in 40–200 µl of RNA storage solution (Ambion), and stored at –80°C.

Total RNA concentration was determined by absorbance at 260 nm and purity by absorbance at 260/280 nm. RNA was diluted in nuclease-free water to a concentration of 0.25 µg µl^{-1}, and the concentration was reconfirmed by spectrophotometer. RNA (0.5 µg) was treated with DNase (DNase 1, Invitrogen) according to the manufacturer's instructions, and tested for DNA contamination by PCR (40 cycles) using lake sturgeon-specific primers designed to amplify DNA coding for lake sturgeon EF-1α (EF-1α RTF1/RTR1, product size 237 bp; see Table 2). Complementary DNA (cDNA) was reverse transcribed from total RNA using oligo (dT) primers and Thermoscript reverse transcriptase (Invitrogen). The quality of the generated cDNAs from all tissues was assessed by employing the primer pair EF-1α RTF1/RTR1 in PCR, running the products on an electrophoresis gel, and visualizing with ethidium bromide and ultraviolet light. cDNA was diluted 1:2 in nuclease-free water and stored at –20°C.

Real-time PCR was conducted using a thermocycler (Miniopticon, Bio-Rad, Mississauga, Ontario, Canada) with iQS SYBR Green Supermix (Bio-Rad) in a 20 µl assay. For ECaC, 8 µl of diluted cDNA template were used, and 1 µl of each primer at a concentration of 10 µmol l^{-1} were used. All primers were designed and optimized for the following PCR reaction conditions: an initial denaturing at 94°C for 4 min, followed by 45 cycles of 3 s at 94°C, 5 s at 64°C and 1 s at 72°C. Elongation factor 1α (EF-1α) was used as a reference gene based on its promise in other fishes (Tang et al., 2007). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a reference gene for gills owing to the presence of treatment effects on EF-1α in gill tissue only. For EF-1α and GAPDH, 2 µl of diluted cDNA template were used. For EF-1α the PCR reaction conditions were: initial denaturing at 94°C for 4 min, followed by 40 cycles of 15 s at 94°C, 20 s at 64°C and 19 s at 72°C. For GAPDH the PCR reaction conditions were: initial denaturing at 94°C for 4 min, followed by 40 cycles of 15 s at 94°C, 20 s at 48°C and 30 s at 72°C. Following the PCR reactions, a melting curve from 40–94°C and subsequent gel verification were conducted to check for the presence of clean target products. Absolute ECaC, EF-1α and GAPDH mRNA levels were also quantified for tissues using carefully constructed standard curves derived from purified gel product (QIAquick gel extraction kit, QIAGEN Inc.), which were run in each real-time PCR assay for ECaC from 10^{-4} to 10^{-12} ng µl^{-1}, and for EF-1α and GAPDH from 10^{-2} to 10^{-8} ng µl^{-1} (a total of N=6 for all tissues except kidney, where N=8). Primers for real-time PCR are listed in Table 2.

Statistical analyses

Statistical analysis was conducted using JMP (version 4.0.4; SAS Institute, Cary, NC, USA). Values presented are means ± s.e.m., and were compared using one-way analysis of variance (ANOVA) followed by Tukey's pairwise comparison. If values could not be transformed ($\log_{10}$) to meet parametric assumptions of normality and homogeneity of variance, a non-parametric Kruskall–Wallis test was conducted followed by a Dunn's pairwise comparison. In all cases differences were considered significant at P<0.05.

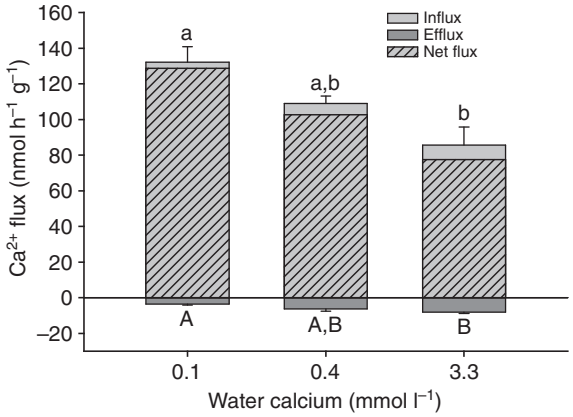

Fig. 1. Whole-body Ca^{2+} influx, efflux and net flux in juvenile lake sturgeon, *Acipenser fulvescens*, acclimated to different water calcium concentrations. Data are means ± s.e.m. (N=8). Significant differences between treatment groups are indicated by either a lowercase letter for influx, or an uppercase letter for efflux (ANOVA, P<0.05, Tukey's *post hoc* test).

RESULTS

Whole-body Ca^{2+} regulation

Whole-body Ca^{2+} influx was significantly greater in the low calcium treatment group than in the high calcium treatment group (Fig. 1). Conversely, Ca^{2+} efflux was lower in the low calcium treatment group than in the high calcium treatment group, and was much lower than influx in all groups. Thus, the resulting net flux was strongly directed inward in all treatments, and increased in a stepwise progression with the lowest values of net flux in the low calcium treatment group and the highest in the high calcium treatment group.

In terms of plasma regulation, ionic or free Ca^{2+} was not different between treatment groups (Table 3). There were no differences between other ions as well, although total plasma ions for the low calcium treatment were significantly greater than for the high calcium treatment.

Intestinal Ca^{2+} flux

Total counts of ^{45}Ca from the GIT of influx fish indicated that there was a treatment Ca^{2+} effect (Fig. 2A). This was supported by the follow-up experiment examining intestinal Ca^{2+} flux, in which it was found that net flux was significantly greater in the low calcium treatment group than in the normal or high calcium treatment groups, with flux directed inward in the low calcium group and outward in the normal and high calcium groups (Fig. 2B).

Table 3. Effects of environmental calcium concentration on plasma ion concentrations

Ion	0.1 mmol l^{-1} Ca	0.4 mmol l^{-1} Ca	3.3 mmol l^{-1} Ca
Na^+	117.9±3.3	113.8±2.6	111.3±1.7
K^+	5.54±0.56	5.48±0.81	3.88±0.31
Ca^{2+}	1.16±0.06	1.19±0.07	0.98±0.09
Mg^{2+}	0.58±0.05	0.57±0.05	0.46±0.04
Cl^-	104.9±1.9	104.4±2.1	101.6±1.5
Br^-	0.08±0.01	0.08±0.01	0.07±0.01
PO_4^{3-}	2.62±0.35	2.68±0.42	2.22±0.20
SO_4^{2-}	2.88±0.29	3.00±0.28	2.90±0.26
Total	235.7±4.3^a	231.3±3.8^{a,b}	223.4±2.9^b

Values are means ± s.e.m. in mmol l^{-1} (N=8).
Different superscript letters indicate significant differences between treatments (Kruskal–Wallis; Dunn's post hoc test; P<0.05).

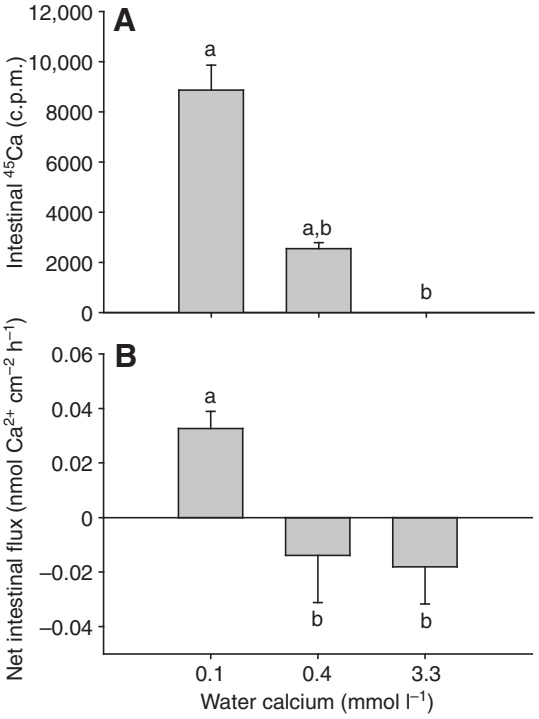

Fig. 2. (A) Mean ^{45}Ca counts per minute (c.p.m.) in the gastrointestinal tract of juvenile lake sturgeon (*A. fulvescens*) acclimated to different water calcium concentrations after a 4 h exposure to ^{45}Ca. Data are means ± s.e.m. (*N*=8). Significant differences between treatment groups are indicated by a lowercase letter (Kruskall–Wallis, *P*<0.05, Dunn's *post hoc* test). (B) Intestinal Ca^{2+} net flux in juvenile lake sturgeon acclimated to different water calcium concentrations. Data are means ± s.e.m. (*N*=4–7). Significant differences between treatment groups are indicated by a lowercase letter (ANOVA, *P*<0.05, Tukey's *post hoc* test).

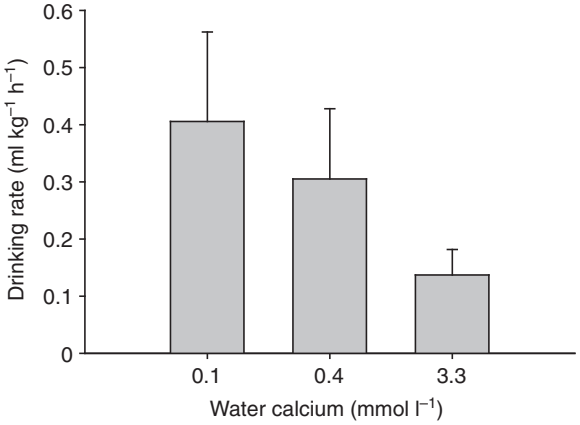

Fig. 3. Mean drinking rate (ml kg^{-1} h^{-1}) in juvenile lake sturgeon (*A. fulvescens*) acclimated to different water calcium concentrations. Data are means ± s.e.m. (*N*=8). The decreasing trend in drinking rate with increasing water calcium concentration was not significant (ANOVA, *P*=0.07).

Drinking rate

Drinking rate was not significantly different between the treatment groups (*P*=0.07; Fig. 3).

ECaC sequence

The lake sturgeon ECaC sequence was obtained through sequencing and cloning steps. The whole gene consisted of 2831 nucleotides with a reading frame of 2049 nucleotides and 683 amino acids (Fig. 4), a predicted molecular mass of 78,917 Da and an isoelectric point at pH 6.994. An ankyrin repeat region, phosphorylation sites, six transmembrane regions and a putative ion pore region were identified in the sequence. A phylogenetic tree based on the amino acid sequence of the lake sturgeon ECaC sequence and other known ECaC sequences was obtained using the neighbor-joining method (Saitou and Nei, 1987). Lake sturgeon ECaC is phylogenetically more distantly related to other fishes studied to date, and fits well into its evolutionary place as a basal bony fish, between the African clawed frog (*Xenopus laevis*) and zebrafish (*Danio rerio*; Fig. 5). The lake sturgeon ECaC amino acid sequence shares 58% identity with zebrafish (Accession number AAQ89712.1.1), 63% identity with rainbow trout (Accession number NP_001117927.1), 66% identity with fugu (*Takifugu rubripes*, Accession number AAP46137.1), 64% identity with crayfish (*Procambarus clarkii*, Accession number AAR19087.1), and 52% identity with *X. laevis* CaT1 (BAC24123.1), based on comparisons with published sequences in GenBank.

Real-time PCR ECaC expression

ECaC mRNA levels were by far the greatest in the gills, measurable in the kidneys, and very low in the GIT tissues (Fig. 6). In the gills, ECaC mRNA levels were greater in high calcium conditions than in normal calcium conditions (Fig. 7). In the kidneys, ECaC mRNA levels were lower in low calcium conditions than in high calcium conditions (Fig. 8A). In the pyloric caeca (Fig. 8B), mid-intestine (Fig. 8C) and spiral intestine (Fig. 8D), mRNA levels were below the level of detectability in many samples, and there were no treatment effects.

DISCUSSION

In the present study, the influence of environmental calcium concentrations on calcium regulation in a freshwater cartilaginous fish and the relative involvement of the intestine and ECaC were examined. Two very interesting findings were noted. First, the surprising result of an increase in gill and kidney ECaC mRNA levels in high calcium conditions. Second, although the intestine had a relatively minor role in Ca^{2+} uptake, unidirectional influx of Ca^{2+} increased in low calcium conditions, suggesting the involvement of other transporters besides ECaC in the GIT. These findings are discussed below.

Influence of environmental calcium on whole-body Ca^{2+} flux

Fish must compensate for the effects of changing external calcium concentrations. Under low environmental calcium concentrations, whole body calcium decreases (Chou et al., 2002; Flik et al., 1986; Hwang et al., 1996). Therefore, teleost fish are known to compensate through increased Ca^{2+} uptake (Chou et al., 2002; Flik et al., 1986; Hwang et al., 1996) and increased circulating concentrations of Ca^{2+} (Flik et al., 1986). Increased Ca^{2+} uptake is primarily facilitated *via* branchial routes because of low levels of drinking in fresh water (Guerreiro et al., 2004). Internally, elevated circulating Ca^{2+} concentrations appear to be maintained through increased concentrations of protein-bound Ca^{2+}, at least in non-vitellogenic lake sturgeon (Allen et al., 2009c). In contrast, under high environmental calcium concentrations, teleost fish may have higher whole-body calcium concentrations (Prodocimo et al., 2007), as external calcium concentration appears to be the main factor contributing to Ca^{2+} uptake in normal to high calcium environments (Guerreiro et al., 2004).

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1     ataagaacagcaattaccagcacctattaatagacgtaggacatatgcatgagccatata  60
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181   agctagacgctacctcagtacctggcatgattgatattgctgatgttcttccatcctgtg  240
241   gatttggctgcctctgaaaacagtaattgcaacaaacaattactgcagtctcacacgcaa  300
301   actgaccaataggagctctgtttgcaggc                                 329

      M  P  P  P  I  G  V  I  S  N  W  W  N  E  L  V  Y  R  F  R
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450   aatgagaatccactgttttatgcagccaaggataatgatgtgtgctccattaacaagcta  509
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510   ctcaagtgtcctttgacagacatctttgagagaggtgctcttggagagactgctctgcat  569
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690   gtggtcaatcagaatgtcaatctggtccgcgagctgattcgccgaggggcttgtgtgacc  749
      T  P  R  V  T  G  S  Y  F  L  K  K  R  G  N  K  V  Y  F  G
750   acgcccagagtaaccgggtcatactttctcaagaagagagggaacaaggtgtacttcggt  809
      E  H  I  L  S  F  A  A  C  I  G  N  E  E  I  V  H  L  L  I
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1890  aaggagttctccagcaccttctttgcgatgaccgagctgtttatgggtctcatagacgtg 1950
      P  V  N  Y  D  V  W  T  P  D  I  V  K  V  L  H  I  I  F  S
1950  cctgtgaactatgacgtttggactccggatattgtgaaggtcctgcatataatcttcagt 2010
      V  F  A  Y  L  L  M  I  N  L  L  I  A  M  M  G  D  T  H  W
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2310  gcatcctggtatgtatgcggtcagacacatagtctgattgttgaccttgaccactggagt 2370
      E  F  E  *
2370  gagtttgaataa                                                  2381

2382  agatatttttttataccaaggtactcagctctacctttttacttccatcacacggcacgcaa 2441
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2642  tatggcagtcgccatttttaatattgctgtaaataattagcatgattgcataattcacaa 2741
2702  ttctctttatatatatatatatatgtgtgtgtgtatccaatacagttgaattaaataaat 2801
2802  attgccaaatggtaaacaaaaaaaaaaaaa                                2831
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Fig. 4. Nucleotide and predicted amino acid sequence of lake sturgeon (*A. fulvescens*) epithelial Ca^{2+} channel (ECaC) cDNA. The coding region is 2049 nucleotides and encodes a 683 amino acid sequence. Lightly shaded region indicates possible ankyrin repeats; lightly shaded and boxed sequences indicate transmembrane regions (six predicted); dashed box indicates ion pore region; boxed and unshaded region indicates N-glycosylation site; bolded, darkly shaded and unboxed sequence indicates N-myristoylation site; underlined amino acids indicate potential phosphorylation sites; underlined nucleotides indicate start codons; and underlined nucleotides with asterisk indicate stop codons.

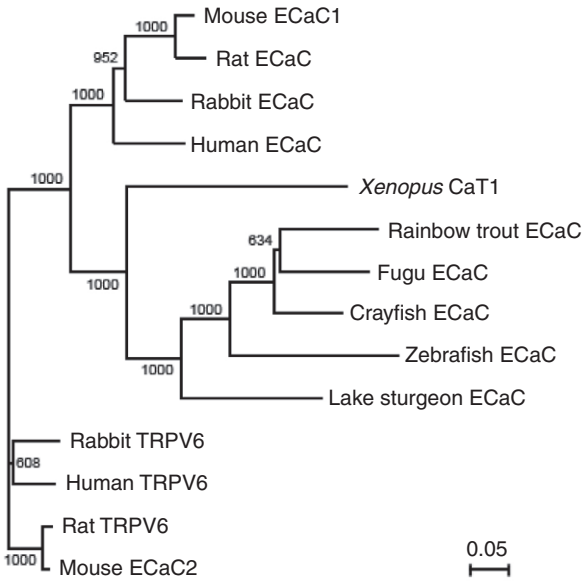

Fig. 5. Phylogenetic analysis of lake sturgeon (*A. fulvescens*) ECaC amino acid sequence. The phylogenetic tree was generated using the neighbor-joining method with bootstrap analysis using 1000 replicates. The scale bar represents the uncorrected proportion of amino acid difference. GenBank Accession numbers of the sequences used are as follows: mouse ECaC1, AAM53408.1; rat ECaC, BAA99541.1; rabbit ECaC, NP_001076126.1; human ECaC, CAB96365.2; *Xenopus* CaT1, BAC24123.1; rainbow trout ECaC, NP_001117927.1; fugu ECaC, AAP46137.1; crayfish ECaC, AAR19087.1; zebrafish ECaC, AAQ89712.1.1; lake sturgeon ECaC, this paper; rabbit TRPV6, AAY34564.1; human TRPV6, NP_061116.2; rat TRPV6, NP_446138.1; mouse ECaC2, AAM53409.1.

In this study, lake sturgeon responded to low environmental calcium concentrations by increasing Ca^{2+} uptake and decreasing efflux, whereas in high environmental calcium concentrations the opposite was true. Ca^{2+} influx rates in lake sturgeon were lower than those reported in freshwater-acclimated larval tilapia [200–250 nmol g^{-1} h^{-1} (Chou et al., 2002; Hwang et al., 1996)], comparable to those reported in juvenile tilapia [~110 nmol h^{-1} g^{-1}; Flik et al. (Flik et al., 1986); Flik et al. (Flik et al., 1985)], and higher than those reported from juvenile rainbow trout [30–60 nmol h^{-1} g^{-1} (Hogstrand et al., 1994; Niyogi and Wood, 2006)], killifish [50–60 nmol h^{-1} g^{-1} (Prodocimo et al., 2007)] and juvenile Adriatic sturgeon [~12 nmol h^{-1} g^{-1} (Fuentes et al., 2007)]. Ca^{2+} efflux rates in lake sturgeon were similar or lower than those in larval tilapia [~9–20 nmol h^{-1} g^{-1} (Chou et al., 2002)] and juvenile tilapia [~15–35 nmol h^{-1} g^{-1} (Flik et al., 1986; Flik et al., 1985)], and higher than those in Adriatic sturgeon [~1 nmol h^{-1} g^{-1} (Fuentes et al., 2007)]. In this study, it is likely that acclimation time also influenced the magnitude of Ca^{2+}-influx, as influx rates in rainbow trout (*Oncorhynchus mykiss*) subjected to low calcium environments have been demonstrated to be initially high, and to gradually decrease with the duration of acclimation (Perry and Wood, 1985). Furthermore, although not measured in this study, influx in different calcium concentrations is likely to be affected by Ca^{2+} influx maximal velocity (J_{max}) and the Michaelis–Menton constant (K_m), which have been found to increase and decrease, respectively, in freshwater larval teleosts in low as compared with high environmental calcium concentrations (Chen et al., 2003).

In lake sturgeon, a 10-fold greater Ca^{2+} influx than efflux rate resulted in a strong inwardly directed net flux, regardless of

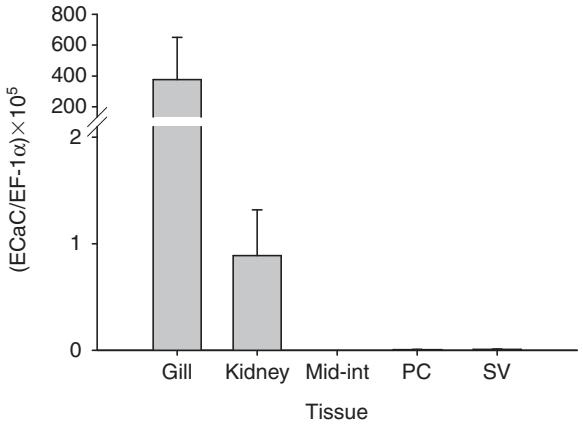

Fig. 6. Lake sturgeon tissue ECaC mRNA levels relative to elongation factor (EF)-1α levels in the normal calcium treatment group (0.4 mmol l^{-1} Ca^{2+}). Data are means ± s.e.m. (*N*=6–8). Mid-int, mid-intestine; PC, pyloric caeca; SV, spiral-valve intestine.

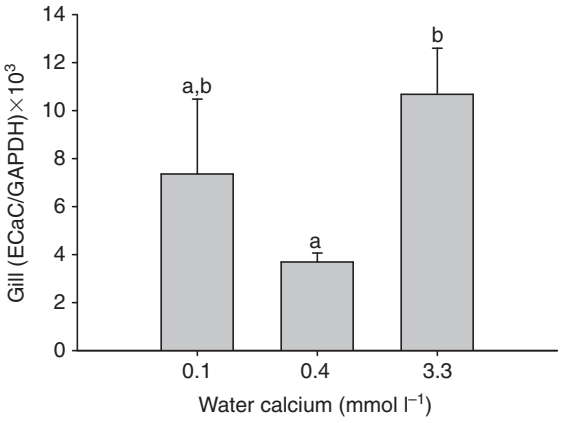

Fig. 7. Lake sturgeon gill ECaC mRNA levels relative to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA levels. Data are means ± s.e.m. (*N*=6). Different lowercase letters indicate significant differences (Kruskal–Wallis, *P*<0.05, Dunn's *post hoc* test).

environmental calcium concentration. This relationship has also been found in the developing stages of other fishes (Chou et al., 2002; Flik et al., 1986; Flik et al., 1985; Fuentes et al., 2007). Thus, lake sturgeon seem very capable of handling low calcium environments, at least at the concentrations tested, although there may be a lower limit of tolerable Ca^{2+} concentrations. In support of this, a pilot experiment found that fish lost equilibrium in calcium concentrations of 0.03 mmol l^{-1}.

An interesting finding of this study was the greater plasma ionic concentration in the low calcium treatment than the high calcium treatment (Table 3). In fishes, elevated environmental calcium is known to buffer against ion loss, and low environmental calcium and low pH can result in decreased osmolality and eventually decreased survival (Gonzalez et al., 2006; McDonald et al., 1980). However, the primary driver for ion loss is low pH rather than low environmental calcium, which causes Ca^{2+} efflux through paracellular tight junctions resulting in increased permeability of the gills (Gonzalez et al., 2006; Marshall, 1985). Thus, the ionic concentrations in the low calcium conditions in this experiment may be explained in part by the similar pH in all treatments. Furthermore, ion uptake capacity and affinity increases have been demonstrated in other fishes in ion-poor, low calcium conditions (Boisen et al., 2003). Thus, mechanisms for ion uptake may be enhanced under low calcium conditions. In addition, mechanisms for ion retention may be increased in ion-poor conditions, as increased bound Ca^{2+} has been found in wild, non-vitellogenic, female lake sturgeon in lower calcium environments (Allen et al., 2009c).

Intestinal involvement in Ca^{2+} regulation

In most freshwater teleost fishes studied, Ca^{2+} uptake occurs predominantly at the gills. It was hypothesized that the intestine would have a larger role in Ca^{2+} uptake in lake sturgeon because of: the limited availability of internal concentrations of Ca^{2+} based on very low circulating concentrations of Ca^{2+} (Allen et al., 2009c); regressive scales with age (Peterson et al., 2007); the largely cartilaginous endoskeleton in which most Ca^{2+} is sequestered into structures such as the cranium (Findeis, 1997), which are less likely to be used for resorption purposes; and the known importance of the intestine to Ca^{2+} and base handling in sturgeons (Allen et al., 2009b). Thus, drinking and diet may play a larger role in Ca^{2+} uptake than is observed in teleost fishes.

In lake sturgeon, compensatory drinking to increase uptake of Ca^{2+} in low calcium conditions or decreased drinking in high calcium conditions does not appear to play a large role in Ca^{2+} homeostasis. Although the amount of ^{45}Ca in the GIT differed between treatment groups in the present study (Fig. 2A), drinking rates did not. In contrast, drinking rates have been shown to be responsive to environmental calcium concentrations in fishes, decreasing in high calcium concentrations in brown trout (*Salmo trutta*) (Oduleye, 1975). In lake sturgeon, drinking rates were comparable to those of other freshwater fishes in normal and high calcium environments, and were higher in low calcium environments, although much lower than those of typical seawater-acclimated teleosts (Hirano, 1974). Furthermore, GIT handling of Ca^{2+} and base, as precipitated $CaCO_3$ (Shehadeh and Gordon, 1969; Wilson et al., 2002), was not present in the different environmental calcium treatments of this experiment, although it has been documented in sturgeon acclimated to brackish water and seawater conditions (Allen et al., 2009b), where drinking plays a large role in preventing dehydration.

In lake sturgeon, net intestinal uptake of Ca^{2+} was occurring in low calcium environments, whereas net excretion was occurring in normal and high calcium environments. However, even in low calcium environments, the estimated proportion of intestinal Ca^{2+} uptake to whole-body Ca^{2+} uptake was small (≤2% either calculated as ^{45}Ca uptake in GIT compared with whole-body ^{45}Ca influx or calculated by drinking rate and water calcium concentration), with most uptake presumably occurring at the gills. Similar proportions of intestinal Ca^{2+} uptake have been found in other freshwater fishes (Flik et al., 1985), whereas in marine Atlantic cod (*Gadus morhua*), the intestine may contribute up to 30% of whole-body Ca^{2+} uptake (Sundell and Björnsson, 1988). When drinking rates are reduced by low salinities in marine larval gilthead seabream (*Sparus auratus*), contribution of the intestine to whole-body Ca^{2+} uptake greatly diminishes (<10%) (Guerreiro et al., 2004). Indeed, based on the drinking rates in this study, drinking does not appear to be a primary mechanism for compensatory Ca^{2+} uptake in lake sturgeon. Interestingly, the capacity for intestinal Ca^{2+} uptake based on the isolated flux studies (Fig. 2B) appears to be higher than that demonstrated by drinking rate. Therefore, the relative role of the intestine for Ca^{2+} uptake may be better elucidated through additional studies that evaluate dietary uptake as well. A number of studies

have shown that intestinal uptake of Ca^{2+} may increase during sexual maturation or under low ambient calcium concentrations in fishes (Berg, 1968; Sundell and Björnsson, 1988; Ichii and Mugiya, 1983). Finally, although only the anterior-middle intestine was examined for Ca^{2+} flux in the present study, the stomach may also be a significant source of Ca^{2+} uptake (Bucking and Wood, 2007), as well as the spiral intestine.

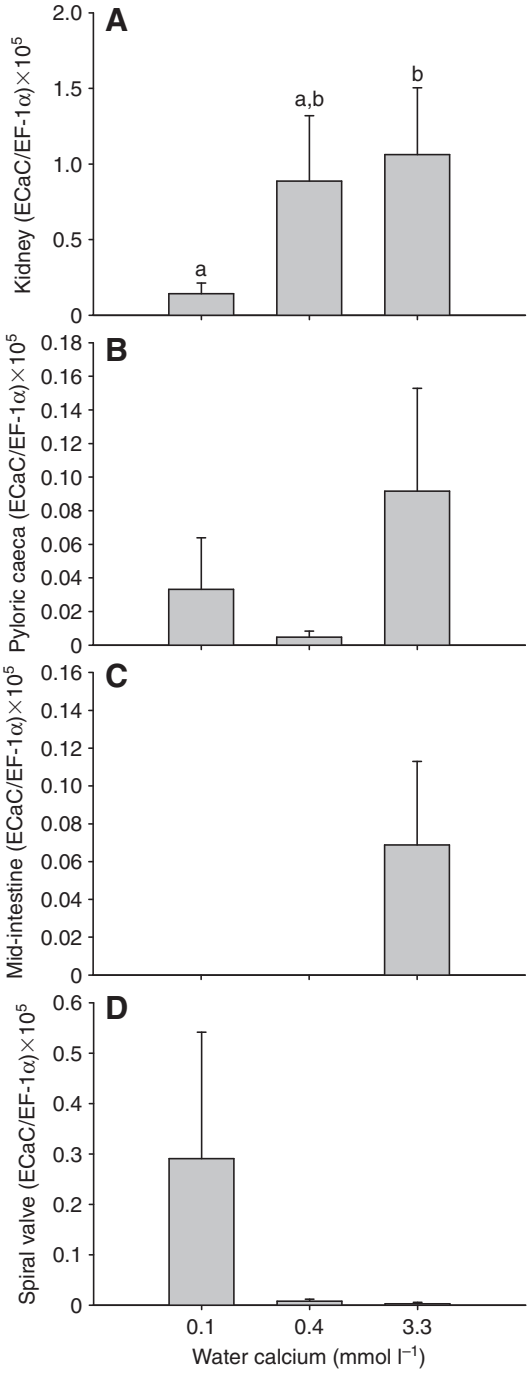

Fig. 8. Lake sturgeon ECaC mRNA levels relative to EF-1α mRNA levels in the kidney (A), pyloric caeca (B), mid-intestine (C) and spiral intestine (D). Data are means ± s.e.m. (*N*=6 for all tissues except kidney, where *N*=8). Different lowercase letters indicate significant differences (ANOVA, $P<0.05$, Tukey's *post hoc* test).

Lake sturgeon ECaC sequence

ECaC is a member of the transient receptor potential (TRP) superfamily and the TRPV (vanilloid) subfamily (den Dekker et al., 2003). Although mammals are known to have two isoforms, ECaC1 (TRPV5) and ECaC2 (TRPV6) (den Dekker et al., 2003), only a single isoform has been identified in teleost fishes (Qiu and Hogstrand, 2004). The teleost fish ECaC shares characteristic structural features with the mammalian forms, having three ankyrin repeats, six transmembrane domains, and a putative hydrophobic Ca^{2+} pore between segments 5 and 6 (den Dekker et al., 2003; Perez et al., 2008; Qiu and Hogstrand, 2004). At the amino acid level, lake sturgeon ECaC is somewhat shorter than other fish ECaCs; however, the phylogenetic relationship of lake sturgeon ECaC fits into its evolutionary place as a basal bony fish, between amphibians and more derived fishes (Fig. 5).

Role of ECaC in Ca^{2+} regulation in the lake sturgeon

Lake sturgeon ECaC mRNA levels were by far the highest in gill tissue as compared with in other tissues, which is consistent with other fishes studied to date (Pan et al., 2005; Qiu and Hogstrand, 2004; Shahsavarani et al., 2006). Current understanding of Ca^{2+} uptake in the gills of fishes involves transcellular transport *via* passive entry by apically located ECaC, and active transport at the basolateral membrane *via* plasma membrane Ca^{2+}-ATPase (PMCA2) and Na^{+}/Ca^{2+} exchangers (NCX1b) (Hwang and Lee, 2007), occurring in mitochondria-rich cells and pavement cells (Shahsavarani and Perry, 2006). Interestingly, in teleost fish ECaC appears to be regulated in response to environmental calcium concentrations (Craig et al., 2007; Liao et al., 2007; Pan et al., 2005; Shahsavarani and Perry, 2006), whereas PMCA and NCX are not (Liao et al., 2007). Indeed, the expression studies on teleost fish ECaC are analogous to those in the mammalian nephron, where ECaC has been described as the 'gatekeeper' for Ca^{2+} transport (Hoenderop et al., 2002). Contrary to this model, ECaC mRNA levels did not increase in low calcium conditions in lake sturgeon, but actually increased in high calcium conditions. Clearly, this finding is interesting, and may relate to several causes. First, ECaC expression has not been studied under high calcium conditions (greater than internal circulating Ca^{2+} concentrations) in other fishes, and so it is unknown how mRNA expression may change in these environments for other fish species. Second, it is possible that the localization of ECaC may change under high calcium conditions, its regulation may change, or that it has a different role in lake sturgeon. Possible indications of the latter may be the uncommon life history strategy of lake sturgeon, having a largely cartilaginous endoskeleton in freshwater, the apparent lack of corpuscles of Stannius (Sasayama, 1999) providing anti-hypercalcemic control (Tseng et al., 2009), and less rigidity for ionic Ca^{2+} regulation (Allen et al., 2009c). There is also the possibility that translational expression of protein, which was not measured in this study, may differ from transcriptional expression of mRNA, or that ECaC expression may change with acclimation time.

Previous studies on fishes have found very low activities of kidney ECaC and did not subsequently investigate changes associated with environmental calcium (Qiu and Hogstrand, 2004; Shahsavarani et al., 2006). In lake sturgeon, kidney ECaC mRNA levels were detectable and varied in response to environmental calcium concentrations. In mammals, ECaC1 (TRPV5) expression predominates in the kidney, where it is located on the apical surface of epithelial cells along the distal convoluted tubules, and it has a role in Ca^{2+} reabsorption (den Dekker et al., 2003). In the lake sturgeon, kidney ECaC expression

decreased in low calcium conditions, which would seem to indicate a role in Ca^{2+} extrusion. When coupled with the environmental expression results from the gills, ECaC expression is the reverse to that previously described in teleost fishes. That is, ECaC expression increases in the gills in high calcium conditions when extrusion would be necessary, and decreases in the kidney in low calcium conditions when one would hypothesize that increased reabsorption would be required.

In lake sturgeon intestinal tissues (pyloric caeca, mid-intestine, and spiral intestine), ECaC mRNA levels were very low, and were below the level of detectability in many of the fish regardless of environmental calcium concentration. Intestinal ECaC, despite high expression levels in mammals (ECaC2, TRPV6) (den Dekker et al., 2003), has very low expression in teleost fishes (Qiu and Hogstrand, 2004; Shahsavarani et al., 2006), which is consistent with the findings in lake sturgeon in this study. Because lake sturgeon were found to regulate Ca^{2+} net flux in the intestine, it is unlikely that ECaC is the main intestinal Ca^{2+} transport mechanism in fishes. An L-type voltage-gated Ca^{2+} channel on the brush border of enterocytes is involved in Ca^{2+} uptake in Atlantic cod (Larsson et al., 1998). Ca^{2+} uptake could occur similar to the transcellular model proposed for the gill, or from a variety of processes, such as vesicular-mediated transport or paracellular pathways (Khanal and Nemere, 2008; Perez et al., 2008).

Synthesis and future directions

The freshwater, cartilaginous, lake sturgeon exhibits compensatory Ca^{2+} uptake in low calcium environments. Although net flux increases in the intestine in low calcium environments, drinking rates are low, and the proportional amount of intestinal Ca^{2+} influx to whole-body influx is low. ECaC plays a role in Ca^{2+} regulation in the lake sturgeon, with the gill being of primary importance, followed by the kidney, and very low expression in the intestine. ECaC expression is different from known models in freshwater fishes, with expression increasing in the gills in high calcium conditions and decreasing in the kidney in low calcium conditions. The uncommon life history strategy of a cartilaginous fish in freshwater, the phylogeny of this ancient fish, the low internal Ca^{2+} concentrations and the lower rigidity of Ca^{2+} regulation all indicate that different regulatory strategies may occur in the lake sturgeon. Future research investigating endocrine-mediated hypercalcemic control may be fruitful owing to the apparent lack of corpuscles of Stannius in sturgeon. Furthermore, localization of ECaC and investigations of other uptake mechanisms in the intestine, and possibly in the gills and kidneys, are needed to clarify potential differences in the Ca^{2+} transport mechanisms for this species.

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