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REVIEW

The physiology of fish at low pH: the zebrafish as a model system

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ABSTRACT

Ionic regulation and acid–base balance are fundamental to the physiology of vertebrates including fish. Acidification of freshwater ecosystems is recognized as a global environmental problem, and the physiological responses to acid exposure in a few fish species are well characterized. However, the underlying mechanisms promoting ionic and acid–base balance for most fish species that have been investigated remain unclear. Zebrafish (*Danio rerio*) has emerged as a powerful model system to elucidate the molecular basis of ionic and acid–base regulation. The utility of zebrafish is related to the ease with which it can be genetically manipulated, its suitability for state-of-the-art molecular and cellular approaches, and its tolerance to diverse environmental conditions. Recent studies have identified several key regulatory mechanisms enabling acclimation of zebrafish to acidic environments, including activation of the sodium/hydrogen exchanger (NHE) and H^+-ATPase for acid secretion and Na^+ uptake, cortisol-mediated regulation of transcellular and paracellular Na^+ movements, and ionocyte proliferation controlled by specific cell-fate transcription factors. These integrated physiological responses ultimately contribute to ionic and acid–base homeostasis in zebrafish exposed to acidic water. In the present review, we provide an overview of the general effects of acid exposure on freshwater fish, the adaptive mechanisms promoting extreme acid tolerance in fishes native to acidic environments, and the mechanisms regulating ionic and acid–base balance during acid exposure in zebrafish.

KEY WORDS: Acid exposure, Acid–base balance, Ionic regulation, Zebrafish

Introduction

Declining fish populations in acidified lakes and streams (see review by McDonald, 1983a) was a significant factor promoting intensive research over the past 40 years on the ecological and physiological consequences of aquatic acidification. In freshwater (FW) ecosystems, acidification is caused largely by atmospheric acidic deposition (i.e. acid rain). Acidification of FW is a global problem, which has been documented in several geographic regions including eastern Europe, the USA and China (Psenner, 1994; Schindler, 1988; Wright et al., 2005). In Canada, it is estimated that about 40% of lakes are in regions susceptible to acid deposition (Kelso et al., 1990). Most aquatic animals, including fish, live in a narrow range of pH near neutrality, and acute or chronic exposure to acidic water can adversely affect their physiological functions (McDonald, 1983a; Wood, 1989). Nevertheless, some species thrive in acidic environments, and thus can serve as useful models for investigating the mechanisms underlying adaptation to acid stress.

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The mechanisms of ionic and acid–base regulation in fish have been summarized in previous reviews (Claiborne et al., 2002; Evans et al., 2005; Gilmour and Perry, 2009; Goss et al., 1992; Haswell et al., 1980; Hwang, 2009; Marshall and Grosell, 2006; McDonald, 1983a; Perry, 1997; Perry and Gilmour, 2006; Wood, 1989). In this review we provide an overview of the general effects of acid exposure on FW fish, with particular emphasis on recent developments linking environmental acidification and ionic regulation in the acid-tolerant zebrafish.

General effects of acid exposure on freshwater fish

Acid exposure can affect the gill in numerous ways, such as by increasing the number and turnover of ion-transporting cells (ionocytes), elevating mucus production and recruiting leukocytes (Balm and Pottinger, 1993; Fromm, 1980; Laurent and Perry, 1991; Leino and McCormick, 1984; Leino et al., 1987; Wendelaar Bonga et al., 1990). Breakdown of gill structure and suffocation caused by mucus accumulation may also occur in fish exposed to extreme acidic water (pH 2.0–3.5) (Packer and Dunson, 1972). At about pH 4.0–4.5, however, the primary effects of acid exposure on most fish species that have been examined are inhibition of active Na^+ uptake coupled with increased rates of passive Na^+ losses, leading to a decrease in plasma Na^+ level (see Table 1 and references therein). Because Na^+ uptake in FW fish is primarily coupled to H^+ secretion through the actions of Na^+/H^+ exchanger (NHE) and H^+-ATPase (Evans et al., 2005), a reduction in environmental pH level would reduce the gradient to drive Na^+ influx. Acid exposure is also known to inhibit Cl^- uptake and decrease plasma Cl^- levels in fish (McDonald, 1983b; McDonald and Wood, 1981; Wood et al., 1998); however, the mechanism for this inhibitory effect is less clear. It was suggested that reductions in plasma bicarbonate (HCO_3^-) concentrations associated with metabolic acidosis may decrease the activity of the Cl^-/HCO_3^- exchanger (Ultsch et al., 1981). Alternatively, elevated H^+ levels in the water may directly impair the structure and/or function of the Cl^-/HCO_3^- exchanger and other Cl^--transporting proteins. Subsequently, reduction of plasma ion levels may promote fluid loss from the vascular compartment, thereby reducing plasma volume and elevating blood viscosity (Milligan and Wood, 1982). Ultimately, mortality may arise from cardiovascular failure (Milligan and Wood, 1982).

The increased ion losses during acid exposure are thought to be largely associated with the disruption of paracellular tight junctions (TJs) (Freda et al., 1991; Kumai et al., 2011; Kwong and Perry, 2013a; McDonald, 1983a). While the precise mechanism of how acid affects TJ integrity is not fully understood, it is believed to be caused by Ca^{2+} displacement from the TJs. Ca^{2+} is essential in the assembly and sealing of TJ proteins between cell contacts (Gonzalez-Mariscal et al., 1990), and thus leaching of Ca^{2+} by acid exposure may disrupt stable cell–cell contacts and increase paracellular permeability to ions (Freda et al., 1991). Indeed, elevation of water Ca^{2+} level reduces diffusive ion losses in fish

List of abbreviations

CA	carbonic anhydrase
DOC	dissolved organic carbon
ECaC	epithelial Ca^{2+} channel
EIPA	5-(*N*-ethyl-*N*-isopropyl)amiloride
ENaC	epithelial Na^+ channel
FW	freshwater
GR	glucocorticoid receptor
HRC	H^+-ATPase-rich cell
KSC	K^+ secreting cell
NaRC	Na^+/K^+-ATPase rich cell
NCC	Na^+/Cl^- co-transporter
NCCC	Na^+/Cl^- co-transporter expressing cell
NCX	Na^+/Ca^{2+} exchanger
NHE	Na^+/H^+ exchanger
PMCA	plasma membrane Ca^{2+}-ATPase
Rhcg1	rhesus glycoprotein 1
SIET	scanning ion-selective electrode technique
TA	titratable acidity
TJ	tight junction

exposed to acidic water (Gonzalez and Dunson, 1989a; McDonald et al., 1980; McWilliams, 1982).

Water hardness may also influence the effects of acid exposure on internal acid–base homeostasis in fish. For example, it is well documented that under soft water conditions (i.e. low environmental Ca^{2+} levels), exposure to acidic water adversely affects ionic balance but does not alter blood acid–base status (Gonzalez et al., 2005; McDonald et al., 1980; Wilson et al., 1999; Wood et al., 1998). The acid–base responses (e.g. metabolic acidosis) during acid exposure are likely attributed to the difference between net cation (Na^+, K^+) and anion (Cl^-) fluxes, as suggested by the strong ion difference theory (Stewart, 1978; Stewart, 1983). Typically, in soft water conditions, acid exposure causes equimolar losses of Na^+ and Cl^-, thus preventing a net influx of H^+ and consequent acid–base disturbance. In hard water, however, acid exposure results in an excess of Na^+ over Cl^- loss, thereby promoting the influx of acid equivalents, which leads to metabolic acidosis. During metabolic acidosis, the kidney may compensate by increasing the excretion of acidic equivalents in the form of both titratable acidity (TA) and ammonium (NH_4^+) ions (King and Goldstein, 1983; McDonald, 1983b; McDonald and Wood, 1981; Wood et al., 1999).

A complicating factor associated with the acidification of water is increased leaching of toxic trace elements from the soil and rock into the water; the increased concentrations of toxic metals may impose additional challenges on fish (Campbel and Stokes, 1985; McDonald et al., 1989; Wood, 1989). In particular, the elevation of Al^{3+} concentrations in acidified lakes and streams maybe an important factor contributing to fish mortality (Schofield and Trojnar, 1980). Additionally, Al^{3+} exposure is known to exacerbate ion losses in fish inhabiting acidic environments (Booth et al., 1988; Playle et al., 1989).

Acid exposure is also known to affect endocrine responses in fish; two particularly well-documented responses are elevated whole-body or plasma levels of prolactin (Wendelaar Bonga and Balm, 1989; Wendelaar Bonga et al., 1984) and cortisol (Brown et al., 1989; Goss and Wood, 1988; Kakizawa et al., 1996; Kumai et al., 2012a; Nagae et al., 2001). Consistent with their increased levels during acid exposure, these hormones are able to promote ion uptake (Flik and Perry, 1989; Flik et al., 1989; Kumai et al., 2012a; Lin et al., 2011), increase H^+-ATPase activity for acid secretion (Lin and Randall, 1993), and reduce epithelial permeability (Chasiotis and Kelly, 2011; Chasiotis et al., 2010; Kelly and Wood, 2001; Kwong and Perry, 2013a; Tipsmark et al., 2009). Therefore, these hormones may play critical roles during the acclimation of fish to acidic environments.

Diverse mechanisms of adaptation in fish natively living in acidic environments

Probably the most extensively studied group of acid-tolerant species of fish are found in the Rio Negro of the Amazon River system. While Rio Negro water chemistry is extremely harsh (pH is around 4.5–5.1; Na^+ and Ca^{2+} levels are ~10 and ~5 μmol l^{-1}, respectively), ~1000 species of fish are estimated to be distributed in these waters (Gonzalez et al., 2005). Previous research identified two major

Table 1. Effects of acid exposure on Na^+ balance and other physiological responses in freshwater fish

Species	pH	Exposure time	Na^+ balance	Gene/protein responses	Others	Reference
Tilapia *Oreochromis mossambicus* (Mozambique tilapia)	3.5–4.0	48–168 h		Somatolactin ↑ Growth hormone ↑ NHE3 ↑ NCC ↑	Ionocyte enlargement	Furukawa et al., 2011; Furukawa et al., 2010
	4.0–4.5	40–90 days	Influx and efflux ↓ Plasma $[Na^+]$ ↓ transiently		Water influx ↑ transiently Prolactin cell activity ↑	Flik et al., 1989; Wendelaar Bonga et al., 1984
Oreochromis niloticus (Nile tilapia)	5.3	36 h		Antioxidant enzymes ↑	Oxidative stress ↑ DNA damage and apoptosis in blood cells	Mai et al., 2010
Shiner *Notropis cornutus* (common shiner)	4.0	4 h	Influx ↓ Efflux ↑ Net $[Na^+]$ loss ↑		Lethal in ~5 h	Freda and McDonald, 1988b
Perch *Perca flavescens* (yellow perch)	4.0	48 h	Influx → Efflux ↑ Net $[Na^+]$ loss ↑			Freda and McDonald, 1988b
Perca fluviatilis (European perch)	3.8–4.5	72 h	Plasma $[Na^+]$ ↓		Haematocrit ↑	Rask and Virtanen, 1986
Sunfish *Lepomis gibbosus* (pumpkinseed sunfish)	4.0	12 h	Influx ↓ Efflux ↑			Gonzalez and Dunson, 1987
	3.5	<24 h	Influx ↓ Efflux ↑ Plasma $[Na^+]$ ↓		Lethal in <24 h	Gonzalez and Dunson, 1987

Table 1. Continued

Species	pH	Exposure time	Na^+ balance	Gene/protein responses	Others	Reference
Enneacanthus obesus (banded sunfish)	3.5	24 h	Influx ↓ (mild) Efflux ↑ (mild)		Haematocrit ↑	Gonzalez and Dunson, 1987
	3.5	1–2 weeks	Bone $[Na^+]$ ↓ Plasma and whole-body $[Na^+]$ ↓		Body water content ↓	Gonzalez and Dunson, 1987; Gonzalez and Dunson, 1989b
	4.0	1 h	Influx ↓ Efflux →			Gonzalez et al., 2005
Trout *Oncorhynchus mykiss* (rainbow trout)	4.0	6 h	Influx ↓ Efflux ↑ Net $[Na^+]$ loss ↑		Lethal in ~6.5 h	Freda and McDonald, 1988b
	4.2	4 days	Net $[Na^+]$ loss ↑ Plasma $[Na^+]$ ↓		Plasma pH and HCO_3^- ↓ Renal H^+ and NH_4^+ secretion ↑	McDonald and Wood, 1981
	4.5	7 days	Plasma $[Na^+]$ ↓	Somatolactin ↑ Growth hormone ↓ Plasma cortisol ↑	Blood pH ↓	Kakizawa et al., 1996
Salmo trutta (brown trout)	4.0	3 h	Influx ↓ Efflux ↑			McWilliams and Potts, 1978
	6.0	6 weeks	Influx and plasma $[Na^+]$ ↓ transiently		Haematocrit ↓	McWilliams, 1980
Salvelinus fontinalis (brook trout)	3.0–3.5	2.6 h	Influx ↓ Net $[Na^+]$ loss ↑ Whole-body $[Na^+]$ ↓		O_2 consumption ↓ Plasma pH ↓ Mortality occurs when plasma pH drops to ~7	Packer and Dunson, 1970
Dace *Tribolodon hakonensis* (Japanese dace)	3.5	48–168 h	Plasma $[Na^+]$ ↓ (mild)	NHE3 ↑ Aquaporin3 ↑ CA II ↑ NBC1 ↑	Blood pH ↓	Hirata et al., 2003
	3.6–3.7 (lake water)	3 days	Plasma $[Na^+]$ ↓		Ionocyte proliferation Blood pH ↓ transiently	Kaneko et al., 1999
Characids *Colossoma macropomum* (Tambaqui)	3.0	Gradual reduction from pH 6.5 to 3.0 in 3 days	Plasma $[Na^+]$ ↓	Plasma cortisol ↑	Plasma ammonia and glucose ↑ Plasma protein levels ↑	Wood et al., 1998
	3.5	24 h	Net $[Na^+]$ loss ↑ transiently		Ammonia excretion ↑	Wilson et al., 1999
Brycon erythropterum (Matrincha)	3.5	24 h	Net $[Na^+]$ loss ↑		Ammonia excretion ↑ Net acidic-equivalent fluxes ↓ Mortality occurs when transfered back to pH 6.0	Wilson et al., 1999
Tetra *Gymnocorymbus ternetzi* (black skirt tetra)	4.0	1 h	Influx ↓ Efflux ↑ Net $[Na^+]$ loss ↑			Gonzalez et al., 1997
	4.5	24 h	Influx and efflux ↑, Net $[Na^+]$ loss ↑ transiently			Gonzalez et al., 1997
Cichlid *Pterophyllum scalare* (angelfish)	3.5	1 h	Influx ↓ Efflux ↑ Net $[Na^+]$ loss ↑		Lethal in ~3 days	Gonzalez and Wilson, 2001
	4.0	6 days	Influx ↓ Efflux → Net $[Na^+]$ loss ↑ transiently			Gonzalez and Wilson, 2001
Catfish *Clarias mossambicus* (North African catfish)	4.0–5.0	4 days	Influx → Efflux ↑ Net $[Na^+]$ loss ↑			Eddy and Maloiy, 1983
Rhamdia quelen (South American catfish)	5.0	10 days			Ammonia excretion ↑ Urea, creatinine and protein excretion ↓	Golombieski et al., 2013
Hoplosternum littorale (Tamoata)	3.5	24 h	Net $[Na^+]$ loss ↑		Lethal ≤18 h Ammonia excretion ↑ Titratable acid fluxes ↑	Wilson et al., 1999
Whitefish *Coregonus clupeaformis* (lake whitefish)	4.1	2 weeks	Plasma $[Na^+]$ ↓	Plasma cortisol ↑	Plasma glucose ↑ Daytime activity ↓ Feeding activity ↓	Scherer et al., 1986

↓, decrease; ↑, increase; →, no change.

strategies for fish to defend Na^+ homeostasis in such an environment: (1) the functioning of a high-capacity, high-affinity Na^+ uptake mechanism that is largely insensitive to acid challenge, and (2) an insensitivity of Na^+ efflux to acid exposure.

Some species of tetra, including neon tetra (*Paracheirodon innesi*) (Gonzalez and Preest, 1999), cardinal tetra (*Paracheirodon axelrodi*) (Gonzalez and Wilson, 2001) and black skirt tetra (*Gymnocorhymbus ternetzi*) (Gonzalez et al., 1997), fall into the first category; they demonstrate insensitivity of Na^+ uptake to water pH as low as 3.5–4.5. In *P. innesi*, Na^+ uptake was found to be inhibited by silver nitrate, which was attributed to inhibition of Na^+/K^+-ATPase activity (Preest et al., 2005). Interestingly, Preest et al. (Preest et al., 2005) also reported that Na^+ uptake in *P. innesi* is insensitive to commonly used pharmacological inhibitors of Na^+ uptake including amiloride [inhibits both epithelial Na^+ channels (ENaCs) and NHE] as well as vanadate (inhibits H^+-ATPase). The lack of an effect of these pharmacological agents may reflect significant differences in the molecular structure of NHE and/or H^+-ATPase, rendering them insensitive to these commonly used inhibitors, or alternatively that under these conditions, these species are absorbing Na^+ through other pathways independent of acid secretion (e.g. Na^+/Cl^- co-transporter, NCC).

Other species, including FW stingray (*Potamotrygon* sp.) (Wood et al., 2002) and several species of teleost including angelfish (*Pterophyllum scalare*) (Gonzalez and Wilson, 2001) and caracid (*Hemigrammus* sp.) (Gonzalez et al., 2002), from the Rio Negro system fall into the second category (see above); they maintain Na^+ efflux at a constant level even when acutely challenged with water of pH 4.0. Unlike tetras, Na^+ uptake in these species is inhibited markedly by exposure to pH 4.0. This response (constant Na^+ efflux with reduced influx) was also observed in some acid-tolerant species distributed in North America, such as banded sunfish (*Enneacanthus obesus*) (Gonzalez and Dunson, 1987; Gonzalez and Dunson, 1989a) and white perch (*Perca flavescens*) (Freda and McDonald, 1988). Remarkably, it has been shown that Na^+ efflux in *E. obesus* was not significantly different from zero after an exposure to water of pH 4, with exposure to pH 3.5 causing only a transient and minor increase in efflux (Gonzalez and Dunson, 1987; Gonzalez and Dunson, 1989a). Because of the probable protective effect of water Ca^{2+} in the regulation of Na^+ efflux (discussed above), it was assumed that the exceptional acid insensitivity of Na^+ efflux in these species is due to the unusually high affinity of TJs to Ca^{2+}. For example, Wood et al. (Wood et al., 2002) demonstrated that acute exposure to pH 4 did not affect the surface-bound Ca^{2+} in *Potamotrygon* sp. Based on the non-linear relationship between Na^+ efflux and ambient Ca^{2+} levels, Gonzalez and Dunson (Gonzalez and Dunson, 1989a) calculated that for *E. obesus*, only 19 μmol l^{-1} of water Ca^{2+} was required to reduce the Na^+ efflux rate by 50% from what was observed in Ca^{2+}-free acidic water. Conversely, Freda and McDonald (Freda and McDonald, 1988) reported the Na^+ efflux in *P. flavescens* was independent of water Ca^{2+} level even at pH 3.25. This result, however, should be interpreted with caution because the lowest Ca^{2+} level in which they measured Na^+ efflux was ~35 μmol l^{-1}. Although the biochemical/molecular basis for differential Ca^{2+} affinities of TJs in different species is unknown, it is nevertheless apparent that certain acid-tolerant fish achieve their ionic homeostasis by controlling Na^+ efflux in acidic water.

Water chemistry may also contribute to measured acid tolerance in some fish species. For example, in *Potamotrygon* sp. (Wood et al., 2003) and *Geophagus* sp. (Gonzalez et al., 2002), Na^+ efflux was significantly higher when measured in acidified non-Rio Negro water than in Rio Negro water. It is believed that the presence of high levels of dissolved organic carbon (DOC) in Rio Negro water may attenuate the effects of acid exposure on ionic balance in these extreme environmental conditions. However, Wood et al. (Wood et al., 2003) observed that supplementing the reference acidic water with commercial humic acid actually increased the Na^+ efflux. The chemical identity of the protective DOC in Rio Negro water and the exact molecular mechanism responsible for Na^+ efflux regulation remain to be elucidated.

Another example of an acid-tolerant species is the Osorezan dace (*Tribolodon hakonensis*), the only fish living in Osorezan lake, where water pH is as low as 3.4 (Hirata et al., 2003; Kaneko et al., 1999). Although *T. hakonensis* migrates to inflowing streams during spawning, where water pH is higher, it otherwise thrives in Osorezan lake (Kaneko et al., 1999). In laboratory conditions, exposure to pH 3.5 water initially reduced both plasma pH and Na^+ levels, but both parameters returned to near-control levels by 48–72 h (Hirata et al., 2003; Kaneko et al., 1999). Because these studies only measured net ionic balance (plasma [Na^+]), it remains to be seen whether *T. hakonensis* regulates its plasma Na^+ level by adjusting passive efflux or active uptake. Nevertheless, Hirata et al. (Hirata et al., 2003) showed that acid exposure led to significant increase in the branchial mRNA expression level of NHE3, but not H^+-ATPase, suggesting that NHE3 may play a role in acid secretion as well as Na^+ uptake. The potential role of NHE3 in acid secretion/Na^+ uptake in this species in acidic water is in contrast to the lack of inhibition of Na^+ uptake by amiloride in neon tetra (Preest et al., 2005). Although the negative results of Preest et al. (Preest et al., 2005) should be interpreted with caution, the difference again highlights the diverse mechanisms of ionic regulation employed by acid-tolerant species.

Recent advances with zebrafish

Zebrafish as an acid-tolerant model

In recent years, zebrafish has become one of the most intensively studied model organisms in the field of ionic and acid–base regulation (Hwang, 2009; Hwang et al., 2011; Hwang and Perry, 2010). Zebrafish naturally inhabit warm water (24–35°C) with a pH ranging from 6.6 to 8.2 (McClure et al., 2006). Under laboratory conditions, zebrafish survive well in acidic water as low as pH 4.0 (Horng et al., 2007; Horng et al., 2009; Kumai et al., 2011; Kwong and Perry, 2013a). Zebrafish also tolerate a wide range of experimental treatments, such as changes in temperature, salinity, P_{CO_2} and P_{O_2}, which allow researchers to study the interactive effects of environmental perturbation. Additionally, there are several advantages to using zebrafish over other species, including an abundance of genetic databases and the applicability of many cellular and molecular physiological approaches (e.g. gene knockdown and overexpression, transgenics, *in vivo* fluorescence imaging, cell transplantation and targeted mutagenesis) (for review, see Ekker and Akimenko, 2010). Recent studies in zebrafish have resulted in the identification and characterization of genes and mechanisms that are involved in ionic and acid–base regulation (Fig. 1). In this section, we discuss the physiological responses of zebrafish to exposure to acidic water, and the potential molecular mechanisms by which they cope with acidic environments.

Effects of acid exposure on TJs and paracellular Na^+ losses

Similar to many other FW species (Freda and McDonald, 1988; Gonzalez and Wilson, 2001; McDonald and Wood, 1981), zebrafish exhibit a significant increase in passive Na^+ efflux and a reduction in whole-body Na^+ content during exposure to acidic water (pH 4.0)

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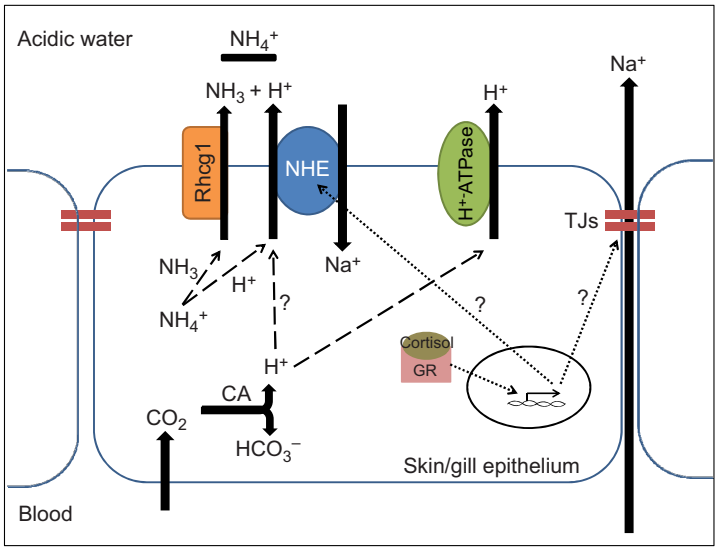

Fig. 1. Proposed model for H^+ and Na^+ handling in zebrafish gills/skin during acclimation to an acidic environment. Acid exposure disrupts the integrity of the epithelial tight junctions (TJs) and increases the paracellular loss of Na^+. The elevated Na^+ loss is compensated for by increasing Na^+ uptake, at least in part via activation the of Na^+/H^+ exchanger (NHE). The excretion of the increased intracellular H^+ levels by acid exposure is mediated primarily by the apical H^+-ATPase. A functional metabolon linking Rhcg1 and NHE partially facilitates H^+ extrusion. Hydrolysis of CO_2 by cytosolic carbonic anhydrase (CA) may also contribute to H^+ production. Acid exposure increases the synthesis of cortisol, which subsequently induces the expression of TJs and NHE through activation of a glucocorticoid receptor (GR) signalling cascade. A question mark indicates that the exact mechanism/pathway remains unclear.

(Kumai et al., 2011; Kwong and Perry, 2013a). Using a paracellular permeability marker, polyethylene glycol (PEG)-400 or -4000, the epithelial permeability was also found to increase during acid exposure (Kumai et al., 2011; Kwong and Perry, 2013a). These results suggest that the increased diffusive Na^+ losses during acid exposure are, at least in part, associated with increased paracellular permeability. In vertebrates including fish, paracellular permeability is governed primarily by TJ proteins, which regulate the intercellular passage of water, ions and neutral solutes (Anderson and Van Itallie, 2009; Chasiotis et al., 2012; Engelund et al., 2012; Kwong and Perry, 2013b; Kwong et al., 2013b). In FW trout, acid exposure leaches surface-bound Ca^{2+}, disrupts the morphology of TJs in the gills and thus increases Na^+ efflux (Freda et al., 1991; McWilliams, 1983). In agreement with this finding, removal of ambient Ca^{2+} caused a more pronounced Na^+ loss in zebrafish exposed to acidic water (Kumai et al., 2011). Acid exposure also modulates the expression of TJ proteins in the zebrafish gill; specifically, the branchial expression of claudin-b, -c and -7, was significantly increased during acid exposure (Kumai et al., 2011). The mammalian orthologs of zebrafish claudin-b and -7 are believed to contribute to epithelial tightness in the renal tubules, where they function as a paracellular barrier to Na^+ (Hou et al., 2006; Van Itallie et al., 2001). In zebrafish, claudin-b is associated with TJs between cells of the lamellar epithelium (e.g. ionocytes and pavement cells) in the gill (Kwong et al., 2013a). Additionally, knockdown of claudin-b in larval zebrafish markedly increased paracellular Na^+ loss (Kwong and Perry, 2013b). Therefore, the induction of barrier-forming TJ proteins (e.g. claudin-b) may minimize paracellular Na^+ losses in zebrafish during acclimation to acidic water.

Regulation of Na^+ balance and H^+ secretion during acid exposure

Through *in situ* hybridization and immunohistochemistry, four types of ionocytes have been identified in larval zebrafish; H^+-ATPase-rich cells (HRCs), Na^+/K^+-ATPase rich cells (NaRCs), Na^+/Cl^- co-transporter expressing cells (NCCC) and K^+ secreting cells (KSCs) (for reviews, see Dymowska et al., 2012; Hwang et al., 2011). Although all four subtypes undoubtedly contribute to body fluid homeostasis in zebrafish, the present review focuses on the NHE- and H^+-ATPase-expressing HRC because it is the main ionocyte responsible for acid secretion and Na^+ uptake. Additionally, the potential role of carbonic anhydrase (CA) in regulating acid–base balance during acid exposure is discussed.

NHE and H^+-ATPase

HRCs, first described by Lin et al. (Lin et al., 2006), express apical H^+-ATPase (*zatp6v1a*) (Lin et al., 2006), NHE3b (*zhne3b*) (Yan et al., 2007), ammonia conducting rhesus glycoprotein (*zrhcg1*) (Nakada et al., 2007), CA-15a (*zCA15a*) (Lin et al., 2008), cytosolic CA2-like a (*zCA2 like a*) (Lin et al., 2008), basolateral anion exchanger-1b (*zslc4a1b*) (Lee et al., 2011) and Na^+/K^+-ATPase subunits *atp1a1a.5* and *atp1b1b* (Liao et al., 2009). Because bafilomycin (a commonly used H^+-ATPase inhibitor) inhibits acid secretion at the surface of this cell (Horng et al., 2007; Lin et al., 2006), HRCs are considered to play an important role in acid secretion and Na^+ uptake coupled to acid secretion (Esaki et al., 2007). Chang et al. (Chang et al., 2009) reported that the branchial mRNA expression level of H^+-ATPase was significantly increased in the zebrafish gill following 7 days of acid exposure. Moreover, studies using the scanning ion-selective electrode technique (SIET) and translational gene knockdown in larvae demonstrated that H^+-ATPase accounts for about 70% of H^+ extrusion from the whole larvae, further supporting the important acid-secreting function of H^+-ATPase (Horng et al., 2007; Lin et al., 2006). In addition to H^+-ATPase, NHE3b is also known to contribute considerably to acid secretion, accounting for about 30% of the total (Shih et al., 2012).

Unlike other FW fish (discussed above), zebrafish compensate for the elevated Na^+ loss during acid exposure by increasing Na^+ uptake, which appears to be associated with activation of H^+-ATPase and NHE3b. Based on the changes in mRNA expression of NHE3b (decrease) and H^+-ATPase (increase) in adult gill following a 7 day acclimation to pH 4, Yan et al. (Yan et al., 2007) suggested that H^+-ATPase probably plays a more prominent role in Na^+ uptake in acidic water. Similarly, treatment with the H^+-ATPase inhibitor bafilomycin was found to inhibit the acid-induced increase in Na^+ uptake in larval zebrafish (Kumai and Perry, 2011). Additionally, Kumai and Perry (Kumai and Perry, 2011) observed that pharmacological treatments with 5-(*N*-ethyl-*N*-isopropyl)amiloride (EIPA; an NHE-selective inhibitor) or NHE3b knockdown prevented the stimulation of Na^+ uptake in larvae reared in acidic water. Together, these findings indicate that NHE3 and H^+-ATPase are essential for increasing Na^+ uptake in zebrafish in acidic environments.

The uptake of Na^+ by FW fish via NHE3 has been challenged based on thermodynamic considerations (Avella and Bornancin, 1989; Parks et al., 2008); theoretically, in an acidic environment,

Na^+ uptake via NHE3 is even more challenging. The thermodynamic barrier limiting Na^+ uptake is likely overcome through an interaction between Rhcg1 and NHE3, where diffusion of NH_3 through Rhcg1 creates a microenvironment in which H^+ is lowered by its combination with NH_3 to form NH_4^+. The resultant H^+ gradient is believed to drive Na^+ uptake via electroneutral NHE3 (Wright and Wood, 2009; Wright and Wood, 2012). Using an *in situ* ligation proximity assay, Ito et al. (Ito et al., 2013) recently demonstrated that NHE3b and Rhcg1 are in close physical proximity in the HRC. Additionally, knockdown of Rhcg1 was found to significantly reduce ammonia excretion and Na^+ uptake in larval zebrafish exposed to acidic (Kumai and Perry, 2011) or low Na^+ (Shih et al., 2012) environments. It was also demonstrated that the usual inhibitory effect of EIPA on Na^+ uptake in acidic water was abolished by Rhcg1 knockdown (Kumai and Perry, 2011). Together, these findings suggest that NHE is not functional in fish experiencing Rhcg1 knockdown, which further supports the notion that Na^+ uptake via NHE3b is linked to ammonia excretion by Rhcg1 via a functional metabolon.

NCC

NCC (*zslc12a10.2*) provides an additional route for Na^+ uptake in zebrafish (Shono et al., 2011; Wang et al., 2009). However, little is known about the functional role of NCC in Na^+ uptake in acidic environments. Although mRNA expression of NCC in acid-exposed zebrafish larvae was unaffected (Y.K. and S.F.P., unpublished results), it remains to be determined whether NCC contributes to overall Na^+ balance in zebrafish acclimated to acidic water.

Carbonic anhydrase

The H^+-ATPase- and NHE3-mediated Na^+ uptake mechanisms require a supply of intracellular H^+ that is typically provided via the hydration of molecular CO_2, a reaction catalysed by CA. Although our understanding of the physiological significance of CA in acid–base/ionoregulation in fish is far from complete, putative roles of CA have been described in several species including zebrafish (for review, see Gilmour, 2012; Gilmour and Perry, 2009). Boisen et al. (Boisen et al., 2003) reported that inhibition of CA with ethoxzolamide reduced Na^+ uptake in adult zebrafish acclimated to hard water, whereas it increased Na^+ uptake in fish acclimated to soft water. Conversely, Craig et al. (Craig et al., 2007) observed a significant increase in mRNA expression of *zCA2-like a* and *zCA2-like b* (also named *CAhz*) in adult zebrafish gill following acclimation to soft water. In another study, Esaki et al. (Esaki et al., 2007) reported inhibition of fluorescent Na^+-green accumulation in zebrafish larvae following ethoxzolamide treatment but, paradoxically, no inhibition in whole-body Na^+ uptake was observed when measured with radiolabelled sodium. Lin et al. (Lin et al., 2008) investigated the molecular physiology of two CA isoforms expressed in HRCs, the extracellular CA15 (*zCA15a*) and cytosolic CA (*zCA2-like a*), and reported that knockdown of zCA2-like a or zCA15a protein reduced H^+ secretion by HRCs. When exposed to acidic water, mRNA expression of *zCA15a* significantly increased, whereas no change was observed for *zCA2-like a*. In contrast, Lee et al. (Lee et al., 2011) observed a significant increase in mRNA expression of anion exchanger (*zslc4a1b*) following acid exposure. Because HCO_3^- that is secreted by zslc4a1b is most likely synthesized as a by-product of the hydration reaction of CO_2 mediated by zCA2-like a, it is possible that the enzymatic activity of CA2 is elevated in HRCs in acidic water despite the lack of any change in mRNA expression. Assessing activity changes associated with any particular CA isoform in specific ionocytes following acid exposure remains a challenge because of the existence of multiple isoforms of CA.

It was shown that knockdown of zCA2-like a or zCA15a expression increases mRNA expression of *znhe3b* in zebrafish larvae reared at normal ambient pH, which appears to be accompanied by an increase in Na^+ uptake (Lin et al., 2008). It was also proposed that absorption of Na^+ via NHE3 is more effective in the CA morphants because the extracellular H^+ concentration at the apical membrane is reduced (Lin et al., 2008). However, whether Na^+ uptake via NHE3b could be further stimulated in the CA morphants during acid exposure remains to be investigated. It is important to note here that a lack of intracellular CA, by slowing the hydration reaction of CO_2, could also potentially lower intracellular H^+ levels, which might ultimately cancel out the effect from changes in H^+ gradient in the environment (unless the uncatalyzed hydration reaction is sufficiently fast to maintain the favourable intracellular H^+ gradient). In contrast to the findings of Lin et al. (Lin et al., 2008), Ito et al. (Ito et al., 2013) demonstrated that knockdown of either zCA2-like a or zCA15a expression resulted in a significant reduction in Na^+-green accumulation in HRCs. Ito et al. (Ito et al., 2013) argued that Na^+-green measures the Na^+ concentration in HRCs, while the radiotracer Na^+ assay performed by Lin et al. (Lin et al., 2008) estimates the whole-body Na^+ uptake, which can occur through additional routes. Interestingly, Ito et al. (Ito et al., 2013) also showed that the localization of *zrhcg1*, *znhe3b*, *zCA2-like a* and *zCA15a* is tightly associated in adult zebrafish gill, addressing the need to consider intracellular H^+ levels in driving NHE3. Notably, in a recent experiment with CA2-like a knockdown, we observed that the morphants could not increase their ammonia excretion in response to acid exposure (Fig. 2), further emphasizing the need to account for interactive effects between cytosolic CA and ammonia secretion.

Effects of acid exposure on other ions

Because acid exposure increases the general paracellular permeability, it is likely that the regulation of other major ions (e.g. Cl^- and Ca^{2+}) is also affected in fish inhabiting acidic water. Interestingly, acid exposure was reported to have no effect on the whole-body Cl^- content in larval zebrafish (Horng et al., 2009), which raises an obvious question about how these fish maintain their Cl^- balance in acidic water. In a preliminary study, acid exposure was found to cause an increase in Cl^- uptake that was strongly inhibited by acetazolamide, a commonly used inhibitor of CA (Y.K. and S.F.P., unpublished results). A similar reduction in Cl^- uptake following CA inhibition was also observed by Boisen et al. (Boisen et al., 2003). In zebrafish (and FW fish in general), several members of the Cl^-/HCO_3^- exchanger protein family (e.g. *zslc26*) (Bayaa et al., 2009; Perry et al., 2009) as well as NCC (*zslc12a10.2*; Wang et al., 2009) have been proposed to play a role in Cl^- absorption. Because Cl^- uptake via the Cl^-/HCO_3^- exchanger requires secretion of a base equivalent (HCO_3^-), one might predict that fish in acidic water rely on NCC for Cl^- uptake, thereby conserving intracellular HCO_3^-. This scenario, however, is difficult to reconcile with the significant reduction in Cl^- uptake that accompanies CA inhibition in acidic water (see above). Furthermore, mRNA expression of an electrogenic Na^+/HCO_3^- co-transporter (*zslc4a4b*) expressed on the basolateral surface of the NCCC, was significantly reduced in response to acid exposure (Lee et al., 2011), hinting that the NCCC might be less active in acidic water.

The uptake of Ca^{2+} in zebrafish occurs via apical epithelial Ca^{2+} channels (*zecac*, or ECaC) (Pan et al., 2005) and basolateral plasma membrane Ca^{2+}-ATPase (*zpmca2*, or PMCA) and Na^+/Ca^{2+}

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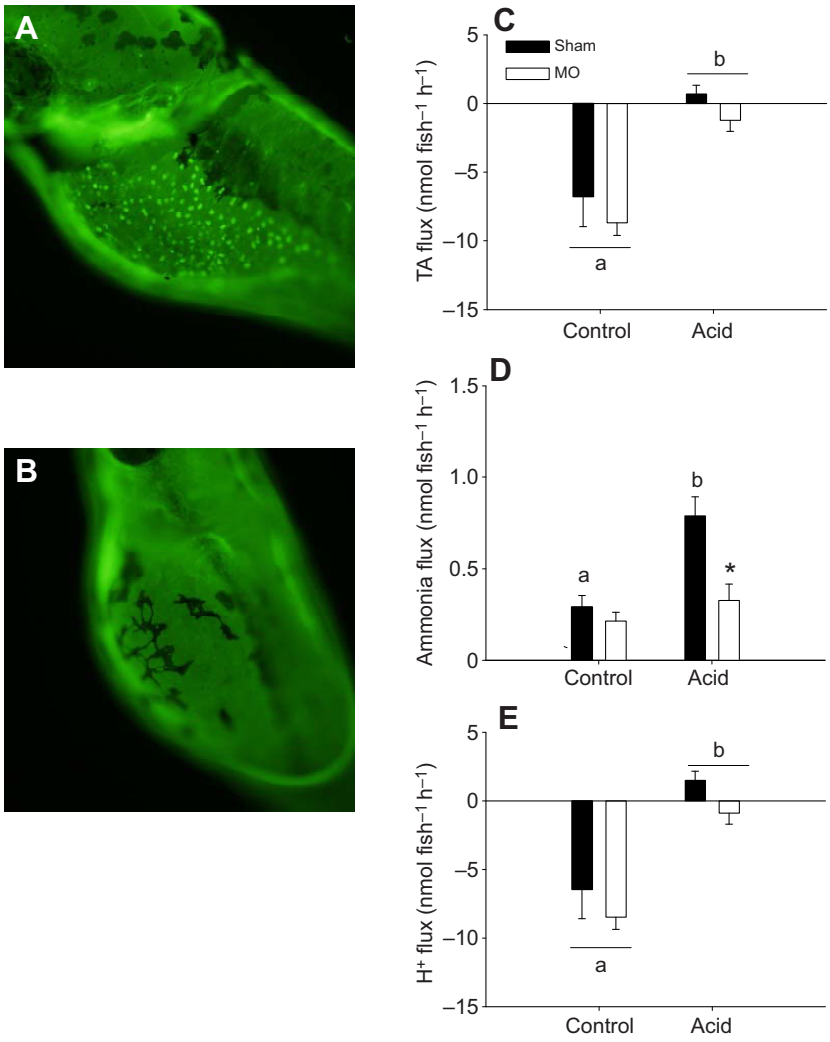

Fig. 2. Role of zCA2-like a in acid–base secretion by larval zebrafish. The potential role of carbonic anhydrase 2-like a (*zCA2-like a*) in acid–base secretion was evaluated in developing zebrafish using a morpholino knockdown approach. The fish were exposed to either control water (pH 7.6) or acidic water (pH 4.0) starting at 2 days post-fertilization (dpf), and measurements were performed at 4 dpf. The effectiveness of the knockdown was confirmed by immunohistochemistry (IHC) using a homologous antibody against zCA2-like a. (A,B) Representative IHC images of zCA2-like a in sham and zCA2-like a morphants, respectively. (C–E) The titratable alkalinity (TA) flux, ammonia secretion rate and H^+ flux in sham and zCA2-like a morphants (MO), respectively. H^+ flux was calculated by adding TA flux and ammonia secretion rate (positive values indicate secretion of acid equivalent). TA flux was measured by the single end-point titration method (water samples were titrated to pH 3.6). TA flux and calculated H^+ flux significantly increased following acid exposure in both sham and *zCA2-like a* morphants, whereas the morphants failed to increase the rate of ammonia secretion following acid exposure. Different letters denote a significant difference between control and acid-exposed fish; asterisk denotes a significant difference between sham and MO (P<0.05). Data are shown as means ± s.e.m., N=4–6. All data were analysed with two-way ANOVA, followed by a *post hoc* Tukey test.

exchanger (*zncx1b*, or NCX) (Liao et al., 2007), with ECaC constituting the rate-limiting step. Unlike Na^+ and Cl^-, which are tightly regulated in acid-exposed zebrafish larvae, the whole-body Ca^{2+} balance was severely disturbed (>80% reduction) in acid-exposed zebrafish larvae (Horng et al., 2009). Those acid-exposed larvae did not exhibit any obvious gross morphological defects, but appeared to be shorter (Horng et al., 2009). Interestingly, the number of NaRCs (ECaC is expressed in a subset of NaRCs) was found to increase in acid-exposed zebrafish larvae (Horng et al., 2009). Similarly, real-time quantitative PCR and *in situ* hybridization revealed that the expression of ecac mRNA and the density of ecac-expressing ionocytes in larval zebrafish were significantly increased following a 2 day exposure to acidic water (Fig. 3). The increase of ecac mRNA level and ecac-expressing cells probably occurred as compensatory responses for the increased Ca^{2+} losses. Notably, however, kinetic analysis of Ca^{2+} uptake in acidic water demonstrated a complete lack of a typical Michaelis–Menten relationship (Y.K., R.W.M.K. and S.F.P., unpublished results). This finding suggests that in acidic water, ECaC may not function effectively for Ca^{2+} transport. This result is in agreement with previous findings from mammalian kidney cortical collecting cells; acidification of apical media alone, rather than subsequent reduction in intracellular pH, was sufficient to inhibit the effective uptake of Ca^{2+} (Bindels et al., 1994). The reduction in whole-body Ca^{2+} content in larval zebrafish following acid exposure raises the possibility of ensuing deleterious consequences on development of bone and/or otolith and hair cells (Cruz et al., 2009).

Effects of acid exposure on cell proliferation

Proliferation of acid-secreting A-type intercalated cells in renal collecting duct is recognized as a key physiological response to chronic acidosis in mammals (Al-Awqati et al., 2000; Welsh-Bacic et al., 2011). Similar cellular remodelling was also found to occur in zebrafish larvae; Horng et al. (Horng et al., 2009) demonstrated that the density of HRCs in the yolk sac was significantly increased in larval zebrafish after acclimation to pH 4 water. Additionally, it was shown that the density of HRCs in the gill increased in adult zebrafish exposed to acidic water (Chang et al., 2009). The increase in the number of HRCs was associated with increased expression of glial cell missing-2 (*gcm2*), a transcription factor regulating HRC differentiation (Chang et al., 2009). These observations suggest that during acid exposure, activation of *gcm2* elicits HRC proliferation, and that these responses likely contribute to facilitating acid secretion and Na^+ uptake. In addition, a recent study by Flynt et al. (Flynt et al., 2009) demonstrated a potentially important role for *miR-8* microRNA in regulating the expression of *znherf1* (NHE regulatory factor 1), which negatively regulates NHE activity by promoting phosphorylation and subsequent internalization of NHE (Murthy et al., 1998; Yun et al., 1997). In zebrafish larvae, knockdown of *miR-8* was found to decrease Na^+ uptake during

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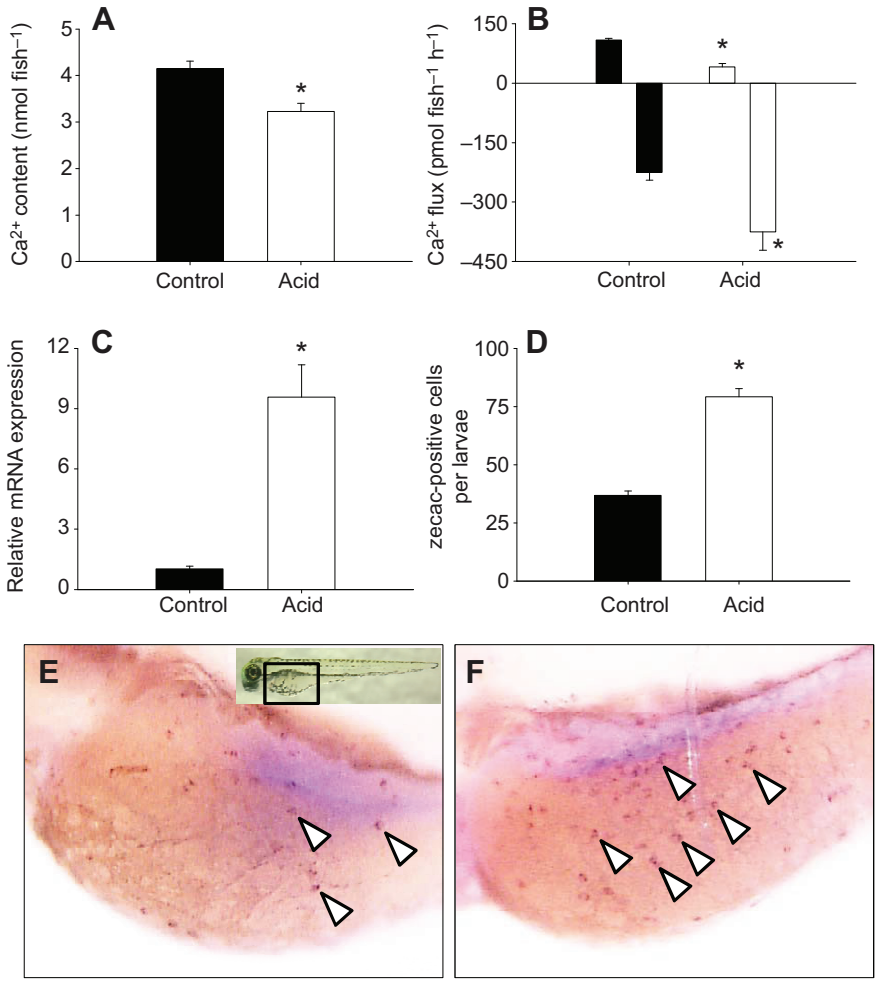

Fig. 3. Disruption of Ca^{2+} homeostasis in larval zebrafish exposed to acidic water. (A) Exposure to acidic water (pH 4.0; control pH 7.6) for 48 h significantly reduced whole-body Ca^{2+} content in larval zebrafish at 4 dpf. (B) This reduction in whole-body Ca^{2+} content was probably a result of decreased Ca^{2+} uptake and increased Ca^{2+} efflux occurring simultaneously. To compensate for the reduced whole-body Ca^{2+} content during acid exposure, zebrafish increased (C) the mRNA expression level of epithelial Ca^{2+} channels (*zecac*) and (D) the density of *zecac*-expressing ionocytes. (E,F) Representative *in situ* hybridization images for *zecac* in control and acid-exposed larvae, respectively. All data were analysed with Student's *t*-test. Asterisk denotes a significant difference between control and acid-exposed fish ($P<0.05$). The mRNA expression of *zecac* was relative to 18S, and data are presented relative to control; all data are shown as means ± s.e.m., $N=5–6$.

exposure to moderately acidic media (pH 5; measured as Na^+-green visualization), which appeared to be a result of *znherf1* overexpression (Flynt et al., 2009). While the specific Na^+ transporter being targeted was not addressed by Flynt et al. (Flynt et al., 2009), their work provided interesting insight into the intracellular signalling within the HRC in response to acid exposure.

Endocrine responses to acid stress

Endocrine regulation of ionic homeostasis in fish has been studied extensively (for reviews, see McCormick, 2001; McCormick and Bradshaw, 2006). In mammals, metabolic acidosis causes a variety of effects on the endocrine system, including suppression of parathyroid and growth hormone secretion, and induction of angiotensin II secretion (Wagner et al., 2011; Wiederkehr and Krapf, 2001). A previous study by Hoshijima and Hirose (Hoshijima and Hirose, 2007) demonstrated that acclimation of zebrafish to ion-poor water increased the mRNA expression of atrial natriuretic peptide, renin, growth hormone and parathyroid hormone; however, their role in ion homeostasis and acid–base balance remain poorly understood. To date, only cortisol (Cruz et al., 2013; Kumai et al., 2012a), isotocin (Chou et al., 2011), prolactin (Breves et al., 2013) and catecholamines (Kumai et al., 2012b) have been implicated in regulating Na^+ uptake in zebrafish. In particular, cortisol is thought to play a major role in regulating Na^+ uptake in acid-exposed zebrafish. For example, expression of glucocorticoid receptor (GR) mRNA (R.W.M.K. and S.F.P., unpublished results) and whole-body cortisol levels (Kumai et al., 2012a) was significantly elevated in larval zebrafish following acid exposure. Importantly, results from pharmacological manipulation or morpholino knockdown have shown that under acidic conditions, cortisol acts through GR to activate Na^+ uptake via a NHE3b–Rhcg1 functional metabolon (Kumai et al., 2012a).

Increasing evidence suggests that elevated cortisol levels can also reduce the epithelial permeability in fish by increasing the abundance of epithelial TJ proteins (Chasiotis et al., 2010; Kelly and Chasiotis, 2011; Kwong and Perry, 2013a; Tipsmark et al., 2009). In larval zebrafish, cortisol treatment increased the mRNA and protein expression of the epithelial TJ proteins claudin-b and occludin-a, which was associated with a reduction in paracelluler permeability (Kwong and Perry, 2013a). The increase in diffusive Na^+ loss and reduced whole-body Na^+ levels following acute acid exposure were mitigated by cortisol treatment (Kwong and Perry, 2013a). Additionally, knockdown of GR abolished the effects of cortisol in reducing paracellular permeability, indicating that activation of GR is an important signalling pathway. These effects of cortisol on epithelial permeability and Na^+ efflux suggest that the increase in whole-body cortisol levels in acid-exposed zebrafish (Kumai et al., 2012a) may contribute to the acid tolerance of this species by reducing paracellular permeability and Na^+ losses (as well as increasing Na^+ uptake). In support of this idea, Kwong and Perry (Kwong and Perry, 2013a) demonstrated that the paracellular permeability following acid exposure was more pronounced in GR morphants. Moreover, the GR morphants exhibited a greater diffusive loss of Na^+ than control fish during acid exposure (Kwong

and Perry, 2013a). Together, these findings suggest that increased cortisol production and its subsequent activation of GR-mediated signalling pathways promote whole-body Na^+ retention, thus attenuating the effects of acid exposure on Na^+ balance in zebrafish.

Cortisol might play yet another role in acid-exposed zebrafish by promoting the proliferation of multiple types of ionocytes (e.g. HRCs and NaRCs). Cruz et al. (Cruz et al., 2013) demonstrated that cortisol treatment increased the proliferation of NaRCs and HRCs in zebrafish larval skin and adult gill through its stimulatory effects on *foxi3a/3b*, transcription factors involved in the regulation of ionocyte differentiation (Esaki et al., 2007; Esaki et al., 2009; Hsiao et al., 2007; Jänicke et al., 2007). Because of the increase in whole-body cortisol levels in acid-exposed larvae, the role of cortisol in ionocyte proliferation/epidermal development offers an attractive mechanism to explain the observed increase in HRCs in response to acid exposure. However, its precise role in ionocyte proliferation specifically under acidic conditions awaits direct confirmation. Similarly, based on knockdown and overexpression experiments, Chou et al. (Chou et al., 2011) showed that isotocin plays a critical role in the proliferation of HRCs and NaRCs, possibly by increasing the expression of *foxi3a*. Although the expression of isotocin was elevated in ion-poor water, it remains to be determined whether a similar elevation occurs in response to acid exposure.

In addition, catecholamines released either from nerves or chromaffin cells (Reid et al., 1998) have long been suspected to play an important role in ionoregulation in FW fish through their action on α- and β-adrenergic receptors (Isaia et al., 1978; Morgan and Potts, 1995; Perry et al., 1984; Vermette and Perry, 1987). A recent study demonstrated that activation of β-adrenergic receptors stimulates Na^+ uptake in larval zebrafish, and through knockdown of selected β-adrenergic receptors, the same study also showed that activation of β-adrenergic receptors contributes to the increase in Na^+ uptake in an acidic environment (Kumai et al., 2012b). Although there was no attempt to identify the specific Na^+ transporter(s) being activated by β-adrenergic receptor stimulation, they are likely to be localized to the HRC, which expresses β-adrenergic receptors (Kumai et al., 2012b). Future studies should be undertaken to elucidate the signalling cascade linking activation of β-adrenergic receptors to the activation of Na^+ transporter(s).

Conclusions and perspectives

Recent advances in molecular and physiological techniques specific to zebrafish have identified various mechanisms that are essential in regulating ionic and acid–base status. The results reveal a complex network of regulatory responses accompanying acid exposure such as modulation of gene expression, regulation of transcellular and paracellular Na^+ movement, hormonal control, intracellular remodelling and cell proliferation. These responses ultimately allow zebrafish to maintain Na^+ balance during acclimation to an acidic environment.

The zebrafish model has proved useful in providing a deeper understanding of the cellular and molecular mechanisms of ionic and acid–base regulation. However, numerous issues remain to be addressed in future studies. For example, (i) the underlying mechanism whereby fish detect changes in the ambient pH, and whether the responses to acid stress are triggered by external versus internal acidosis remain unclear; (ii) although our understanding of the regulation of Na^+ balance in an acidic environment has greatly improved using the zebrafish model, the effects of acid exposure on development and metabolism, as well as on the regulation of other major ions (e.g. Cl^-, K^+ and Mg^{2+}) await further investigation; (iii) the precise mechanism by which acid exposure affects TJ integrity and epithelial permeability, and the role of TJ proteins in regulating paracellular ion movement during acid exposure have not been fully characterized; (iv) the potential compensatory role of the kidney in promoting ion reabsorption, and the molecular mechanism for increasing the renal excretion of acid equivalents during acid exposure remain to be investigated; (v) knowledge of the hormonal response to acid exposure is currently limited to cortisol – whether other stress/ionoregulatory hormones (e.g. thyroid hormones, prolactin, isotocin and calciotropic hormones) also play a role in acid–base and ionic regulation during acid exposure is unclear; (vi) additionally, the functional involvement of CA, NCC and Cl^-/HCO_3^- exchangers in acid–base balance needs to be clarified; (vii) finally, because of the likelihood of diverse physiological strategies of adaptation to acidic water in different species and habitats, future research should be expanded to a range of acid-tolerant and intolerant species. This information may help expand our understanding of the evolutionary basis of physiological adaptation to acidic environments. Ultimately, we hope that models will emerge which integrate across multiple interacting systems to help explain how ionic and acid–base balance are achieved in fish inhabiting acidic water.

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Competing interests
The authors declare no competing financial interests.

Author contributions
R.W.M.K., Y.K. and S.F.P. conceived and designed the experiments; R.W.M.K. and Y.K. performed the experiments; R.W.M.K. and Y.K. analysed the data; R.W.M.K., Y.K. and S.F.P. drafted and revised the paper.

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