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RESEARCH ARTICLE

The role of environmental calcium in the extreme acid tolerance of northern banjo frog (*Limnodynastes terraereginae*) larvae

Coen Hird, Craig E. Franklin and Rebecca L. Cramp*

ABSTRACT

Many aquatically respiring animals acutely exposed to low pH waters suffer inhibition of ion uptake and loss of branchial (gill) epithelial integrity, culminating in a fatal loss of body Na^+. Environmental calcium levels ($[Ca^{2+}]_e$) are pivotal in maintaining branchial junction integrity, with supplemental Ca^{2+} reversing the negative effects of low pH in some animals. Tolerance of some naturally acidic environments by aquatic animals is further complicated by low $[Ca^{2+}]_e$, yet many of these environments are surprisingly biodiverse. How animals overcome the damaging actions of low pH and low environmental Ca^{2+} remains unknown. We examined the effects of $[Ca^{2+}]_e$ on the response to low pH in larvae of the highly acid-tolerant frog *Limnodynastes terraereginae*. Acute exposure to low pH water in the presence of low (5 μmol l^{-1}) $[Ca^{2+}]_e$ increased net Na^+ efflux. Provision of additional $[Ca^{2+}]_e$ reduced net Na^+ efflux, but the effect was saturable. Acclimation to both low and high (250 μmol l^{-1}) $[Ca^{2+}]_e$ improved the resistance of larvae to Na^+ efflux at low pH. Exposure to the Ca^{2+} channel inhibitor ruthenium red resulted in an abrupt loss of tolerance in low pH-acclimated larvae. Acclimation to acidic water increased branchial gene expression of the intracellular Ca^{2+} transport protein calbindin, consistent with a role for increased transcellular Ca^{2+} trafficking in the tolerance of acidic water. This study supports a role for $[Ca^{2+}]_e$ in promoting branchial integrity and highlights a potential mechanism via the maintenance of transcellular Ca^{2+} uptake in the acid tolerance of *L. terraereginae* larvae.

KEY WORDS: Amphibian, Tadpole, Low pH, Wallum, Epithelia, Calcium transport

INTRODUCTION

Life in freshwater environments is complicated by the fact that animals are hyper-ionic with respect to the environment and ions tend to move out of the animal along their diffusion gradients. In order to offset ion losses, freshwater animals actively take up ions from the environment via specialised cells in the gill epithelia, integument (in larvae and embryos) and across the gut (Edwards and Marshall, 2012; Evans et al., 1999). The physiological challenges of living in freshwater environments can be further compounded by low pH. Most aquatically respiring animals are intolerant of water pH <5 (low pH). In many animals, low pH water substantially impairs both ion uptake capacity and epithelial integrity, leading to a rapid loss of homeostasis that can be fatal (Freda and Dunson, 1984; McDonald et al., 1984; Meyer et al., 2010, 2020; Robinson, 1993).

The effect of low pH water on branchial epithelial integrity drives its toxicity in many species. In acid-sensitive animals, exposure to low pH water reduces transepithelial resistance through the disruption of paracellular tight junctions (Daye and Garside, 1976; Meyer et al., 2010; Rosseland and Staurnes, 1994). Tight junctions (TJs) are composed of a complex of transmembrane and associated proteins which function in cell–cell adhesion and control epithelial permeability by acting as a selective barrier to ions and small molecules (Schneeberger and Lynch, 2004). In doing so, TJs regulate and maintain key transcellular ion gradients necessary to facilitate transepithelial ion uptake. Low extracellular pH has been shown to reduce the resistance of the paracellular pathway by changing the conformation of TJ proteins, which alters their gating properties (Schneeberger and Lynch, 1992, 2004).

Environmental calcium concentration ($[Ca^{2+}]_e$) is essential to maintaining the integrity of the epithelial TJ (Schneeberger and Lynch, 1992). Increased $[Ca^{2+}]_e$ has been shown to ameliorate the effects of low pH on epithelial integrity in some fish and amphibian species (Dalziel et al., 1986; Matsuo and Val, 2002; McDonald et al., 1983; Meyer et al., 2020). This may be because low $[Ca^{2+}]_e$ causes dissociation of the paracellular junctions, increasing epithelial permeability (Bhat et al., 1993; Ma et al., 2000; O'Keefe et al., 1987). In some fishes and larval amphibians acutely exposed to low pH, $[Ca^{2+}]_e$ correlates negatively with the rate of net Na^+ loss (Cummins, 1988; Freda et al., 1991; Gascon et al., 1987; Gonzalez and Dunson, 1989; Kumai et al., 2011; McDonald and Rogano, 1986; McDonald et al., 1983). Ca^{2+} may act on the TJ directly, but also indirectly through Ca^{2+}-dependent proteins in adherens junctions (AJs) which sit basally to the TJ (Brown and Davis, 2002). Removal of Ca^{2+} from the extracellular space has been shown to reduce cytosolic $[Ca^{2+}]$ (González-Mariscal et al., 1990), cause the detachment and internalisation of extracellular AJ and TJ proteins (Volberg et al., 1986), and reduce transepithelial resistance (González-Mariscal et al., 1990). AJs function primarily in cell–cell adhesion, but also in the regulation of the actin cytoskeleton and as transcriptional regulators (Hartsock and Nelson, 2008). Extracellular Ca^{2+} can regulate paracellular permeability by interacting directly with calcium-dependent junctional proteins on the cell surface and/or through active transcellular Ca^{2+} uptake and cytosolic Ca^{2+} signalling pathways (Stuart et al., 1996). Although $[Ca^{2+}]_e$ is important in determining the effects of low pH exposure in fish and amphibians, the specific mechanism through which this occurs remains unknown.

Transcellular uptake of Ca^{2+} from the surrounding water via the gills constitutes the major route by which Ca^{2+} is taken up in fish (Baldwin and Bentley, 1980; Chasiotis et al., 2012; Flik and Verbost, 1995). Active branchial Ca^{2+} uptake occurs at ionocytes in

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the branchial epithelium. Extracellular Ca^{2+} enters ionocytes through non-voltage-gated epithelial Ca^{2+} channels (ECaC) in the apical membrane (Edwards and Marshall, 2012; Flik and Verbost, 1995) and is then shuttled to the basolateral membrane by Ca^{2+}-binding proteins such as calbindin for extrusion via Na^+/Ca^{2+} exchangers (NCX) and plasma membrane Ca^{2+}-ATPase transporters (PMCA). Once in the basolateral extracellular space, Ca^{2+} can directly interact with the extracellular Ca^{2+}-binding domain of E-cadherin in AJs to influence its properties and those of the overlying TJ (Pokutta et al., 1994; Zhang et al., 2009). Branchial Ca^{2+} absorption is tightly controlled through the regulation of ECaC activity and changes in the abundance of intracellular Ca^{2+} transport proteins (Cai et al., 1993; Kelly and Wood, 2008; Khanal and Nemere, 2008; Shahsavarani and Perry, 2006; Verbost et al., 1993; Wongdee and Charoenphandhu, 2013). This, in turn, may reduce the transcellular movement of Ca^{2+} to the basolateral extracellular space, which could limit its availability to E-cadherin in the AJ and compromise the permeability of the overlying TJ. Factors that compromise the maintenance of transcellular Ca^{2+} transport pathways could influence the Ca^{2+}-sensitive aspects of junctional stability.

Given that highly acidic water is toxic to most aquatic animals, naturally acidic freshwater bodies are surprisingly biodiverse. Among the most acidic freshwater ecosystems in the world is the Wallum along the eastern coast of southern Queensland and northern New South Wales in Australia. Wallum ecosystems are characterised by highly acidic waters, ranging from pH 2.8 to 5.5 (Hines and Meyer, 2011). Compounding the difficulties of living at low pH, Wallum waters are also dilute (low in salts) and soft (low in Ca^{2+} and Mg^{2+}; Bayly, 1964). Despite these challenges, larvae of some Wallum frog species can tolerate exceptionally acidic waters (Hines and Meyer, 2011; Meyer, 2004; Meyer et al., 2020). One such species is the northern banjo frog, *Limnodynastes terraereginae*, populations of which can be found throughout eastern Australia inhabiting aquatic environments which range in pH from circumneutral to pH 3.0, making it one of the most highly acid-tolerant vertebrate species known. Meyer (2004) showed that *L. terraereginae* larvae from Wallum environments are highly acid tolerant in part as a result of their capacity to withstand the acute disruption of epithelial integrity and associated loss of body Na^+. Moreover, $[Ca^{2+}]_e$ was implicated in underpinning the resistance of larvae to acid-induced Na^+ efflux in acutely exposed *L. terraereginae* larvae but not in acid-acclimated larvae (Meyer, 2004). The role of $[Ca^{2+}]_e$ in facilitating acid tolerance in acid-acclimated larvae and the mechanistic basis by which this occurs remain unclear. Given that $[Ca^{2+}]_e$ is limited in Wallum environments and that Ca^{2+} uptake is typically inhibited by low pH (Malley, 1980; Yeh et al., 2003), understanding how amphibian larvae manage Ca^{2+} transport in low pH water is likely central to understanding the mechanistic basis of their tolerance to these extreme environments.

To determine the importance of $[Ca^{2+}]$ in Na^+ homeostasis at low pH, we examined whole-animal Na^+ and Ca^{2+} flux following both acute and chronic exposure to low pH and different $[Ca^{2+}]_e$ in *L. terraereginae* larvae. To understand the role of transcellular Ca^{2+} uptake for the maintenance of epithelial integrity at low pH, *L. terraereginae* larvae reared at low pH were exposed to the calcium channel antagonist ruthenium red (RR). We also measured gene expression patterns of four key Ca^{2+} transport proteins (ECaC, calbindin, NCX and PMCA) and E-cadherin in the gill epithelia. We hypothesised that acute exposure to low pH would result in increased net Na^+ efflux under low $[Ca^{2+}]_e$, but that chronic exposure (acclimation) to low $[Ca^{2+}]_e$ would reduce net Na^+ efflux with low pH exposure and increase Ca^{2+} influx. In addition, we hypothesised that the acute impairment of apical Ca^{2+} uptake via ECaC (with RR) would increase net efflux of Na^+ at low pH, consistent with a role for transcellular Ca^{2+} transport in the maintenance of intercellular junction integrity. Finally, acclimation to both low pH and low $[Ca^{2+}]_e$ was hypothesised to correspond to an increase in the expression of the four key Ca^{2+} transporters (ECaC, calbindin, NCX and PMCA) and E-cadherin, consistent with an increased rate of transcellular Ca^{2+} uptake and reinforcement of Ca^{2+}-dependent AJs to protect junctional integrity in acid-acclimated larvae.

MATERIALS AND METHODS

Experimental animals and general methods

All animals were collected under the Queensland Department of Environment and Heritage Protection Scientific Purposes Permit (WITK15563515), and all procedures were approved by The University of Queensland's Animal Ethics Welfare Unit (SBS/484/17) and the University of Queensland's Animal Ethics Committee (approval number: SBS/460/14/ARC). *Limnodynastes terraereginae* Fry 1915 egg masses were collected in January 2018 from Bribie Island National Park (water pH 3.3–4.4), QLD, Australia. Eggs were allocated to circumneutral (pH 6.5) or low (pH 3.5) pH artificial soft water (ASW; Freda and Dunson, 1984): distilled water plus (in µmol l^{-1}) 40 $CaCl_2{\cdot}2H_2O$, 40 $MgSO_4{\cdot}7H_2O$, 120 NaCl, 50 NaOH and 20 KCl; pH was adjusted with 0.1 mol l^{-1} H_2SO_4. Hatched tadpoles were housed in 5 l plastic tanks connected to two 200 l filtered recirculating aquarium systems (15 tanks per system). Each system was connected to a canister filter for biological, mechanical and chemical filtration (Fluval G6). System pH was monitored daily (LAQUA P-22, Horiba Instruments, Singapore) and regulated as necessary through the addition of 0.1 mol l^{-1} H_2SO_4. Water $[Na^+]_e$ and $[Ca^{2+}]_e$ were measured weekly using flame photometry (BWB Technologies, Newbury, UK). Tadpoles were fed every second day with thawed frozen spinach, and each system underwent a 20% water change weekly. Room temperature was maintained at 22±1°C with fluorescent overhead lighting programmed to a 12 h:12 h light:dark photoperiod (06:00–18:00 h). The following experiments were replicated once within the laboratory.

Whole-animal Na^+ and Ca^{2+} flux

The Na^+ and Ca^{2+} concentration (µmol l^{-1}) in water samples was measured using a flame photometer (BWB Technologies). Na^+ and Ca^{2+} detection ranges were calibrated using 5, 50, 500 and 1000 µmol l^{-1} standards. To buffer potential spectral interference of sulphites and phosphates on Ca^{2+} readings, 50 µmol l^{-1} $[Ca^{2+}]_e$ samples were diluted to 50% and 250 µmol l^{-1} $[Ca^{2+}]_e$ samples were diluted to 25% (Thiers and Hviid, 1962; Welch et al., 1990). Water samples containing 5 µmol l^{-1} $[Ca^{2+}]_e$ were under the limit of detectability for our flame photometer and Ca^{2+} flux could not be reliably measured in this treatment; Na^+ concentrations remained within the limits of detectability for all $[Ca^{2+}]_e$ exposures and were recorded for all treatments. Ion concentrations were converted to total ion levels by multiplying the recorded ion concentrations with the volume of the test chamber. All net Na^+ and Ca^{2+} flux measures (in nmol l^{-1} h^{-1}) were calculated by comparing changes in total Na^+ and Ca^{2+} abundance over the exposure period as follows:

$$\text{Net ion flux} = \left(\frac{\text{Initial ion} - \text{Final ion}}{t}\right), \quad (1)$$

where t is the time of the exposure period in hours (6 h).

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Experiment 1: effects of acute exposure to high or low Ca^{2+} levels on whole-animal net Na^+ and Ca^{2+} flux at low pH

To assess the effect of acute exposure to high or low $[Ca^{2+}]_e$ on acid-induced net Na^+ and Ca^{2+} flux, *L. terraereginae* larvae (n=36; Gosner stages 26–38; Gosner, 1960) were randomly allocated to three 5 l tanks ($n \leq 12$ per tank to obtain adequate statistical power, based on previous ion flux work with the species in Meyer, 2004) containing 50 µmol l^{-1} $[Ca^{2+}]_e$ ASW (Table 1). Water pH was maintained at pH 6.5 and larvae were acclimated to these conditions for 4 weeks. Water pH was monitored daily and regulated as necessary via addition of 0.1 mol l^{-1} H_2SO_4. Tadpoles were fed every second day with thawed frozen spinach, and each system underwent a 100% water change weekly. Ammonia levels were monitored using an API® Ammonia Test Kit (Mars Fishcare, Chalfont, PA, USA). Larvae were fasted for 2 days and then placed into individual 200 ml glass beakers containing 50 ml of 5, 50 or 250 µmol l^{-1} $[Ca^{2+}]_e$ ASW (n=12 per treatment), 30 min prior to testing. Water pH in half of the beakers in each treatment (n=6) was acutely lowered to pH 3.5 through the addition of dilute H_2SO_4 (0.1 mol l^{-1}). A 5 ml water sample was collected from all beakers 1 and 7 h following pH adjustment for the measurement of Na^+ and Ca^{2+} concentration. Net ion flux was determined by subtracting the ion concentration at the start of the exposure (1 h sample) from that at the end (7 h sample). Following experimentation, all larvae were lightly blotted dry, weighed (mean mass Na^+ model 0.68 g, Ca^{2+} model 0.7 g), then euthanised by immersion in 0.25 mg l^{-1} buffered MS222 (Ramlochansingh et al., 2014) and pithing for assessment of total body $[Na^+]$ and $[Ca^{2+}]$. Larvae were dissolved in 2 ml of 70% NHO_3 and 5 ml 1:100 aliquots were prepared by filtering the sample through a 45 µmol l^{-1} filter syringe (Fisherbrand™, Loughborough, UK) before $[Na^+]$ and $[Ca^{2+}]$ were measured in duplicate using a flame photometer as described above.

Experiment 2: effects of chronic exposure to high or low Ca^{2+} levels on whole-animal net Na^+ and Ca^{2+} flux at low pH

To assess the effect of chronic exposure (acclimation) to high or low $[Ca^{2+}]_e$ on whole-animal net Na^+ and Ca^{2+} flux following acute exposure to low pH, *L. terraereginae* larvae (n=24; Gosner stages 26–38) were transferred to tanks containing either 5 or 250 µmol l^{-1} $[Ca^{2+}]_e$ ASW 4 weeks prior to sampling. Control larvae were maintained in 50 µmol l^{-1} ASW. Thirty minutes prior to testing, larvae were placed into individual 200 ml glass beakers containing 50 ml of ASW with the same $[Ca^{2+}]$ as their holding tanks (n=12 per treatment). Water pH in the beakers in each treatment was then acutely lowered to pH 3.5 through the addition of 0.1 mol l^{-1} H_2SO_4. Water samples were then collected from all beakers at 1 and 7 h and analysed as detailed above for $[Na^+]$ and $[Ca^{2+}]$. Larvae were blotted dry and weighed (mean mass Na^+ model 0.64 g, Ca^{2+} model 0.59 g).

Table 1. Nominal ion concentrations (in µmol l^{-1}) in three $[Ca^{2+}]_e$ artificial soft water treatments used for acclimation and exposure

$[Ca^{2+}]_e$	$[Na^+]$	$[Cl^-]$	$[Mg^{2+}]$	$[K^+]$
5	513	520	40	20
50	513	520	40	20
250	513	520	40	20

Measured Na^+ levels were within a 15% margin of error compared with nominal values in 250 µmol l^{-1} $[Ca^{2+}]_e$ treatments and within a 3% margin of error in 5 and 50 µmol l^{-1} $[Ca^{2+}]_e$ treatments. $[Ca^{2+}]_e$ treatments were composed of distilled water with the following additions (in µmol l^{-1}): for 5 µmol l^{-1} $[Ca^{2+}]_e$: 5 $CaCl_2 \cdot 7H_2O$, 40 $MgSO_4 \cdot 7H_2O$, 490 NaCl, 23 NaOH, 20 KCl; for 50 µmol l^{-1} $[Ca^{2+}]_e$: 50 $CaCl_2 \cdot 7H_2O$, 40 $MgSO_4 \cdot 7H_2O$, 400 NaCl, 113 NaOH, 20 KCl; and for 250 µmol l^{-1} $[Ca^{2+}]_e$: 250 $CaCl_2 \cdot 7H_2O$, 40 $MgSO_4 \cdot 7H_2O$, 513 NaOH, 20 KCl.

Experiment 3: effects of Ca^{2+} uptake inhibition on whole-animal Na^+ and Ca^{2+} flux acclimated to low pH

RR was used to inhibit apical Ca^{2+} transport. RR does not penetrate TJs and is commonly used as a histochemical marker of the barrier formed by epithelial TJs (González-Mariscal et al., 1989; West et al., 2002). *Limnodynastes terraereginae* larvae (n=12; Gosner stages 26–38) were randomly allocated to an isolated 5 l tank containing 50 µmol l^{-1} $[Ca^{2+}]_e$ ASW at pH 3.5 and maintained for 4 weeks as detailed in experiment 1. Thirty minutes prior to testing, larvae (n=12) were placed into individual 200 ml glass beakers containing 50 ml of 50 µmol l^{-1} $[Ca^{2+}]_e$ ASW at pH 3.5. RR was added to half of the beakers to a concentration proven to induce an approximately half-maximal response in ECaC activity *in vitro* (10 µmol l^{-1}; Hoenderop et al., 2001). Water samples were collected at 1 and 7 h post-exposure and analysed as described above. Larvae were then removed from beakers, blotted dry and weighed (mean mass 0.86 g).

Gene expression of Ca^{2+} transport and AJ proteins

To assess whether $[Ca^{2+}]_e$ exposure and low pH influences the expression of branchial Ca^{2+} transport proteins and E-cadherin, *L. terraereginae* larvae (n=36; Gosner stages 26–38) were randomly allocated to six isolated 5 l tanks (n=6 per tank) containing 5, 50 and 250 µmol l^{-1} $[Ca^{2+}]_e$ ASW. Water pH was maintained at pH 6.5 (n=18) or reduced to pH 3.5 (n=18). Larvae were maintained under these conditions for 4 weeks. *Limnodynastes terraereginae* larvae were then euthanised by immersion in 0.25 mg l^{-1} buffered MS222 (Ramlochansingh et al., 2014) and pithing. Both branchial baskets were dissected free and stored in RNAlater (Ambion Inc.) at 4°C for 24 h, before being moved into a −20°C freezer. Total RNA was extracted from *L. terraereginae* gills using an RNeasy Mini Kit following the manufacturer's instructions (Qiagen, Valencia, CA, USA). Total RNA was eluted from the silicon spin column in ultrapure water and its concentration quantified using a Qubit fluorometer (ThermoFisher Scientific, Waltham, MA, USA). Any residual genomic DNA contamination was removed, and RNA was reverse transcribed using an iScript gDNA Clear cDNA Synthesis Kit (Bio-Rad Laboratories Inc., Hercules, CA, USA) following the manufacturer's guidelines. Appropriate no-reverse transcriptase controls were generated by replacing reverse transcriptase with water.

The transcripts for target genes (*ECaC*, *Calb1*, *PMCA*, *NCX*, *E-cadherin*) and house-keeping genes (*β-actin*, *GAPDH*, *RPS*, *TUB*) were identified using an in-house *L. terraereginae* transcriptome with homologous sequences from other amphibians as the reference query. Reference sequences were compared against the *L. terraereginae* transcriptome using the 'blastn' tool in Galaxy Australia (Jalili et al., 2020). Putative *L. terraereginae* gene sequences were then compared against the National Centre for Biotechnology Information (NCBI) database using the 'blastn' tools default parameters to confirm their identity. PrimerQuest (Integrated DNA Technologies, Caraville, IA, USA) was used to design specific qPCR primers (Table S1). All primer pairs were evaluated for specificity and to ensure that they produced only a single band of the appropriate length using MyTaq DNA Polymerase (Bioline, Alexandria, NSW, Australia) and agarose gel electrophoresis.

Quantitative PCR assays were conducted using iTaq™ Universal SYBR® Green Supermix (Bio-Rad Laboratories Inc.) in a Mini

Opticon detection system (MJ Mini Cycler, Bio-Rad Laboratories Inc.). Samples were analysed in triplicate and each plate included appropriate no-template controls. No-reverse transcriptase controls were assessed independently for each biological sample to confirm the absence of genomic DNA contamination. Cycling parameters were as follows: 95°C for 1 min followed by 40 cycles of 95°C for 15 s and 60°C for 30 s. Melt (dissociation) curves (65–90°C) were conducted after each run. Reaction efficiencies were calculated using a serially diluted pooled cDNA standard. All PCR efficiencies were greater than 90% with an R^2 of over 0.99. All assays produced unique and single peak dissociation curves. Data were exported to MS Excel using Bio-Rad CXF Manager (version 3.1). The stability of the candidate house-keeping genes was calculated using the geNorm algorithm via the *NormqPCR* package (Perkins et al., 2012). All four genes were found to be highly stable across the six treatment groups and met the criteria for designation as appropriate housekeeping genes. A combination of housekeeping genes was used as a pseudo-housekeeper (Rocha-Martins et al., 2012; Vandesompele et al., 2002). The geNorm algorithm revealed the two most stable housekeeping genes (*GAPDH* and *RPS*) combined were an effective housekeeping control. To combine the housekeeping genes, the geometric mean of the raw amplification threshold (Ct) values and the reaction efficiencies for *GAPDH* and *RPS* were calculated for use in analyses. The following calculations for target gene expression were conducted following Pfaffl (2001). To account for differing reaction efficiencies between primers, adjusted Ct values for the pseudo-housekeeper and the genes of interest were calculated using the following formula:

$$\mathrm{Ct}_{\mathrm{adjusted}} = \log_2(E^{\mathrm{Ct}}), \quad (2)$$

where E is the reaction efficiency for the gene and Ct is the raw Ct value of the sample. ΔCt was calculated for each sample for statistical analyses:

$$\Delta\mathrm{Ct}_{\mathrm{sample}} = \mathrm{Ct}_{\mathrm{adjusted}}(\mathrm{GOI}) - \mathrm{Ct}_{\mathrm{adjusted}}(\mathrm{HKG}), \quad (3)$$

where $\mathrm{Ct}_{\mathrm{adjusted}}$(GOI) is the adjusted Ct value for the gene of interest, and $\mathrm{Ct}_{\mathrm{adjusted}}$(HKG) is the adjusted Ct value for the combined housekeeping genes. ΔCt in each target gene was quantified as fold-change relative to the expression of a reference group (the 50 µmol l^{-1} $[Ca^{2+}]_e$ at pH 6.5 group).

Statistical analyses

All analyses were conducted in the R statistical environment (http://www.R-project.org/). α was set at 0.05 for all statistical tests. Models were two-tailed and assumed a Gaussian error structure, and data satisfied assumptions of hypothesis tests. The effects of body size on rates of ion flux were accounted for by considering wet body mass as a covariate in statistical models.

Differences in net Na^+ and Ca^{2+} flux between treatment groups were tested by fitting analysis of covariance (ANCOVA) models using the *car* package (Fox and Weisberg, 2018). For experiment 1, a two-way ANCOVA was fitted using exposure $[Ca^{2+}]_e$ and test pH as independent variables. A separate two-way ANCOVA was fitted for whole-body $[Na^+]$ and $[Ca^{2+}]$ in these larvae using exposure $[Ca^{2+}]_e$ and test pH as independent variables (Fig. S1). To determine whether acclimation to low or high Ca^{2+} affected Na^+ and Ca^{2+} flux at low pH in experiment 2, larvae reared at 50 µmol l^{-1} $[Ca^{2+}]_e$ and exposed to low pH at 5 and 250 µmol l^{-1} $[Ca^{2+}]_e$ from experiment 1 were compared with larvae reared at 5 or 250 µmol l^{-1} $[Ca^{2+}]_e$ and exposed to low pH at 5 and 250 µmol l^{-1} $[Ca^{2+}]_e$. A two-way ANCOVA was fitted modelling test $[Ca^{2+}]_e$ and rearing $[Ca^{2+}]_e$ (equimolar to test $[Ca^{2+}]_e$ versus 50 µmol l^{-1} $[Ca^{2+}]_e$ control) as independent variables. For experiment 3, a one-way ANCOVA was fitted with RR treatment as the independent variable. For all experiments, one-sample Student's *t*-tests were used to test whether Na^+ and Ca^{2+} flux in control groups was significantly different from zero, suggesting a departure from homeostasis. Net Na^+ and Ca^{2+} flux was assessed in separate models. Water evaporation from beakers over the course of the experiment was small (0.5–1.5 ml in 7 h) and did not affect our measures of ion flux. *Post hoc* analyses for all ion flux experiments were conducted with the *emmeans* package (https://CRAN.R-project.org/package=emmeans) using the Tukey method of *P*-value adjustment for multiple comparisons. All data reported are estimated marginal means adjusted for the effect of the covariate.

Differences in target gene expression between treatment groups was analysed by comparing ΔCt values in analysis of variance models using the *car* package (Fox and Weisberg, 2018). A two-way analysis of variance model was fitted using acclimation $[Ca^{2+}]_e$ and pH as independent variables. This model was fitted for all genes of interest. *Post hoc* analyses were conducted using Tukey's honestly significant difference test for multiple comparisons.

RESULTS

Effect of acute $[Ca^{2+}]_e$ exposure on Na^+ and Ca^{2+} flux at low pH

Baseline net Na^+ and Ca^{2+} flux in control larvae (pH 6.5, 50 µmol l^{-1} $[Ca^{2+}]_e$ acclimated) at pH 6.5 was close to zero, although there was a small but significant net loss of Na^+ (t_5=−5.21, P<0.01; Fig. 1). Net Ca^{2+} flux in control larvae was not significantly different from zero. There was a significant interaction between pH and $[Ca^{2+}]_e$ on net Na^+ efflux in *L. terraereginae* larvae ($F_{29,2}$=6.18, P<0.01). Irrespective of $[Ca^{2+}]_e$, all larvae exposed acutely to pH 3.5 water experienced a substantial net loss of Na^+, indicating that Na^+ efflux rates were considerably higher than Na^+ uptake rates. The magnitude of the effect of acute low pH exposure on Na^+ flux was greatest in larvae exposed to 5 µmol l^{-1} $[Ca^{2+}]_e$. There was less effect of low pH on Na^+ efflux in larvae exposed to 50 µmol l^{-1} (t_{29}=3.2, P<0.01) and 250 µmol l^{-1} $[Ca^{2+}]_e$ (t_{29}=2.56, P=0.041). There was no effect of pH or $[Ca^{2+}]_e$ on net Ca^{2+} flux.

Effect of chronic $[Ca^{2+}]_e$ exposure on Na^+ and Ca^{2+} flux at low pH

Relative to larvae simultaneously exposed to low pH and acute alterations to $[Ca^{2+}]_e$, 4 weeks of acclimation to both low and high calcium levels reduced the impact of acute low pH exposure on net Na^+ flux ($F_{19,1}$=9.59, P<0.01; Fig. 2). Similarly, there was a significant effect of acclimation to 250 µmol l^{-1} $[Ca^{2+}]_e$ on net Ca^{2+} flux, with larvae acclimated to high $[Ca^{2+}]_e$ experiencing a significant net Ca^{2+} influx compared with larvae in 50 µmol l^{-1} $[Ca^{2+}]_e$ ($F_{19,1}$=12.56, P<0.01).

Effect of inhibition of apical Ca^{2+} uptake on Na^+ and Ca^{2+} flux in larvae acclimated to low pH

In *L. terraereginae* larvae reared from hatching at pH 3.5 and with 50 µmol l^{-1} $[Ca^{2+}]_e$ ASW, baseline net Na^+ efflux was small but significantly lower than zero (t_5=−4.1833, P<0.01; Fig. 3). Larvae had a baseline net Ca^{2+} influx that was slightly but significantly higher than zero (t_5=2.7916, P=0.038). Acute exposure of *L. terraereginae* larvae to RR resulted in a substantial increase in both net Na^+ efflux ($F_{9,1}$=71.174, P≤0.001) and net Ca^{2+} efflux ($F_{9,1}$=38.14, P<0.001) over the 6 h exposure period.

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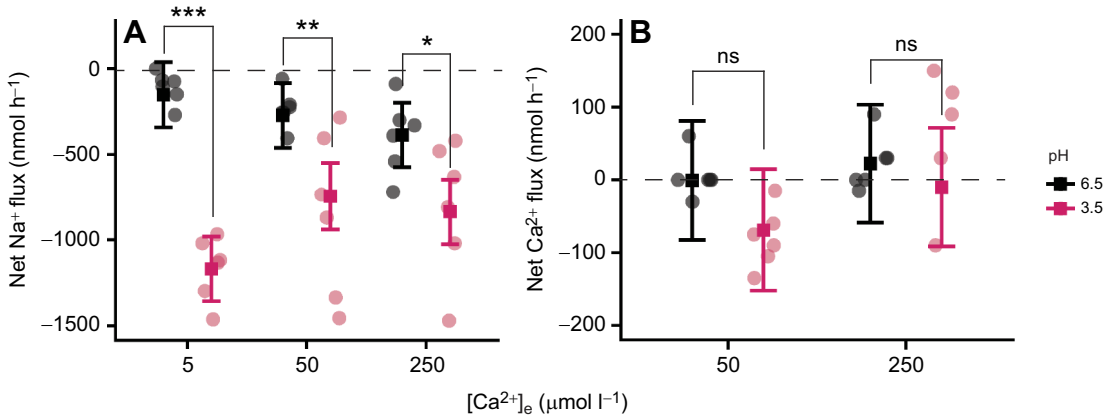

Fig. 1. Effect of acute $[Ca^{2+}]_e$ exposure on whole-animal Na^+ and Ca^{2+} flux at low pH. Net Na^+ (A) and Ca^{2+} (B) flux is shown for *Limnodynastes terraereginae* larvae (n=12 within each treatment group in experiment 1) reared in 50 μmol l^{-1} environmental Ca^{2+} ($[Ca^{2+}]_e$) at pH 6.5 and acutely exposed to low pH (pH 3.5) at 5, 50 and 250 μmol l^{-1} $[Ca^{2+}]_e$. Points indicate the estimated marginal means adjusted for body mass, and error bars represent 95% confidence intervals for the fitted models. Positive net flux indicates a net ionic influx, whereas negative net flux indicates a net ionic efflux. Asterisks indicate statistically significant differences between groups (*P<0.05, **P<0.01; ***P<0.001; ns, not significant).

Expression of Ca^{2+} transport and AJ genes

The expression of key Ca^{2+} transport proteins and E-cadherin in the gills of *L. terraereginae* was compared across larvae reared at both pH 6.5 and pH 3.5 in low, moderate or high $[Ca^{2+}]_e$. There was no significant effect of acclimation pH or $[Ca^{2+}]_e$ on the gene expression of branchial ECaC, PMCA or NCX channels (Fig. 4). However, branchial calbindin gene expression was significantly higher in larvae reared at pH 3.5 versus pH 6.5 ($F_{1,24}$=5.640, P=0.026) but was not influenced by $[Ca^{2+}]_e$.

DISCUSSION

Highly acidic waters pose a major threat to transcellular Ca^{2+} uptake pathways, which may play a role in the acute and potentially fatal branchial Na^+ loss experienced by acid-sensitive aquatic animals exposed to low pH. Conversely, acid-tolerant animals may employ a suite of mechanisms that enable them to protect Ca^{2+} uptake capacity, which in turn allows them to resist the negative effects of low pH on junctional integrity. Consistent with our hypothesis, acute exposure to low pH water in the presence of low $[Ca^{2+}]_e$ increased net Na^+ efflux, but not net Ca^{2+} flux in acid-tolerant *L. terraereginae* larvae. Provision of additional $[Ca^{2+}]_e$ reduced net Na^+ efflux rates, but this effect was saturable. Acclimation to both low and high $[Ca^{2+}]_e$ improved the resistance of larvae to Na^+ efflux at low pH and resulted in an increased net Ca^{2+} influx. Inhibition of apical epithelial calcium uptake by RR resulted in the complete loss of tolerance to low pH in larvae acclimated to low pH water, consistent with our hypothesis that acclimation to low pH involves the protection of Ca^{2+} uptake capacity. Acclimation to low pH water increased branchial gene expression of the intracellular Ca^{2+} transport protein calbindin independent of $[Ca^{2+}]_e$. Given that 90% of the transcellular Ca^{2+} flux is transported by calbindin (Bronner, 2001), and the maximum Ca^{2+} flux through rat duodenal

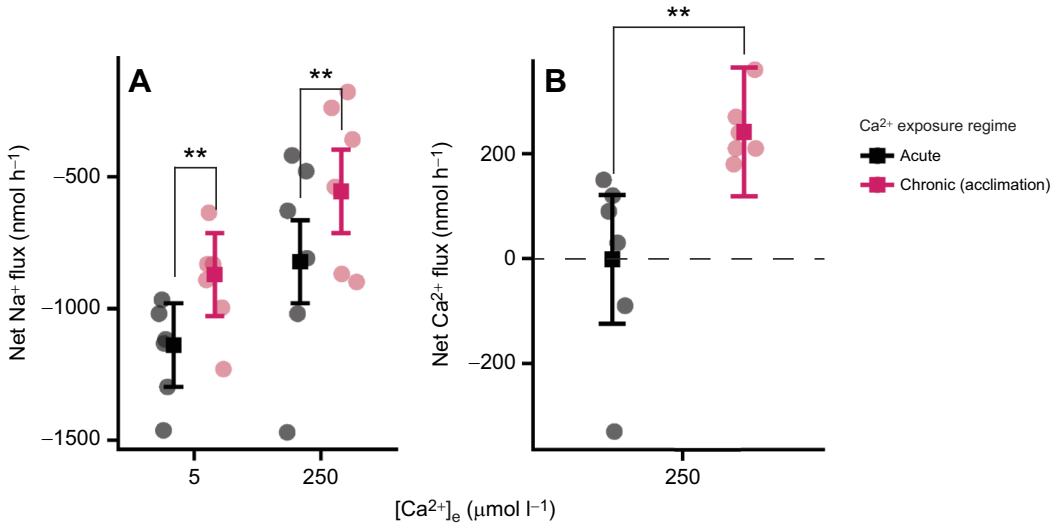

Fig. 2. Effect of acute or chronic exposure to low and high $[Ca^{2+}]_e$ on whole-animal Na^+ and Ca^{2+} flux at low pH. Net Na^+ (A) and Ca^{2+} (B) flux is shown for *L. terraereginae* larvae (n=12 within each treatment group in experiment 1) reared in 50 μmol l^{-1} $[Ca^{2+}]_e$ and acutely exposed to low pH at either 5 or 250 μmol l^{-1} $[Ca^{2+}]_e$ (Acute) compared with that for larvae (n=12 within each treatment group in experiment 2) reared in 5 or 250 μmol l^{-1} $[Ca^{2+}]_e$ and acutely exposed to low pH at equimolar $[Ca^{2+}]_e$ (Chronic). Points indicate the estimated marginal means adjusted for body mass, and error bars represent 95% confidence intervals for the fitted models. Positive net flux indicates a net ionic influx, whereas negative net flux indicates a net ionic efflux. Asterisks indicate statistically significant differences between groups (**P<0.01).

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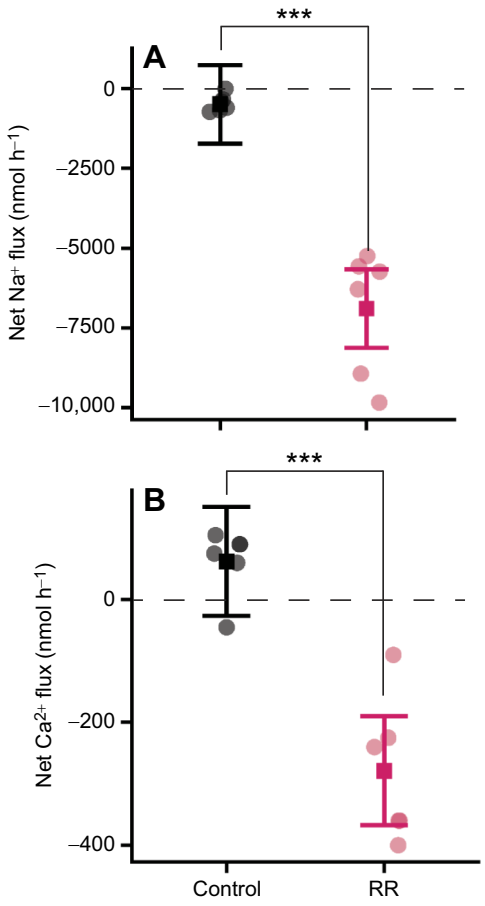

Fig. 3. Effect of inhibition of apical Ca^{2+} uptake on whole-animal Na^+ and Ca^{2+} flux following acclimation to low pH. Net Na^+ (A) and Ca^{2+} (B) flux is shown for *L. terraereginae* larvae (n=12 within each group) reared at pH 3.5 with 50 µmol l^{-1} $[Ca^{2+}]_e$, acutely exposed to 10 µmol l^{-1} ruthenium red (RR) at pH 3.5. Points indicate the estimated marginal means adjusted for mass, and error bars represent 95% confidence intervals for the fitted model. Net Na^+ and Ca^{2+} flux was significantly higher in *L. terraereginae* acutely exposed to 10 µmol l^{-1} RR for 6 h. Asterisks indicate statistically significant differences between groups (***P<0.001).

cells is a linear function of the cellular calbindin content (Bronner et al., 1986), we contend that the finding of calbindin upregulation at low pH is consistent with a role for increased transcellular Ca^{2+} trafficking in the tolerance of low pH water. These results establish a potential role for the security of Ca^{2+} uptake capacity in the tolerance of *L. terraereginae* larvae living in highly acidic waters.

Acid-naive *L. terraereginae* larvae reared at circumneutral pH and acutely exposed to low pH had a greater rate of net Na^+ efflux than larvae reared and tested at pH 6.5, consistent with an acute negative effect of low pH on intercellular junction integrity. The high rate of net Na^+ efflux was substantially reduced in animals acclimated to low pH, consistent with an acid-tolerant phenotype. In acid-naive *L. terraereginae*, the high rate of net Na^+ efflux was exacerbated in the presence of low $[Ca^{2+}]_e$, indicating a protective effect of $[Ca^{2+}]_e$ on epithelial junction integrity. This is consistent with previous studies showing that $[Ca^{2+}]_e$ plays a significant role in determining net Na^+ efflux rates and mortality (acid tolerance) with low pH exposure in a range of fish and amphibian larvae (Brown, 1981, 1982, 1983; Cummins, 1988; Freda and Dunson, 1984; Freda et al., 1991; Gascon et al., 1987; Gonzalez and Dunson, 1989; Gonzalez et al., 1998; Kullberg et al., 1993; Kumai et al., 2011; McDonald and Rogano, 1986; McDonald et al., 1983; Meyer et al., 2010; Riesch et al., 2015; Wright and Snekvik, 1978). However, environmental Ca^{2+} was only beneficial for controlling Na^+ efflux up to a point: exposure of larvae to 250 µmol l^{-1} $[Ca^{2+}]_e$ did not further reduce net Na^+ flux beyond that of larvae exposed to control (50 µmol l^{-1}) levels. This may indicate the influence of elevated Ca^{2+} on Na^+ efflux is saturable, and 50 µmol l^{-1} $[Ca^{2+}]_e$ completely saturates the gill epithelium. This is consistent with the findings of Meyer (2004), who demonstrated that 80 µmol l^{-1} $[Ca^{2+}]_e$ reduced Na^+ efflux during low pH exposure in *L. terraereginae* larvae, but raising $[Ca^{2+}]_e$ to 400 µmol l^{-1} had no further effect. Caution must be taken in extrapolating this finding directly to the natural setting, where dissolved organic compounds may alter the effects of H^+ on the ion transport processes (Picker et al., 1993; Wood et al., 2003).

The protective effect of $[Ca^{2+}]_e$ on branchial junction permeability has been attributed to extracellular Ca^{2+} bound to the gill epithelium, specifically to the TJ (Freda and McDonald, 1988; Gonzalez and Dunson, 1989; McDonald, 1983; McWilliams, 1983; Reid et al., 1991; Yu et al., 2010). However, these studies did not account for the possibility that low pH affects junctional stability by impairing transcellular Ca^{2+} uptake pathways. Exposure of *L. terraereginae* larvae to water containing 10 µmol l^{-1} RR, a potent ECaC channel inhibitor (Nilius et al., 2001), resulted in a large (7500–17,500 nmol h^{-1}) increase in net Na^+ efflux. This suggests that the inhibition of the transcellular Ca^{2+} uptake pathway has an immediate and severe effect on junctional stability, leading to increased junctional permeability to Na^+. Similar findings have been observed in studies of goldfish and tetras exposed to La^{3+}, a Ca^{2+} analogue that, unlike RR, also has the capacity to penetrate TJs and interact with AJs directly (Eddy and Bath, 1979; Gonzalez et al., 1997; Lacaz-Vieira and Marques, 2004). The inhibition of transcellular Ca^{2+} uptake using RR has not been performed previously on whole animals but does show that inhibition of Ca^{2+} uptake can have catastrophic impacts on homeostasis, consistent with the loss of junctional stability. We posit that the inhibition of apical Ca^{2+} uptake disrupts AJ stability, with consequences for the maintenance of TJ stability, and that any effect of acid on net Na^+ flux is at least in part mediated by calcium ions. This is consistent with the finding that Ca^{2+} channel inhibitors increased amphibian embryo mortality at low pH, but Na^+ channel blockers did not (Shu et al., 2015). As ECaC activity is inhibited by low pH (Vennekens et al., 2001), we propose that a loss of Ca^{2+} uptake capacity following acute exposure to low pH may underpin the loss of junctional stability and resulting Na^+ efflux in acid-sensitive organisms. Conversely, adaptations that counter ECaC inhibition at low pH may protect Ca^{2+} uptake capacity in acidophilic species. While the effects of RR on Na^+ efflux in acid-acclimated larvae were rapid and extreme, an investigation of its effects on junctional morphology would be needed to demonstrate that high Na^+ losses were the result of junctional disruption and not the inhibition of other Na^+ and Ca^{2+} transport pathways. While RR is a potent inhibitor of ECaC, it can also affect other Ca^{2+} transport proteins which may otherwise affect the maintenance of ion balance (Hajnóczky et al., 2006; Vincent and Duncton, 2011), or act as a systemic toxicant.

Unlike Na^+ flux, acute exposure to low pH water did not affect net Ca^{2+} flux when larvae were acutely exposed to high or control $[Ca^{2+}]_e$. In both circumneutral and low pH water, Ca^{2+} flux was not significantly different from zero, suggesting that rates of influx and efflux were balanced. This was unexpected as low pH has been shown to inhibit Ca^{2+} uptake (ECaC activity) in multiple cell lines (Bindels et al., 1994; Hoenderop et al., 1999; Vennekens et al.,

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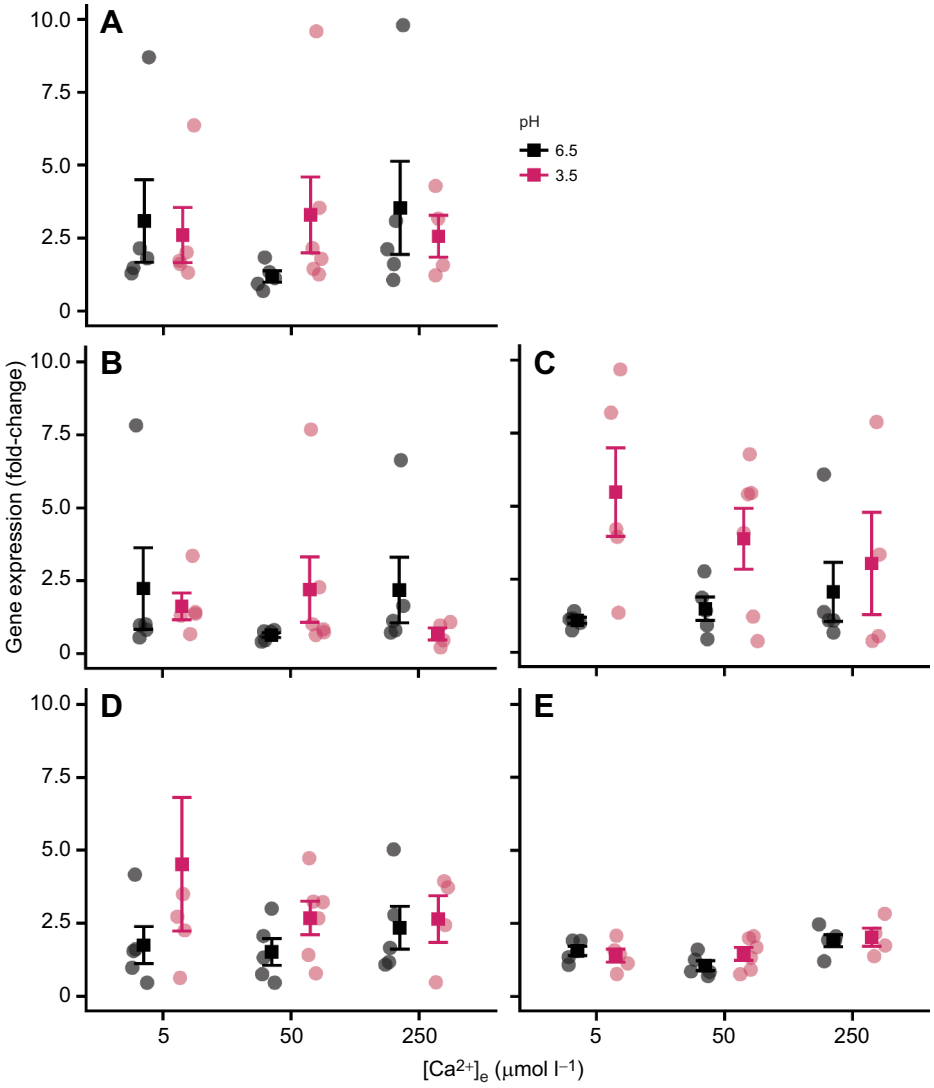

Fig. 4. Effect of $[Ca^{2+}]_e$ exposure and low pH on expression of Ca^{2+} transport and adherens junction (AJ) genes. Expression of (A) *ECaC*, (B) *NCX*, (C) *Calb1*, (D) *PMCA* and (E) *E-cadherin* in *L. terraereginae* larvae (n=6 per group) reared in 5, 50 and 250 μmol l^{-1} $[Ca^{2+}]_e$ at circumneutral (pH 6.5) and low pH (pH 3.5). Change in expression is represented as fold-change relative to the mean value for the control group (pH 6.5, 50 μmol l^{-1} $[Ca^{2+}]_e$). Squares and error bars indicate mean fold-change and s.e.m.

2001). If low pH also inhibited ECaC activity in *L. terraereginae*, it should have manifested as a net increase in the rate of Ca^{2+} loss. In fact, *L. terraereginae* larvae reared in high $[Ca^{2+}]_e$ and acutely exposed to low pH experienced a net Ca^{2+} influx (~250 nmol h^{-1}) compared with animals reared at control $[Ca^{2+}]_e$ levels. In low pH reared larvae, there was an apparent net increase in Ca^{2+} uptake. This influx suggests that ECaC in the branchial epithelium of *L. terraereginae* larvae is not substantially inhibited by protonation, and that transcellular environmental Ca^{2+} uptake is maintained at low pH and may play a role in facilitating acid tolerance in *L. terraereginae*. This may highlight an adaptation for the prevention of Ca^{2+} uptake inhibition at low pH and could be linked to the expression of a less pH sensitive ECaC isoform. Consistent with this hypothesis, inhibition of apical Ca^{2+} uptake by RR did result in a large increase in net Ca^{2+} efflux in larvae. Clearly, the disturbance of transcellular Ca^{2+} uptake has serious implications for the maintenance of transepithelial resistance via the loss of junction stability at low pH.

The maintenance of transcellular Ca^{2+} transport is potentially important in promoting acid tolerance of acidophilic animals. Ca^{2+} might influence tolerance of low pH via association with the Ca^{2+}-dependent AJ, which is directly responsible for the stability of the TJ and thus epithelial permeability, a major factor in preventing Na^+ loss at low pH in acidophilic species (Gumbiner et al., 1988; Kwong et al., 2014; Watabe-Uchida et al., 1998). Lowering intracellular $[Ca^{2+}]$ in MDCK cells has been shown to interfere with the formation of TJs (Stuart et al., 1996). Ca^{2+} also has many signalling functions such as hormone regulation (Clapham, 2007; D'Souza-Li, 2006), which may potentially alter the expression of genes involved in maintaining junctional integrity at low pH. Calbindin and other intracellular Ca^{2+}-binding proteins function to buffer intracellular Ca^{2+} concentration by facilitating the basolateral extrusion of Ca^{2+} taken up across the apical membrane (Christakos et al., 1989). The finding that calbindin mRNA was upregulated in *L. terraereginae* larvae reared at pH 3.5 is suggestive of increased transcellular Ca^{2+} movement in the gill epithelium of *L. terraereginae* larvae acclimated to low pH. Interestingly, cytosolic Ca^{2+} is critical in regulating ECaC activity (Hoenderop et al., 1999). This finding is consistent with the idea that transcellular Ca^{2+} transport is involved in acid tolerance to some degree and that Ca^{2+} uptake is not inhibited by low pH in *L. terraereginae* larvae.

In contrast to our hypotheses, mRNA expression of the key transcellular Ca^{2+} transport proteins ECaC, NCX and PMCA was not influenced by environmental pH or $[Ca^{2+}]_e$ in *L. terraereginae* larvae despite the observation that acclimation to low pH was

accompanied by an apparent net increase in Ca^{2+} uptake. If this effect was indeed the result of an increase in Ca^{2+} uptake and not a reduction in efflux, then it is possible that it was facilitated by an increase in the activity of the existing channels as opposed to the *de novo* production of more channels. Likewise, the lack of increase in E-cadherin mRNA expression with chronic low pH exposure suggests that E-cadherin function was unaffected by low pH. E-cadherins bind Ca^{2+} from the extracellular environment; our data suggest that maintenance of the transcellular Ca^{2+} transport pathway allows the maintenance of favourable Ca^{2+} concentrations in the extracellular space to prevent E-cadherin disfunction. Consistent with this idea, increased expression of calbindin mRNA in acid-acclimated larvae provides some evidence that increased transcellular Ca^{2+} transport plays a role in promoting extreme acid tolerance. Increased Ca^{2+} uptake at the apical membrane may be evidenced by increased intracellular Ca^{2+} shuttling rates (and an associated increased abundance of calbindin proteins) to maintain Ca^{2+}-dependent junction dynamics in low pH environments. The finding that low pH-acclimated larvae upregulated calbindin mRNA and had a net Ca^{2+} influx supports a role for increased transcellular $[Ca^{2+}]_e$ movement in facilitating acid tolerance in *L. terraereginae* larvae. However, mRNA expression levels do not always correlate well with actual levels of protein expression, so care must be taken when interpreting mRNA expression patterns in the absence of corresponding protein expression levels. Differential post-translational processing of mRNA and other factors can be responsible for the low correlation between an organism's transcriptome and its proteome (Ghazalpour et al., 2011; Marguerat et al., 2012). Although we cannot rule out a paracellular route for the uptake of Ca^{2+} into the extracellular space, studies in fish suggest that more than 97% of branchial (gills) Ca^{2+} uptake is active (transcellular) (Flik et al., 1995). The hormonal control of Ca^{2+} transport protein function and abundance is a potentially overlooked factor in understanding how branchial Ca^{2+} transport is influenced by extracellular pH and $[Ca^{2+}]_e$ in *L. terraereginae*.

This study showed that environmental Ca^{2+} has a protective effect on the control of Na^+ efflux at low pH in *L. terraereginae* larvae. Furthermore, it demonstrated that larvae have a capacity for acclimation to low pH via changes in Na^+ and Ca^{2+} flux, which appear to involve the transcellular pathway (i.e. increased calbindin mRNA upregulation). Given that *L. terraereginae* larvae can maintain or increase Ca^{2+} uptake at low pH, this suggests that protonation of the branchial epithelium likely does not outwardly inhibit Ca^{2+} uptake. However, we only examined net Na^+ and Ca^{2+} flux, which does not reveal details about the behaviour of separate uptake and efflux pathways. For example, increases in efflux rates that are compensated for by commensurate increases in uptake rates may be missed by measuring just net ion flux. Radioactive isotopes or fluorescent ion analogues could be used to better resolve changes in influx and efflux pathways and how they contribute to net ion flux. Inhibition of apical Ca^{2+} uptake by RR supports a role for the maintenance of transcellular Ca^{2+} uptake in the control of branchial junction stability at low pH in *L. terraereginae* but does not reveal the specific site of action for Ca^{2+} in preventing Na^+ loss at low pH. Using intracellular Ca^{2+} markers to track Ca^{2+} movement during acclimation to low pH may help to elucidate this mechanism. As *L. terraereginae* larvae are exceptionally acid tolerant, their ability to maintain Ca^{2+} uptake in very soft and acidic waters may be a unique adaptation. Comparing transcellular Ca^{2+} transport capabilities with those of other acid-sensitive species might reveal the mechanistic adaptations employed by *L. terraereginae* in the maintenance of epithelial stability at low pH. The current study highlights a role for transcellular Ca^{2+} transport and the prevention of Ca^{2+} uptake inhibition by low pH in the extreme acid tolerance of *L. terraereginae* larvae.

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Author contributions
Conceptualization: C.H., C.E.F., R.L.C.; Methodology: C.H., C.E.F., R.L.C.; Software: C.E.F., R.L.C.; Validation: C.H., C.E.F., R.L.C.; Formal analysis: C.H., C.E.F., R.L.C.; Investigation: C.H., C.E.F., R.L.C.; Resources: C.E.F., R.L.C.; Data curation: C.H., C.E.F., R.L.C.; Writing - original draft: C.H., C.E.F., R.L.C.; Writing - review & editing: C.H., C.E.F., R.L.C.; Visualization: C.H., C.E.F., R.L.C.; Supervision: C.E.F., R.L.C.; Project administration: C.E.F., R.L.C.; Funding acquisition: C.E.F., R.L.C.

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Data availability
The complete datasets and R scripts used for analysing the data are publicly available at UQ eSpace: https://doi.org/10.14264/18c7301

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