MECHANISMS OF Na^+ HOMEOSTASIS

BY ZEBRAFISH (*DANIO RERIO*) IN ACIDIC WATER

By

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ABSTRACT

Zebrafish, *Danio rerio*, are able to survive exposure to extreme acidity (pH 4). Because previous studies demonstrated that disruption of ionic balance during exposure to acidic water is the major cause of mortality in acid-sensitive freshwater species, the focus of this thesis was to characterize the molecular mechanisms enabling zebrafish to maintain their Na^+ homeostasis following exposure to acidic water. Initial findings (Chapter 2) demonstrated that branchial mRNA expression of selected isoforms of claudins, major components of tight junctions, are altered in an isoform-dependent manner, suggesting the potential regulation of epithelial permeability to minimize ion loss. Concurrently, a marked stimulation of Na^+ uptake was observed in adults and larvae following acid-exposure. Because of the uniqueness of this response (increasing Na^+ uptake in acidic water) among freshwater teleosts, the mechanisms related to Na^+ uptake and its stimulation were investigated further (Chapters 3 - 7). Pharmacological treatments and gene knockdown approaches revealed that a functional metabolon consisting of an apically expressed Na^+-H^+-exchanger (NHE3b) in association with an apically expressed ammonia-conducting channel (Rhcg1), enables Na^+ uptake in acidic water.

During chronic (>1 day) exposure to acidic water, cortisol (via glucocorticoid receptors) and catecholamines (via β-adrenergic receptors) are involved in stimulating Na^+ uptake. Although catecholamines may act on both NHE3b and Na^+-Cl^- co-transporter (NCC), the effects of cortisol on Na^+ uptake are mediated primarily by activation of NHE3b. On the other hand, during acute (<3 h) exposure to acidic water, cortisol does not appear to affect Na^+ uptake; rather, the stimulation of Na^+ uptake appears to be mediated by angiotensin II and catecholamines. Cyclic AMP (cAMP), a signalling molecule synthesized following the activation of β-adrenergic receptors, is critically involved in stimulating Na^+ uptake, likely via

activation of NHE3b and NCC. In agreement with this idea, ionocytes that express NHE3b also express high levels of β-adrenergic receptor (propranolol binding sites) as well as trans-membrane adenylyl cyclase (forskolin binding sites).

Taken together, the results of this thesis provide fresh insight into the mechanisms of osmoregulation in freshwater (FW) fish. In particular, the data reveal the presence of complex pathways regulating Na^+ uptake in zebrafish exposed to acidic water. The relative importance of the various pathways depends in part on the duration of exposure; acute versus chronic.

RÉSUMÉ

Les poissons zèbre, *Danio rerio*, peuvent survivre à une exposition à une extrême acidité (pH 4). Des études antérieures ont démontré que la perturbation de l'équilibre ionique lors de l'exposition à l'eau acide est la cause majeure de mortalité chez les espèces d'eau douce sensibles à l'acidité. Ainsi, le but de cette thèse fut de caractériser les mécanismes moléculaires permettant aux poissons zèbre de maintenir l'homéostasie sodique suite à l'exposition à une eau acide. Les premiers résultats (Chapitre 2) ont démontré que l'expression de l'ARNm branchial de certaines isoformes de claudines, composantes principales des jonctions serrées, est régulée de façon spécifique selon l'isoforme. Ces résultats suggèrent qu'il y a une régulation potentielle de la perméabilité épithéliale permettant de minimiser la perte d'ions. En même temps, une stimulation marquée de l'absorption de Na^+ a été observée chez les adultes et les larves, suite à une exposition à une eau acide. En raison de cette réponse unique (l'augmentation de l'absorption de Na^+ en eau acide) chez les téléostéens d'eau douce, les mécanismes reliés à l'absorption de Na^+ ainsi que sa stimulation ont été étudiés plus en détails (Chapitres 3-7). Des traitements pharmacologiques et l'utilisation d'une approche d'inactivation génique ont révélé qu'un complexe protéique composé d'un échangeur Na^+-H^+ (NHE3b) exprimé apicalement, en association avec un transporteur d'ammoniac exprimé apicalement (Rhcg 1), permet l'absorption de Na^+ en eau acide.

Lors d'une exposition chronique (>1 jour) à l'eau acide, le cortisol (par l'entremise les récepteurs de glucocorticoïdes) et les catécholamines (par l'entremise des récepteurs β-adrénergiques) sont impliqués dans la stimulation de l'absorption de Na^+. Bien que les catécholamines peuvent agir sur le NHE3b et le co-transporteur Na^+-Cl^- (NCC), les effets du cortisol sur l'absorption de Na^+ sont régulés principalement par l'activation de NHE3b. En

revanche, le cortisol ne semble pas avoir d'effet sur l'absorption de Na^+ lors d'une exposition aiguë (< 3 h) à l'eau acide. Plutôt, la stimulation de l'absorption de Na^+ semble être régulée par l'angiotensine II et les catécholamines en situation d'exposition aiguë. L'AMP cyclique (AMPc), un messager secondaire synthétisée suite à l'activation des récepteurs β-adrénergiques, est impliquée de façon critique dans la stimulation de l'absorption de Na^+, probablement à travers l'activation de NHE3b et de NCC. En accord avec cette idée, les ionocytes qui expriment le NHE3b expriment aussi un haut niveau de récepteurs β-adrénergiques (sites de liaison pour le propanolol) ainsi que de l'adénylate cyclase transmembranaire (sites de liaison pour la forskoline).

Ensemble, les résultats de cette thèse offrent de nouvelles perspectives quant aux mécanismes d'osmorégulation chez les poissons d'eau douce. En particulier, les données révèlent la présence d'axes de régulation complexes modulant l'absorption de Na^+ chez les poissons zèbre lors d'une exposition à l'eau acide. L'importance relative de ces divers axes de régulation semble dépendre de la durée de l'exposition soit aiguë ou chronique.

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5-HT	serotonin
AC	adenylyl cyclase
ACE	angiotensin converting enzyme
AE	anion exchanger
ANG-II	angiotensin II
ANOVA	analysis of variance
AT_1	type-I angiotensin II receptor
AT_2	type-II angiotensin II receptor
atp1	adenosine triphosphatase 1
atpv6	v-type adenosine triphosphatase 6
BCA	bicinchoninic acid
CA	carbonic anhydrase
cAMP	cyclic adenosine monophosphate
cDNA	complementary deoxyribonucleic acid
cm	centimeter
ConA	concanavalin A
cpm	count per minute
ct	cycle threshold
D	dark
DMSO	dimethyl sulfoxide
DNase	deoxyribonuclease
DOC	dissolved organic carbon
dpf	days post fertilization
ECL	enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EIA	enzyme immunoassay
EIPA	5-(N-Ethyl-N-isopropyl)amiloride
ENaC	Epithelial Na^+ channel
Epac	exchange protein directly activated by cyclic adenosine monophosphate
ERK1/2	extracellular signal-regulated kinase 1/2
FITC	Fluorescein isothiocyanate
FW	freshwater
g	gram
gcm2	glial cell missing 2
GR	glucocorticoid receptor
GRE	glucocorticoid response element
h	hour
H^+-ATPase	H^+ adenosine triphosphatase
HEA	high external ammonia
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HMA	5-(N,N-Hexamethylene)amiloride
HRC	H^+ adenosine triphosphatase rich cell
hpf	hours post fertilization

IHC	immunohistochemistry
ISH	*in situ* hybridization
IRRR	insulin receptor related receptor
$J_{MAX}Na^+$	maximum transport rate of Na^+
J_{out}^{Na}	rate of Na^+ efflux
K_m	affinity constant for transport kinetics
Kir	inward rectifying K^+ channel
L	light
MDCK	Madin-Darby canine kidney
min	minute
ml	millilitre
mM	millimolar
mOsmol	milliosmolar
MR	mineralocorticoid receptor
MR/MRC	mitochondrion rich cell
mRNA	messenger ribonucleic acid
MT	microtubule
mV	millivolt
Na^+-K^+-ATPase	Na^+ K^+ adenosine triphosphatase
NaR/NaRC	Na^+ K^+ adenosine triphosphatase rich cell
NCC	Na^+ Cl^- co-transporter
ng	nanogram
NHE	Na^+ H^+ exchanger
NHERF1	Na^+ H^+ exchanger regulatory factor 1
nM	nanomolar
nmol	nanomole
OKP	opossum kidney clone P cell
PBS	phosphate buffered saline
PEG	polyethylene glycol
PI-3	phosphoinositide-3 kinase
PKA	protein kinase A
PKC	protein kinase C
Pyk2	protein tyrosine kinase 2
qPCR	quantitative polymerase chain reaction
RAAS	renin-angiotensin-aldosterone system
real time PCR	real time polymerase chain reaction
Rh	Rhesus glycoprotein
RM	repeated measures
ROMK	renal outer medullarly K^+ channel
RU-486	mifepristone
sAC	soluble adenylyl cyclase
SEM	standard error of the mean
SIET	scanning ion electrode technique
SGK	serum glucocorticoid regulated kinase
slc	solute carrier family
TEP	transepithelial potential

TJ	Tight junction
tmAC	transmembrane adenylyl cyclase
tUT	toadfish urea transporter
µCi	micro Currie
µg	microgram
µl	microliter
µM	micromolar
zecac	zebrafish epithelial Ca^{2+} channel
zncx	zebrafish Na^{+}-Ca^{2+} exchanger
zpmca	zebrafish plasma membrane Ca^{2+} adenosine triphosphatase

CHAPTER 1
General Introduction

For most vertebrates, maintaining a constant body fluid osmolarity is critical to survival. Teleosts living in freshwater (FW) maintain their body fluid osmolarity at approximately 250 mOsmol/L owing to the accumulation of salts with the body fluids. Consequently, a large ionic gradient leads to the continuous passive loss of salts to the environment. FW teleosts achieve ionic homeostasis by regulating both passive efflux and active absorption of ions from the environment. The molecular mechanisms underlying these strategies for osmoregulation in FW fish have been investigated for nearly a century beginning with the pioneering work by Krogh (1938) and are summarized in several recent reviews (Chasiotis et al., 2012; Dymowska et al., 2012; Evans, 2011; Hwang, 2009; Hwang and Perry, 2010; Hwang et al., 2011; Kumai and Perry, 2012).

The fundamental question of this thesis is how zebrafish (*Danio rerio*), a FW teleost and model organism, regulates its whole body Na^+ balance when exposed to extreme acidity, a condition known to disrupt Na^+ homeostasis. The overall approach is to describe, in significant detail, how ionic homeostasis is defended in an acidic environment through the actions of multiple endocrine mechanisms and signalling molecules. It is hoped that this thesis will provide a useful point of comparison for studies using other model species, such as goldfish, *Carassius auratus* and medaka, *Oryzias latipes*.

In the remainder of the Introduction, I will review three key points; 1) the general consequences of acid-exposure on whole body Na^+ balance in FW fish, and how some acid-tolerant species overcome this challenge, 2) endocrine regulation of two acid-secreting proteins, Na^+-H^+ exchanger (NHE) and H^+-ATPase, and 3) the power of the zebrafish model for physiological research and the current state of knowledge on mechanisms of Na^+ homeostasis in this species.

Na^+ homeostasis in acidic water

Responses to acid exposure by acid-sensitive species

The classic work by (Krogh, 1938) demonstrated that Na^+ uptake in FW goldfish, *Carassius auratus*, is coupled with secretion of acid-equivalent (either H^+ or NH_4^+), and uptake of Cl^- is coupled with secretion of base-equivalent (HCO_3^-) ions. Although recent studies (Hiroi et al., 2008; Inokuchi et al., 2009; Wang et al., 2009) demonstrated the presence of Na^+-Cl^- co-transport mediated via a thiazide sensitive Na^+-Cl^- co-transporter, (NCC; Hebert et al., 2004), the initial model of Krogh has stood the test of time. Currently, two mechanisms of Na^+ uptake coupled with acid-secretion are proposed to operate in FW fish; 1) direct exchange of acid and Na^+ mediated by NHE2 and 3, two apically oriented isoforms of the NHE gene family (Craig et al., 2007; Edwards et al., 2005; Hirata et al., 2003; Ivanis et al., 2008b; Scott et al., 2005b), and 2) uptake of Na^+ via an apical Na^+ conducting channel facilitated by an electrical gradient generated by active secretion of H^+ via H^+-ATPase (Avella and Bornancin, 1989; Fenwick et al., 1999; Lin et al., 1994; Lin and Randall, 1991). The NHE model has been challenged because electroneutral Na^+ uptake in dilute FW is difficult to reconcile thermodynamically (Avella and Bornancin, 1989; Parks et al., 2008) and the Na^+ channel model suffers from the apparent lack of an epithelial Na^+ channel analog in any annotated teleost genome. Regardless, it is beyond doubt that Na^+ uptake in FW teleosts is linked to acid secretion. This linkage raises an obvious interest in how FW fish regulate their Na^+ balance under low pH conditions.

Owing in part to the concern about the environmental impact of acid rain, the physiological consequences of acid exposure on aquatic animals received considerable research interest between 1970 and 1980 (for review see McDonald, 1983a). Results from these early studies suggested that the primary cause of mortality from acid exposure in acid-sensitive fish,

such as rainbow trout, *Oncorhynchus mykiss*, and brown trout, *Salmo trutta*, is a reduction in plasma [Na^+]. The plasma [Na^+] is significantly lowered because of inhibition of Na^+ uptake and to a greater extent, uncontrolled increase in Na^+ efflux following acid-exposure (McDonald, 1983a; McWilliams and Potts, 1978; Milligan and Wood, 1982). Ultrastructural analysis using transmission electron microscopy demonstrated that acid-exposure disrupts the integrity of tight junctions (TJs) between epithelial cells in fish gills (Marshal, 1985; Freda et al., 1991) as well as amphibian tadpole (Meyer et al., 2010). The disruption of TJs is considered to be the main cause of increased Na^+ efflux in acidic water. These early studies also demonstrated a significant role for water [Ca^{2+}] in controlling Na^+ efflux in acidic water. Exposure of both rainbow and brown trout to acidic water with elevated [Ca^{2+}] reduced Na^+ efflux, as the apparent branchial permeability to Na^+ was reduced in response to elevated [Ca^{2+}] (McDonald, 1983b; McWilliams and Potts, 1978). Because the disruption of TJs (and subsequent increase in Na^+ efflux) in acid-exposed fish was attributed to the leaching of Ca^{2+} from TJs, it is likely that the apparent protective effect of higher [Ca^{2+}] in acidic water is a result of the stabilization of TJs.

The studies discussed above demonstrate the significant challenge associated with maintaining ionic (but mainly Na^+) balance in acidic water. However, there are a number of FW fish species that thrive in extremely acidic environments, where pH may range from 3.4 ~ 4.0.

Species native to extremely acidic environments; diversity of adaptation

Probably the most extensively studied group of acid-tolerant species of fish are found in the Rio Negro from the Amazon River system. While Rio Negro water chemistry is extremely harsh, with pH around 4.5 - 5.1, [Na^+] and [Ca^{2+}] ~ 10 and ~ 5 µM, respectively, approximately 1000 species of fish are estimated to be distributed in these waters (for review see Gonzalez et al., 2005) . Previous research identified two major strategies to defend Na^+ homeostasis in acidic

water; 1) 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 (Gonzalez et al., 2005). Some species of tetra, including neon tetra, *Paracheirodon innesi* (Gonzalez and Preest, 1999), cardinal tetra, *Paracheirodon axelrodi* (Gonzalez and Wilson, 2001), and Blackskirt tetra, *Gymnocorhymbus ternetzi* fall into the first category; they demonstrate insensitivity of Na^+ uptake to water pH as low as 3.5 - 4.5. Interestingly, (Preest et al., 2005) demonstrated that Na^+ uptake in *P. innesi* also is insensitive to commonly used pharmacological inhibitors of Na^+ uptake including amiloride [inhibits both epithelial Na^+ channels (ENaC) and NHE] as well as vanadate (inhibits H^+-ATPase). Additionally, Na^+ uptake in *P. innesi* was inhibited by silver nitrate, which was attributed to inhibition of Na^+-K^+-ATPase activity (Preest et al., 2005). The lack of an effect of these pharmacological agents may reflect significant differences in the molecular structure of NHE3 and/or H^+-ATPase rendering them insensitive to these commonly used inhibitors or alternatively that under these conditions, these species are absorbing Na^+ through an alternative pathway independent of acid-secretion (e.g. NCC).

Other species, including FW stingray (*Potamotrygon sp.*; Wood et al., 2002b) 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) also was observed in some acid-tolerant species distributed in North America, such as banded sunfish, *Enneacanthus obesus* (Gonzalez and Dunson, 1987; Gonzalez and Dunson, 1989) and white perch, *Perca flavescens* (Freda and McDonald, 1988). Remarkably, (Gonzalez and Dunson,

1987; Gonzalez and Dunson, 1989) reported that Na^+ efflux in *E. obesus* was not significantly different from zero even after an exposure to water of pH 4, with exposure to pH 3.5 causing only a transient and minor increase in efflux. Based on the protective effect of Ca^{2+} in regulation of Na^+ efflux (see above), it was assumed that the exceptional acid-insensitivity of Na^+ efflux in these species is due to unusually high affinity of TJs to Ca^{2+}. For example, (Wood et al., 2002b) 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+}], (Gonzalez and Dunson, 1989) calculated that for *E. obesus*, only 19 µM of [Ca^{2+}] was required to reduce the Na^+ efflux rate by 50% from what was observed in Ca^{2+}-free acidic water. On the other hand, (Freda and McDonald, 1988) reported the Na^+ efflux in *P. flavescens* was independent of [Ca^{2+}] even at pH 3.25. The latter result, however, should be interpreted with caution because the lowest [Ca^{2+}] in which they measured Na^+ efflux was ~ 35 µM. 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.

Specific water chemistry might also contribute to the 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. (2003) observed that supplementing the reference acidic water with commercial humic acid actually increased the Na^+ efflux. The

chemical identity of protective DOC in Rio Negro water, and the exact molecular mechanism responsible for Na^+ efflux regulation remain to be elucidated.

Another example of 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). 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 experiments, exposure to pH 3.5 water initially reduced both plasma pH and [Na^+], but both parameters returned to near-control levels by 48 - 72 h (Hirata et al., 2003; Kaneko et al., 1999). Because previous studies only measured net ionic balance (plasma [Na^+]), it remains to be seen whether *T. hakonensis* regulates its plasma [Na^+] by adjusting passive efflux or active uptake. However, (Hirata et al., 2003) also 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 on Na^+ uptake from pharmacological treatment with NHE3 inhibitor in neon tetra (Peest et al., 2005). Although the negative results by (Preest et al. 2005) should be interpreted with caution, the difference again highlights the diverse mechanisms of ionic regulation employed by acid-tolerant species.

These previous studies using acid-tolerant species have demonstrated a variety of adaptive mechanisms that allow them to defend their Na^+ homeostasis in acidic water. In some cases, NHE3 is thought to underlie such adaptation. On the other hand, data regarding the molecular mechanisms that are responsible for activating NHE3 and H^+-ATPase in acidic water are largely unknown for fish. Because a significantly larger body of literature is available, the ensuing discussion will focus largely on studies using mammalian renal epithelia, tissues often

compared (on a functional basis) to the FW teleost fish gill, with particular emphasis on two proteins, NHE3 and H^+-ATPase, two proteins intricately involved in acid-secretion and Na^+ transport in mammalian kidney and fish gill/skin epithelia.

Physiological responses to acidosis

Sensing

For animals to respond properly to a change in their internal environment, deviations from homeostatic set points must first be detected by one or more biological sensors to trigger downstream responses. For a protein to function as an acid-base sensor it must be responsive to physiologically relevant changes in pH, pCO_2 or $[HCO_3^-]$ and upon activation should quickly initiate downstream signalling cascades that ultimately result in the activation of acid-base transporting proteins (Preisig, 2007). To date, the identification of pH sensors (and the physicochemical substrates being detected) remains elusive (for recent reviews see Brown et al, 2012; Brown and Wagner, 2012; Skelton et al, 2010). Although multiple receptors, such as G-protein coupled receptor 4 (Sun et al., 2010) and insulin receptor-related receptor (IRRR; (Deyev et al., 2011; Petrenko et al., 2013), have been suggested to act as sensors, the two most well-characterized acid-base sensors are tyrosine kinase in proximal tubule and soluble adenylyl cyclase in collecting duct (CD) (Boron, 2006; Brown and Wagner, 2012; Preisig, 2007). In OKP cells (a mammalian kidney proximal tubule cell line), non-receptor type tyrosine kinase (Pyk2) is activated within 30 s exposure to acidic media (Li et al., 2004). In the same study, it was demonstrated that Pyk2, once activated, interacts with another type of non-receptor tyrosine kinase, c-Src and that the formation of this protein complex is critically required for the acid-mediated activation of NHE3. While the study by (Li et al., 2004) demonstrated a significant role of Pyk2, it remains to be clarified whether this kinase is directly sensing alteration in pH or

changes in other variables, such as pCO_2. Indeed, (Zhou et al., 2005) demonstrated that within a short time frame, acid-base regulation (measured as the reabsorption of HCO_3^-) in isolated proximal tubules is strongly dependent on pCO_2 and $[HCO_3^-]$ of basolateral fluid, but not pH.

Cyclic adenosine monophosphate (cAMP), which is synthesized by adenylyl cyclase (AC), has long been recognized as one of several secondary messengers in intracellular signalling. Although AC initially was thought to occur only as a trans-membrane protein, (Buck et al., 1999) successfully purified a novel, soluble isoform of AC (sAC). Because the catalytic activity of sAC is stimulated in response to a rise in $[HCO_3^-]$ within a physiologically-relevant range (Chen et al., 2000), the potential role of sAC as an acid-base sensor has received considerable attention over the past several years (Tresguerres et al., 2010a; Tresguerres et al., 2011). One of the most well-documented roles of sAC in acid-base regulation is to mediate the translocation of H^+-ATPase toward the apical membrane in type A (acid secreting) intercalated cells (Păunescu et al., 2008; Păunescu et al., 2010) and epididymal clear cells (Pastor-Soler et al., 2003; Pastor-Soler et al., 2008), thus facilitating the secretion of H^+ into the lumen. It is now recognized that cAMP can activate two major downstream targets; protein kinase A (PKA) and exchange proteins directly activated by cAMP (Epac) (Cheng et al., 2008; Gloerich and Bos, 2010; Métrich et al., 2010). Based on the pharmacological inhibition of these two proteins, the stimulatory effect of cAMP on H^+-ATPase in these tissues is likely to be mediated by PKA (Pastor-Soler et al., 2008; Păunescu et al., 2010).

Activation of transporters

While the sensing mechanisms discussed above initiate the response to acid-base disturbance, chronic internal acidosis may influence other physiological processes including the endocrine systems. Angiotensin II (ANG-II), two steroid hormones (cortisol and aldosterone),

and endothelin are hormones known to play an important role in regulating acid-base balance in the kidney, (Preisig, 2007; Wagner et al., 2006). Angiotensin II is a biologically active peptide synthesized by angiotensin converting enzyme (ACE), from angiotensin I, and is one of the major hormones controlling systemic water and salt balance in mammals. ANG-II interacts with Type I and II ANG-II receptors (AT_1 and AT_2). Whereas the majority of studies focused on the physiological role of AT_1 (the expression level of AT_2 declines after birth), it is now known that AT_2 also is involved in adults, especially under pathophysiological conditions (Crowley and Coffman, 2012; Stegbauer and Coffman, 2011). Traditionally, ANG-II has been regarded as part of the renin-angiotensin-aldosterone system (RAAS) but recent studies revealed aldosterone independent modulation of salt/water balance by ANG-II (van der Lubbe et al., 2011). With respect to responses of ANG-II to internal acidosis, ANG-II is also known to activate both H^+-ATPase and NHE3 (see below). Based on the rate of intracellular pH recovery in Na^+-free media, it was demonstrated that ANG-II can activate H^+-ATPase activity in both proximal tubule and collecting duct isolated from mouse and rat, respectively (Wagner et al., 1998; Wagner et al., 2011). Rothenberger et al. (2007) demonstrated that the AT_1 receptor plays a quantitatively larger role than AT_2 in mediating the stimulatory effect of ANG-II on H^+-ATPase activity, which is dependent on H^+-ATPase trafficking via microtubules. The same study suggested that downstream signalling activated by ANG-II includes, but is not limited to, a rise in intracellular Ca^{2+}, protein kinase C (PKC), extracellular signal-regulated kinase 1/2 (ERK1/2) and phosphatidyinositol-3 (PI-3) kinase (Rothenberger et al., 2007). Equally, the stimulatory role of ANG-II on NHE3 is well established although when applied at high concentration (Houillier et al., 1996), ANG-II may inhibit NHE3 activity. It is likely that the stimulatory effect of ANG-II on NHE3 is induced through multiple pathways, including activation of tyrosine kinase, c-Src

(Tsuganezawa et al., 1998), PKC (Houillier et al., 1996) and PI-3 kinase [increases the surface insertion of NHE3 in OKP cell lines (Du Cheyron et al., 2003)]. In addition to ANG-II acting as an endocrine/paracrine factor, recent studies revealed a significant role of ANG-II as an intracellular signalling molecule. Thus, ANG-II may be synthesized within a cell or be internalized once bound to AT_1 receptor (Zhuo and Li, 2007). The intracellular pool of ANG-II found in proximal tubule or kidney cell line was shown to stimulate directly the mRNA expression of NHE3, further emphasizing the complexity of ANG-II mediated regulation on NHE3 (Li et al., 2012; Li and Zhuo, 2008)

Aldosterone and cortisol are steroid hormones. They bind to mineralocorticoid receptor (MR) and glucocorticoid receptor (GR), respectively, which then acts as a ligand-dependent transcription factor that binds to hormone response elements within the promoter region of target genes (Falkenstein et al., 2000). Alternatively, these steroids can also trigger downstream signalling and rapid physiological responses by directly activating signalling kinase(s) (Dooley et al., 2012). For example, within the collecting duct, it was suggested (Winter et al., 2004) that aldosterone can activate H^+-ATPase in a non-genomic manner; this conclusion was based on the speed of response and its insensitivity to actinomycin D/cyclohexamide treatment. The downstream signalling cascade was further elucidated by demonstrating a critical role for PKC and intracellular Ca^{2+} in mediating the stimulatory effect of aldosterone (Winter et al., 2011). In addition, chronic (7 days) treatment of rabbits with aldosterone led to a significant increase in H^+-ATPase activity in the collecting duct (Garg and Narang, 1988). Because of the long duration of treatment in the latter study, it is possible that the stimulatory effect of aldosterone on H^+-ATPase activity also included the classic, genomic pathway. The regulation of NHE3 by glucocorticoid hormones also is well-characterized and occurs through multiple mechanisms

including genomic induction (Ambuhl et al., 1999; Baum et al., 1994) and non-genomic modulation. The non-genomic modulation of NHE3 occurs via phosphorylation mediated by serum/glucocorticoid regulated kinase 1 (SGK-1) (Wang et al., 2005; Yun et al., 2002) and SGK-3 (He et al., 2011).

Cell differentiation and proliferation

In addition to changes occurring within a single cell, prolonged acidosis leads to the differentiation and proliferation of specific renal epithelial cells responsible for acid secretion. Within the renal collecting duct, A- and B-intercalated cells are thought to be responsible for acid- and base- secretion, respectively, although recent studies suggest the intercalated cells are also involved in electrolyte transport (Eladari et al., 2011). Interestingly, during systemic acidosis, the proportional density of A-intercalated cells increased, while that of B-intercalated cells decreased within the collecting duct, whereas there is no change in the density of total intercalated cells (Welsh-Bacic et al. 2012). A potential explanation for these data is that during acidosis, B-intercalated cells de-differentiate and are transformed into A-intercalated cells. A 230-kDa protein secreted by B-intercalated cells undergoing conversion to A-intercalated cells was first purified in 1996 (Takito et al., 1996), and is now referred to as hensin (or DMBT1 in the Mouse Genome Project nomenclature). More recently, (Gao et al., 2010) showed that when hensin was conditionally deleted in intercalated cells of transgenic mice, no A-intercalated cells were observed in the medullary CD, providing further evidence for the critical role played by hensin in the formation of A-intercalated cells (for recent reviews see: Al-Awqati, 2008; Al-Awqati, 2011; Al-Awqati and Gao, 2011). Clearly, animals are capable of remodelling renal epithelial to better handle chronic pH disturbances.

The capacity to sense acidosis, mobilize acid-secreting transporters, and epithelial remodelling, constitute an effective global response to acidosis. To identify and characterize similar molecular systems in fish have been the major driving force of my research. To investigate this complex physiological question most effectively, I selected zebrafish as a model organism.

Zebrafish as a model system to study osmoregulation

Zebrafish, a small (about 2.5 cm in length) cyprinid species native to India and Bangladesh, has become a powerful model to study a variety of biological questions. While its size hinders certain classical physiological measurements, such as direct determinations of plasma electrolyte and gas chemistry and of trans-epithelial potential (TEP), zebrafish offers a number of advantages, including its relative ease to maintain in the laboratory, high fecundity, rapid development (zebrafish embryos, when reared at 28.5° C, hatch at around 48 - 72 hours post fertilization; hpf), and the availability of molecular tools such as annotated genomic data and established techniques for reverse genetics and generation of transgenic lines (Bill et al., 2009; Eisen and Smith, 2008; Ekker and Akimenko, 2010; Suster et al., 2009). Although they are often found in slow-moving streams or rice paddles in India and Bangladesh,where water pH is between 6 - 8 (Engeszer et al., 2007), in laboratory zebrafish are able to tolerate a wide range of experimental treatments, including exposure to waters of pH ranging from 4 to 10 (Horng et al., 2007), acclimation to water of highly variable ionic composition (Hoshijima and Hirose, 2007), temperature (Chou et al., 2008), PO_2 and PCO_2 (Vulesevic and Perry, 2006). Owing to their tolerance of harsh experimental treatments, combined with other advantages discussed above, the zebrafish has emerged as an excellent platform to investigate mechanisms, plasticity and regulation of ion uptake.

Overview of zebrafish osmoregulation

Four types of ion transporting cells (ionocytes) have been identified in zebrafish larvae (for recent reviews see: Dymowska et al., 2012; Hwang et al., 2011): those enriched with H^+-ATPase (atpv6; H^+-ATPase rich cell or HRC), those enriched with Na^+-K^+-ATPase (atp1; Na^+-K^+-ATPase rich cell or NaRC), those that express Na^+ Cl^- co-transporter (*slc12a10.2*; NCC cell or NCCC) and those that express inwardly rectifying K^+ channels (*Kir1.1*) , an ortholog of mammalian renal outer medullary K^+ channels (ROMK; Abbas et al., 2011). Furthermore, it is possible that NaR cells consist of further subtypes, some of which express Ca^{2+} transporting genes, including epithelial Ca^{2+} channel (*zecac*), plasma membrane Ca^{2+}ATPase (*zpmca2*) and Na^+-Ca^{2+} exchanger (*zncx1b*); (Liao et al., 2007) as well as those expressing selected isoform(s) of apical anion exchanger, such as *zslc26a3* (Bayaa et al., 2009; Perry et al., 2009). The existence of functionally distinct ionocytes in adult gill and larval skin has been demonstrated in other fish species including rainbow trout, *Oncorhynchus mykiss* (Galvez et al., 2002; Goss et al., 2001; Reid et al., 2003) and tilapia, *Oreochromis mossambicus* (Hiroi et al., 2005; Hiroi et al., 2008; Inokuchi et al., 2009). Although all ionocytes undoubtedly contribute to overall ionic homeostasis in zebrafish, this thesis focuses on HRC and NCCC because they are the ionocytes responsible for acid secretion (HRC only) and Na^+ uptake (both) in zebrafish, the central topics of my studies.

The HRC, first identified by Lin et al. (2006), is the most well-studied and well-characterized zebrafish ionocyte. Using non-invasive scanning ion selective electrode technique (SIET), it was shown that the HRC exhibits significant bafilomycin-sensitive H^+ secretion (Lin et al., 2006). The putative role of H^+-ATPase in acid secretion was confirmed by Horng et al., (2007), who observed a significant reduction in acid secretion in larvae after translational

knockdown of the A subunit of H^+-ATPase (*zatp6v1a*). Based on data from immunohistochemistry (IHC) or in situ hybridization (ISH), HRCs are now known to express, in addition to H^+-ATPase, apical NHE3b (Yan et al., 2007), ammonia conducting rhesus glycoprotein (Rhcg1) (Nakada et al., 2007a), carbonic anhydrase (CA) 15a (*zCA15a*) (Lin et al., 2008) and cytosolic CA2 like a (*zCA2 like a*) (Lin et al., 2008), basolateral anion exchanger (AE)1b (Lee et al., 2011) and Na^+-K^+-ATPase subunits, *atp1a1a.5* and *atp1b1b* (Liao et al., 2009). Current models propose that the HRC is the site of ammonia secretion [via apical Rhcg1; Nakada et al., 2007a; Shih et al., 2008) and Na^+ uptake (via NHE3b and an as yet unidentified Na^+ conducting channel coupled to H^+-ATPase; Yan et al., 2007) as well as a key site of acid secretion via H^+-ATPase and NHE3b.

Although the pioneering studies of Maetz and colleagues (Garcia Romeu and Maetz, 1964) demonstrated independence of Na^+ and Cl^- uptake in goldfish, *Carassius auratus*, a link between Na^+ and Cl^- uptake was subsequently proposed because of the presence in FW-acclimated tilapia of a subset of ionocytes apically expressing NCC (Hiroi et al., 2008; Horng et al., 2009a; Inokuchi et al., 2009; Inokuchi et al., 2008). In zebrafish larvae, NCCCs were first identified in Wang et al. (2009). Somewhat surprisingly, NCC knockdown results in a significant increase in Na^+ uptake and whole body Na^+ content, a response that was attributed to a compensatory increase in the activity of NHE3b (Wang et al., 2009). The functional redundancy of HRC and NCCC with regard to Na^+ uptake was further demonstrated with the discovery of a transcription factor *glial cell missing 2* (*gcm2*), which plays a critical role in HRC differentiation (Chang et al., 2009). When *gcm2* was knocked down, thus preventing HRC differentiation, the larvae exhibited an increase in NCCC density (Shono et al., 2011), associated with a significantly higher whole body Na^+ content. The results of these studies, when taken

together, clearly demonstrate redundancy in the functions of HRC and NCCC with respect to Na^+ uptake, which may contribute to the ability of zebrafish to tolerate a wide range of environmental conditions.

Physiological consequences of acid-exposure in zebrafish.

In zebrafish, acclimation to an acidic environment is known to increase the expression of several genes thought to be involved in acid secretion/Na^+ uptake, including H^+-ATPase (*zatp6v1a*; Chang et al, 2009; Yan et al., 2007), zAE1b (Lee et al., 2011) and *zCA15a* (but not *zCA2 like a*) (Lin et al., 2008). Furthermore, exposure to acidic water significantly increased the apical openings of individual HRCs, potentially as a compensatory mechanism to increase the exposure of membrane-bound H^+-ATPase and facilitate acid secretion (Horng et al., 2009b). Indeed, when measured with SIET, acid-exposed zebrafish larvae secreted acid at a significantly higher rate, both at the level of yolk sack and individual HRC (Horng et al., 2009b). Although it remains unknown whether zebrafish larvae in acidic water suffer from internal acidosis, these data clearly indicate a compensatory response to facilitate acid-secretion during exposure to extreme acidity.

Similar to the proliferation of acid-secreting A-type intercalated cells in the collecting duct during acidosis (see discussion above), acid-exposure causes the proliferation of HRCs in larval zebrafish skin (Horng et al., 2009b). As expected, mRNA expression of *gcm2* was elevated and HRC density was increased in adult gill after 4- and 7-day acclimation to pH 4 (Chang et al., 2009). These observations further support the critical role of *gcm2* in HRC differentiation, and also imply that the same signalling pathways exist between larval skin and adult gill in the differentiation/proliferation of HRCs.

Rationale and goals of the thesis

Previously, the questions shaping the research on the physiological consequences of acid-exposure in zebrafish focused on acid-base homeostasis and the regulation of ionocyte differentiation. Surprisingly, however, there are virtually no data concerning the consequences of acid-exposure on Na^+ homeostasis. Although Horng et al (2009b) reported that acid-exposed larval zebrafish are able to defend their overall Na^+ balance, the underlying mechanisms have not been elucidated. Therefore, the overall goal of this thesis was to investigate the physiological mechanisms that maintain Na^+ balance during exposure of zebrafish to acidic environments. The main goals of Chapter 2 were to determine 1) if adult zebrafish defend their whole body Na^+ balance during prolonged exposure to acidic water, as reported in larvae and 2) if so, whether Na^+ balance is achieved by regulating passive efflux or active uptake. The surprising outcome of Chapter 2 was that adult zebrafish, when exposed to acidic water, significantly increase their Na^+ uptake to counteract the concurrent increase in Na^+ efflux. The overarching theme of the remainder of the thesis was to characterize the molecular mechanisms allowing Na^+ uptake in acidic water (Chapter 3) and the physiological mechanisms responsible for inducing Na^+ uptake in acidic environments (Chapters 4 - 7). Four endocrine or neuroendocrine systems were investigated; cortisol (Chapter 4), angiotensin (Chapter 5), α- and β-adrenergic receptors (Chapter 6) and cAMP (Chapter 7). Chapter 8 attempts to synthesize the general principles gained from these studies while providing several new directions for future research.

CHAPTER 2
Strategies for maintaining Na^+ balance in zebrafish (*Danio rerio*) during prolonged exposure to acidic water.

Notes on Chapter

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This study was conceived by SFP. AB performed RT-qPCR under the supervision of SS. YK performed all the flux experiments and drafted the manuscript under the supervision of SFP.

Abstract

The objective of the present study was to characterize the capacity of zebrafish (*Danio rerio*) to regulate whole body Na^+ levels during exposure to acidic (pH 3.8-4.0) water. Exposure to acidic water significantly affected the mRNA levels of 14 claudin and two occludin isoforms, tight junction proteins thought to be involved in regulating paracellular efflux. Despite these changes, Na^+ efflux as well as uptake of polyethylene glycol (PEG), a marker for paracellular pathway, was persistently elevated during the 2-week period of acid-exposure, although there was a transient recovery between 12- and 72-h. Pre-exposing fish to acidic water for 2 weeks failed to attenuate the increase in Na^+ efflux associated with acute exposure to acidic water of low [Ca^{2+}]. However, during recovery in water of circumneutral pH following exposure to acidic water, normal rates of Na^+ efflux were restored within 5 h. The rate of Na^+ uptake was significantly elevated between 4 and 7 days of exposure to acidic water; the increase was associated with significant increases in maximal Na^+ uptake capacity ($J_{MAX}Na^{+)}$ and affinity constant (K_M). These results demonstrate that in acidic water, zebrafish maintain their whole body Na^+ balance primarily by regulating Na^+ uptake, rather than Na^+ efflux.

Introduction

Living in a hypoionic medium, freshwater (FW) teleost fish are faced with the challenge of maintaining their body fluids hyperionic to the environment. Ion homeostasis is defended by actively absorbing salts from the environment and by regulating the obligatory, diffusive loss of ions across the gill. Previous studies have focused largely on the regulation of active ionic uptake (for recent reviews see: Evans, 2008; Evans et al., 2005; Hwang, 2009; Hwang and Lee, 2007; Hwang and Perry, 2010; Marshall and Grosell, 2005), owing in part to a limited understanding of the molecular mechanisms controlling the transepithelial efflux of ions. Theoretically, efflux could occur either via transcellular or paracellular pathways. Although the relative contributions of transcellular and paracellular Na^+ efflux to the total Na^+ efflux is still under debate (Wood et al., 2009), it has been theorized that in some environmental conditions such as acidic water, paracellular Na^+ efflux becomes dominant (McDonald et al., 1983 and see references therein).

Tight junctions (TJs), distributed to the apical regions of epithelial and endothelial cells of vertebrates, are the primary regulators of paracellular flux (Schneeberger and Lynch, 2004; Tsukita et al., 2001). Claudins (and to a lesser extent occludins), transmembrane proteins associated with TJs, are considered to be primarily responsible for determining the permeability of a given TJ (Saitou et al., 2000). For example, over-expression of claudin-2 in Madin-Darby Canine Kidney (MDCK) cells increased the permeability to cations (Amasheh et al., 2002; Furuse et al., 2001) whereas over-expression of claudin-4 in MDCK cells reduced cation permeability (Van Itallie et al., 2001). Subsequently, isoforms such as claudins-8 (Yu, 2003), -11 (Van Itallie et al., 2003) and -14 (Ben-Yosef et al., 2003) have been shown to increase the "tightness" of membranes to cations whereas claudin-7 (Alexandre et al., 2007) and -15 (Van Itallie et al., 2003) have been shown to reduce permeability to cations or increase permeability to

anions (for a general review see: Van Itallie and Anderson, 2006). These charge-selective properties of specific claudin isoforms are responsible for maintaining steep ion concentration gradients across epithelia in various vertebrate transporting tissues, such as distal colon, where claudin 8 defends a steep concentration gradient of Na^+ (Amasheh et al., 2009).

Research on the roles of claudins and occludins in teleosts was triggered by the identification of 56 claudin isoforms in *Fugu rubripes* genome (Loh et al., 2004). Unlike in zebrafish, *Danio rerio*, where the emphasis of research on TJ proteins has been embryo (Siddiqui et al., 2010) or organ development (Bagnat et al., 2007; Hardison et al., 2005), research on TJ proteins in other teleosts has largely focused on their role in osmoregulation. Both the transcriptional and translational levels of claudin isoforms or occludin have been shown to be affected by external salinity in goldfish, *Carassius auratus* (Chasiotis et al., 2009), pufferfish, *Tetraodon nigroviridis* (Bagherie-Lachidan et al., 2009; Bagherie-Lachidan et al., 2008; Clelland et al., 2010), tilapia *Oreochromis mossambicus* (Tipsmark et al., 2008a) Atlantic salmon, *Salmo salar* (Tipsmark et al., 2008b) and southern flounder, *Paralichthys lethostigma* (Tipsmark et al., 2008c). In addition, these changes in expression level of TJ proteins are likely to be under endocrine regulation, most notably cortisol. In recent *in vitro* studies utilizing cell culture derived from FW-acclimated rainbow trout (*Oncorhynchus mykiss*) and from a SW fish, *T. nigroviridis*, cortisol treatment increased occludin mRNA expression and reduced paracellular permeability (Chasiotis et al., 2010) and altered transcript levels of some claudin isoforms, though in this case permeability was not measured (Bui et al., 2010). Overall, it seems likely that fish would be capable of regulating their gill epithelial permeability depending on the environment. Thus, the objective of the present study was to characterize the response of zebrafish (*Danio rerio*) to a severe reduction of ambient pH, an environmental condition

previously shown to affect branchial permeability. Specifically, exposure to acidic conditions is known to induce a significant increase in Na^+ efflux in variety of FW fishes (Freda and McDonald, 1988; McDonald et al., 1983) which generally has been attributed to a disruption of TJ proteins. In support of this interpretation, (Meyer et al., 2010) showed that increasing Na^+ loss in aquatic larval Eastern dwarf tree frog (*Litoria fallax*) exposed to acidic water was associated with a significant reduction in the depth of gill epithelial TJs. Thus, a first objective of the present study was to assess the effect of acidic water on the transcriptional expression of TJ proteins. To do so, the mRNA expression levels were analyzed using RT-qPCR of 18 TJ proteins in adult zebrafish gill over a two-week period of exposure to acidic water (3.8 – 4.0). A second objective was to directly assess the effects of acidic water on Na^+ efflux and to compare with its effects on non-ionic paracellular permeability (as assessed by measuring the whole body uptake of PEG 400). To more fully understand how zebrafish respond to a disturbance in Na^+ homeostasis caused by acid-exposure, whole body Na^+ levels and Na^+ uptake were also measured. Results from this study suggest that zebrafish are able to regulate whole body Na^+ balance in the face of prolonged and uncompensated Na^+ loss, primarily by increasing Na^+ uptake. While complex changes in TJ mRNA levels were observed, they apparently have no long term impact on rates of Na^+ efflux.

Materials and Methods

Animal care and experimental treatment

Adult zebrafish (*Danio rerio* Hamilton-Buchanan 1822) were purchased from Big Al's Aquarium Services (Ottawa ON, Canada) and kept in the University of Ottawa Aquatic Care Facility where they were maintained in plastic tanks supplied with aerated, dechloraminated City of Ottawa tap water at 28° C (mean weight = 0.44 ± 0.008 (SEM) g; N = 665). Fish were subjected to a constant 14 h L:10 h D photoperiod and fed daily with No.1 crumble-Zeigler^{TM} (Aquatic Habitats, Apopka, FL, USA) until satiation. To prepare acidic water, H_2SO_4 was added to the City of Ottawa tap water to lower pH to the desired level of 3.8-4.0. Experimental tanks were placed in a temperature-controlled environment to maintain water temperature at 28-28.5° C. During the two-week period of acid water treatment, approximately 1/3 of the water was changed daily to prevent accumulation of nitrogenous waste and to maintain the desired water pH. Water pH was kept within 3.80-4.10 (mean water pH = 3.93). The experiments were conducted in compliance with guidelines of the Canadian Council of Animal Care (CCAC) and after the approval of the University of Ottawa Animal Care Committee (Protocol BL-226).

Experimental protocols

To investigate the effect of exposure of zebrafish to acidic conditions on Na^+ homeostasis, the following series of experiments were performed:

Series 1. Effect of acid exposure on whole body Na^+ content

For this Series, the same fish used in Series 3 (unidirectional Na^+ efflux) were used to calculate the whole body Na^+ content. Following the measurement of unidirectional Na^+ efflux as described in Series 3, fish were pulverized in liquid nitrogen. Half of the pulverized samples was placed into a pre-weighed vial, weighed, and digested with 7% perchloric acid overnight at 65° C. Once digestion was complete, samples were supplemented with deionised water to a total

volume of 20 ml and Na^+ concentration was measured by flame emission spectrophotometry (model AA260, Varian, Palo Alto, CA, USA) to calculate the whole body Na^+ content. This information was used to calculate the internal Na^+ specific activity for Series 3.

Series 2. mRNA expression profiles of claudin and occludin isoforms

Tissues (brain, liver, gut, heart, kidney, eye, gill and muscle) were dissected from adult zebrafish and snap frozen in liquid nitrogen to assess the tissue distribution patterns of 18 TJ proteins (16 claudins and 2 occludins), which included all claudin/occludin isoforms listed in ZFIN when this study was conceived. The nomenclature published on ZFIN (http://zfin.org/cgi-bin/webdriver?MIval=aa-ZDB_home.apg) was adopted. Concurrently, fish were subjected to a 2-week exposure to acidic or regular aquarium water (controls). After 1, 3, 6, 12, 24, 48, 72, 168 and 336 h of exposure, fish were killed with an overdose of ethyl 3-aminobenzoate methanesulfonate (MS-222; N = 6 at each time point) and their gills were dissected and snap-frozen in liquid nitrogen for later RNA extraction for real-time PCR.

RNA extraction and tissue distribution of TJ proteins

Total RNA was extracted from the tissue using TRIzol® reagent (Invitrogen) following the manufacturer's instructions. After quantifying the extracted RNA using a spectrophotometer (model ND-1000, NanoDrop, Wilmington, DE) with NanoDrop ND-1000 software (version 3.3.0), cDNA was synthesized by treating the 2 µg of extracted RNA with DNase (Invitrogen) and RevertAid^{TM} M-MNuLV reverse transcriptase (Fermentas, Burlington, ON, Canada) according to the manufacturer's instructions. To establish the tissue distribution patterns of TJ proteins, RT-PCR was performed with the primers listed in Table 1. Primers were designed using Primer3 software and amplicons were sequenced to verify the product. Conditions of PCR were as follows: initial denaturation at 94° C for 30 s followed by 40 cycles of 94° C for 30 s,

58° C for 60 s, and 72° C for 45 s, with final extension for 5 min at 72° C. PCR products were run on 2% agarose gels and images were acquired using GelDoc equipped with Quantity-One 1-D analyzer software (BioRad, Mississauga, ON, Canada).

RT-qPCR

Fish were killed and cDNA was synthesized from gill tissue as described above. RT-qPCR was performed using a MX3000P qPCR system with analysis completed on MXPro 4.0 (Stratagene). Each reaction consisted of 4.06-5.06 µl of sterile water, 6.3 µl of Brilliant II SYBR Green Master Mix (Stratagene), 0.25-0.75 µl of both forward and reverse primer (Table 2.1) and 0.19 µl of diluted reference dye. PCR conditions were identical to those described above. cDNA templates were added to the final volume of 12.5µl. 18S ribosomal RNA was used as the internal control, as 18S has been recognized as a stable normalizing gene in zebrafish across developmental stages and in a variety of experimental treatments (McCurley and Callard, 2008). Consistent with the relatively constant 18S expression levels, in the present study majority of samples had cycle threshold (ct) values for 18S between 18 and 21. To determine the efficiency of PCR reactions, standard curves for each gene were constructed by serial dilution of pooled templates. The final real-time PCR data were analyzed using the modified cycle threshold ($\Delta\Delta C_T$) method as described by (Pfaffl, 2001).

Series 3. Effect of water pH on unidirectional Na^+ efflux.

The same fish used in series 1 were used to calculate the unidirectional Na^+ efflux. Unidirectional Na^+ efflux was measured by standard radiotracer methods using ^{22}Na. Fish were subjected to exposure to acidic or control water for two weeks as described above, and measurements were taken at the same time points as in Series 2. For Na^+ efflux measurements, 0.5 µCi of ^{22}Na in the form of NaCl (Perkin Elmer, Waltham, MA, USA, activity = 0.1 µCi/µl)

was injected intraperitoneally into adult zebrafish (N ≥ 6 for each time point). Each fish was then placed into a 60-ml, aerated flux chamber connected to an overhead reservoir, from which the chamber was flushed continuously with either control or acidic water. Fish were allowed to recover from handling stress for 5 h before any measurements were performed. After the recovery period, flow into the flux chamber was stopped, and 2-ml water samples were collected for 1 h at 15-min intervals. Subsequently, fish were removed from the chamber, briefly rinsed in isotope-free water to remove residual ^{22}Na on the external surfaces, and pulverized in liquid nitrogen. Pulverized samples were separated into two pre-weighed vials, weighed, and digested with either Solvable™ (Perkin Elmer) or 7% perchloric acid overnight at 65° C. Once digestion was complete, samples digested with Solvable™ were supplemented with 450 µl of glacial acetic acid and added to 15 ml of scintillation cocktail (BioSafe-II, RPI co. Mt. Prospect, IL, USA). Water samples were added to 10 ml of the same cocktail. Radioactivity of samples was measured using a liquid scintillation counter (model LS-6500 Beckman Coulter, Co. Mississauga ON, Canada). Perchloric-acid digested samples were handled as described in series 1 to calculate the internal Na^+ specific activity. The efflux rate (J_{out}^{Na}) was calculated as follows:

$$J_{out}^{Na} = \frac{1}{SA_F \cdot w} \frac{dW}{dt}$$

, where SA_F = internal specific activity of Na^+ (cpm/nmol), w = wet weight of the fish (g), W = total radioactivity of the medium (cpm) and t = duration of measurement (h). Backflux was not considered for the calculation of the unidirectional flux rate as the external specific activity was less than 5% of internal specific activity.

Series 4. Effect of water pH on permeability to PEG.

Paracellular permeability (Curtis and Wood, 1991) was measured as the influx rate of [^{3}H]PEG (polyethylene glycol). Fish were subjected to the 2-week exposure to acidic conditions

as described above, and measurements were taken at the same time points as in Series 1. Fish (N ≥ 6) were placed in flux chambers and allowed to recover from the handling stress as in Series 2. After the recovery period, water flow to the chamber was stopped and 0.3 µCi of [^{3}H]PEG (MW: 400, American Radiolabeled Chemicals Inc. St. Louis, MO, USA, 0.13 µCi/µl) was added to the chamber. Water samples (2 ml) were collected 5 and 60 min after the addition of isotope. Subsequently, fish were removed from the chamber, rinsed and digested as described above, with the exception that the entire fish was digested in one vial using Solvable™. Radioactivity of the digested fish and water samples were measured by liquid scintillation counting. The rate of [^{3}H]PEG influx (J_{in}^{PEG}) was calculated as follows:

$$J_{in}^{PEG} = \frac{F}{N \cdot SA \cdot w \cdot t}$$

, where F = total radioactivity incorporated into the fish (cpm), N = index to normalize radioactivity of water (unitless), SA = specific activity of PEG (dpm/µmol), w = wet weight of the fish (g) and t = time of exposure (h). Cpm was converted to dpm (disintegrations per minute) by the liquid scintillation counter taking quenching and counting efficiency into consideration.

Series 5. Effect of acclimation to low pH on resistance to Ca^{2+} removal.

The objective of this series was to investigate whether the 2-week acclimation to acidic conditions would render zebrafish more resistant to a subsequent treatment known to increase Na^+ efflux, a reduction in ambient [Ca^{2+}] (Freda and McDonald, 1988).

The following five groups were set up; (1) control group (N = 6), in which the rate of Na^+ efflux was measured at normal pH (pH 7.3-7.6) and [Ca^{2+}], typically around 200 µM; (2) acute low pH group (N = 6), in which fish were acutely exposed to acidic water (pH 4.0) with normal [Ca^{2+}]; (3) low pH/low[Ca^{2+}] group (N = 6), in which fish were acutely exposed to acidic (pH 4.0) and soft (final [Ca^{2+}] ~ 10 µM) water; (4) acclimated group (N = 5), in which fish were

exposed to acidic conditions with normal [Ca^{2+}] for 2 weeks before being exposed to acidic soft water and (5) "normal pH acclimated" group (N = 6) in which fish were kept in normal water for 2 weeks and then acutely exposed to acidic soft water. In addition, to assess the effect of Ca^{2+} removal in normal pH condition, Na^+ efflux was also measured in (1) water of normal pH and normal [Ca^{2+}] and (2) normal pH but low [Ca^{2+}] (N = 5 - 6). In all "acute" transfers, intended new water chemistry was reached within 15-20 min and fluxes were started immediately afterwards.

Na^+ efflux was measured and calculated as described in Series 3. To lower the ambient [Ca^{2+}], chambers were flushed for 20 - 30 min with soft water after the 5-h recovery period. Low [Ca^{2+}] water was prepared by dissolving NaCl and $NaHCO_3$ in deionised water to adjust [Na^+] and [Cl^-] to typical Ottawa tap water levels ([Na^+] = 600 – 700 µM; [Cl^-] = 200 µM) and by lowering the pH to the desired level (3.8-4.0) using H_2SO_4.

Series 6. Effect of acclimation to low pH on Na^+ efflux upon return to circumneutral pH.

The objective of this series was to further assess whether acclimation to low pH was reducing the paracellular permeability. If so, a significant decrease in Na^+ efflux would be expected to accompany the return of fish to circumneutral pH. Fish were acclimated to the acidic water and placed in the flux chamber as described above. The first group of fish (N = 6) was allowed to recover from the handling in circumneutral water for 5 h before the Na^+ efflux rate was measured as described above. The second group (N = 8) was allowed to recover from the handling in acidic water and were exposed to Ottawa tap water approximately 15 min before Na^+ efflux rate was measured. The third group (N = 11) was kept in Ottawa tap water for 2 weeks and the rate of Na^+ efflux was measured in the same water as the handling control group.

Series 7. Effects of acidic conditions on Na^+ uptake.

Fish were acclimated to acidic or control conditions (N = 6 - 18) for up to one week as described in Series 3 and Na^+ uptake rate was measured 3, 4, 5 ,6 and 7 days after the onset of acclimation (see Discussion regarding why uptake was measured for only one week). On the day of Na^+ uptake measurement, fish were placed in a flux chamber and allowed to recover from the handling stress as described in Series 3. After the recovery period, water flow to the chamber was stopped and 0.5 µCi of ^{22}Na was added to the chamber. Two sets of 2 ml water samples were collected 5 and 60 min after the addition of isotope. Subsequently, fish were removed from the chamber and digested as described in Series 3, with the exception that whole fish was digested in Solvable™. Radioactive samples of fish and water were prepared and handled as described in Series 3. The second set of water samples was used to measure the total $[Na^+]$ in the external medium by flame emission spectrophotometry. The rate of Na^+ uptake (J_{in}^{Na}) was calculated as follows:

$$J_{in}^{Na} = \frac{F}{SA \cdot w \cdot t}$$

,where F = total incorporated radioactivity of fish (cpm), SA = external specific activity of Na^+ (cpm/nmol), w = wet weight of the fish (g) and t = duration of the measurement (h). Backflux was not considered for the calculation of the unidirectional influx rate as the internal specific activity was less than 5% of external specific activity.

Series 8. Effects of low pH exposure on Na^+ uptake kinetics.

Fish were acclimated to acidic or control conditions (N = 3 – 7) for 5 days before the assessment of Na^+ uptake kinetics. External concentration of Na^+ was adjusted to the desired level by dissolving NaCl in deionised water. $CaSO_4$ (Sigma) was also added to the medium to yield a final concentration of 160-200 µM, representative of $[Ca^{2+}]$ found in Ottawa tap water.

After adjusting the ion concentration of the medium, pH was adjusted to the desired level with H_2SO_4 if necessary.

After 5 days of acclimation to either control or acidic water, fish were transferred to the 60-ml flux chamber and were allowed to recover from the handling stress for 5 h as described in Series 2. After 5 h of recovery, the chambers were flushed with water of appropriate Na^+ concentration for 30 min to reach the target [Na^+] concentration. After the flux period, the flow was stopped and the rate of Na^+ uptake was measured as described in Series 7.

Statistical analysis

All statistical analyses were performed using SigmaPlot (v. 11, Systat Inc. Chicago, IL, USA). Changes in mRNA expression level between control and treatment groups (Series 2) and the effect of Ca^{2+} removal on Na^+ efflux rate in normal pH (Series 5) were assessed by Student's *t*-test or Mann-Whitney-*U* test depending on whether the data were normally distributed (determined automatically during each statistical analysis). Data on whole body Na^+ content (series 1), Na^+ efflux and influx (Series 3 and 7) and PEG uptake (Series 4) were analyzed by two-way ANOVA. The effect of Ca^{2+} removal on Na^+ efflux in low pH conditions (Series 5) as well as the effect of acclimation to low pH upon transfer to circumneutral pH (Series 6) was assessed by one-way ANOVA. For Series 8, parameters associated with uptake kinetics were calculated by fitting one-site saturation kinetics to the dataset using SigmaPlot. K_M and $J_{MAX}Na^+$ were compared with Student's *t*-test, using the estimated value and SE calculated by SigmaPlot (Motulsky, 1998). In all cases, when significance was detected, ANOVA tests were followed by Holm-Sidak post hoc tests. For all ANOVA tests, when necessary the data were either ln or square-root transformed to satisfy the requirement of normal distribution. Correlation between

PEG uptake and Na^+ efflux was analyzed with Pearson's correlation. In all cases, statistical significance was set at $p<0.05$.

Results

Series 1. Whole body Na^+ content changes following transfer to acidic water

The exposure to acidic water caused a significant reduction in whole body [Na^+] (Fig. 2.1) which was first detected 3 h after the onset of exposure, and persisted for up to 72 h. Whole body Na^+ levels in the acid-treated group were restored to control levels after 1- and 2-weeks of exposure (N = 6 - 12; Fig. 2.1; two-way ANOVA).

Series 2. mRNA expression profiles of occludin and claudin isoforms.

The tissue distribution patterns of 18 isoforms of TJ proteins (16 claudins and 2 occludins) are shown in Fig. 2.2. These isoforms showed tissue specific distribution patterns although some isoforms, such as claudins-2, -7 and -12, appeared to be expressed ubiquitously. In the gill, transcripts of all isoforms were detected, though isoforms such as claudin-A, -D, -I and occludin A appeared to be more highly expressed than other isoforms including claudin-C and -J.

RT-qPCR revealed complex temporal changes in TJ protein mRNA levels in response to the exposure to acidic water. In total, mRNA levels of both occludins and 14 of the 16 claudin isoforms were significantly affected at some time point during the 2-week long experiment. Based on the overall pattern of changes in expression level, claudins were classified into three groups. Expression levels of claudin-B, -E, -G and -7 increased predominantly during the first 24 h of acid exposure (represented by claudin –B in Fig. 2.3A; N = 4 - 6; two-way ANOVA). On the other hand, expression levels of claudin-C, -D, -H and both occludins increased predominantly during the later stages of exposure to acidic water (represented by claudin-H in Fig. 2.3B; N = 4 - 6; two-way ANOVA). The remaining isoforms, claudin-A, -F, -I, -J, -2, -8, -11, -12 and -19, showed no clear temporal trend in their changes in mRNA expression level (represented by claudin-A in Fig. 2.3C; N = 4 - 6; two-way ANOVA). Typically, mRNA levels

were significantly increased except for claudin-G, -I, -2 and -8 which were significantly reduced between 48 and 72 h of exposure to low pH water. The global changes in TJ mRNA levels over the 2-week period of exposure to acidic water are summarized in Table 2.2.

Series 3. Effect of water pH on unidirectional Na^+ efflux.

Exposure of fish to acidic water caused a significant increase in Na^+ efflux after immediate (1 and 6 h after the onset) and chronic (72, 168 and 336 h after the onset) exposure to acidic water (N = 6 - 12; Fig. 2.4A; two-way ANOVA).

Series 4. Effect of low pH on paracellular permeability

Exposure of fish to acidic water caused a significant increase in PEG influx. influx rate was elevated for the initial 12 h exposure to acidic water. Following a brief period of recovery between 24 and 72 h of exposure, PEG influx rate again was significantly elevated in acid-exposed fish between 168 and 336 h of exposure (N = 6; Fig. 2.4B; two-way ANOVA).

Using all of the data gathered from control and acid-exposed zebrafish, there was a statistically significant correlation between PEG uptake and Na^+ efflux ($R^2 = 0.49$, $p < 0.05$, N = 18; Figure 2.4C). However, neither the control nor acid-exposed groups, when analyzed separately, exhibited a significant positive correlation (p = 0.49 and 0.07 for controls and acid-exposed fish, respectively).

Series 5. Effect of acclimation to low pH on resistance to Ca^{2+} removal.

Removal of Ca^{2+} had no impact on the rate of Na^+ efflux under normal water pH conditions (N = 5 - 6; Fig. 2.5A; Student's t-test). However, removal of Ca^{2+} under acidic conditions significantly increased the rate of Na^+ efflux (N = 5 - 7; Fig. 2.5B; one-way ANOVA). Acclimating fish to low pH water for 2 weeks prior to acute exposure to low ambient $[Ca^{2+}]$ did not prevent or diminish the massive loss of Na^+ (N = 5 - 7; Fig. 2.5B; one-way

ANOVA). The normal pH acclimated group, which were kept in the control water for 2 weeks before acutely exposed to acidic, low [Ca^{2+}] water, suffered the equally massive loss of Na^+ (N = 5 - 7; Fig. 2.5B; one-way ANOVA).

Series 6. Effect of acclimation to acidic water on Na^+ efflux.

The rate of Na^+ efflux was significantly higher in fish that were acutely transferred to circumneutral pH water after two-week acclimation to low pH water compared to the handling control group (N = 6 - 11; Fig. 2.6; one-way ANOVA; Fig. 2.6). However, following a 5-h recovery in circumneutral pH water, the Na^+ efflux rate was no longer different from the control level (N = 6 - 11; Fig. 2.6; one-way ANOVA; Fig. 2.6).

Series 7. Effect of acidic conditions on Na^+ uptake.

The rate of Na^+ uptake was significantly elevated in acid exposed fish between 12 and 168 h, with the largest difference observed after 5 days (624 ± 63 nmol/g/h in control group versus 1365 ± 86 nmol/g/h in the acid-exposed group (N = 6 - 18; Fig. 2.7; two-way ANOVA).

Series 8. Effect of acidic conditions on Na^+ uptake kinetics.

Fig. 2.8 shows the kinetic curves of Na^+ uptake in both control and acid-water acclimated zebrafish. The dataset fitted to the typical Michaelis-Menten curve ($R^2_{adj.}$ for the fitted curve = 0.71 for normal pH and 0.90 for low pH). When $J_{MAX}Na^+$ and K_M were compared between acid-acclimated and control groups, both parameters were significantly elevated in acid-exposed groups. Specifically, $J_{MAX}Na^+$ of low-pH acclimated fish and control fish was 1488 ± 178 and 835 ± 78 nmol/g/h, respectively ($p < 0.01$, Student's t-test). Calculated K_M was 703 ± 260 and 112 ± 58 μM for low pH acclimated group and control group, respectively ($p < 0.05$, Student's t-test).

Figure 2.1. Effect of low-pH exposure on whole body Na^+ content in zebrafish (*Danio rerio*). Whole body Na^+ levels were significantly reduced for up to 72 h in acid-treated fish. Asterisks denote significant differences in Na^+ content or Na^+ efflux rate between control (filled circles) and acid (open circles) treated fish; different letters denote significant differences within the acid treated group. N = 6 - 12. Data are presented as means ± SEM.

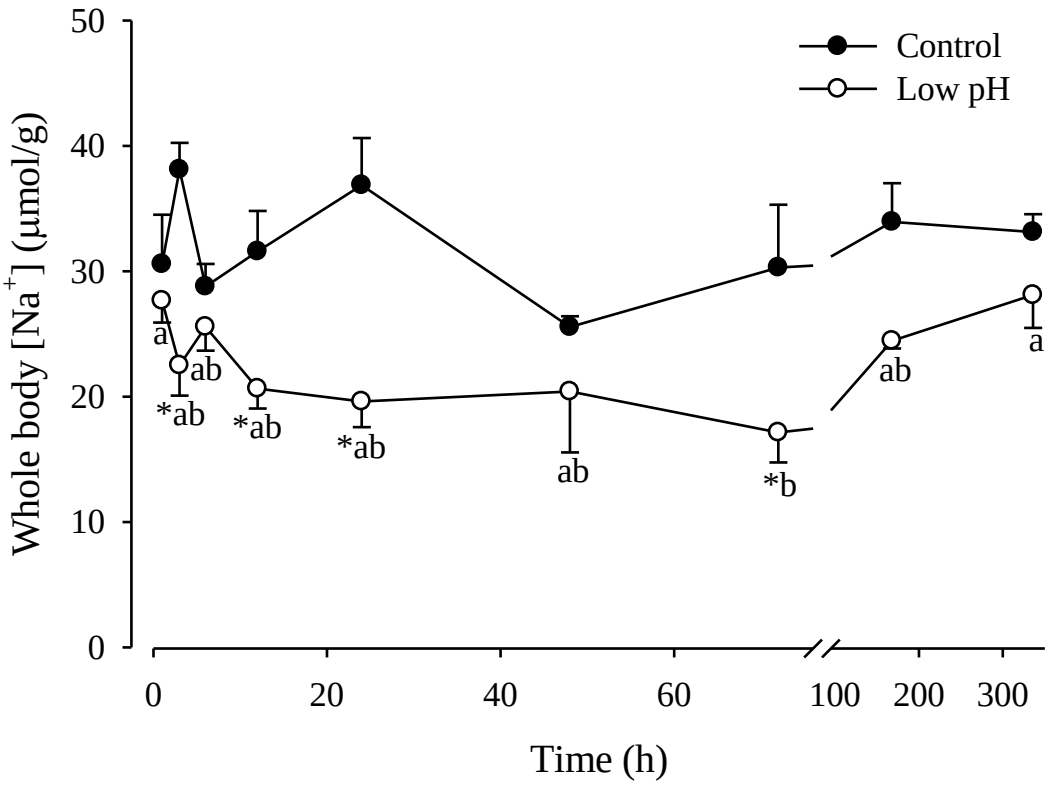
Control
Low pH
Whole body [Na+] (µmol/g)
50
40
30
20
10
0
0
20
40
60
100
200
300
Time (h)
a
*ab
ab
*ab
*ab
ab
*b
ab
a

Figure 2.2. Representative tissue distribution patterns of 18 tight junction genes in adult zebrafish (*Danio rerio*). 18S ribosome RNA was used as a reference gene.

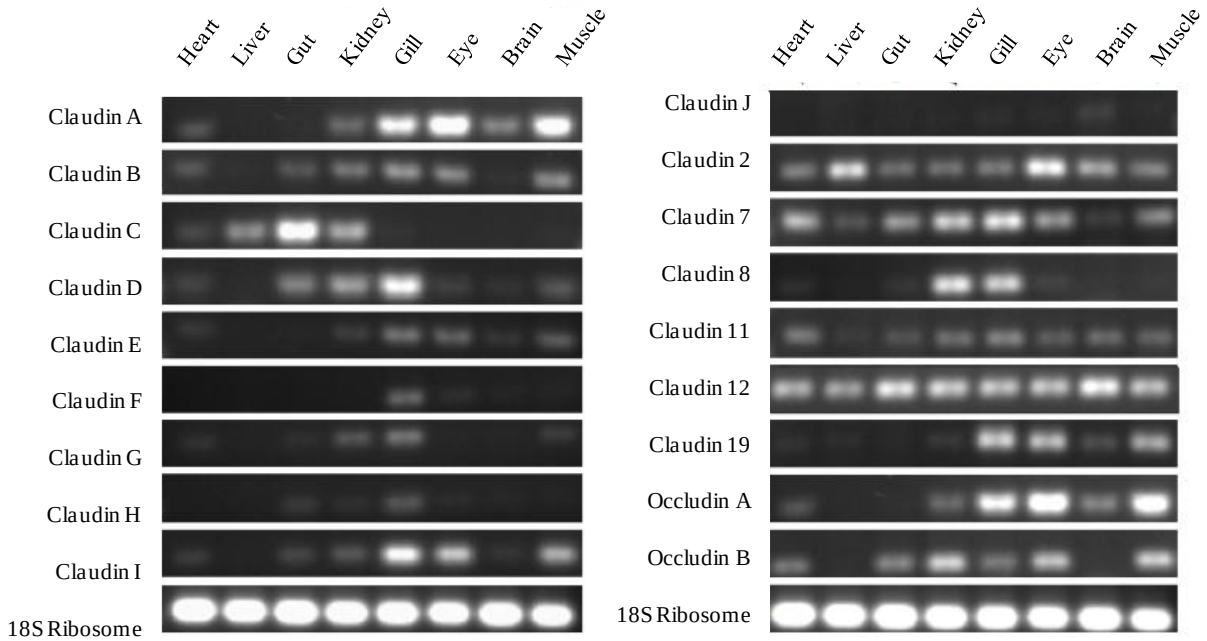
Heart
Liver
Gut
Kidney
Gill
Eye
Brain
Muscle
Claudin A
Claudin B
Claudin C
Claudin D
Claudin E
Claudin F
Claudin G
Claudin H
Claudin I
18S Ribosome
Heart
Liver
Gut
Kidney
Gill
Eye
Brain
Muscle
Claudin J
Claudin 2
Claudin 7
Claudin 8
Claudin 11
Claudin 12
Claudin 19
Occludin A
Occludin B
18S Ribosome

Figure 2.3. Effect of low-pH exposure on branchial mRNA expression of selected claudin isoforms. Changes in relative mRNA expression of claudin B (Fig 2.3A; representing "early response" group), claudin H (Fig 2.3B; representing "late response" group) and claudin 7 (Fig 2.3C; representing "no clear trend" group) in control fish (filled circles) and fish exposed to low pH (unfilled circles). N = 4-6. Asterisks denote significant differences from the control fish at any particular sampling time. Data are presented as means ± SEM.

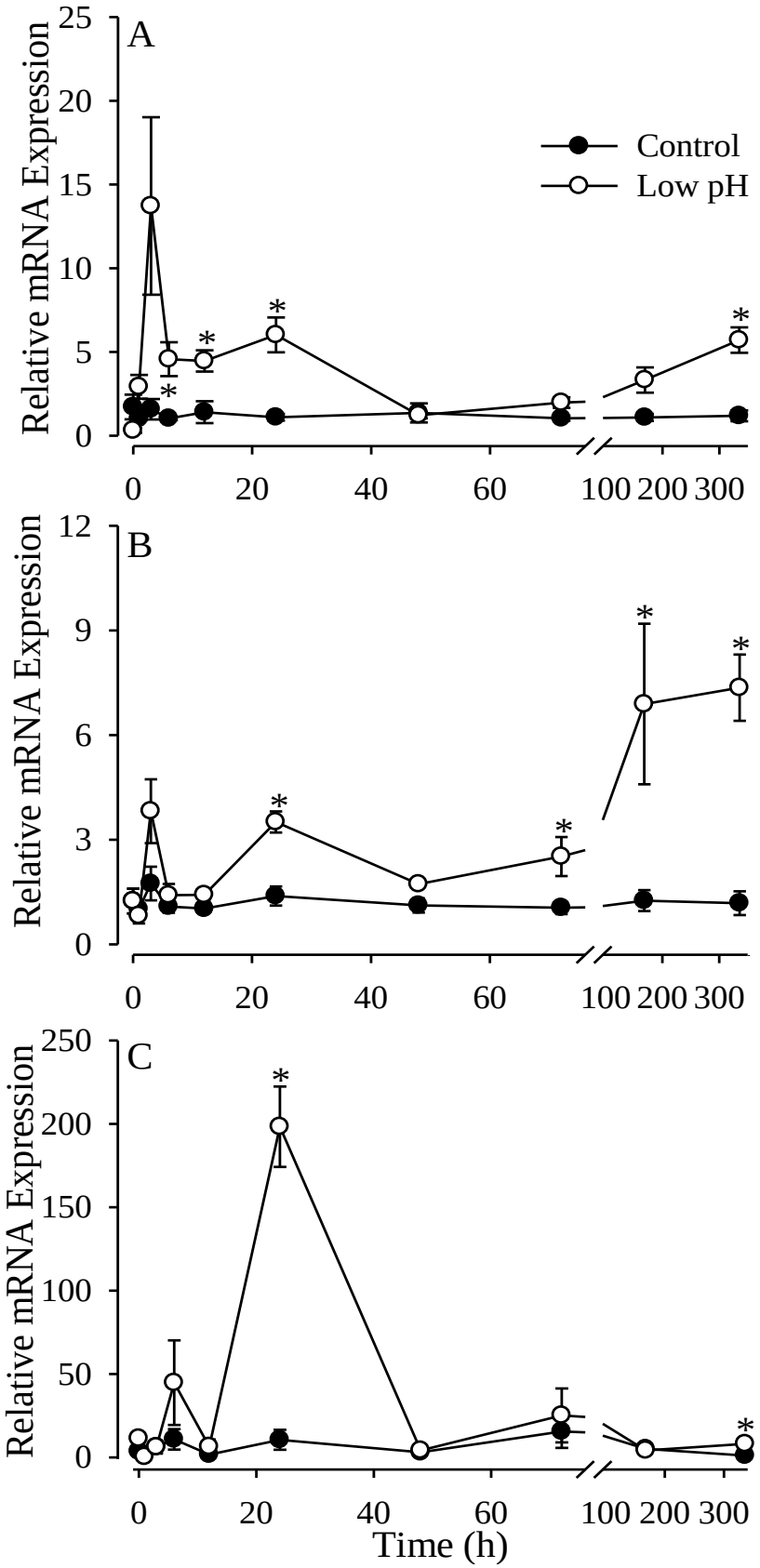
A
B
C
Control
Low pH
Relative mRNA Expression
Relative mRNA Expression
Relative mRNA Expression
Time (h)
0
20
40
60
100
200
300
0
5
10
15
20
25
0
3
6
9
12
0
50
100
150
200
250
*

Figure 2.4. Effect of low-pH exposure on Na^+ efflux and PEG uptake rate. Changes in the unidirectional Na^+ efflux rate (N = 6 - 12; Fig.2.4A), PEG uptake rate (N = 6; Fig.2.4B) and the correlation between Na^+ efflux and PEG uptake (Fig 2.4C) in zebrafish (*Danio rerio*) exposed to control (filled circles) or low pH (3.8 - 4.0) conditions (unfilled circles) for 2 weeks. Both Na^+ efflux and PEG uptake rates remained significantly elevated throughout the 2-week experimental period, with a transient respite between 12 and 72 h. Asterisks denote significant differences in Na^+ efflux or PEG uptake between control and acid treated fish. Data are presented as means ± SEM. For Fig. 2.4C, the linear correlation ($R^2 = 0.49$, $P < 0.05$) was obtained by plotting the mean data (without the standard errors) from Figs. 2.4A and B.

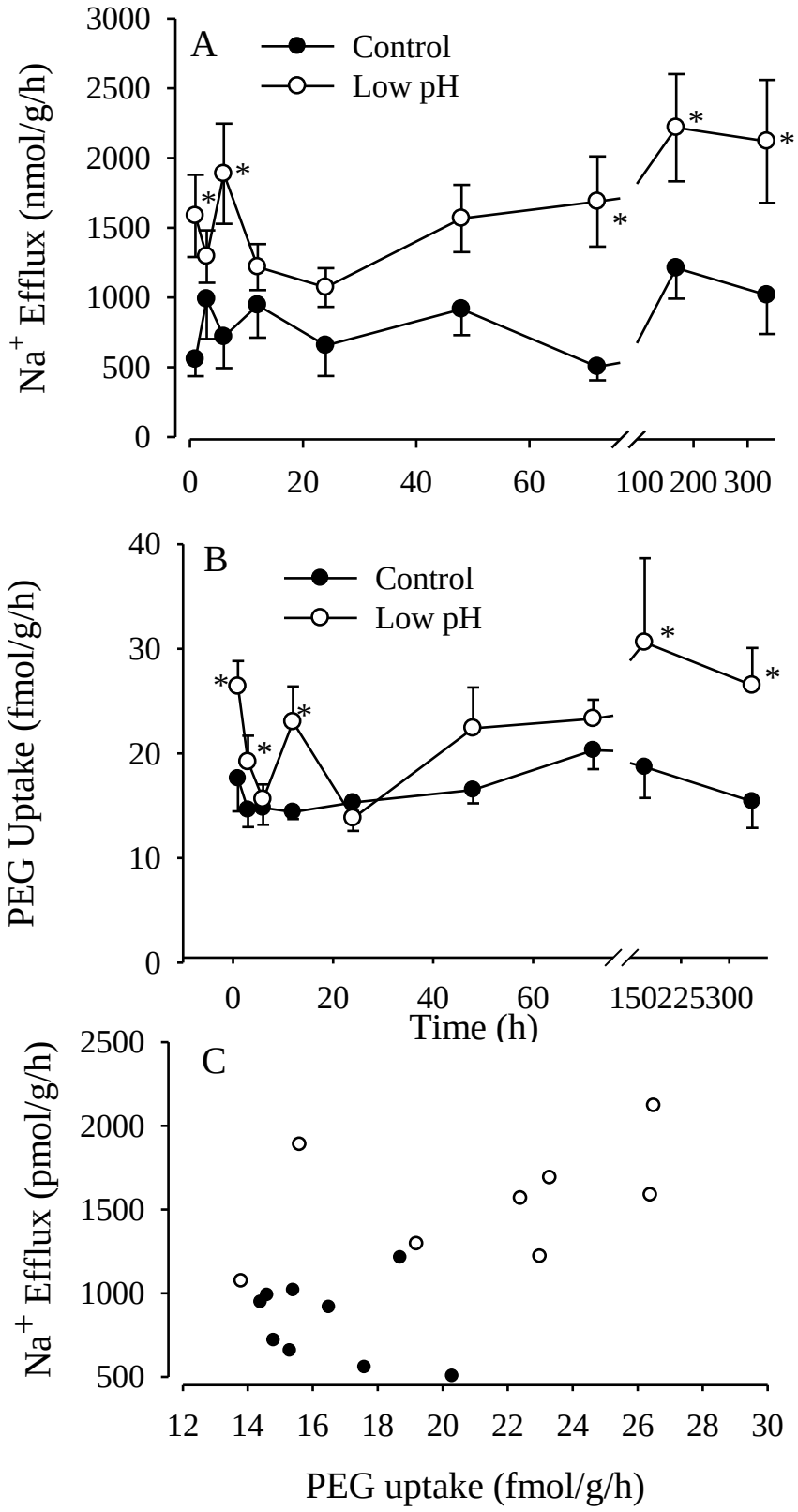
A
Control
Low pH
Na^+ Efflux (nmol/g/h)
3000
2500
2000
1500
1000
500
0
0
20
40
60
100
200
300
B
Control
Low pH
PEG Uptake (fmol/g/h)
40
30
20
10
0
0
20
40
60
150
225
300
Time (h)
C
Na^+ Efflux (pmol/g/h)
2500
2000
1500
1000
500
12
14
16
18
20
22
24
26
28
30
PEG uptake (fmol/g/h)

Figure 2.5. The pH-dependent effect of [Ca^{2+}] removal on the rate of Na^+ efflux rate in adult zebrafish (*Danio rerio)*. Removal of Ca^{2+} did not affect Na^+ efflux when measured in control pH water (N = 5 - 6; Fig 2.5A) but significantly increased Na^+ efflux when measured in low pH water (N = 5 - 7; Fig. 2.5B). In addition, 2-week acclimation to acid water (low pH 2 wks group) or normal pH (control 2wks group) water did not reduce increase in Na^+ efflux following exposure to Ca^{2+}-depleted low pH water (N = 5 - 7; Fig. 2.6B). Different letters denote significant differences in the rate of Na^+ efflux among the treatment groups. Data are presented as means ± SEM.

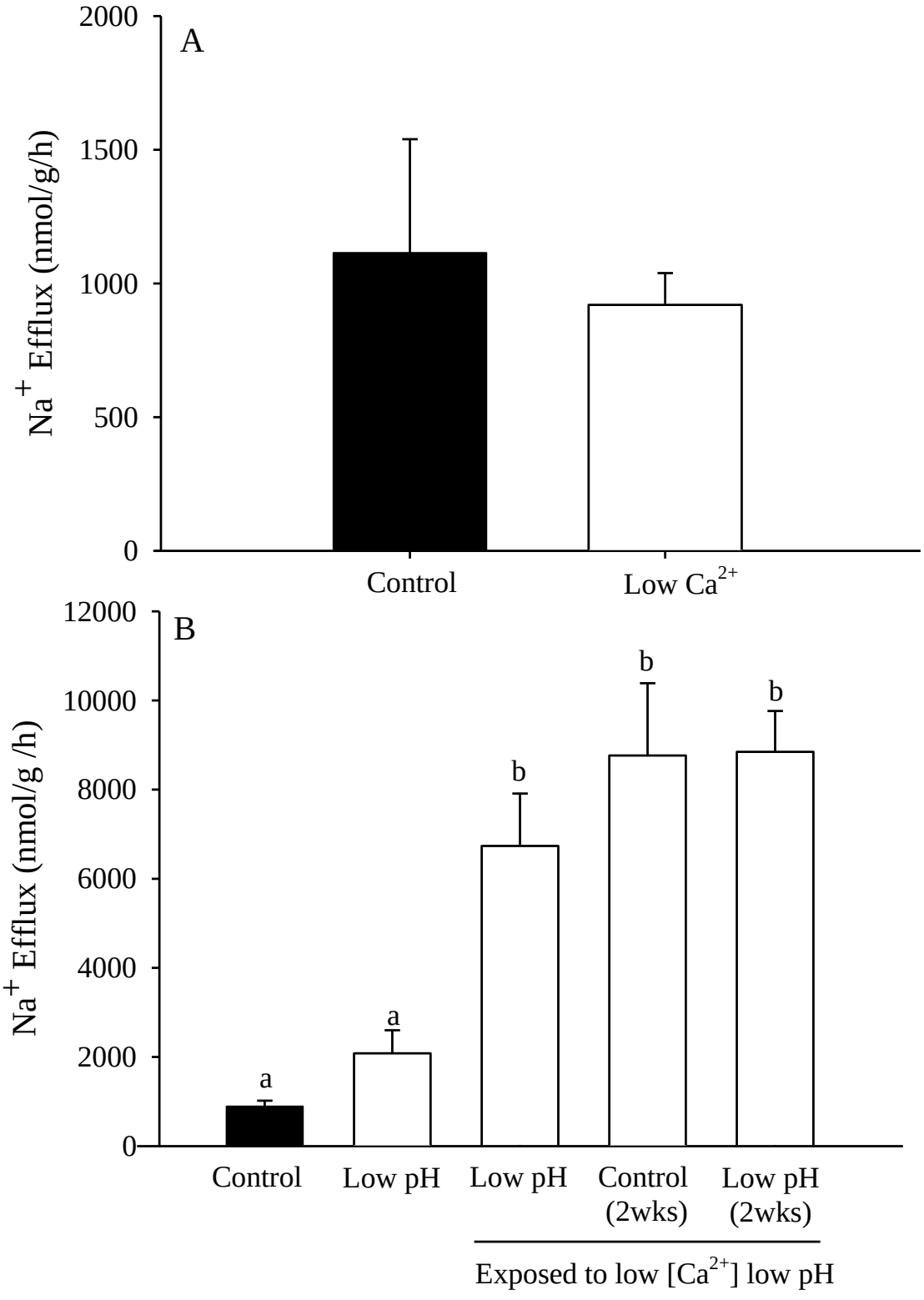
A
2000
1500
1000
500
0
Na+ Efflux (nmol/g/h)
Control
Low Ca2+
B
12000
10000
8000
6000
4000
2000
0
Na+ Efflux (nmol/g /h)
a
a
b
b
b
Control
Low pH
Low pH
Control (2wks)
Low pH (2wks)
Exposed to low [Ca2+] low pH

Figure 2.6. The effect of 2-week acclimation to low pH water on Na^+ efflux rate during recovery in circumneutral pH water. N = 6 – 11. Different letters denote significant differences in the rate of Na^+ efflux among the treatment groups. Data are presented as means ± SEM.

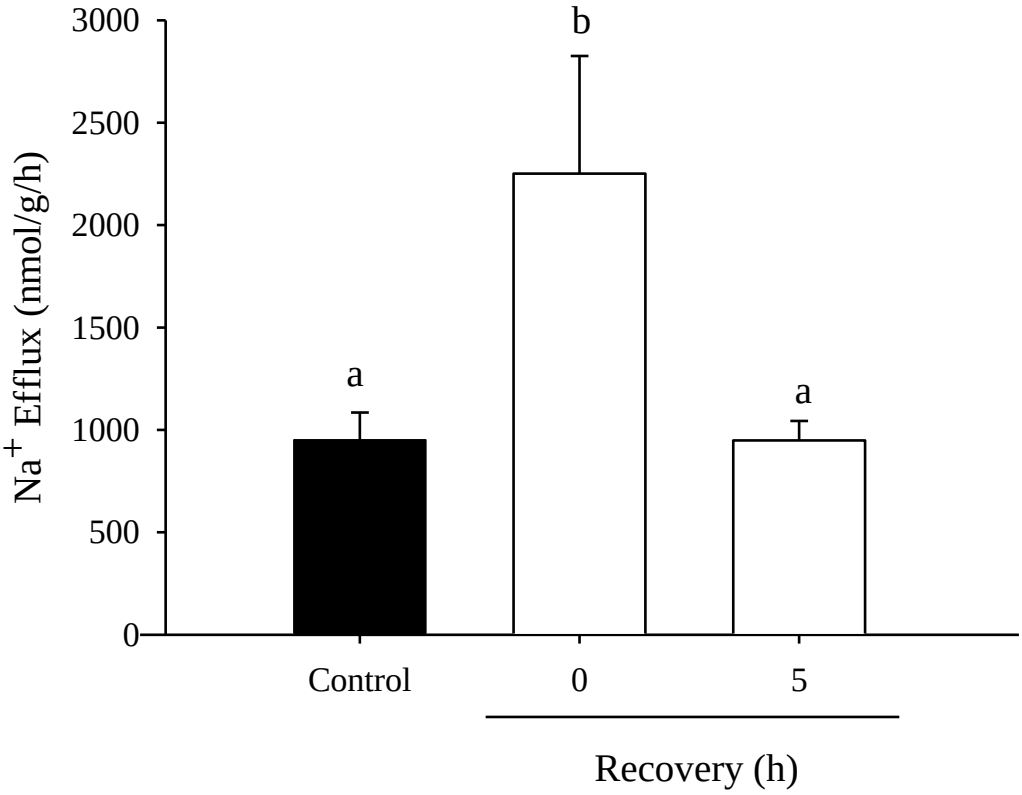
3000
2500
2000
1500
1000
500
0
Na+ Efflux (nmol/g/h)
a
b
a
Control
0
5
Recovery (h)

Figure 2.7. The effect of exposure to low pH water (pH 3.8 - 4.0) on the rate of Na^+ uptake in adult zebrafish (*Danio rerio*). N = 6 - 18. Different letters represent significant differences in Na^+ uptake rates within control (filled circles) or low pH water acclimated (unfilled circles) groups and asterisks denote significant differences between control and acid-treated groups. Data are presented as means ± SEM.

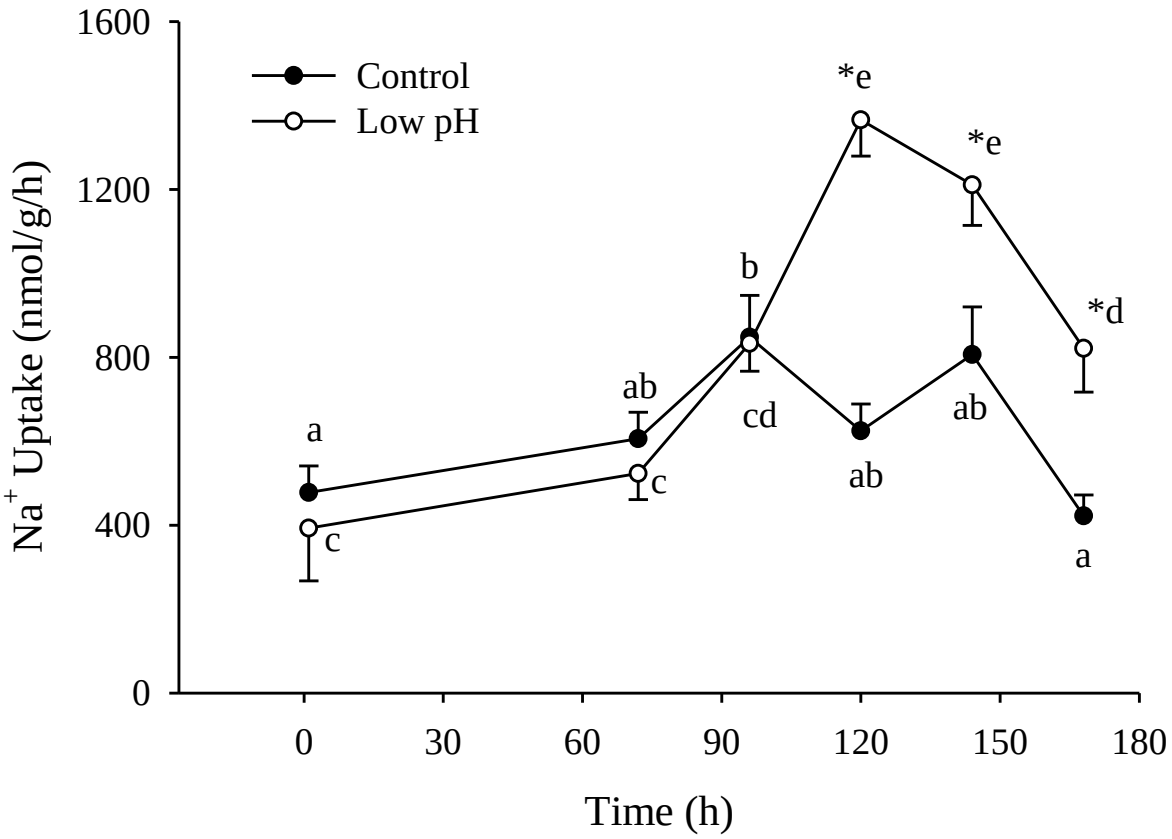
1600
1200
800
400
0
Control
Low pH
Na+ Uptake (nmol/g/h)
0
30
60
90
120
150
180
Time (h)
a
ab
b
*e
*e
*d
c
c
cd
ab
ab
a

Figure 2.8. The effect of 5-day exposure to low pH water (pH 3.8-4.0) on Na^+ uptake kinetics by adult zebrafish (*Danio rerio*). N = 3 – 7 for all data points. One-site saturation kinetic curve was fitted using SigmaPlot. For control group (filled circles), $J_{MAX}Na^+$ was 835 ± 78 µmol/g/h and affinity constant (K_M) was 112 ± 58 µM. In acid-exposed group (unfilled circles), $J_{MAX}Na^+$ was 1488 ± 178 µmol/g/h and K_M was 703 ± 260 µM. Both $J_{MAX}Na^+$ and K_M were significantly elevated in low pH-exposed group than in control group. Data are presented as means ± SEM.

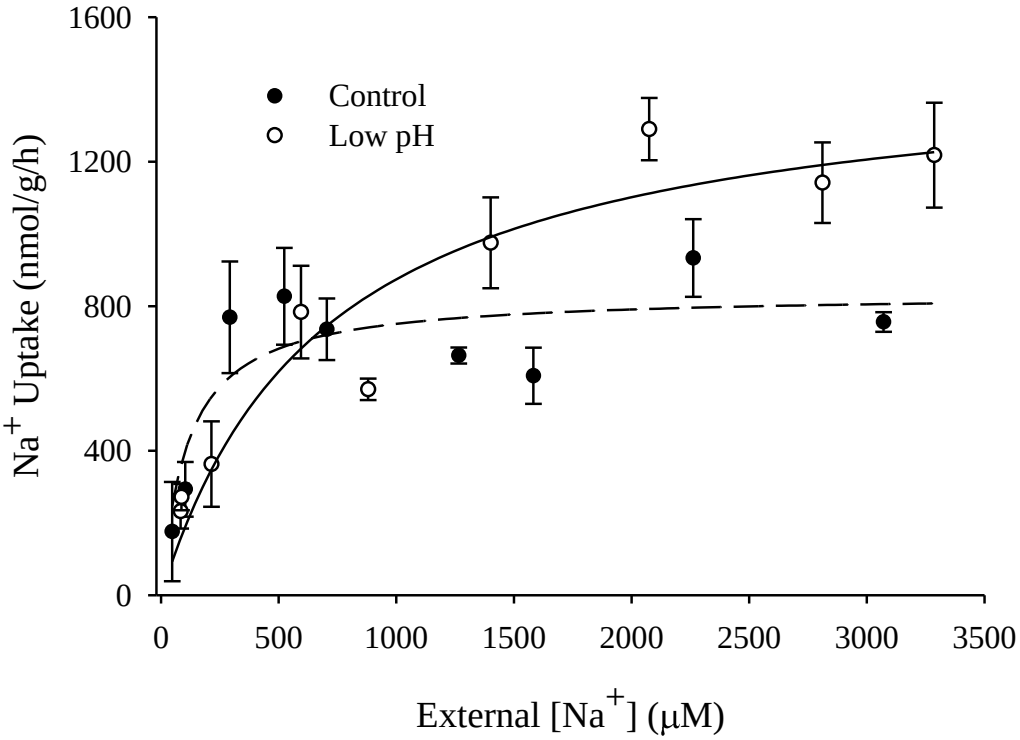
1600
1200
800
400
0
Control
Low pH
Na^+ Uptake (nmol/g/h)
0
500
1000
1500
2000
2500
3000
3500
External [Na^+] (μM)

Table 1.1: List of primers for RT-qPCR.

Gene	Product Length (bp)	Primer Sequence
Claudin A	141	FWD: 5'-TAA TTG CCC TCC ACA AGA CC-3'
		REV: 5'-AAA GCT GTC CAG CAT CCA AT-3'
Claudin B	148	FWD: 5'-AGA CAG CGG AAA ATA CAC AGC-3'
		REV: 5'-TGA GCC TCA ATG TCC AAC AA-3'
Claudin C	130	FWD: 5'-GTA CCC TCC GCA AAG TCG TA-3'
		REV: 5'-CTT TCA AGG AAA GAC TGA CAG C-3'
Claudin D	109	FWD: 5'-GGG TCG CGC TTA TTC TGT TA -3'
		REV: 5'-TTC CTA CAC AAA CAG GAG ACG AT-3'
Claudin E	145	FWD: 5'-CTG CCC TCC AAA AGA TGA AA-3'
		REV: 5'-TGG CAA GTC TTA GAA CGA AAG A-3'
Claudin F	145	FWD: 5'-CCG TAT CAT CCT CCT GTT GC-3'
		REV: 5'-TGT CTC CCC AAA AAC TCA AGA-3'
Claudin G	144	FWD: 5'-GGG TCG GTA TAC ACC AGC TT-3'
		REV: 5'-TCT GCT TTA CAA AGA CGA TCT CA-3'
Claudin H	108	FWD: 5'-GAA TGG GCT ATT CTG CTC CA-3'
		REV: 5'-TCA CCC TTT TCA TCC GTC TT-3'
Claudin I	130	FWD: 5'-GAG CCG CCA AAT ACT ACA GC-3'
		REV: 5'-TTC GCT ACC TTA GAC GGG TTA-3'
Claudin J	126	FWD: 5'-TCT GGC ACT CAC AGT GGC TA-3'
		REV: 5'-CAG CCC ATA TGA ATA GTT TAC CC-3'
Claudin 2	137	FWD: 5'-GCC CAG GAT TCA AGG AAA AT-3'
		REV: 5'-GAG CCT TTC AGC AAT CCA AG-3'
Claudin 7	122	FWD: 5'-CTT GCT CAA AGG GTC AGT CA -3'
		REV: 5'-GTC CTT TCC AGC TCG TGA AC -3'
Claudin 8	113	FWD: 5'-CGT TCA TAC AGC CCT CTC GT-3'
		REV: 5'-CAC ACA AAC ATG CTT GCA CA-3'
Claudin 11	136	FWD: 5'-CCA CGA TGG AGT TAC CAG CTA-3'

		REV: 5’-TGT GTC TGT GTG AGT TTG AGT GTT-3’
Claudin 12	133	FWD: 5’-TGC TCC ATC ACA AAG CTA CG -3’
		REV: 5’-TGT GTC TGT GTG AGT TTG AGT GTT-3’
Claudin 19	146	FWD: 5’-GAA AAA CCC TCG TAT CCT CCA-3’
		REV: 5’-ATC AGG AGA ACA GGC GAA GA-3’
Occludin A	107	FWD: 5’-GGG TCT GCT GGC TGA CTA TC-3’
		REV: 5’-GAA TCT CCA CGG GAC TTT CA-3’
Occludin B	113	FWD: 5’-GAC CAT TAA GGA TGG CCT CA-3’
		REV: 5’-GCT GAG CAG CAC TGA CTT TG-3’
18S	117	FWD: 5’-GGC GGC GTT ATT CCC ATG ACC-3’
		REV: 5’-GGT GGT GCC CTT CCG TCA ATT C-3’

Table 2.1: Changes in TJ mRNA expression levels. NS: no significant change, +; significant increase compared to control level, -; significant decrease compared to the control level.

	Exposure to acidic water (h)									
	0	1	3	6	12	24	48	72	168	336
Claudin A	NS	NS	NS	NS	NS	+	NS	NS	NS	+
Claudin B	NS	NS	+	+	+	+	NS	+	+	+
Claudin C	NS	NS	+	+	+	NS	NS	NS	+	+
Claudin D	NS	NS	NS	NS	NS	+	NS	NS	+	+
Claudin E	NS	NS	NS	+	+	+	NS	NS	NS	+
Claudin F	NS	NS	NS	NS	NS	+	NS	NS	NS	+
Claudin G	NS	NS	NS	+	+	+	-	-	NS	NS
Claudin H	NS	NS	NS	NS	NS	+	NS	+	+	+
Claudin I	NS	NS	+	NS	NS	+	-	NS	NS	NS
Claudin J	NS	NS	NS	NS	+	NS	NS	NS	NS	NS
Claudin 2	NS	NS	NS	NS	NS	NS	-	NS	NS	NS
Claudin 7	NS	NS	+	+	NS	+	NS	+	NS	+
Claudin 8	NS	NS	NS	NS	NS	NS	NS	-	NS	NS
Claudin 11	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Claudin 12	NS	NS	+	NS	NS	NS	NS	NS	NS	+
Claudin 19	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Occludin A	NS	NS	+	NS	NS	+	NS	NS	NS	+
Occludin B	NS	+	NS	NS	NS	+	NS	NS	NS	+

Discussion

The objective of the present study was to investigate how zebrafish respond to exposure to acidic water and the consequent reduction in the whole body Na^+ content. The novel findings presented here are that 1) exposure to acidic water significantly disturbed whole body Na^+ balance, but that it was restored within the 2-week experimental period; 2) exposure to acidic water induced complex and time-dependent changes in mRNA levels of TJ isoforms in the gill; 3) despite the significant changes in mRNA levels of 16 TJ isoforms, Na^+ efflux, elevated immediately by exposure to acidic water, was not strongly regulated during the ensuing 2-weeks of exposure; 4) PEG influx also was increased during acid-water exposure and there was a significant correlation between PEG uptake and Na^+ efflux across the range of PEG fluxes measured in control and acid-exposed fish, and finally; 5) adult zebrafish were capable of defending (and even augmenting) Na^+ uptake during exposure to acidic water, which may be the major mechanism of regulating whole body Na^+ levels.

On the method to measure PEG uptake rate

Radiolabeled PEG has been used as a marker of paracellular permeability in both *in vivo* and *in vitro* studies (Chasiotis et al., 2010; Curtis and Wood, 1991; Kim, 1996; Mitrovic and Perry, 2009; Wood and Pärt, 1997). Conventionally, for *in vivo* studies, PEG is injected into the fish and after equilibration the rate of appearance in the water is measured as an indicator of epithelial paracellular permeability, assuming that the large size of PEG molecules restricts their transcellular movement. However, to accurately assess epithelial paracellular permeability using this method it is imperative to prevent the entry of PEG into the medium via the urine because a previous study in rainbow trout, *Oncorhynchus mykiss* showed that 80-90% of PEG could originate via urinary excretion (Curtis and Wood, 1991). Because the small size of zebrafish (~0.5 g) prevented cannulation of the urinary bladder or sealing the vent (Mitrovic and Perry,

2009) (without significantly stressing the fish), PEG was added to the external medium in this study and the rate of PEG entry into the fish was assessed as an indicator of epithelial paracellular permeability. The underlying assumption is that the paracellular movement of PEG is strictly dependent on the concentration gradient of the solute and independent of directionality. Indeed, in preliminary experiments, there was a significant positive correlation between the external PEG concentration and the rate of PEG influx ($N = 54$, Pearson's $r = 0.413$, $p < 0.01$; data not shown). Given that the entry of PEG into the fish via drinking is likely to be negligible in adult FW fish and the likely absence of a PEG-specific transcellular transporter, this observation suggests that PEG influx is likely to be a reasonable surrogate measurement of paracellular permeability in the efflux direction.

Environmental effects on mRNA expression of tight junction proteins

For the present study, the tissue distribution patterns as well as the change in mRNA expression levels of 18 TJ proteins (16 claudins and 2 occludins) was analyzed. The selection of these isoforms was based on the number of annotated TJ protein in the zebrafish genome at the time this study was initiated. Currently (June 2013), ZFIN (http://zfin.org/cgi-bin/webdriver?MIval=aa-ZDB_home.apg) lists 31 claudin isoforms from the zebrafish genome. Given the presence of more than 50 claudin isoforms in the *F. rubripes* genome (Loh et al., 2004), additional claudin isoforms are likely to be discovered in zebrafish.

Generally, TJ proteins (Fig. 2.2) exhibited tissue specific distribution patterns although some isoforms such as claudin-2, -7, -11 and 12 were expressed ubiquitously (at least among the tissues examined in this study). On the other hand, claudin-J was expressed at low levels in the tissues investigated in the present study. Although translational knockdown of claudin-J using antisense morpholinos has been shown to lead to defects in otolith development in larval

zebrafish (Hardison et al., 2005), its function in adult stage zebrafish remains to be elucidated. Recently, (Clelland and Kelly, 2010) reported a distribution pattern of 20 isoforms of TJ proteins in zebrafish tissues. Although there is agreement in the expression patterns of several of the isoforms between the two studies, there are also some marked differences. For example, (Clelland and Kelly, 2010) failed to detect claudin-7 mRNA in brain, eye, gill, gut, liver, kidney and muscle, whereas in this study claudin-7 was detected in all of these organs (Fig. 2.2). The reason for the observed difference is unknown.

Two week exposure to acidic water led to dynamic changes in levels of most (14 out of 16) claudin genes, and in most cases the changes were the result of *increasing* mRNA levels (Table 2.2). However, a reduction of TJ mRNA levels was observed at 48- and 72-h of exposure. Of the four genes that were reduced, claudin-2 is orthologous to mammalian claudin-2, which increases the paracellular permeability to cations (Amasheh et al., 2002). Thus, reduced expression of mRNA for claudin-2 (if manifested at the protein level), would be an appropriate response to limit Na^+ loss during exposure to acid water. On the other hand, based on the orthology analysis by Ensemble (http://uswest.ensembl.org/Danio_rerio/Info/Index), the remaining three genes that were reduced, claudin-G, -I, -8, appear to be orthologous to mammalian claudin-4 and -8, which contribute to reducing paracellular permeability (Amasheh et al., 2009; Van Itallie et al., 2003). Obviously, reduced mRNA expression of claudin-G, -I, and -8, thought to be relatively impermeable to cations, is counterintuitive when fish are losing excess Na^+. However, as posttranslational modifications of claudins, such as phosphorylation, palmitoylation and glycosylation (for review, see Van Itallie and Anderson, 2006) may also occur, observed reductions in the mRNA level might not necessarily indicate a corresponding reduction in the level of functional protein. Although some claudin antibodies raised against

mammalian isoforms are available and have been used in fish (Tipsmark et al., 2008a), such antibodies could recognize multiple claudin isoforms of zebrafish and thus were not used in the present study. Western blot analysis using homologous isoform-specific antibodies (currently unavailable) will be useful in addressing this issue. Another point to consider is that a given claudin isoform does not interact with all other claudin isoforms equally. For example, (Furuse et al., 1999) reported that claudin-1 failed to interact with claudin-2, whereas claudin- 3 interacted with both claudin-1 and -2. Thus, it is possible that the changes in mRNA levels (and presumably protein) levels occurring during the first 24 h of exposure to acidic water (Table 2.2) might have changed the relative composition of claudins in TJs, possibly increasing claudin isoforms with which claudin-G, -I and -8 could not interact with. Regardless, the present study provides the first evidence that exposure to an acidic condition strongly influences the transcription level of TJ proteins in zebrafish gill. Although no strong regulation of Na^+ efflux during the acid exposure was found (discussed below), it is possible that without such changes in mRNA expression level of these isoforms, Na^+ efflux during the acid exposure would have been even more severe, as seen when the fish were exposed to acidic, low [Ca^{2+}] water (Fig. 2.5B). Assessing the physiological consequences of functional knockdown of selected TJ isoforms would provide useful information to address this issue.

Na^+ loss and changes in permeability in fish exposed to acidic water

Previous studies on physiological responses of FW fish to acid water exposure showed a significant increase in Na^+ loss and consequent reduction in plasma [Na^+] in species such as rainbow trout (McDonald et al., 1980; McDonald and Wood, 1981) and common shiner, *Notropis cornutus* (Freda and McDonald, 1988), although species that are native to acidic water,

such as yellow perch, *Perca flavescens* (Freda and McDonald, 1988) and banded sunfish, *Enneacanthus obesus* (Gonzalez and Dunson, 1987) control their Na^+ efflux rate in acidic water.

Unlike *P. flavescens* and *E. obesus*, where Na^+ efflux rate was not severely affected by exposure to acidic water, zebrafish exhibited a significant increase in Na^+ efflux during the acid exposure, although there was a transient respite between 12 and 72 h (Fig.2.4A). Given the considerable change in transcription levels of TJ isoforms that occurred within the first 24 h exposure, it is possible that this transient recovery in Na^+ loss was the result of modification in TJs, such as changes in their overall depth or in composition of claudin isoforms constituting TJ strands. Regardless of the physiological basis for the transient recovery in Na^+ uptake, it obviously could not be maintained during prolonged exposure to acidic conditions (Fig. 2.4A). Thus, the eventual restoration of whole body Na^+ balance presumably was related to increased Na^+ uptake (discussed below).

The decrease in Na^+ efflux during the 5-h recovery in circumneutral pH water following the two-week acclimation to acidic medium (Fig. 2.6) suggests rapid regulation of TJ permeability which is consistent with the dynamic nature of TJ regulation (Shen et al., 2008). It remains to be determined whether the reduction in Na^+ efflux during recovery was due to the rapid biogenesis of new TJ strands, or re-arrangement of TJ protein isoforms within the pre-existing strands. The ultrastructural observation of TJ strands using electron microscopy during acid exposure and recovery should provide interesting information on this issue.

In agreement with the Na^+ efflux data, changes in paracellular permeability as estimated from measurements of PEG influx, also showed a significant and prolonged increase during exposure to acidic water (Fig. 2.4B). The parallel increases in Na^+ efflux and PEG influx (Fig. 2.4C) suggest that the majority of Na^+ efflux occurs via a common paracellular pathway, at least

in zebrafish exposed to acidic conditions, which contrasts with the recent proposition of (Wood et al., 2009) of a transcellular route for Na^+ efflux in the Amazonian oscar (*Astronotus ocellatus*). The argument that Na^+ moves through a paracellular pathway in acidic water is in agreement with the damage to overall TJ integrity seen in the gills of acid-exposed tadpoles (Meyer et al., 2010). At the same time, when fish are not in acidic water, transcellular efflux of Na^+, as suggested by (Wood et al., 2009) is theoretically feasible. Assuming external $[Na^+]$ = 800 µM, transmembrane potential = -40 mV, the Nernst equilibrium would be reached with ~ 4 mM $[Na_i]$, which would be considerably lower than previously reported $[Na_i]$ of FW fish gill, as (Morgan et al., 1994) reported $[Na_i]$ between 62 - 66 mM in brown trout, *Salmo trutta* gill cells. Although the technique used by (Morgan et al., 1994) did not distinguish between bound and free Na^+, which contributes to the ion activity and transmembrane potential, and no information on $[Na_i]$ is available for zebrafish gill cells, $[Na_i]$ of gill cells in other fish species suggests transcellular Na^+ efflux, at least in normal pH water, is a possibility. Demonstration of transcellular and/or paracellular effluxes of ions from FW fish, quantifications of the proportion of those two routes contributing to the overall ion loss and characterizing environmental effects on these proportions are interesting avenues for future research.

Interaction between Ca^{2+} and pH on Na^+ efflux.

The biogenesis and maintenance of TJs is critically dependent on both extracellular and intracellular $[Ca^{2+}]$ (Gonzalez-Mariscal et al., 1990; Stuart et al., 1994). Previous studies with goldfish *C. auratus* (Eddy, 1975) and brown trout, *S. trutta* (McWilliams, 1982) also showed that a reduction in ambient $[Ca^{2+}]$ leads to a significant increase in Na^+ efflux in circumneutral as well as acidic water, although some fish species, such as yellow perch, *P. flavescens*, are capable of defending Na^+ efflux even in acidic water with extremely low ambient $[Ca^{2+}]$ (Freda and

McDonald, 1988). In the current study, removal of external [Ca^{2+}] had markedly different consequences on Na^+ efflux depending on water pH. Specifically, removal of [Ca^{2+}] did not have any impact on Na^+ efflux in normal pH water, but removal of [Ca^{2+}] significantly increased Na^+ efflux in acidic water. If the removal of extracellular Ca^{2+} disrupts the structure and maintenance of TJs (Gonzalez-Mariscal et al., 1990; Stuart et al., 1994), the observed effect of Ca^{2+} removal in acidic condition is consistent with the notion (discussed above) that paracellular flux of Na^+ mediated by TJs is playing a more dominant role in determining total Na^+ efflux in acidic water. However, as the removal of external Ca^{2+} was shown to induce Na^+ loss even in circumneutral pH in other FW species (Eddy, 1975), it is possible that significance of the paracellular Na^+ efflux might be species-dependent.

The original intention was to determine whether a 2-week period of exposure to low pH and the associated changes in TJ isoform expression would confer any advantage (with respect to Na^+ efflux) to zebrafish acutely exposed to a reduction of ambient [Ca^{2+}]. Clearly, this was not the case because prior exposure did not attenuate the massive loss of Na^+ associated with lowered ambient Ca^{2+} at low pH. Thus, it would appear that the complex changes in TJ isoform expression occurring during the two-week period of exposure to acidic water did not confer any resistance to Ca^{2+} withdrawal.

Increased Na^+ uptake in acidic conditions: acclimation to acidic conditions?

Perhaps the most surprising finding of the present study was the marked increase in Na^+ uptake measured after 5 days of exposure to acidic conditions (Fig. 2.7). Na^+ uptake was measured during exposure to acidic water for one week rather than the usual 2-week period because given that whole body Na^+ balance was restored between 3 and 7 days of exposure to acidic water (Fig 2.1) without sustained regulation of Na^+ efflux (Fig. 2.4A), it was reasoned that

Na^+ uptake must be increased during this period. The observed increase in Na^+ uptake (Fig. 2.7) supports this prediction. It is worth noting that Na^+ uptake rate under the control condition (around 500 nmol/g/h) was considerably lower than the efflux rate observed in this study (around 1000 nmol/g/h, Fig. 2.4A). Although such difference seems contradictory to the relatively stable whole body Na^+ balance (Fig. 2.1), it is possible that due to the stress from injection of radioisotope, Na^+ efflux was elevated in comparison to the handling stress. Similar increase in ion loss in comparison to uptake (and thus overall net loss) was seen in previous study, such as (Mitrovic et al., 2009), where goldfish were injected with $^{36-}$Cl.

The complete lack of inhibition of Na^+ uptake following transfer to low ambient pH is in stark contrast to the prolonged and severe inhibition of Na^+ uptake observed when rainbow trout are exposed to acidic conditions (McDonald, 1983b), and raises the question about the nature of the Na^+ uptake mechanism operating in the zebrafish gill. As an initial step to determine the nature of the mechanism, Na^+ uptake kinetic parameters were estimated using zebrafish acclimated to control or acidic water for 5 days, as the largest increase in Na^+ uptake was observed at this time point (Fig. 2.8). Although the significant increase in J_{MAX} following the transfer to acidic water was expected, the analysis also revealed a significant increase in K_M, which suggests reduced affinity of the Na^+ uptake mechanism. It is possible that this increase in K_M is the result of a switch in Na^+ uptake mechanism between neutral and acidic water. One possibility is an increased reliance on NHE (Na^+/H^+ exchanger) in the acidic water, as the K_M of Na^+ transport by mammalian NHE can be as high as 4.7 - 10 mM (Orlowski, 1993). Supporting the possible role of NHE in Na^+ uptake in acidic water, (Hirata et al., 2003) demonstrated a significant increase in NHE3 mRNA expression in the gill of Osorezan dace, *Tribolodon hakonensis*, following their exposure to extremely acidic water (pH 3.5). On the other hand,

currently there is no clear agreement on whether NHEs are involved in Na^+ uptake by zebrafish or in any FW fish (Evans, 2008). For example, (Boisen et al., 2003) demonstrated that treatment with 5-(N-Ethyl-N-isopropyl)-amiloride (EIPA), a specific NHE inhibitor, did not inhibit Na^+ uptake in adult zebrafish, whereas (Esaki et al., 2007) demonstrated that EIPA strongly inhibited Na^+ uptake in zebrafish larvae. In support of the role of NHE in Na^+ uptake by zebrafish in acidic medium, (Flynt et al., 2009) reported that knocking down miRNA8 reduced Na^+ uptake in zebrafish larvae reared in acidic medium (pH 5.0) and suggested this could be due to the over expression of NHE regulatory factor 1 (NHERF1), which is responsible for internalizing NHE3 in zebrafish. On the other hand, (Yan et al., 2007) reported significant reduction in mRNA level of NHE3b, one of NHE isoforms that could be responsible for Na^+ uptake, in adult zebrafish gill following 2-week acclimation to acidic water (pH 4.0), although other isoforms, such as NHE2, might be responsible for Na^+ uptake (Craig et al., 2007). Another issue, as recently reviewed by (Parks et al., 2008), is that in acidic FW water where external [Na^+] is low and [H^+] is high, thermodynamics dictates the direction of Na^+ transport mediated by NHE would be in the efflux rather than the influx direction. Although the kinetic data reported in the present study and (Hirata et al., 2003) suggests the possible role of NHE in Na^+ uptake by zebrafish in acidic water, direct evidence would be required to demonstrate its function, and certainly it would be necessary to address how NHE could function in the face of the proposed thermodynamic constraints. Research is currently underway in our laboratory to address this question.

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CHAPTER 3
Ammonia excretion via Rhcg1 facilitates Na^+ uptake in larval zebrafish, *Danio rerio*, in acidic water

Notes on Chapter

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YK conceived the project and did all experiments under the supervision of SFP. The manuscript was drafted by YK and edited by SFP.

Abstract

The involvement of a Na^+/H^+ exchanger (NHE) in mediating Na^+ uptake by freshwater (FW) fish is currently debated. Although supported indirectly by empirical molecular and pharmacological data, theoretically its operation should be constrained thermodynamically owing to unfavourable chemical gradients. Recently, there has been an increasing focus on ammonia channels (Rh proteins) as potentially contributing to Na^+ uptake across the FW fish gill. In this study, the hypothesis that Rhcg1, a specific apical isoform of Rh protein, is critically important in facilitating Na^+ uptake in zebrafish larvae via its interaction with NHE, was investigated. Treating larvae (4 day post fertilization; dpf) with 5-(N-Ethyl-N-isopropyl)-amiloride (EIPA, an inhibitor of NHE), caused a significant reduction in Na^+ uptake in fish reared in acidic water (pH ~4.0). A role for NHE in Na^+ uptake was further confirmed by translational knockdown of NHE3b, an isoform of NHE thought to be responsible for Na^+/H^+ exchange in zebrafish larvae. Exposing the larvae reared in acidic water to 5 mM external ammonium sulfate or increasing the buffering capacity of the water with 10 mM HEPES caused concurrent reductions in ammonia excretion and Na^+ uptake. Furthermore, translational knockdown of Rhcg1 significantly reduced ammonia excretion and Na^+ uptake in larvae chronically (4 days) or acutely (24 h) exposed to acidic water. Unlike in sham-injected larvae, EIPA did not affect Na^+ uptake in fish experiencing Rhcg1 knockdown. Additionally, exposure of larvae to bafilomycin A1 (an inhibitor of H^+-ATPase) significantly reduced Na^+ uptake in fish reared in acidic water. These observations suggest the existence of multiple mechanisms of Na^+ uptake in larval zebrafish in acidic water; one in which Na^+ uptake via NHE3b is linked to ammonia excretion via Rhcg1 and another facilitated by H^+-ATPase.

Introduction

Mechanisms of Na^+ uptake by freshwater (FW) organisms have received considerable attention over the past 75 years. Krogh (1938) initially proposed the link between Na^+ uptake and ammonia excretion, though it was also implied that Na^+ is not directly exchanged with NH_4^+. The results of subsequent studies have either supported this original idea about the link between Na uptake and ammonia excretion (Maetz and Garcia Romeu, 1964) or provided evidence that Na^+ is exchanged with H^+, rather than NH_4^+ (Kerstetter et al., 1970). Most current models of Na^+ uptake across the FW fish gill incorporate a flexible electroneutral exchanger that can exchange H^+ and/or $NH4^+$ for Na^+ (Evans, 2008). In a pivotal publication, (Avella and Bornancin, 1989) challenged the model of Na^+ uptake via electroneutral Na^+-H^+ exchange because of thermodynamic constraints and instead suggested that the uptake of Na^+ occurs through a Na^+ channel that is indirectly coupled to the active excretion of H^+ via a H^+-ATPase. They argued effectively that an unfavourable chemical gradient for Na^+ entry across the apical membrane of gill epithelial cells would not permit the operation of an electroneutral Na^+/H^+ exchanger in the dilute FW environment. Instead, it was proposed that a favourable electrochemical gradient for diffusive Na^+ entry across the apical membrane could be established by the development of a sufficiently negative membrane potential, in turn a consequence of outward H^+ pumping (Avella and Bornancin, 1989). Subsequent studies incorporating measurements of branchial H^+-ATPase activity (Lin and Randall, 1991) and protein/mRNA levels (Sullivan et al., 1995; Sullivan et al., 1996; Wilson et al., 2000) or employing pharmacological inhibitors (Fenwick et al., 1999; Reid et al., 2003) further strengthened the case for a role for H^+-ATPase in Na^+ uptake. However, the channel that conducts Na^+ in association with H^+-ATPase is still unknown in fish. Indeed, the most likely candidate, the epithelial Na^+

channel (ENaC) has yet to be found in any sequenced fish genome; clearly, the topic of Na^+ uptake in FW fish remains controversial (Evans, 2008).

Though challenged for theoretical reasons (Avella and Bornancin, 1989; Parks et al., 2008), there is abundant pharmacological and molecular evidence supporting a role for Na^+/H^+ exchangers (NHEs; members of the slc9a protein family) in Na^+ uptake by FW fish. For example, a significant increase in NHE3 mRNA expression level was detected in Osorezan dace (*Tribolodon hakonensis*) following a 7-day acclimation to extremely acidic conditions (pH 3.5) (Hirata et al., 2003). Similarly, significant increases in NHE2-like protein levels (Edwards et al., 2005) or NHE2 mRNA levels (Ivanis et al., 2008b) were reported during hypercapnic acidosis in FW-acclimated killifish (*Fundulus heteroclitus*) and rainbow trout (*Oncorhynchus mykiss*) gill, respectively. The mRNA level of NHE3b (Yan et al., 2007) and NHE2 (Craig et al., 2007) in zebrafish (*Danio rerio*) gill significantly increased after exposure to soft water for 1-2 weeks. Similarly, NHE2 mRNA expression in killifish gill transiently increased following transfer from seawater (SW) to FW (Scott et al., 2005b). In addition, using a homologous antibody, NHE2 was localized to the apical region of a sub-population of mitochondrion rich cells (MRCs) in rainbow trout gill (Ivanis et al., 2008b); similar apical localization of NHE3 and NHE3b was demonstrated for Osorezan dace (Hirata et al., 2003) and zebrafish (Yan et al., 2007) respectively. Furthermore, amiloride analogs (e.g. 5-(N-ethyl-N-isopropyl) amiloride; EIPA) with increased specificity for Na^+/H^+ exchangers, have been shown to reduce Na^+ uptake in goldfish (*Carassius auratus*; Preest et al., 2005) and zebrafish (Esaki et al., 2007); note however that in one study (Boisen et al., 2003), EIPA did not inhibit Na^+ uptake by adult zebrafish acclimated to soft or hard water. While these experimental results (with the exception of Boisen et al., 2003), support a role for Na^+/H^+ exchange in Na^+ uptake/acid excretion by FW fish, its

operation must abide by thermodynamic constraints, which at least macroscopically, would appear to be unfavourable (Parks et al., 2008). The basolateral excretion of Na^+ via Na^+-K^+-ATPase might be generating a microenvironment where $[Na^+]_i$ is kept sufficiently low to allow Na^+/H^+ exchange (Hirata et al., 2003; see also) (Perry and Gilmour, 2006); to date, however, no direct experimental demonstration of such a "microenvironment" is available. Furthermore, the relatively low expression of Na^+/K^+/ATPase was noted in H^+-ATPase rich cells (HRCs), a subset of ionocytes responsible for Na^+ uptake in zebrafish (Horng et al., 2007; Horng et al., 2009b; Lin et al., 2006; for review see : Hwang, 2009; Hwang and Perry, 2010), thereby making the Na^+-K^+-ATPase based microenvironment model less attractive.

The discovery of ammonia conducting Rh proteins in fish (Nakada et al., 2007b; for review see Weihrauch et al., 2009; Wright and Wood, 2009) has shed new light on the potential origin of a favourable gradient for Na^+/H^+ exchange across the FW fish gill apical membrane. Although controversies still remain as to whether Rh proteins conduct ammonia in the gaseous form as NH_3 (Mak et al., 2006) or the ionic form as NH_4^+ (Nakhoul et al., 2005), as well as the possibility of facilitating trans-membrane fluxes of other solutes such as CO_2 (Nawata and Wood, 2008; Perry et al., 2010a; Soupene et al., 2004), a recent study (Nawata et al., 2010) supports the role of fish Rh proteins as NH_3 carriers. Since the initial report of Rh proteins in pufferfish, *Takifugu rubripes* (Nakada et al., 2007b), gill, isoforms of Rh proteins have been detected in gills of both FW and SW species, including zebrafish (Braun et al., 2009a; Braun et al., 2009b; Nakada et al., 2007a; Shih et al., 2008), goldfish (Perry et al., 2010b), hagfish (*Eptatretus stoutii*) (Braun and Perry, 2010), mangrove killifish (*Kryptolebias marmoratus*) (Hung et al., 2007), rainbow trout (Nawata et al., 2007) toadfish (*Opsanus beta*) (Rodela et al 2012), midshipman (*Porichthys notatus*) (Bucking et al., 2013) and medaka (*Oryzias latipes*)

(Wu et al., 2010). Of particular interest is the Rhcg1 isoform, which plays a significant role in facilitating ammonia excretion in zebrafish larvae (Braun et al., 2009a; Shih et al., 2008) where it is co-localized with NHE3b in HRCs (Nakada et al., 2007a). Assuming that Rhcg1 conducts gaseous ammonia, and close proximity between Rhcg1 and NHE, Rhcg1 could potentially provide a gradient for H^+ to facilitate the operation of NHE through; 1) the donation of intracellular H^+ (localized acidification), as H^+ is removed from NH_4^+ as simultaneously generated NH_3 is conducted through Rhcg1 and 2) removal of external H^+ (localized alkalization), as the excreted NH_3 reacts with extracellular H^+ to form NH_4^+. Such an interaction between Rh proteins and Na^+ uptake (i.e. a functional "metabolon") was suggested in (Wright and Wood, 2009) with experimental evidence following soon thereafter (Tsui et al., 2009; Zimmer et al., 2010). Using medaka, Wu et al. (2010) demonstrated a strong correlation between ammonia excretion and Na^+ uptake as well as localized alkalization near the apical membrane of ionocytes, further implying the involvement of a metabolon.

A first objective of the present study was to test whether a functional metabolon linking Na^+ uptake to ammonia excretion facilitates the functioning of NHE (presumably NHE3b) (Yan et al., 2007) in zebrafish larvae. Specifically, translational knockdown of Rhcg1 was employed and its consequence on both ammonia excretion and Na^+ uptake in larval zebrafish was determined. In addition, control larvae and larvae experiencing Rhcg1 knockdown were treated with EIPA to further clarify the link between Rhcg1 and a functional NHE. Given the possibility of multiple Na^+ uptake mechanisms in FW fish, a second objective was to assess whether zebrafish employ other mechanism(s) of Na^+ uptake, more specifically the electrical coupling of H^+-ATPase and diffusive Na^+ entry as previously reported (Horng et al., 2007). A final objective was to directly implicate NHE in Na^+ uptake by using an antisense morpholino oligonucleotide

designed to splice out a functionally significant exon (exon 2). It was recently demonstrated that unlike in other species examined (McDonald and Wood, 1981), Na^+ uptake in zebrafish is significantly increased during exposure to acidic conditions and that this increased uptake is associated with a decreased affinity (increased K_M) for Na^+, indicative of NHE operation (Kumai et al., 2011). For these reasons, experiments were performed in water of circumneutral pH and in acidic water. The results demonstrated that Na^+ uptake via NHE3b in larval zebrafish exposed to acidic water is reliant on ammonia excretion by Rhcg1 whereas H^+-ATPase facilitates a portion of Na^+ uptake regardless of water pH.

Materials and Methods

Experimental animals and husbandry

Adult zebrafish (*Danio rerio* Hamilton-Buchanan 1822) were purchased from Big Al's Aquarium Services (Ottawa, ON, Canada) and kept in the University of Ottawa Aquatic Care Facility where they were maintained in plastic tanks supplied with aerated, dechloraminated City of Ottawa tap water at 28° C. Fish were subjected to a constant 14 h L:10 h D photoperiod and fed daily until satiation with No.1 crumble-ZeiglerTM (Aquatic Habitats, Apopka, FL, USA). Embryos were collected following the standard method (Westerfield, 2000). Collected embryos were reared in 50 ml Petri dishes supplemented with either dechloraminated City of Ottawa tap water (pH 7.3-7.5) or acidified water (water pH was lowered to 3.9-4.0 by adding H_2SO_4 to the Ottawa tap water) supplemented with 0.05% ethylene blue. The Petri dishes were kept in incubators set at 28.5° C. Dead embryos were removed and water was changed daily. As all experiments were performed on 5 day post fertilization (dpf) fish or younger, they were not fed for the duration of the experiment. The experiments were conducted in compliance with guidelines of the Canadian Council of Animal Care (CCAC) and after the approval of the University of Ottawa Animal Care Committee (Protocol BL-226). Unless otherwise stated, all chemicals used for this study were purchased from Sigma.

Experimental protocols

To investigate the mechanisms of Na^+ uptake in acidic water, the following series of experiments were performed.

Series 1. Pharmacological inhibition of NHE (slc9a) and its consequences on Na^+ uptake

At 4 dpf, 6 zebrafish larvae (yielding N = 6) reared in control pH were transferred to a 2 ml micro-centrifuge tube. Following the transfer, the fish were exposed to either 100 µM 5-(N-Ethyl-N-isopropyl) amiloride (EIPA) dissolved in 0.2% DMSO for 30 min. Throughout this

study, whenever DMSO was used to prepare inhibitor solutions, vehicle control experiments were performed. To measure the rate of Na^+ uptake, 0.5 µCi ^{22}Na in the form of NaCl (Perkin Elmer, Woodbridge, ON, Canada) was added to each tube to a final activity of 0.25 µCi/ml. Water samples (50 µl) were collected at 5 min and 2 h after the addition of radioisotope. At the end of the 2 h flux period, larvae were killed with overdose of ethyl 3-aminobenzoate methanesulfonate (MS222) and briefly washed in isotope-free water containing high levels of Na^+ (>200 mM) to remove any residual radioisotope attached to the surface of the fish. The remaining radioactive water in the tube was stored separately for later measurement of the total [Na^+]. Pilot experiments demonstrated that Na^+ uptake was unaffected by EIPA in larvae reared (and assayed) in acidic water. To account for the possibility that acidic water, itself, is interfering with the action of EIPA, the following modified protocol was used throughout the study to assess the effect of EIPA on Na^+ uptake by larvae reared in acidic water. Immediately before the measurement of Na^+ uptake, six 4 dpf larvae raised in acidic water were acutely transferred to regular holding water (pH 7.3-7.5). Fish were then exposed to 100 µM EIPA, and ^{22}Na was added to the water immediately. The duration of the flux period was reduced to 1 h to minimize the effects of acutely changing the water pH. Water samples and larvae were collected as described above. For the calculation and the handling of radioactive samples, refer to "analytical tools and calculations" section.

Series 2. Ammonia and Na^+ fluxes during exposure to high external ammonia (HEA).

To assess the consequences of inhibiting ammonia excretion on Na^+ uptake, 5 dpf larvae reared in neutral or acidic water were acutely exposed to the HEA [5 mM $NH_4(SO_4)_2$] conditions and ammonia and Na^+ fluxes were measured immediately after the exposure (N = 4-6). All measurements were completed within 3.5 h of transfer to HEA. Water pH was measured once

the ammonia was added and adjusted to the desired acidity for larvae reared in acidic water. For the ammonia excretion measurements, 3-4 larvae (5 dpf) were transferred to 2 ml micro-centrifuge tubes (yielding N = 1). Water samples (300 µl) were collected at the beginning and end of the 3 h flux period, and were frozen at -20° C for the later measurement of ammonia concentration. To measure Na^+ uptake, ^{22}Na was added as described in Series 1 immediately after larvae were exposed to HEA; the flux period was shortened to 1 h. Ammonia excretion and Na^+ uptake were measured using different individuals, but they were collected on the same day. Samples were collected and prepared as described in Series 1.

Series 3. Ammonia excretion and Na^+ uptake during exposure to highly buffered conditions.

To further assess the consequence of reduced ammonia excretion on Na^+ uptake, 4 dpf larvae were acutely exposed to highly buffered water and their ammonia excretion and Na^+ uptake were measured immediately (N = 4-9). As in the previous series, all measurements were completed within 3.5 h of transfer to the buffered condition. Regardless of the rearing condition, fish were exposed to the buffered water at control pH (see Discussion). The buffered water was prepared by dissolving HEPES in Ottawa tap water to a final concentration of 10 mM (Nawata and Wood, 2008). Because HEPES significantly reduced the water pH, it was necessary to adjust the water pH with KOH. Ammonia excretion was measured as described in Series 2. Although Na^+ uptake by larvae reared in control pH was measured only in 1) non-buffered control pH water or 2) buffered control pH water (N = 6 each), the following three groups were prepared to assess Na^+ uptake by larvae reared in acidic water (N = 6 per group); group 1) exposed to non-buffered control pH water; group 2) exposed to buffered control pH water; and group 3) exposed to buffered control pH water and 100 µM EIPA. The final experimental group

was used to assess whether NHE remained functional even after the external medium was buffered. Na^+ uptake was measured as described in Series 1, with 1-h flux periods.

Series 4. Na^+ uptake and ammonia excretion following transfer from acidic to water of circumneutral pH.

Larvae were reared in acidic water until 4 dpf; for ammonia excretion measurements, 10 larvae were placed in a 2 ml centrifuge microtube (yielding N = 1) and the ammonia excretion rate was measured as described above, with the exception that the flux period was shortened to 1 h. Following the initial measurement period in acidic water, the micro-centrifuge tube was washed twice with the control pH water to ensure complete changeover of water chemistry. Ammonia excretion was measured for 1-h intervals at 0, 1, 2, 3, 5 and 6 h after the exposure to control pH water. After each flux period, tubes were washed with fresh control pH water to ensure stable water chemistry.

For Na^+ uptake, 4 dpf larvae reared in acidic water were transferred to a Petri dish containing control pH water. Na^+ uptake was measured as described in Series 1 (with 1 h flux) at times matching the ammonia excretion determinations.

Series 5. Consequences of Rhcg1 knockdown on ammonia excretion and Na^+ uptake - morpholino design and microinjection

A morpholino oligonucleotide intended to bind to the translation start site of zebrafish Rhcg1 (accession no: AB286865) ([5'-TTGGTGTTTTTGACCATTTTTGATC-3']) was designed by GeneTools (http://www.gene-tools.com/). The identical morpholino was successfully used and validated in a previous study to knockdown the expression of Rhcg1 (Braun et al., 2009a); the same protocol was used in this study. Briefly, the morpholino was prepared to a final concentration of 4 ng/nl

in 1x Danieau buffer [58 mM NaCl, 0.7 mM KCl, 0.4 mM $MgSO_4$, 0.6 mM $Ca(NO_3)_2$, and 5.0 mM HEPES (pH 7.6)] and 0.05% phenol red. Injections were performed using a microinjector system (model IM 300, Narishige, Long Island, NY). To control for possible consequence of microinjection, a "sham" group was injected with a standard control morpholino from GeneTools (5'-CCTCTTACCTCAGTTACAATTTATA-3') prepared as the Rhcg1 morpholino. Morpholino was injected at a dose of 4 ng/embryo at 1-2 cell stages. After injection, embryos were reared as described above. At 24 h post fertilization (hpf), injected embryos were observed with a model SMZ1500 microscope (Nikon Instruments, Melville, NY) to screen for the embryos that successfully incorporated the injected morpholino based on the presence of widely-distributed carboxyfluorescein. Only carboxyfluorescein-positive embryos were kept and used for subsequent experiments. Morphant and sham groups were reared in either control or low pH water as described above.

Series 5.1. Effect of chronic exposure to acidic water on ammonia excretion and Na^+ uptake by Rhcg1 morphants

To confirm the efficacy of Rhcg1 knockdown, ammonia excretion was measured at 4 dpf in shams or morphants reared in either control pH or low pH conditions as described above (N = 4-6). For the measurement of Na^+ uptake, larvae reared in both neutral and low pH conditions were divided into the following three groups (N = 5-17); group 1) sham fish; group 2) Rhcg1 morphants; and group 3) Rhcg1 morphants treated with 100 µM EIPA. Na^+ uptake was measured as described in Series 1.

Series 5.2. Effects of acutely changing water pH on Na^+ uptake by Rhcg1 morphants

Rhcg1 morphants and sham-injected larvae were subjected to the following treatments; 1) rearing in acidic water until 4 dpf followed by transfer to control pH water for 6 h (N = 5-6) and

2) rearing in control water until 3 dpf followed by transfer to acidic water for 24 h (N = 4-6). For treatment group 1, Na^+ uptake was measured both before and after the transfer to control pH water as described in series 1, but without any pharmacological blockers. For treatment group 2, sham and Rhcg1-morpholino injected larvae were exposed to 100 µM EIPA as described in series 1 following the 24 h exposure to acidic water. In addition, ammonia excretion was measured in randomly selected sham and Rhch1 morphants (reared in control pH water until 4 dpf) to assess the effectiveness of Rhcg1 knockdown (N = 6). These 3 experiments were done using sham and morpholino-injected fish collected on the same day.

Series 6. Pharmacological inhibition of H^+-ATPase and its consequences on Na^+ uptake and ammonia excretion

Larvae were treated with bafilomycin A1 (final concentration = 1 µM; LC Laboratories, Woburn, MA, USA), a known inhibitor of H^+-ATPase. Larvae (4 dpf; N = 5-6) reared in either neutral or low pH conditions were transferred to micro-centrifuge tubes and then exposed to bafilomycin A1 for 30 min. After 30 min, 0.5 µCi/ml of ^{22}Na was added to the medium. The rate of Na^+ uptake was measured as described in Series 1 with 1 h flux periods.

To assess the effect of bafilomycin treatment on ammonia excretion in acidic water, larvae were exposed to 1 µM bafilomycin (LC Laboratories, Woburn, MA, USA) dissolved in 100 % ethanol as vehicle (final concentration of ethanol in water was 0.1 %). Ethanol was used as a vehicle for this experiment as DMSO considerably interfered with the ammonia assay protocol. Preliminary experiment indicated no effect of ethanol treatment on ammonia excretion (data not shown). For ammonia flux, 10 larvae were placed in 2-ml microtube and 400 µl of water samples were collected at the beginning and end of the 1-h flux period and analyzed as

described below to calculate the ammonia flux. For the bafilomycin treated group, the initial water sample was collected immediately after the addition of the drug.

Series 7. NHE3b expression levels in zebrafish larvae in acidic water and the effect of its knockdown on Na^+ uptake

Although both NHE2 and NHE3 have been implicated in Na^+ uptake in teleosts including zebrafish (Craig et al., 2007; Yan et al., 2007), given its known apical expression in adult H^+-ATPase rich cells (HRCs) of zebrafish gill (Yan et al., 2007), the expression level of NHE3b in larval zebrafish in low pH water was analyzed. Using TRIzol® (Invitrogen), total RNA was extracted from 4 dpf larvae reared in either control or low pH water and cDNA was synthesized by treating 2 µg of extracted RNA with DNase (Invitrogen) and RevertAid^{TM} M-MNuLV reverse transcriptase (Fermentas, Burlington, ON, Canada) according to the manufacturer's instructions. For the RT-qPCR, the following primers were used for NHE3b; forward 5'-TGCAGACAGCGCCTCTAGC-3' and reverse 5'-TGTGGCCTGTCTCTGTTTGC-3' (Yan et al., 2007). The PCR product was confirmed by direct sequencing. RT-qPCR was performed using Brilliant II SYBR Green Mastermix (Stratagene, La Jolla, CA) (10 µl final volume) with the following compositions: 6.25 µl Brilliant II SYBR Green Mastermix, 0.2 µl of each primer, 0.25 µl of cDNA and sterile water. The thermal profile for PCR reactions was as follows; initial denaturation at 95° C for 10 min followed by 40 cycles of 95° C for 30 s, 58° C for 1 min, and 72° C for 1 min (MXPro software ver. 4.10 (Stratagene). The expression level of NHE3b was normalized to that of 18S using a modification of the ΔΔCt method (Pfaffl, 2001). 18S was chosen as the normalizing gene because it has been successfully used as a standard for adult zebrafish subjected to the same acid stress (Kumai et al., 2011) along with other experimental treatments (McCurley and Callard, 2008). For 18S, the following primers were used; forward

5’-GGCGGCGTTATTCCCATGACC-3’ and reverse GGTGGTGCCCTTCCGTCAATTC-3’. PCR conditions were the same as NHE3b.

To further elucidate the role of NHE3b in Na^+ uptake in acidic water, translational knockdown was performed using a morpholino designed to splice out exon 2, which contains a the putative Na^+-binding site. The morpholino (5’-AGCTCAGTGACTGGAAAGAGAAATA-3’) was designed and microinjected as described above, along with the same control morpholino as in Series 5. Due to high mortality observed in morphants chronically raised in acidic water, shams and morphants were raised in control water until 3 dpf and subsequently transferred to acidic water for 24 h before the measurement of Na^+ uptake.

To confirm the effectiveness of morpholino knockdown, the following PCR primers were designed using Primer3 software (http://frodo.wi.mit.edu/primer3/); forward 5’-TCCTGAAACACCACGATTCA-3’ and reverse 5’- ACAGGGTCCACAGCAGACAT-3’. Primers were designed to amplify exons 1 through 3 of NHE3b, which should result in a shift of amplicon size in the morphants. Total RNA was extracted and cDNA synthesized as described above from 4 dpf sham and NHE3b-morphants. Conditions for PCR were as follows: initial denaturation at 94° C for 30 s followed by 40 cycles of 94° C for 30 s, 62° C for 60 s, and 72° C for 45 s, with final extension for 5 min at 72° C. PCR products were run on 1% agarose gel and imaged with GelDoc software equipped with Quantity-One 1-D analyzer software (BioRad, Mississauga, ON, Canada). PCR product was confirmed with direct sequencing.

Analytical tools and calculations

Ammonia excretion was determined colorimetrically using a method (Verdouw, 1978) modified for 96-well microplate using an absorbance of 650 nm. Ammonia excretion (J_{out}^{Amm}, nmol/fish/h) was calculated as:

$$J_{out}^{Amm} = \frac{([Amm_F] - [Amm_I])V}{t}\frac{1}{n}$$

, where n = number of larvae in the tube and $[Amm]_F$ and $[Amm]_I$ = the measured concentration of ammonia (µM) at the end and beginning of a flux period, V = volume of water in the microtube (L) and t = duration of flux period (h).

To determine Na^+ uptake, all collected water samples were supplemented with 5 ml of scintillation cocktail (Biosafe-II, RPI Corp, Mt. Prospect, IL., USA) and their radioactivity was measured with a liquid scintillation counter (model LS-6500 Beckman Coulter, Co. Mississauga, ON, Canada). After being rinsed in an isotope-free medium, larvae were digested in a tissue solubilizer Solvable^{TM} (Perkin Elmer) overnight at 65° C. After the complete digestion of the fish, samples were supplemented with 10 ml of the same scintillation cocktail. Samples were then neutralized by adding 450 µl of glacial acetic acid before measuring their radioactivity. The concentration of total Na^+ in the water was measured using flame emission spectrophotometry (model AA260, Varian, Palo Alto, CA, USA). Due to the limited volume of water, $[Na^+]$ was measured only at the end of the flux period to calculate the external specific activity. It was assumed that, given the typical $[Na^+]$ of the experimental water (700 - 1000 µM), Na^+ influx rate (on the scale of 1 nmol fish^{-1} h^{-1}) and even smaller expected net flux of Na^+ (difference between influx and efflux), changes in total $[Na^+]$ during the flux period would be negligible. The rate of Na^+ uptake (J_{in}^{Na} , pmol/fish/h) was calculated as follows:

$$J_{in}^{Na} = \frac{F}{SA \cdot n \cdot t}$$

, where F= total incorporated radioactivity (DPM, disintegration per minute), SA= specific activity of the medium (DPM/pmol), n = number of larvae (typically 1) and t = the

duration of the incubation (h). DPM was calculated by the liquid scintillation counting program after taking quenching and counting efficiency into consideration.

Statistical analysis

All statistical analyses were performed with SigmaPlot (v. 11, Systat Inc. Chicago, IL, USA). Two-way ANOVA was used to analyze data from Series 1, Series 2, Series 5.2. (Na^+ uptake following transfer to control pH water), Series 6 and Series 7. Whenever significant interactions were detected, a Holm-Sidak post-hoc test was used to identify significant differences among treatment groups. One-way ANOVA was used to analyze data from Series 3 (Na^+ uptake by larvae reared in acidic water), Series 4 (Na^+ uptake following transfer to control pH), and Series 5.1. and 5.2. (Na^+ uptake data). One way RM ANOVA was used to analyze ammonia flux data from Series 4 followed by a Holm-Sidak post-hoc test. Student's *t*-test was used to analyze the ammonia flux data from Series 3 and 5, Na^+ uptake data from larvae reared in neutral pH water from Series 3, ammonia excretion data from Series 6 and gene expression data from Series 7. When assumptions of normality or equal variance were violated, data were transformed using natural log- or square-root transformation. For all analyses, the level of statistical significance was set at $p<0.05$.

Results

Series 1. Pharmacological inhibition of NHE (slc9a) and its consequences on Na^+ uptake.

Exposing the fish to DMSO was without effect on Na^+ uptake regardless of the rearing condition (data not shown). Na^+ uptake was significantly higher in larvae reared in acidic water than in control water (N = 6; two-way ANOVA; Fig. 3.1). EIPA treatment did not affect Na^+ uptake in control larvae reared in water of circumneutral pH (N = 6, two-way ANOVA; Fig. 3.1). However, Na^+ uptake was significantly reduced by EIPA treatment in larvae reared in acidic water (N = 6, two-way ANOVA; Fig. 3.1).

Series 2. Ammonia and Na^+ fluxes during exposure to high external ammonia (HEA).

Though there was a strong trend (P = 0.06), there was no difference in rates of ammonia excretion between larvae reared in low and control pH water (N = 5 – 6; two-way ANOVA; Fig. 3.2A). Regardless of rearing conditions, subjecting the larvae to acute HEA, caused a significant influx (negative net flux) of ammonia during the 3 h measurement period (N = 5 - 6; two way ANOVA; Fig. 3.2A), thus effectively blocking the excretion of ammonia.

When the 5 dpf larvae were challenged with HEA, Na^+ uptake was significantly reduced regardless of rearing conditions. The rate of Na^+ uptake remained significantly higher in larvae reared in low pH conditions than those reared in control pH, both with and without HEA treatment (N = 4 - 6; Fig. 3.2B; two-way ANOVA).

Series 3. Ammonia excretion and Na^+ uptake during exposure to highly buffered conditions.

As expected, exposure to buffered conditions significantly reduced the rate of ammonia flux in larvae reared at both control pH and in low pH water (N = 4 - 9; Figs. 3.3A, C; Student's t-test).

The effects of external buffering on Na^+ uptake were dependent on the rearing conditions (Figs. 3.3B, D). External buffering had no effect on Na^+ uptake by 4 dpf larvae reared in control pH conditions (N = 6; Fig. 3.3B; Student's t-test). On the other hand, external buffering significantly reduced the rate of Na^+ uptake in larvae reared in acidic water (N = 6; Fig. 3.3D; one-way ANOVA). When the larvae were treated with EIPA in buffered water, the treatment further reduced Na^+ uptake (N = 6, Fig. 3.3D; one-way ANOVA).

Series 4. Na^+ uptake and ammonia excretion following transfer from acidic water to water of circumneutral pH.

Ammonia excretion and Na^+ uptake were rapidly reduced following the transfer from acidic to control pH water. Ammonia excretion remained reduced for up to 6 h after transfer to water of control pH (N = 6; Fig. 3.4A; one-way RM ANOVA). The reduction in Na^+ uptake was complete within 3 h of the transfer to control pH, after which there was no further change in Na^+ uptake (N = 6; Fig. 3.4B; one-way ANOVA).

Series 5. Consequences of Rhcg1 knockdown on ammonia excretion and Na^+ uptake

Knockdown reduced ammonia excretion in larvae reared in control pH water by 50% (N = 4 - 6; Fig. 3.5A; Student's t-test). In larvae raised in acidic water, Rhcg1 knockdown reduced ammonia excretion by about 75% (N = 5 - 6; Fig. 3.5C; Student's t-test).

Rhcg1 knockdown or treatment of Rhcg1 morphants with 100 µM EIPA did not affect Na^+ uptake by larvae reared in control pH water (N > 5 for all groups; Fig. 3.5B; one-way ANOVA). Although Na^+ uptake was significantly reduced by Rhcg1 knockdown in larvae raised in acidic water, EIPA treatment did not further reduce the rate of Na^+ uptake ($N \geq 10$ for all three groups; Fig. 3.5D; one-way ANOVA).

To further elaborate the role of Rhcg1, sham and Rhcg1-morhants were subjected to 6 h exposure to control pH water following 4 days of exposure to acidic water (Fig. 3.6B) or 24 h exposure to acidic water following 3 days of exposure to control water (Fig. 3.6C). Ammonia excretion was significantly lower in morpholino-injected larvae (N = 6 Fig. 6A, Student's t-test), again supporting the effective knockdown of Rhcg1.

In agreement with the data in Fig. 3.5D, the rate of Na^+ uptake was significantly lower in 4 dpf Rhcg1 morphants reared in acidic water before transfer to control pH water (N = 4 - 6; Fig. 3.6B; two way ANOVA). Exposure to control pH for 6 h significantly reduced Na^+ uptake in both shams and Rhcg1 morphants, and interestingly, the rate of Na^+ uptake was significantly higher in Rhcg1 morphants than in sham after the transfer (N = 4 - 6; Fig. 3.6B; two way ANOVA).

In agreement with Fig. 3.5B, there was no difference in Na^+ uptake between 3 dpf sham and Rhcg1-morphants reared in control water (N = 5 - 6; Fig. 3.6C; one-way ANOVA). Additionally, in 4 dpf sham-injected fish reared in control pH water, there was no change in Na^+ uptake from the 3 dpf sham-injected larvae (N = 5 - 6; Fig. 3.6C; one way ANOVA). In both sham- and Rhcg1 morpholino-injected larvae, Na^+ uptake was significantly stimulated following 24 h exposure to acidic water (N = 5 - 6; Fig, 3.6C; one-way ANOVA,). Consistent with Fig. 3.5D, Na^+ uptake rate was significantly lower in Rhcg1 morphants than in sham-injected larvae (one-way ANOVA, Fig. 3.6C). In further agreement with Fig. 3.5D, treatment with 100 µM EIPA reduced Na^+ uptake only in sham-injected larvae exposed to acidic water (N = 5 - 6; Fig. 3.6C; one-way ANOVA).

Series 6. Pharmacological inhibition of H^+-ATPase and its consequences on Na^+ uptake.

Bafilomycin treatment reduced Na^+ uptake in larvae reared in control water by about 30% (N = 6, Fig. 3.7; two-way ANOVA) and by approximately 75% in larvae reared in low pH water (N = 6; Fig. 3.7; two-way ANOVA).

Bafilomycin treatment also significantly reduced ammonia excretion by larvae in acidic water from 1.02 ± 0.19 to 0.42 ± 0.08 nmol/larvae/h (N = 5 – 6; Student's t-test).

Series 7. NHE3b mRNA expression in zebrafish larvae and effect of its knockdown on Na^+ uptake.

There was no significant different in the mRNA expression level of NHE3b between larvae reared in control and low pH conditions (N = 4 - 6; data not shown).

The NHE3b morpholino successfully removed the intended target of exon 2, as shown by the shift in the PCR product size from roughly 700 bp to 400 bp (Fig. 3.8A). As a further confirmation, when PCR was performed with a set of primers designed to amplify regions within exon 2, the amplicon of the expected size was observed only in the sham-injected group (data not shown). NHE3b knockdown did not affect Na^+ uptake rate by larvae raised in control pH water (N = 5; Fig. 3.8B; two-way ANOVA). However, the knockdown of NHE3b significantly reduced Na^+ uptake rate by larvae acutely exposed to low pH water (N = 5; Fig. 3.8B; two way ANOVA).

Figure 3.1. The effect of 100 µM EIPA treatment on the Na^+ uptake rate by zebrafish larvae reared in neutral and low pH conditions. Acid-exposure significantly induced Na^+ uptake in zebrafish larvae, which was inhibited by treatment with EIPA. Data are presented as means ± SEM. N = 6. Different letters indicate significant difference between groups.

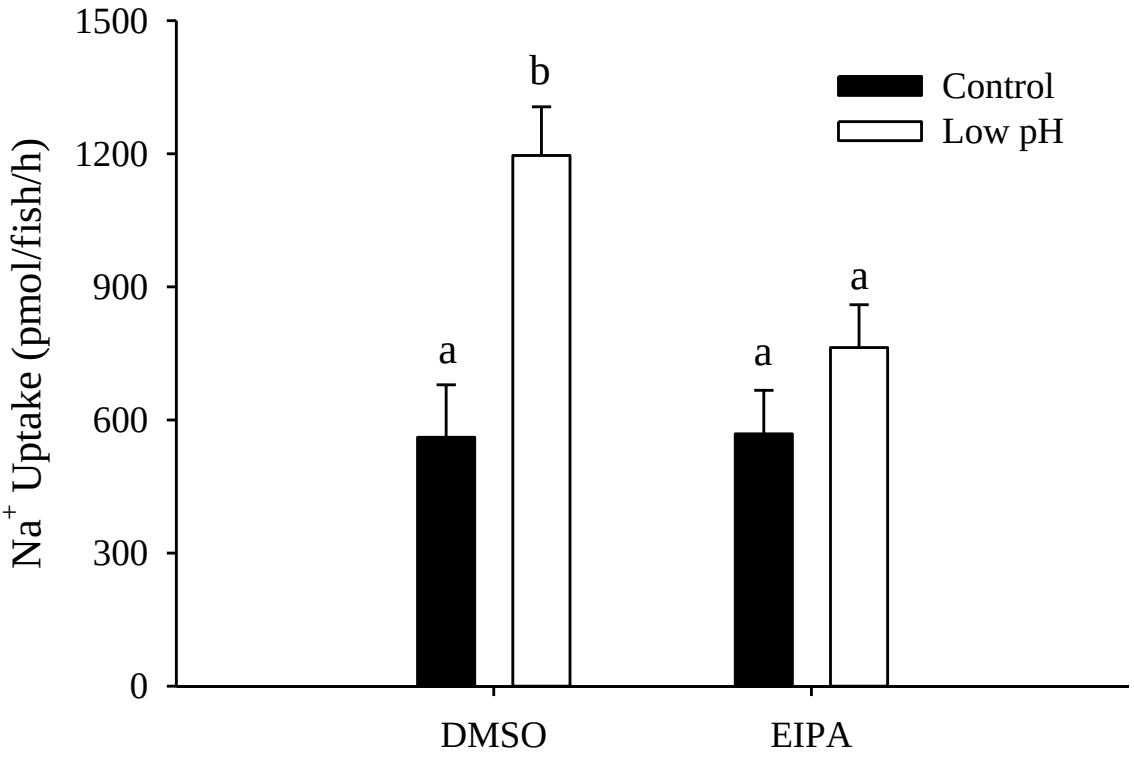

1500
1200
900
600
300
0
Na+ Uptake (pmol/fish/h)
b
a
a
a
Control
Low pH
DMSO
EIPA

Figure 3.2. The effect of HEA treatment on ammonia secretion and Na^+ uptake by larval zebrafish. HEA treatment caused significant influx of ammonia (N = 5 - 6; Fig. 3.2A) and reduction in Na^+ uptake (N = 4 - 6; Fig. 3.2B) regardless of the rearing conditions. Data are presented as means ± SEM. Different letters indicate significant difference between groups.

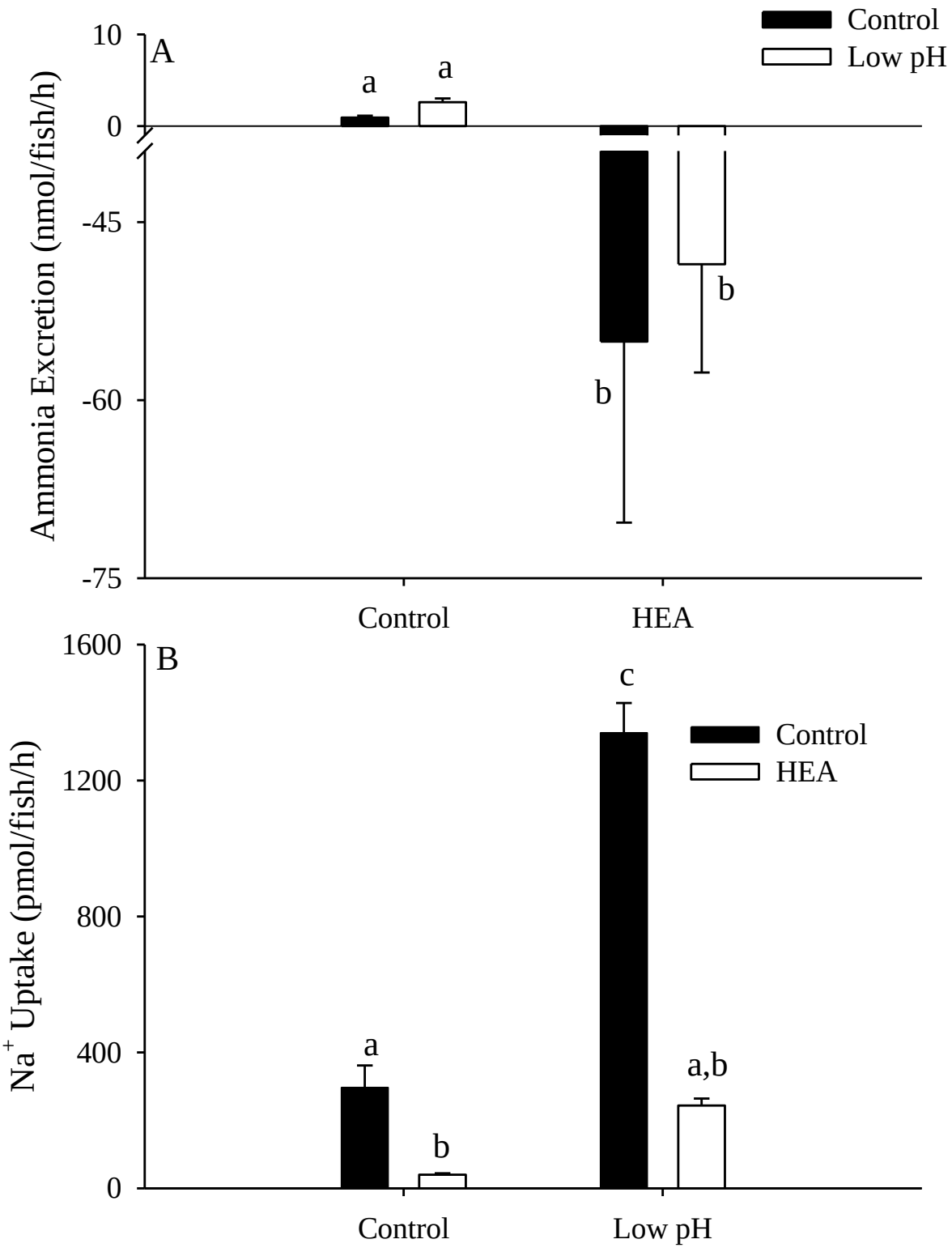

A
Control
Low pH
10
0
-45
-60
-75
Ammonia Excretion (nmol/fish/h)
a
a
b
b
Control
HEA
B
1600
1200
800
400
0
Na+ Uptake (pmol/fish/h)
Control
HEA
a
b
c
a,b
Control
Low pH

Figure 3.3.The effect of external buffering on ammonia secretion and Na^+ uptake by zebrafish larvae. External buffering equally reduced ammonia flux in larvae reared in control pH (N = 4 – 9; Fig. 3.3A) and low pH (N = 6; Fig. 3.3C), but affected Na^+ uptake only in those reared in low pH water (N = 6; Fig. 3.3D), not in control pH water (N = 6; Fig. 3.3B). Data are presented as means ± SEM. Asterisks in Figs. 3.3A,C indicate significant difference from the control group. Different letters in Fig. 3.3D indicate significant difference between groups.

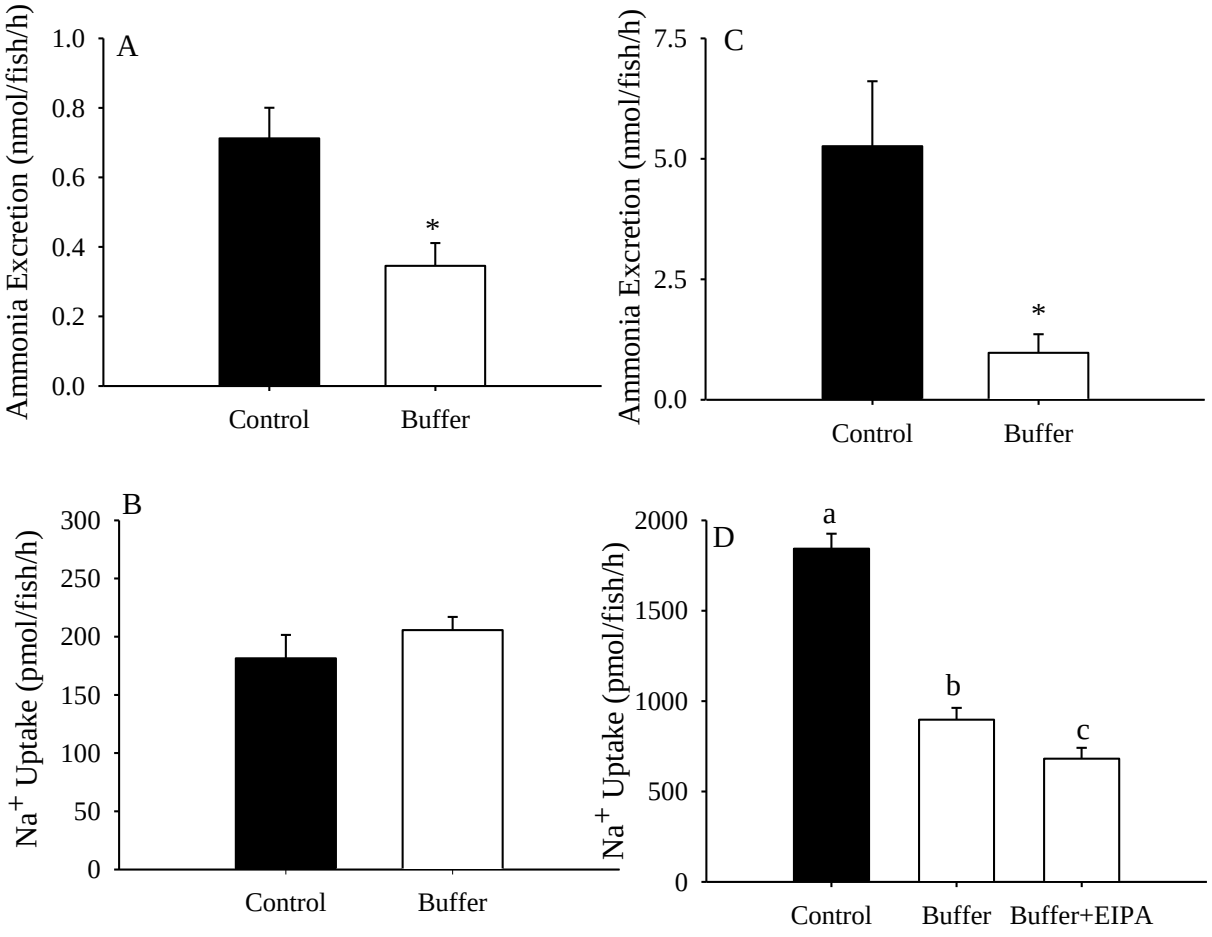
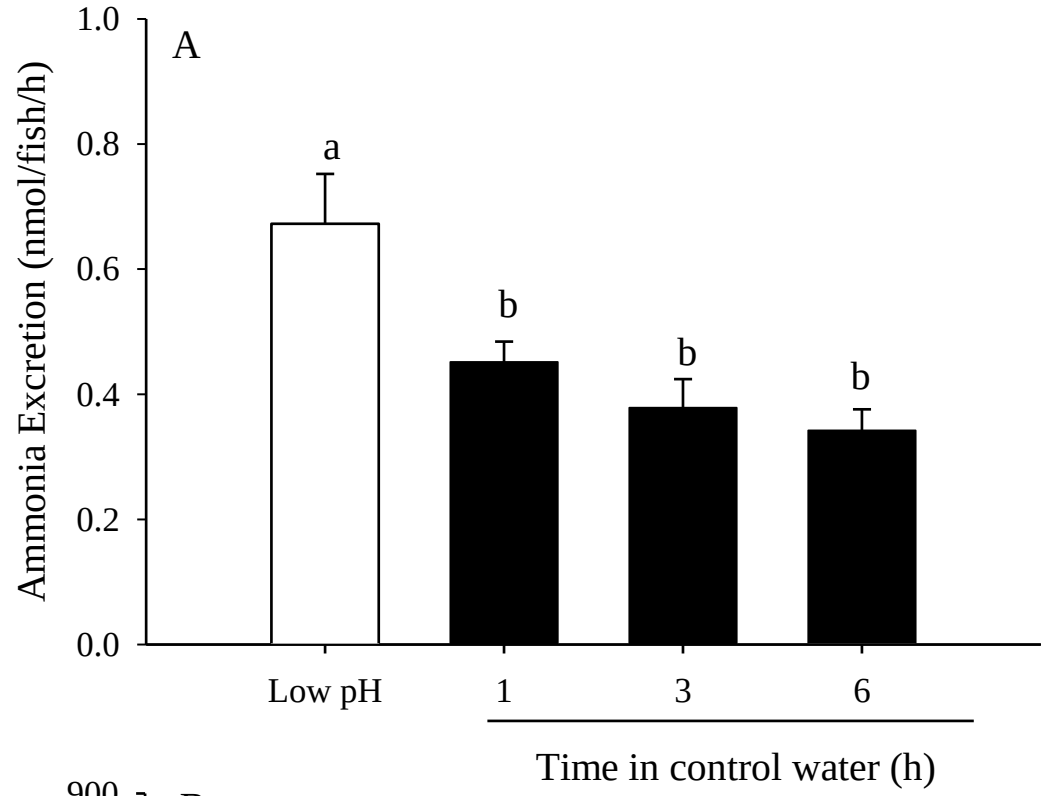

A
Ammonia Excretion (nmol/fish/h)
1.0
0.8
0.6
0.4
0.2
0.0
*
Control
Buffer
C
Ammonia Excretion (nmol/fish/h)
7.5
5.0
2.5
0.0
*
Control
Buffer
B
Na+ Uptake (pmol/fish/h)
300
250
200
150
100
50
0
Control
Buffer
D
Na+ Uptake (pmol/fish/h)
2000
1500
1000
500
0
a
b
c
Control
Buffer
Buffer+EIPA

Figure 3.4. The effect of acute transfer of zebrafish larvae from low to control pH water on ammonia secretion and Na^+ uptake. Following the transfer both ammonia excretion (N = 6; Fig. 3.4A) and Na^+ uptake (N = 6; Fig. 3.4B) were significantly reduced. Data are presented as means ± SEM. Different letters denote significant difference between groups.


A
Ammonia Excretion (nmol/fish/h)
1.0
0.8
0.6
0.4
0.2
0.0
a
b
b
b
Low pH
1
3
6
Time in control water (h)

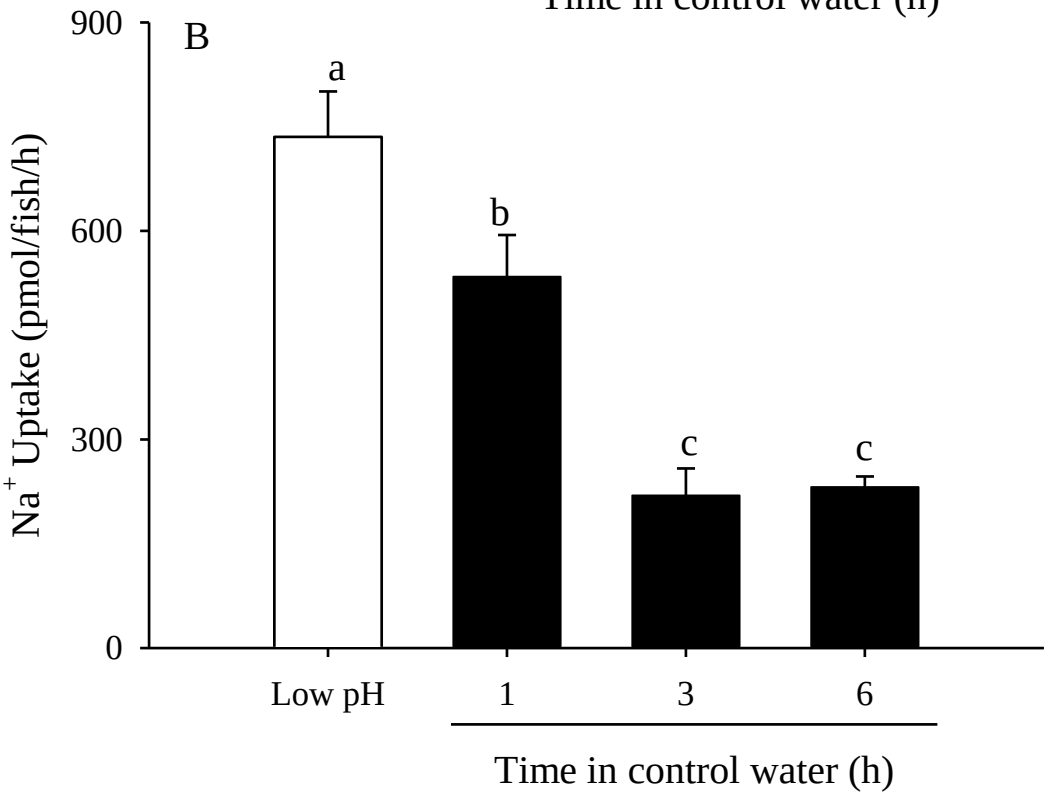

B
Na+ Uptake (pmol/fish/h)
900
600
300
0
a
b
c
c
Low pH
1
3
6
Time in control water (h)

Figure 3.5. The effect of Rhcg1 knockdown on ammonia secretion and Na^+ uptake by zebrafish larvae. Rhcg1 knockdown equally reduced ammonia excretion by larvae reared in control (N = 4 – 6; Fig. 3.5A) and low pH (N = 5 - 6; Fig. 3.5C); on the other hand, knockdown affected Na^+ uptake only in larvae reared in low pH (N = 10 - 17; Fig. 3.5D) water, not in control pH (N = 5 - 7; Fig.3.5B). EIPA treatment did not inhibit Na^+ uptake in Rhcg1 morphants regardless of their rearing conditions (N = 5 - 17; Figs. 3.5B, D). Data are presented as mean ± SEM. Different letters and asterisks indicate significant difference between groups.

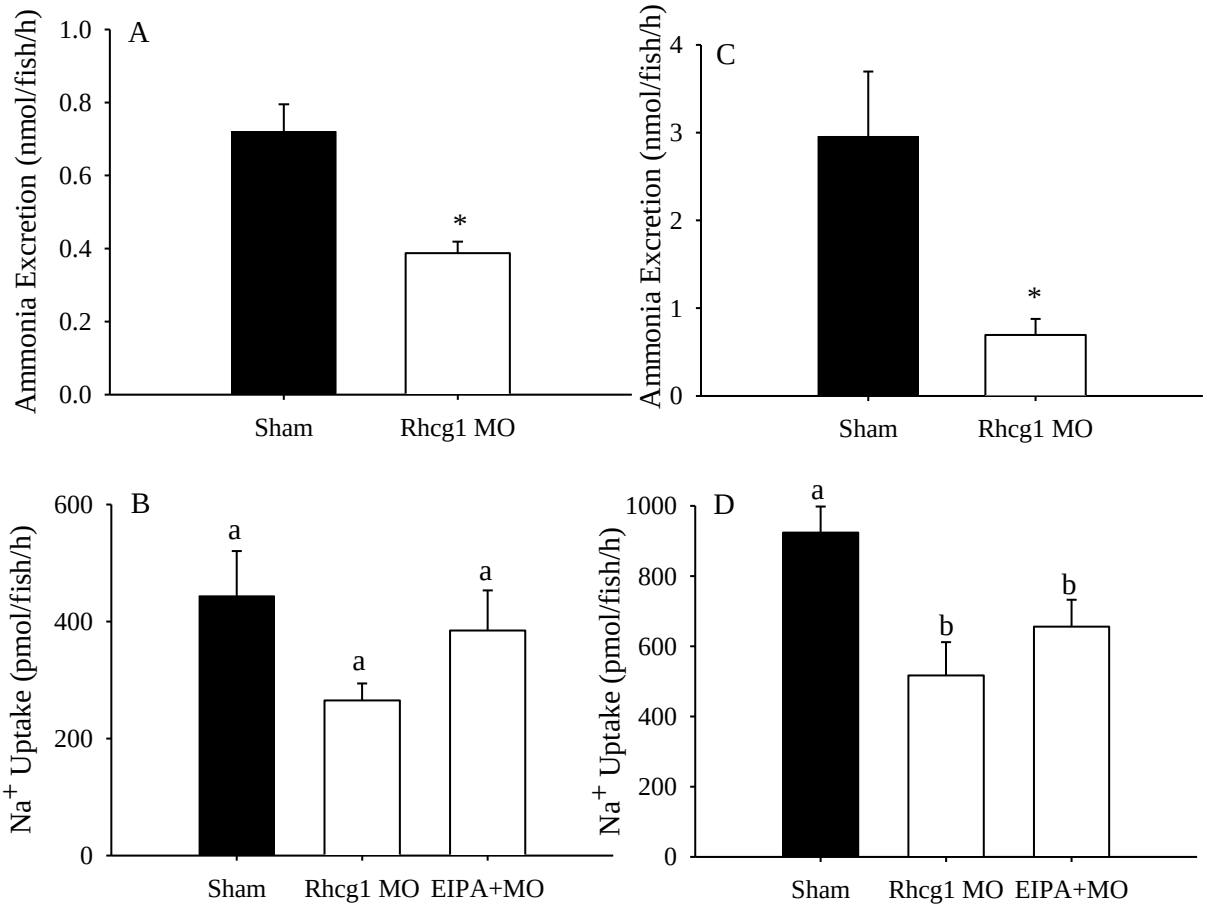

A
Ammonia Excretion (nmol/fish/h)
1.0
0.8
0.6
0.4
0.2
0.0
*
Sham
Rhcg1 MO
C
Ammonia Excretion (nmol/fish/h)
4
3
2
1
0
*
Sham
Rhcg1 MO
B
Na+ Uptake (pmol/fish/h)
600
400
200
0
a
a
a
Sham
Rhcg1 MO
EIPA+MO
D
Na+ Uptake (pmol/fish/h)
1000
800
600
400
200
0
a
b
b
Sham
Rhcg1 MO
EIPA+MO

Figure 3.6. The effect of Rhcg1 knockdown on ammonia secretion and Na^+ uptake in zebrafish larvae following acute exposure to control and low pH water. Rhcg1 knockdown reduced ammonia excretion by larvae reared in control pH (N = 6; Fig. 3.6A). Following transfer to control water, Na^+ uptake was reduced in both sham and Rhcg1 morpholino injected larvae equally (N = 4 – 6; Fig. 3.6B). Knockdown of Rhcg1 prevented the complete stimulation of Na^+ uptake following transfer to low pH water, which was insensitive to EIPA treatment (N = 5 - 6; Fig. 3.6C). Data are presented as means ± SEM. An asterisk in Fig. 3.6A indicates significant difference from the control group. Different letters in Figs. 3.6B,C indicate significant difference between groups.

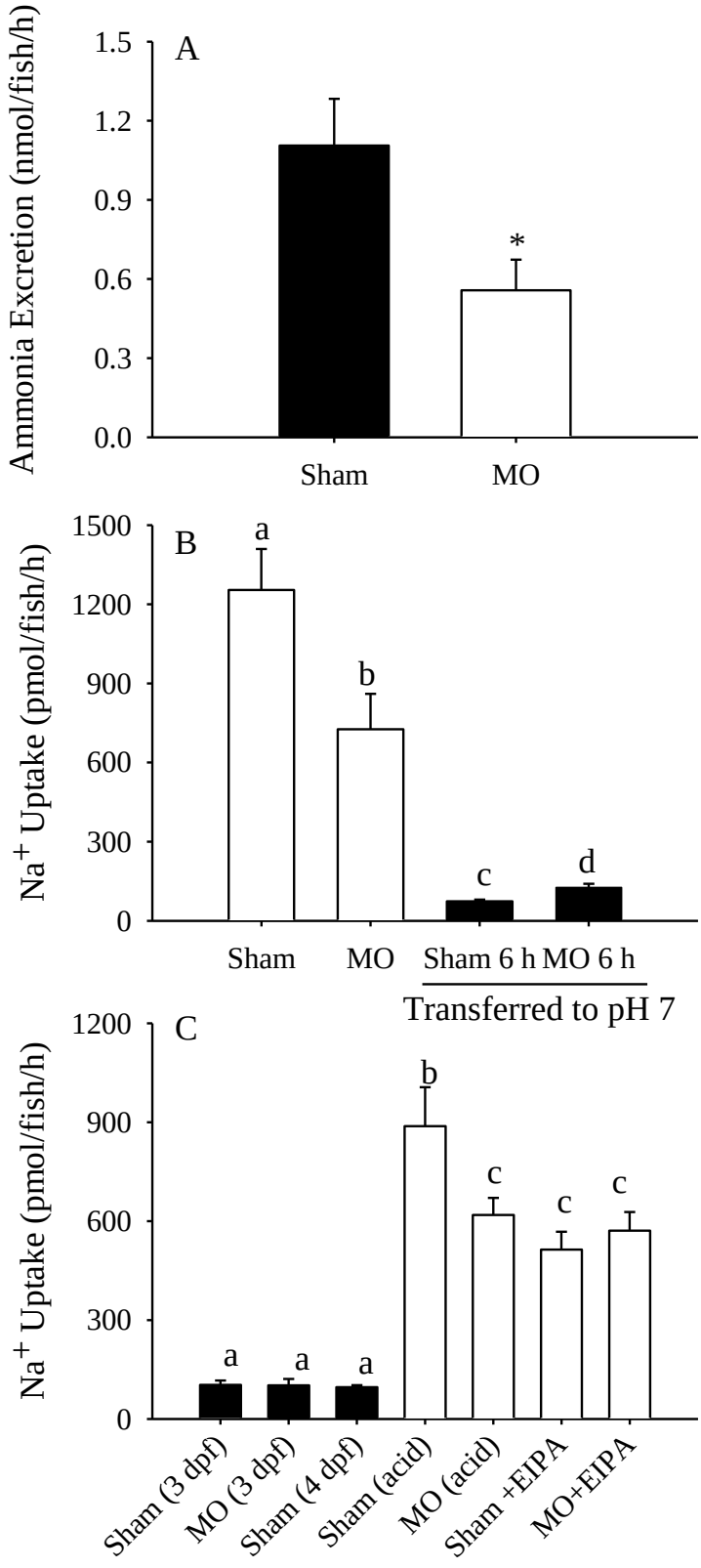

A
Ammonia Excretion (nmol/fish/h)
1.5
1.2
0.9
0.6
0.3
0.0
*
Sham
MO
B
Na^+ Uptake (pmol/fish/h)
1500
1200
900
600
300
0
a
b
c
d
Sham
MO
Sham 6 h
MO 6 h
Transferred to pH 7
C
Na^+ Uptake (pmol/fish/h)
1200
900
600
300
0
a
a
a
b
c
c
c
Sham (3 dpf)
MO (3 dpf)
Sham (4 dpf)
Sham (acid)
MO (acid)
Sham +EIPA
MO+EIPA

Figure 3.7. The effect of bafilomycin A1 treatment on Na^+ uptake by zebrafish larvae. The bafilomycin treatment significantly reduced the Na^+ uptake in larvae reared in neutral and low pH conditions (N = 6). Data are presented as means ± SEM. Different letters indicate significant differences between groups.

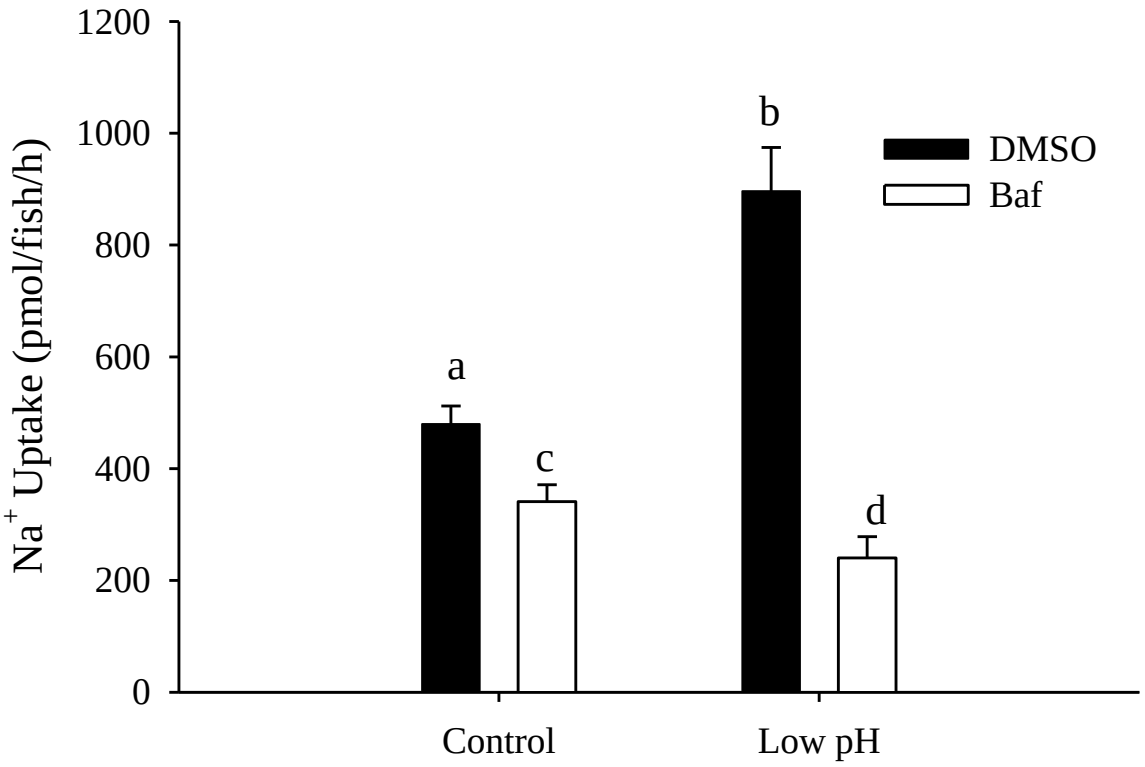
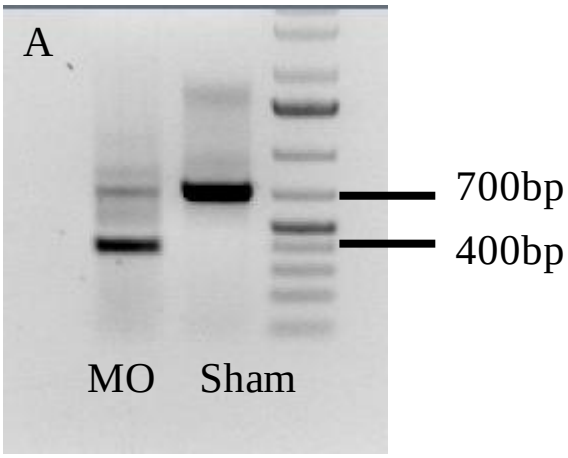
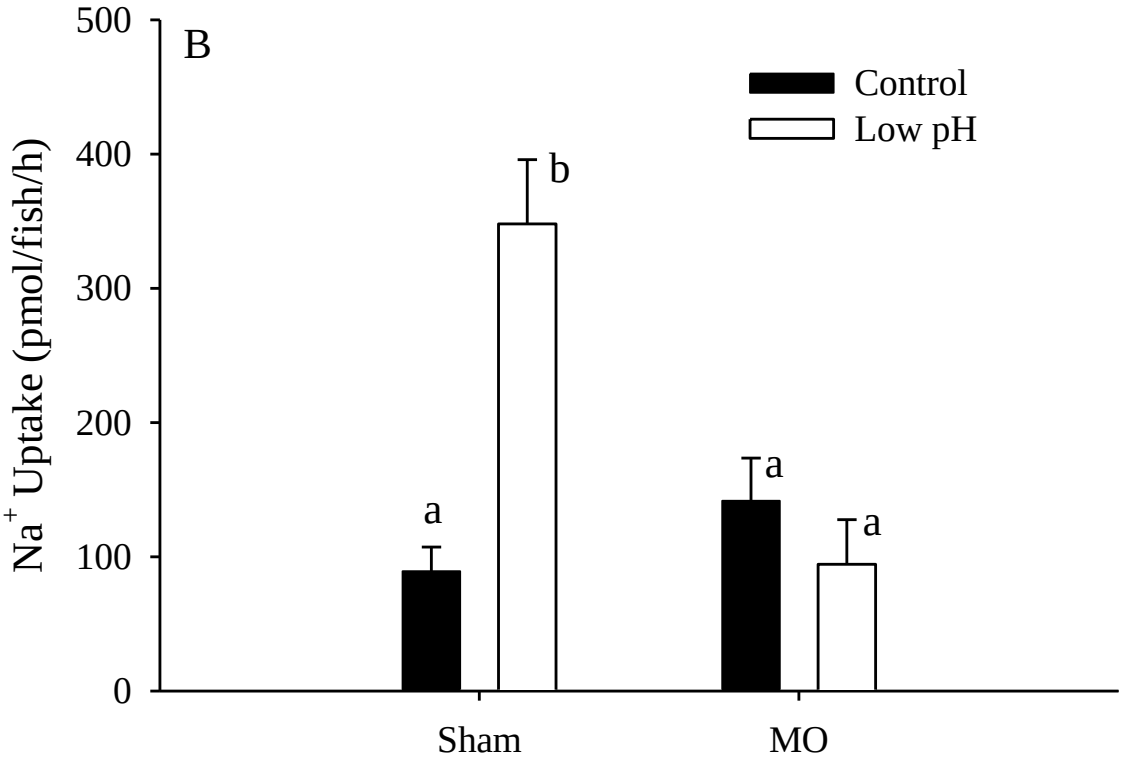

1200
1000
800
600
400
200
0
Na+ Uptake (pmol/fish/h)
a
c
b
d
DMSO
Baf
Control
Low pH

Figure 3.8. The consequence of NHE3b knockdown on Na^+ uptake by zebrafish. The morpholino effectively removed exon 2 of the target (Fig. 3.8A). Na^+ uptake rate was significantly lower in NHE3b morphants exposed to acidic water for 24 h (N = 5; Fig. 3.8B), whereas the gene knockdown had no effect on Na^+ uptake by larvae reared in control pH (Fig. 3.8B). Data are presented as means ± SEM. Different letters indicate significant difference between groups.


A
700bp
400bp
MO
Sham


B
Control
Low pH
Na^+ Uptake (pmol/fish/h)
500
400
300
200
100
0
a
b
a
a
Sham
MO

Discussion

The objectives of the present study were to experimentally test for the involvement of Na^+/H^+ exchange, in particular NHE3b, in Na^+ uptake in zebrafish larvae reared in circumneutral or acidic water conditions and to examine if ammonia excretion via Rhcg1 is critically important for facilitating its function. Although it remains to be seen if any other isoforms of NHEs known to be expressed on zebrafish gill or larval skin are also responsible for Na^+ uptake, the data clearly indicate an involvement of NHE3b in Na^+ uptake by larvae reared and assayed in acidic conditions and its dependence on ammonia excretion via Rhcg1. The results also reveal the existence of a second Na^+ uptake mechanism mediated by H^+-ATPase, in agreement with previous reports (Boisen et al., 2003; Horng et al., 2007).

NHE plays a role in Na^+ uptake by zebrafish in acidic water

It was recently reported that, unlike many other FW fishes investigated to date (see Introduction), zebrafish respond to chronic exposure to acidic water by increasing their Na^+ uptake rate (Kumai et al., 2011). Results from the present study confirm that zebrafish larvae defend their Na^+ homeostasis during acid-exposure using this strategy. Given the increase in Na^+ uptake in acidic water, the initial objective was to determine the mechanism(s) of Na^+ uptake operating under this environmental condition. Based on the study of Hirata et al. (2003), which reported an increase in NHE expression in the gill of acid-exposed Osorezan dace, *T. hakonensis*, the potential involvement of NHE in acid-exposed zebrafish was investigated.

Numerous studies have indirectly implicated NHE in branchial Na^+ uptake in FW teleosts (see Introduction). Theoretically however, NHE should function as an exporter (not importer) of Na^+ in the dilute FW environment (for review see Parks et al., 2008). The thermodynamic constraints on NHE-mediated Na^+ uptake presumably are even more significant in acidic water because the chemical gradients resulting from the combination of low external [Na^+] and high

[H^+] would favour outward Na^+ flux. Thus, it was particularly intriguing that EIPA treatment (Fig. 3.1) as well as knockdown of NHE3b (Fig. 3.8) caused a significant reduction in Na^+ uptake uniquely in zebrafish larvae reared in acidic water. In their analysis on the effects of acidic water on the mRNA levels of eight NHE isoforms in zebrafish gill, Yan et al., (2007) reported that acclimation to acidic water led to a significant reduction in the levels of NHE3b mRNA, the isoform believed to be primarily responsible for Na^+ uptake in FW fish (Evans, 2008). On the other hand, the results of the current study showed no such reduction in the expression of NHE3b mRNA following 4-day of acclimation to acidic water. The differences in the results of the two studies may be related to the different ages of the animals sampled (larvae versus adults) and lengths of exposure (4 days versus 1 week]). Because it was proposed that NHE3b plays a more important role in zebrafish larvae exposed to low pH water for 4 days, the lack of any increase in NHE3b mRNA expression was unexpected. However, because RNA was extracted from entire larvae, rather than from the ionocytes responsible for Na^+ uptake, it is possible that any change in the expression of NHE3b within ionocytes was masked by lack of (or opposite) expression changes in other cells. Furthermore, it is possible that the expression levels of NHE3b mRNA do not correspond to the levels of functional protein, given that NHE3 is one of the NHE isoforms that undergoes extensive intracellular trafficking (Chow et al., 1999). Unfortunately, it was not possible to quantify NHE3b protein levels in zebrafish larvae or assess the localization of NHE3b within ionocytes owing to the unavailability of suitable antibodies. Development of such molecular tools would allow a better understanding of the functional significance of NHEs, in particular NHE3b, in Na^+ uptake by adult and larval zebrafish, and their dependence on environmental pH.

Interactions between ammonia excretion and Na^+ uptake.

Given the evidence supporting a role for NHE3b in Na^+ uptake in acidic water, the next objective was to determine how the apparent thermodynamic limitations associated with its function in acidic FW might be overcome. Considering the recent focus on possible interactions between Na^+ uptake and ammonia excretion through Rh proteins in FW fish (Tsui et al., 2009; Wu et al., 2010; Zimmer et al., 2010; for review see Wright and Wood, 2009), it was predicted that ammonia excretion via Rhcg1, an apical isoform of Rh protein co-localized with NHE3b and H^+-ATPase in zebrafish HR cells (Nakada et al., 2007a); for review on zebrafish ionocyte subtypes see (Hwang, 2009; Hwang and Perry, 2010) facilitates the function of NHE. To test this idea, zebrafish were challenged with two environmental conditions known to reduce the rate of ammonia excretion (HEA and increased buffering) and the consequences on Na^+ uptake were observed.

As reported previously (e.g. Wilson and Taylor, 1992), exposure to HEA caused a significant influx of ammonia during the 3 h measurement period, most likely owing to the imposed reversal of the P_{NH3} gradient (Fig. 3.2A). The abolition of ammonia excretion was accompanied by a significant reduction in Na^+ uptake in larvae reared in either neutral or acidic water (Fig. 3.2B). While it is attractive to argue that the observed reduction in Na^+ uptake is due to the disruption of an Rhcg1-NHE3b metabolon, it is puzzling that HEA treatment significantly reduced Na^+ uptake even in larvae reared in neutral water because they are insensitive to inhibition of NHE using EIPA. As an alternate explanation, it is possible that the observed inhibition was simply caused by competition between NH_4^+ and Na^+ for binding sites on a Na^+ channel-like protein. For example, the permeability ratio for NH_4^+ and Na^+ (pNH_4^+/pNa^+) through myelin cell epithelial Na^+ channels in *Rana pipiens* was 0.16 (Hille, 1971). Because the $[NH_4^+]/[Na^+]$ was approximately 6 in the present study, a Na^+ channel, if present, could conduct

NH_4^+ almost as effectively as Na^+. It should be noted, however, that the epithelial Na^+ channel responsible for Na^+ uptake in FW teleost has not yet been indentified, and therefore it is possible that teleost Na^+ channels have much higher selectivity for Na^+ than for other monovalent cations compared to mammalian homologs.

As an alternative, exposure of fish to water with increased buffering capacity was employed to reduce ammonia excretion. Previous studies in rainbow trout (Nawata and Wood, 2008) and zebrafish (Shih et al., 2008) demonstrated that external buffering reduced ammonia excretion. It has been theorized that buffering prevents the effective conversion of NH_3 to NH_4^+ within a "boundary layer" adjacent to the gill epithelium thereby raising external pNH_3 and reducing the gradient for NH_3 diffusion (Wright et al., 1989). On the other hand, by comparing the inspired and expired water pH, (Randall and Wright, 1989), concluded that when external water pH is below approximately 5, boundary layer pH is higher than the bulk water pH, as dehydration of HCO_3^- plays a more important role in determining the boundary layer pH (Randall and Wright, 1989). As the purpose of external buffering was to raise boundary layer pH and reduce the ammonia flux, in this experiment larvae were exposed to a buffered water of neutral pH, regardless of the rearing conditions.

Consistent with previous findings, external buffering significantly reduced ammonia excretion in larvae reared in both neutral and acidic water (Figs. 3.3A, C). In contrast, however, the effect of increased external buffering on Na^+ uptake was dependent on the rearing conditions. For larvae reared in neutral water, increased buffering did not affect Na^+ uptake (Fig. 3.3B) whereas exposure to buffered water reduced Na^+ uptake by about 50% in larvae reared in acidic water (Fig. 3.3D). The observation that reduction in ammonia excretion affects Na^+ uptake only

in the condition where NHE is proposed to be playing an important role is consistent with the idea of increased importance of ammonia-dependent Na^+ uptake in acidic water.

When the larvae reared in acidic water were treated with EIPA after acute transfer to highly buffered water, Na^+ uptake was further reduced, suggesting that NHE was still functional. This observation suggests either 1) an exchange of NH_4^+ and Na^+ mediated by NHE, as NH_4^+ excretion would not be hindered by external buffering and/or 2) the presence of the proposed metabolon, though to a lesser extent, as the larvae still managed to excrete some ammonia even in the buffered water. Regardless of the Na^+ uptake mechanism inhibited by EIPA treatment in acidic water, the results suggest a strong connection between ammonia excretion and Na^+ uptake in zebrafish larvae.

Gene knockdown suggests the involvement of an Rhcg1-NHE metabolon

The results of the HEA and increased buffering experiments simply demonstrated a linkage between ammonia excretion and Na^+ uptake in zebrafish larvae; a similar link has been reported in previous studies using other species (Maetz and Garcia Romeu, 1964; Salama et al., 1999; Wright and Wood, 1985; Wu et al., 2010). However, given the multiple mechanisms proposed for ammonia excretion (Weihrauch et al., 2009; Wilkie, 2002) and Na^+ uptake (Evans, 2008) in FW fish, the results from these experiments, alone, provide little information on the molecular nature of the proposed interaction. To provide insight into the molecular mechanism(s) of this interaction, the consequences of Rhcg1 knockdown on Na^+ uptake were assessed. This was accomplished by using a morpholino antisense oligonucleotide targeted against zebrafish Rhcg1 that was used previously (Braun et al., 2009a), where its effectiveness was confirmed by western blot analysis of Rhcg1 protein.

The effects of Rhcg1 knockdown on Na^+ uptake differed between the two rearing conditions. Specifically, Na^+ uptake was unaffected by knockdown in fish reared in control water whereas Rhcg1 knockdown reduced the rate of Na^+ uptake by almost 50% in fish raised in acidic water (Fig. 3.5B,D). This observation is in agreement with the contention that ammonia excretion facilitates Na^+ uptake only under acidic conditions, where it was hypothesized that fish are more reliant on NHE proteins. To further confirm that the function of NHE depends on the excretion of ammonia via Rhcg1, Rhcg1 morphants reared in acidic water were briefly transferred to control water and treated with 100 µM EIPA. EIPA treatment had no effect on the rate of Na^+ uptake in Rhcg1 morphants (Fig. 3.5D) suggesting that the component of Na^+ uptake normally inhibited by EIPA in acidic water (Fig. 3.1) is no longer functional in Rhcg1 morphants.

The results from acute transfer experiments (Figs. 3.4, 3.6B and 3.6C) further confirm the significance of Rhcg1 on Na^+ uptake in acidic water as well as the extreme plasticity of Na^+ uptake mechanisms in zebrafish. Within 6 h of transfer from acidic water to control conditions, the rate of Na^+ uptake was significantly reduced to a level that was roughly comparable to Na^+ uptake in larvae chronically raised in neutral pH (Fig. 3.4). Recently, using SIET (scanning ion electrode technique), it was demonstrated individual MR cells from euryhaline medaka larvae could alter their functions rapidly in responses to changes in external salinity (Shen et al., 2011). The rapid reduction in Na^+ uptake observed in this study might indicate a similar functional plasticity in zebrafish HRCs. It is also of interest to note that increases in Na^+ uptake following transfer to acidic water were observed only after 24 h of exposure (Fig. 3.6C), a significantly slower response in comparison to what was seen in larvae transferred from acidic to control pH (Figs. 3.4, 3.6B). Although changes in intracellular pH was not measured due to the small size

of larvae, it is possible that such long periods of acid-exposure were required to induce acidosis in those larvae, a response required to increase Na^+ uptake. Indeed, mobilization of NHE is a mechanism to restore the acidosis associated with hypercapnia treatment in fish (Edwards et al., 2005; Ivanis et al., 2008b). The response to acidosis might be one of the underlying causes for activating Na^+ uptake in acidic water. Further analysis on the acid-base balance of acid-exposed zebrafish would shed useful information on this possibility.

Taken together, the results of the present study clearly support the model proposed by Wright and Wood (2009) whereby an Rhcg1-NHE metabolon plays an important role in removing the thermodynamic constraints limiting the function of NHE.

Other mechanisms of Na^+ uptake in zebrafish

As shown in Fig. 3.6B, Rhcg1 morphants still exhibited a stimulation of Na^+ uptake, although to a lesser extent than in sham-injected larvae, following 24 h exposure to acidic water. This observation suggests that, although NHE is playing an important role in Na^+ uptake, there is likely to be an additional mechanism of Na^+ uptake in zebrafish larvae in acidic water. Because of the involvement of H^+-ATPase in acid excretion by HRCs in zebrafish larvae (Horng et al., 2007; Lin et al., 2006) and its proposed role in Na^+ uptake (Boisen et al., 2003; Horng et al., 2007), the possible contribution of H^+-ATPase to Na^+ uptake was assessed using bafilomycin A1, a specific inhibitor of V-type H^+-ATPase. Interestingly, treatment with bafilomycin reduced Na^+ uptake in acidic water to a greater extent than EIPA treatment (Fig. 3.7). Although this result appears contradictory to the suggested dominant role of NHE, it is possible that bafilomycin is inhibiting Na^+ uptake via a mechanism indirectly impeding NHE function in addition to its possible direct effect on Na^+ entry through channels electrically coupled to H^+-ATPase. Because bafilomycin indirectly inhibits ammonia excretion in zebrafish larvae in

circumneutral pH water (Shih et al., 2008) and given the demonstrated reliance of NHE on ammonia excretion, it is conceivable that bafilomycin is secondarily inhibiting Na^+ uptake via NHE. Indeed, in the present study, treating zebrafish larvae in acidic water with 1 µM bafilomycin reduced ammonia excretion by almost 50 %, implying the potential carryover effect of bafilomycin treatment on the metabolon model. To clearly distinguish the two potential effects of bafilomycin treatment on Na^+ uptake, it would be necessary to render the Na^+ channel associated with H^+-ATPase non-functional, and then subsequently treat such larvae with bafilomycin and assess the extent of inhibition on Na^+ uptake. Given that identity of such Na^+ channel in teleost fish remains unknown (Evans, 2008), such experiments are not possible at present. However, the near complete inhibition of Na^+ uptake in acidic water by bafilomycin strongly suggests that the majority of Na^+ uptake takes place through NHE and/or H^+-ATPase coupled Na^+ channel mechanisms. Indeed, when Na^+-Cl^- co-transporter, another mechanism of Na^+ uptake in some species of fish (Hiroi et al., 2008; Inokuchi et al., 2008) was inhibited with 1 µM metolazone (Wang et al., 2009), such treatment had no effect on Na^+ uptake by larvae either in low or control pH conditions. To test for the presence of a Na^+ uptake pathway through an ENaC-like channel, larvae reared in either control or low pH water were exposed to phenamil (a specific inhibitor of ENaC) at concentrations ranging from 1 to 100 µM. Treatment with phenamil had no effect on the overall Na^+ uptake rate, in disagreement with the results of Reid et al. (2003) who reported phenamil-sensitive Na^+ uptake in a subset of mitochondrion rich cells (MRCs) from rainbow trout gill. Given that an ortholog of ENaC has not yet been identified in any fish genome, it is possible that a Na^+ channel, if even present in zebrafish, is insensitive to phenamil.

Conclusions and Perspectives

The findings of this study provide new insight on the long-standing debate surrounding the mechanisms of Na^+ absorption in FW fish, and how ammonia excretion is coupled to Na^+ uptake, at least in acidic water. The contradictory results that have been reported in previous studies with respect to the link between Na^+ uptake and ammonia excretion may partly reflect the multiple mechanisms for ammonia excretion and Na^+ uptake combined with strong environmental effects favouring one or more of these processes.

Although the results of the present study strongly support the role of NHE3b in Na^+ uptake by zebrafish in acidic water through association with Rhcg1, a fundamental question remains; "why do fish rely on NHE in acidic water, where its function appears even more thermodynamically constrained than in neutral pH water?". Although not yet demonstrated experimentally, two explanations are possible; 1) a conserved physiological response to acidosis caused by exposure to acidic water, and 2) inefficiency of H^+-ATPase to create (and maintain) a sufficient electrical gradient to meet the increasing need for Na^+ uptake in acidic water. Furthermore, the molecular mechanisms that orchestrate the stimulation of Na^+ uptake during chronic acid-exposure in zebrafish are virtually unknown. Research is currently underway in our laboratory to describe the hormonal as well as molecular signalling cascades responsible for this increase in Na^+ uptake.

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CHAPTER 4
Cortisol regulates Na^+ uptake by zebrafish, *Danio rerio*, larvae via the glucocorticoid receptor.

Notes on Chapter

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YK conceived and performed experiments under supervision from SFP. DN and MMV provided assistance with western blotting. The manuscript was written by YK and edited by SFP.

Abstract

Unlike other freshwater fish previously examined, zebrafish are capable of increasing their rate of Na^+ uptake during chronic exposure to acidic water (pH 4). In the present study, the potential role of cortisol in the induction of Na^+ uptake during acid-exposure was investigated. When zebrafish larvae (4 days post-fertilization; dpf) were treated with waterborne cortisol, the rate of Na^+ uptake was significantly increased; this effect was blocked by co-incubating larvae with RU-486, an antagonist selective for the glucocorticoid receptor (GR). A similar induction in Na^+ uptake, which was also blocked by RU-486, was observed when larvae were treated with dexamethasone, a selective GR agonist. Conversely, treating larvae with aldosterone, a selective agonist for the mineralocorticoid receptor (MR) had no effect on Na^+ uptake. Acid-exposure increased whole body cortisol levels and translational knockdown of GR using antisense morpholinos prevented the full induction of Na^+ uptake during exposure to acidic water, further confirming the role of cortisol and GR in Na^+ uptake stimulation. Using immunohistochemistry, GR was localized to ionocytes known to be responsible for Na^+ uptake (HRCs). Knockdown of Rhcg1, an apical membrane ammonia channel or Na^+/H^+ exchanger 3b (NHE3b), proteins known to play an important role in facilitating Na^+ uptake in acidic water, prevented the stimulatory effects of cortisol treatment on Na^+ uptake, suggesting that cortisol regulates Na^+ uptake by stimulating an Rhcg1-NHE3b "functional metabolon".

Introduction

Cortisol, through its glucocorticoid and mineralocorticoid actions, plays an important role in regulating a wide range of physiological processes in fish including metabolism, immune responses, growth, reproduction and osmoregulation (Mommsen et al., 1999; Wendelaar Bonga, 1997). Although initially recognized as a key osmoregulatory hormone promoting acclimation to seawater (SW) in euryhaline teleosts (Hwang and Wu, 1993; for review see McCormick, 2001; McCormick and Bradshaw, 2006), there is increasing acknowledgement of its involvement in freshwater (FW) fish (Evans et al., 2005). For example, cortisol treatment was shown to stimulate Na^+, Cl^- and Ca^{2+} uptake (Laurent and Perry, 1990; Lin et al., 2011; Perry et al., 1992), while increasing the functional surface area of chloride cells (Bindon et al., 1994a; Bindon et al., 1994b; more recently referred to as ionocytes), specialized subtypes of branchial epithelial cells responsible for ion absorption in FW fish (Hwang, 2009; Hwang and Lee, 2007; Hwang and Perry, 2010; Hwang et al., 2011; Perry, 1997). Moreover, exogenous cortisol stimulates, either at the enzyme activity or protein/mRNA expression levels, the molecular transporters thought to be mediating ionic uptake, including H^+-ATPase (Lin and Randall, 1993), Na^+/H^+ exchanger 2 (Ivanis et al., 2008b), epithelial Ca^{2+} channel (Lin et al., 2011; Shahsavarani and Perry, 2006) and plasma membrane Ca^{2+}-ATPase (Flik and Perry, 1989). In addition, cortisol can influence gill cell permeability in several species (Chasiotis et al., 2010; Kelly and Chasiotis, 2011; Wood et al., 2002a) by regulating the expression of several tight junction associated proteins (Bui et al., 2010; Chasiotis et al., 2010; Tipsmark et al., 2009), and potentially affect the rate of passive loss of ions to the environment.

Although there is clear evidence for a role of cortisol in modulating osmoregulation by FW fish, the signalling pathways through which these effects are exerted remain more

controversial. Because aldosterone is lacking in fish (Colombo et al., 1972), it was traditionally assumed that cortisol acts through a single receptor which confers both "glucocorticoid" and "mineralocorticoid" functions in teleosts. With the discovery in rainbow trout of a mineralocorticoid receptor (MR) like gene (Colombe et al., 2000), as well as alternative hormones (e.g. 11-deoxycorticosteroid) that could potentially activate MR (Kiilerich et al., 2011b; Sturm et al., 2005), a significantly more complicated mechanism is emerging for corticosteroid signalling in teleosts (for recent reviews see Bury and Sturm, 2007; Prunet et al., 2006). Sloman et al. (2001) demonstrated that treating rainbow trout with the presumed MR antagonist, spironolactone, prevented branchial chloride cell proliferation following their exposure to soft water, suggesting a role for the MR in osmoregulation. Similarly, Scott et al. (2005a) demonstrated that spironolactone injections prevented the increase in gill mRNA expression levels of the α1a subunit of Na^+-K^+-ATPase in killifish, *Fundulus heteroclitus,* following FW transfer. However, GR also has been implicated in osmoregulation in FW fish. For example, in FW Atlantic salmon, *Salmo salar*, cortisol treatment is associated with increased mRNA expression of the α1a subunit of Na^+-K^+-ATPase, which is partially inhibited by RU-486 (Kiilerich et al., 2007; McCormick et al., 2008). Lin et al. (2011) demonstrated that knockdown of GR, but not MR, significantly reduced the whole body Ca^{2+} content of zebrafish larvae following transfer to a low Ca^{2+} environment. Using a pharmacological approach, Kelly and Chasiotis (2011) demonstrated a role for GR in influencing the expression levels of tight junction protein (occludin) and paracellular permeability in cultured trout gill cells. Clearly, the mechanism(s) by which cortisol affects various aspects of osmoregulation in FW fish is complex and likely dependent on environmental conditions and species.

Recently, it was demonstrated that zebrafish are able to increase their rate of Na^+ uptake when exposed to acidic water (pH 4), a response partially attributed to activation of a functional metabolon linking Na^+ uptake via NHE3b and ammonia excretion via Rhcg1, an apically distributed ammonia-conducting channel (Kumai et al., 2011; Kumai and Perry, 2011). Given that cortisol is known to 1) increase in zebrafish larvae at 3-4 days post fertilization (dpf) in response to external stressors such as handling and salinity challenge (Alderman and Bernier, 2009; Alsop and Vijayan, 2008), 2) stimulate ion uptake in FW fish (see above), 3) induce NHE3 expression or increase the surface activity of NHE3 in mammalian cells (Bobulescu et al., 2005; Kinsella et al., 1984) and rainbow trout kidney (Ivanis et al., 2008a), and 4) activate compensatory mechanisms facilitating acid-excretion in trout during metabolic acidosis (Gilmour et al., 2011), it was hypothesized that cortisol is partially responsible for inducing the rise in Na^+ uptake in acid-exposed zebrafish larvae.

Thus, the principal objective of the present study was to test the hypothesis that cortisol is responsible for increasing Na^+ uptake by zebrafish larvae, in particular under acidic conditions by stimulating NHE3b and if so, to determine whether the effect is mediated by GR or MR (or both). The results clearly demonstrated that increasing levels of cortisol stimulate Na^+ uptake in zebrafish larvae via the specific interaction of cortisol with GR. The results suggest a potentially widespread role of cortisol under a variety of stress-inducing environmental conditions, including low pH, in regulating Na^+ uptake by zebrafish.

Materials and Methods

Experimental animals and husbandry

Adult zebrafish (*Danio rerio* Hamilton-Buchanan 1822) were purchased from Big Al's Aquarium Services (Ottawa, ON, Canada) and kept in the University of Ottawa Aquatic Care Facility where they were maintained in plastic tanks supplied with aerated, dechloraminated City of Ottawa tap water at 28° C. Fish were subjected to a constant 14 h L:10 h D photoperiod and fed daily until satiation with No. 1 crumble-Zeigler^{TM} (Aquatic Habitats, Apopka, FL, USA). Embryos were collected following the standard method (Westerfield, 2000). Collected embryos were reared in 50 ml Petri dishes supplemented with either dechloraminated City of Ottawa tap water (pH 7.3-7.5) or acidified water (water pH was lowered to 3.9-4.0 by adding H_2SO_4 to the Ottawa tap water) supplemented with 0.05% ethylene blue. The Petri dishes were kept in incubators set at 28.5° C. Dead embryos were removed and water was changed daily. As all experiments were performed on 4 dpf fish, they were not fed for the duration of the experiment. The experiments were conducted in compliance with guidelines of the Canadian Council of Animal Care (CCAC) and after the approval of the University of Ottawa Animal Care Committee (Protocol BL-226). Unless otherwise stated, all chemicals used for this study were purchased from Sigma.

To test the role of cortisol on regulation of Na^+ uptake in zebrafish, the following series of experiments were performed.

Series 1. Effects of cortisol on Na^+ uptake and whole body Na^+ content

To determine whether Na^+ uptake is affected by cortisol, hatched 2 dpf larvae were transferred and maintained in zebrafish medium containing 500 nM cortisol (hydrocortisone) for 2 days. A comparable, physiologically relevant dose of cortisol was successfully used to induce physiological responses in previous studies on *Xenopus laevis* tadpole (500 nM; Yao et al., 2008)

and cell culture derived from rainbow trout gill (150 – 1500 nM; Chasiotis et al., 2010). Stock solutions were prepared by dissolving cortisol in dimethyl sulfoxide (DMSO); the concentration of DMSO never exceeded 0.1% in the final working solution. In all series of experiments, controls were performed in which DMSO, alone, was dissolved in water. To measure the rate of Na^+ uptake, 0.25 µCi ^{22}Na in the form of NaCl (Perkin Elmer, Woodbridge, ON, Canada) was added to each tube to a final activity of 0.15 µCi/ml. Water samples (50 µl) were collected at 5 min and 2 h after the addition of radioisotope. At the end of the 2 h flux period, larvae were killed with overdose of ethyl 3-aminobenzoate methanesulfonate (MS-222) and briefly washed in isotope-free water containing high levels of Na^+ (>200 mM) to remove any residual radioisotope attached to the surface of the fish. The remaining radioactive water in the tube was stored separately for later measurement of the total [Na^+]. For the processing of samples and calculation of influx rate, see "analytical methods and calculation" section below.

To measure the whole body Na^+ content, larvae were treated with waterborne cortisol as described above. When they reached 4 dpf, 10 larvae were pooled to yield one (1) sample for whole body ion content measurement. Larvae were killed with MS-222 overdose, briefly washed in ion-free water and digested in 1 N HNO_3 solution at 65° C. The digest was then supplemented with ion-free water and the Na^+ concentration was measured using a flame emission spectrophotometer (model AA260, Varian). The ion content was expressed as nmol/larvae.

Series 2 Effect of pharmacological manipulation of GR and MR on Na^+ uptake

To determine whether the effects of cortisol were being mediated by GR or MR (or both), 2 dpf larvae were administered the following treatments for 2 days; 1) 500 nM dexamethasone (a GR-selective agonist), 2) 500 nM dexamethasone combined with 1 µM RU-486 (a GR-selective

antagonist) , 3) 500 nM cortisol, 4) 500 nM cortisol combined with 1 µM RU-486, and 5) 500 nM aldosterone (Steraloids Inc. Newport, RI, USA). All chemicals were dissolved in DMSO and the rate of Na^+ uptake was measured at the end of the treatment using 4 dpf larvae.

To determine whether cortisol signalling was required to induce Na^+ uptake during exposure to acidic water, 2 dpf larvae were treated with either 1 µM RU-486 or 10 µM eplerenone (a selective MR antagonist; see Discussion for details) for 24 h in the control zebrafish medium. After the initial exposure to the drugs, larvae were transferred to acidified zebrafish medium supplemented with the same concentration of antagonists and kept there for another 24 h. A previous study showed that 24-h exposure to acidic water is sufficient to stimulate Na^+ uptake by zebrafish larvae (Kumai and Perry, 2011). In addition, two control groups were also monitored in which fish were exposed to DMSO for 48 h without any changes in water pH or exposed to DMSO for 48 h with the latter 24 h being spent in acidic water. In all groups Na^+ uptake was measured at the end of the treatment using 4 dpf larvae.

Series 3. Effect of GR knockdown on Na^+ uptake

To provide further evidence that GR is playing a significant role in stimulating Na^+ uptake, an oligonucleotide morpholino targeting the translation start site of zebrafish GR (5'-CTCCAGTCCTCCTTGATCCATTTTG-3') was designed by GeneTools (http://www.gene-tools.com/). The morpholino was prepared to a final concentration of 1 ng/nl in 1x Danieau buffer [58 mM NaCl, 0.7 mM KCl, 0.4 mM $MgSO_4$, 0.6 mM $Ca(NO_3)_2$, and 5.0 mM HEPES (pH 7.6)] and 0.05 % phenol red. Injections were performed using a microinjector system (model IM 300, Narishige, Long Island, NY). The same morpholino was successfully used to knockdown GR in a previous study (Nesan et al., 2012). Control groups were injected with a standard control morpholino (5-CCTCTTACCTCAGTTACAATTTATA-3) from GeneTools prepared as

the GR morpholino. Morpholino was injected at a dose of 2 ng/embryo at 1-2 cell stages. No significant mortality or deformities were observed in injected larvae.

Both control and GR morphants were raised in the control medium until 3 dpf, then directly transferred to acidified medium for 24 h. The Na^+ uptake rate was measured as described above. As a control, a separate group of larvae were kept in control medium until 4 dpf. The extent of knockdown was assessed by staining the sham and GR morphants using a GR antibody as described below.

Series 4. Whole-body cortisol measurements

4 dpf larvae raised in control pH water or exposed to acidic water for 24-h prior to sampling were killed by MS-222 overdose and 20 fish from each group were pooled and immediately frozen on dry ice to yield one (1) sample for cortisol extraction. Frozen samples were stored at – 80 ° C until cortisol extraction. To extract cortisol, each sample was homogenized in 3-ml of 2:1 chloroform-methanol. Samples were left at room temperature for 45 min. Subsequently, 1 ml of 2 M KCl-5 mM EDTA solution was added to each sample and vigorously mixed. After 15 min, the aqueous layer was collected and fluid was evaporated under a continuous flow of nitrogen gas. After the complete evaporation of the solution, precipitate was dissolved in 500 µl of 2-methoxyethanol and stored at -80° C. The cortisol concentration of the sample was measured using a commercial EIA kit (Cayman chemical, Burlington, ON) according to the manufacturer's instructions. Because it was not possible to measure the concentration of proteins in the extracted samples, the measured concentration was converted to pg cortisol/larva.

Series 5. Localization of GR by immunohistochemistry

To determine whether GR was expressed in HRCs, 4 dpf larvae were incubated with zebrafish medium containing 50µg/L of Alexa-488 conjugated concanavalin-A (conA; Invitrogen, Burlington, ON, Canada) for 30 min. ConA has been repeatedly used as a marker for HRCs in zebrafish larvae (Esaki et al., 2007; Lin et al., 2006). After incubation with conA, larvae were killed with overdose of MS-222 and fixed overnight at 4° C in a 4% paraformaldehyde solution prepared in PBS. After fixation, larvae were briefly rinsed in PBS and stored in 100% ethanol at – 20° C. Larvae were rehydrated in 50 and 100% PBS and subjected to heat induced antigen retrieval by heating at 65° C in 150 mM Tris buffer (set at pH 9) for 10 min. Subsequently, larvae were washed in PBS and incubated with 5% Triton-X mixed in PBS (PBST) with gentle shaking overnight at room temperature. Larvae were incubated with GR antibody (P-20; Santa Cruz Biotech, 1:100) overnight at 37 ° C in PBST. This antibody was raised against the C-terminal region of human GR (alpha subtype), which shares relatively high sequence identity (72%) with zebrafish GR. In addition, the same antibody was successfully used in a previous study to detect GR in zebrafish (Dickmeis et al., 2007; Lin et al., 2011). The specificity was confirmed by performing western blot on protein extract from larvae with the GR antibody (see below). In addition, pre-absorption of the antibody with the antigen peptide (Santa Cruz Biotech) completely prevented the staining in immunohistochemistry (data not shown). After several washes in PBS, larvae were incubated with alexa-546 conjugated anti rabbit secondary antibody (1:500, Invitrogen, Burlington, ON) for 1 h at 37 ° C and mounted onto a glass slide. Samples were observed using confocal microscopy (Leica ZM-510) and images acquired using Zen program (Zeiss).

To determine whether GR is also expressed in other type of ionocytes (for general discussion on zebrafish ionocyte subtypes, see Hwang, 2009; Hwang et al., 2011), larvae were

stained with both GR and Na^+-K^+-ATPase antibody (alpha 5, diluted 1:300 in PBST; Developmental Studies Hybridoma Bank, University of Iowa) using the identical protocol as for GR. Alpha 5 was labeled with alexa-488 conjugated anti mouse secondary antibody (Invitrogen, diluted 1:500 in PBS)

For western blotting, larval samples (n=25) were homogenized and then sonicated in 70 uL of 50 mM Tris buffer (pH 7.5) supplemented with protease inhibitors (Roche, Mannheim, Germany). Total protein concentration was determined using the bicinchoninic acid (BCA) method with bovine serum albumin as the standard. Samples were size-fractionated on 8% polyacrylamide gel and the proteins were transferred onto nitrocellulose membranes. Membranes were blocked with 5% skimmed milk in TBS-T (20 mM Tris, 300 mM NaCl and 0.1% Tween 20 with 0.02% sodium azide, pH 7.5) for 1-h at room temperature followed by an overnight incubation at 4 ° C with the same anti-GR primary antibody (1:500 dilution). Membranes were washed with TBS-T (3 × 10 min) and TBS (1 × 10 min) and incubated in secondary antibody (horseradish-peroxidase goat anti-rabbit IgG, 1:3636; Bio-Rad, Hercules, CA, USA) for 1 h at room temperature and washed as before. Band detection was carried out with the ECL-Plus Western blotting detection system (GE Healthcare Life Sciences, Ptscataway, NJ, USA) and the image was scanned using PharosFX imager (Bio-Rad) using chemiluminescence ECL-Plus settings.

Series 6. Assessing the mechanisms of action of cortisol on Na^+ uptake stimulation

Zebrafish were treated with waterborne cortisol as described in Series 1. Following the 2-day exposure to cortisol, larvae were treated with either DMSO as vehicle control or 1 µM bafilomycin A1 (LC Laboratories, Woburn, MA) dissolved in DMSO. Immediately after addition of inhibitor to the medium, ^{22}Na was added to measure the Na^+ uptake rate as described

above, with the flux period shortened to 1 h to minimize the potentially detrimental effects of bafilomycin treatment.

To assess the effects of cortisol on ammonia excretion, zebrafish were treated with waterborne cortisol as described in Series 1. However, as it was previously noted that DMSO significantly interfered with color development in the colorimetric ammonia assay (Kumai and Perry, 2011), cortisol was dissolved in ethanol, rather than DMSO. A vehicle control with ethanol was also performed. After 2-day exposure to cortisol, 3 larvae were placed in a 2-ml microtube (yielding N = 1). Water samples (500 µl) were collected at the beginning and end of the 3-h flux period and were immediately frozen at -20° C until later analysis.

A previous study (Kumai and Perry, 2011) showed that ammonia excretion by zebrafish larvae was significantly elevated in acidic water. To determine if the induction in ammonia excretion was under the control of cortisol, larvae were pre-treated with 1 µM RU-486 for 24-h prior to exposure to acidic water. RU-486 was dissolved in ethanol, and fish were pre-treated and exposed to acidic water as described in Series 2. The rate of ammonia excretion was determined in the following three groups; 1) vehicle control group chronically raised in control water (usual aquarium water pH); 2) vehicle control group exposed to pH 4 for 24 h and 3) a group treated with RU-486 for 48-h, with the latter 24-h in low pH water.

To determine whether cortisol was influencing Na^+ uptake by interacting with Rhcg1 and/or NHE3b, morpholinos against Rhcg1 (5'-TTGGTGTTTTTGACCATTTTTGATC-3') or NHE3b (5'-AGCTCAGTGACTGGAAAGAGAAATA-3') were injected into 1- or 2- cell stage embryos. Both morpholinos have been used previously with success (Braun et al., 2009a; Kumai and Perry, 2011). The Rhcg1 and NHE3b morpholinos were mixed with Danieau buffer to a final concentration of 4 ng/nl and injected at a dose of 4 ng/embryo (Kumai and Perry, 2011). A

control morpholino (see above) was injected (4 ng/embryo) into a separate group of fish. 24-h after injection, embryos were screened using a SMZ1500 microscope (Nikon Instruments, Melville, NY) for the presence of widely-distributed carboxyfluorescein. Only carboxyfluorescein-positive embryos were raised to 2 dpf in control medium. Upon reaching 2 dpf, fish were treated with waterborne cortisol as described in Series 1 and their rate of Na^+ uptake was measured.

Series 7. Assessment of cortisol treatment on gene expression of Rhcg1 and NHE3b.

Larvae were exposed to 500 nM cortisol as described above. Total RNA was extracted with TRIzol® (Invitrogen), following manufacturer's instructions. cDNA was synthesized by treating 1 µg of extracted RNA with DNase (Invitrogen) and RevertAid^{TM} M-MNuLV reverse transcriptase (Fermentas, Burlington, ON, Canada) according to the manufacturer's instructions. For the RT-qPCR, the following primers were used: NHE3b; forward 5'-TGCAGACAGCGCCTCTAGC-3' and reverse 5'-TGTGGCCTGTCTCTGTTTGC-3' (Yan et al., 2007) and Rhcg1; forward 5'-CTGTTTGGGATCACGCTGTA -3' and reverse 5'-CCCTTGAGATGGAGAGACCA -3' (Braun et al., 2009a). RT-qPCR was performed using Brilliant III SYBR Green Mastermix (Stratagene, La Jolla, CA) (10 µl final volume) with the following compositions: 5 µl Brilliant III SYBR Green Mastermix, appropriate concentration of primers (100 nM for Rhcg1, 150 nM for 18S and 50 nM for NHE3b), cDNA template (1 µl for Rhcg1 and NHE3b and 0.5 µl for 18S) and sterile water. The thermal profile for PCR reactions was as follows; initial denaturation at 95° C for 3 min followed by 40 cycles of 95° C for 20 s, 58° C for 20 s min (MXPro software ver. 4.10 (Stratagene). Following the 40 cycles, SYBR green dissociation curves were used to confirm the amplification of a single product. The expression level of NHE3b was normalized to that of 18S using a modification of the ΔΔCt

method (Pfaffl, 2001). 18S was chosen as the normalizing gene because it has been successfully used as a standard for zebrafish under various experimental treatments (McCurley and Callard, 2008). For 18S, the following primers were used; forward 5'-GGCGGCGTTATTCCCATGACC-3' and reverse 5'- GGTGGTGCCCTTCCGTCAATTC-3'.

Series 8. Assessing the effects of cortisol in larvae lacking HRCs.

To determine whether cortisol is able to target more than one type of ionocyte known to function in Na^+ uptake, the formation of HRCs was prevented by injecting morpholino against a transcription factor, *glial cell missing 2* (*gcm-2*), which plays a crucial role in regulating the formation of HRCs in zebrafish (Chang et al., 2009; Shono et al., 2011). Na^+ uptake is maintained (and even increased) in *gcm-2* morphants owing to an increasing contribution from NCCCs (Shono et al., 2011). The morpholino against *gcm-2* was designed and injected as previously reported, with the exception that the morpholino was injected at 1 ng/embryo. A separate sham injected group, which was injected with 1 ng of control morpholino, was also evaluated. The successful knockdown was confirmed by staining the larvae with conA as described above.

Total RNA was extracted and cDNA synthesized from 4dpf sham and *gcm-2* morphants as described above. RT-qPCR for GR was performed using the following primer sets: forward: 3'-CCGGTGTTCTCCTGTTTGAT-5' and reverse: 3'-ACAGCTTCTTCCAGCCTCAG-5' (Lin et al., 2011), with 18S as the normalizing gene. The PCR sample composition and thermal cycling profile was the same as Rhcg1.

To assess whether *gcm-2* morphants are still capable of responding to exogenous cortisol, sham and *gcm-2* morphants were treated with 500 nM cortisol and their Na^+ uptake was measured at 4 dpf as described above.

Analytical methods and calculations

To determine Na^+ uptake, all collected water samples were supplemented with 5 ml of scintillation cocktail (Biosafe-II, RPI corp, Mt. Prospect, IL, USA) and their radioactivity was measured with a liquid scintillation counter (model LS-6500 Beckman Coulter, Co. Mississauga ON, Canada). After being rinsed in an isotope-free medium, larvae were digested in a tissue solubilizer (Solvable^{TM}, Perkin Elmer) overnight at 65° C. After complete digestion, samples were supplemented with 10-ml of the same scintillation cocktail. Samples were then neutralized by adding 450 µl of glacial acetic acid before measuring their radioactivity. The concentration of total Na^+ in the water was measured using flame emission spectrophotometry (model AA260, Varian, Palo Alto, CA, USA). Owing to the limited volume of water, [Na^+] was measured, and hence external specific activity was determined, only at the end of the flux period. It was assumed that, given the typical [Na^+] of the experimental water (700 - 1000 µM), Na^+ influx rate (on the scale of 1 nmol/fish/h or less) and even smaller expected net flux of Na^+ (difference between influx and efflux), changes in total [Na^+] during the flux period would be negligible. The rate of Na^+ uptake (J_{in}^{Na} , pmol/fish/h) was calculated as follows:

$$J_{in}^{Na} = \frac{F}{SA \cdot n \cdot t}$$

, where F = total incorporated radioactivity (DPM, disintegration per minute), SA = specific activity of the medium (DPM/pmol), n = number of larvae (typically 1) and t = the duration of the incubation (h). DPM was calculated by the liquid scintillation counting program after taking quenching and counting efficiency into consideration. Ammonia excretion was determined colorimetrically at an absorbance of 650 nm (Verdouw et al., 1978) using a modified 96-well microplate assay. Ammonia excretion (J_{out}^{Amm} , nmol/fish/h) was calculated as:

$$J_{out}^{Amm} = \frac{([Amm_F] - [Amm_I])V}{t}\frac{1}{n}$$

, where n = number of larvae in the tube and $[Amm]_F$ and $[Amm]_I$ = the measured concentration of ammonia (μM) at the end and the beginning of the flux period, V = volume of water in the microtube (L) and t = duration o the flux period (h).

Statistical analysis

All statistical analyses were performed with SigmaPlot (v. 11, Systat Inc. Chicago, IL, USA). Student's *t*-test was used to analyze data from Series 2.1, 2.3 (the effect of aldosterone treatment), 2.4, 2.6 (the effect of cortisol treatment on ammonia excretion), 2.7 and series 2.8 (RT-qPCR data). One-way ANOVA followed by Tukey post hoc test was used to analyze data from Series 2.2 and 2.6 (effect of bafilomycin on cortisol-treated larvae, effect of RU-486 treatment on ammonia excretion). Two-way ANOVA followed by Tukey post hoc test was used to analyze data from Series 2.4, 2.6 and 2.8 (knockdown experiments). When assumptions of normality or equal variance were violated, data were transformed using natural log- or square-root transformation. For all analyses, the level of statistical significance was set at $p<0.05$.

Results

Series 1 Effect of cortisol on Na^+ uptake and whole body content

Exposure to 500 nM cortisol for 2 days significantly increased Na^+ uptake (N = 6; Fig. 4.1 A; Student's t-test). The cortisol treatment also significantly elevated the whole body Na^+ content (N = 7; Fig. 4.1B; Student's t-test).

Series 2 Effect of pharmacological manipulation of GR and MR on Na^+ uptake

Exposure to 500 nM dexamethasone increased Na^+ uptake (N = 6; Fig. 4.2A; one-way ANOVA). However, when the dexamethasone treatment was combined with 1 µM RU-486, the stimulatory effect of dexamethasone was prevented (N = 6; Fig. 4.2A; one-way ANOVA). Treating the larvae with 1 µM RU-486 also eliminated the stimulation of Na^+ uptake observed in cortisol-treated larvae (N = 6; Fig. 4.2B; one-way ANOVA). However, treating larvae with 500 nM aldosterone, a selective MR agonist, was without effect on Na^+ uptake (N = 5 - 6; Fig. 4.2C; Student's t-test).

Na^+ uptake was significantly elevated in acid-exposed zebrafish, a response which was abolished in the presence of RU-486 (N = 6 - 12; Fig. 4.3A; one-way ANOVA). The stimulatory effect of acidic water on Na^+ uptake was unaffected by 10 µM (and up to 30 µM; data not shown) eplerenone (N = 6 - 12; Fig. 4.3B; one-way ANOVA) or 10 µM spironolactone (data not shown).

Series 3 Effect of GR knockdown on Na^+ uptake

There was no effect of GR knockdown on Na^+ uptake in zebrafish larvae reared in control medium (N = 6; Fig. 4.4A; two-way ANOVA). Similar to Series 2, exposure to acidic water significantly induced Na^+ uptake (N = 6; Fig. 4.4A; two-way ANOVA). However, the stimulation of Na^+ uptake, though still observed, was significantly diminished when GR

morphants were exposed to acidic water (N = 6; Fig. 4.4A; two-way ANOVA). Sham and GR-morphants were stained with GR antibody to assess the effectiveness of GR knockdown. GR primarily stained cells on the yolk sack (Fig. 4.4B; see below for the discussion on specific localizations), whereas no staining was seen in GR morphants, suggesting that the injection of GR morpholino was indeed successful at preventing the expression of GR in those larvae (Fig. 4.4C).

Series 4 Whole-body cortisol measurements

Whole body cortisol levels were significantly higher in larvae exposed for 24 h to low pH water than in the control group (N = 6 - 8; Fig. 4.5; Student's t-test).

Series 5 Localization of GR by immunohistochemistry

Immunohistochemistry results revealed a strong colocalization of GR with conA positive HRCs (Figs. 4.6A, B). ConA stains the apical opening of HRCs, whereas the staining of GR appeared to be spread throughout the cytosol. In contrast, GR was not colocalized with Na^+-K^+-ATPase rich cells (NaRC) (Fig. 4.6C, 4.6D), suggesting that GRs are likely to be expressed only very weakly, if at all, in NaRCs of zebrafish larvae. In agreement with previous studies (Dickmeis et al., 2007; Lin et al., 2011), the GR antibody detected ~90 kDa band in western blot (Fig. 4.6E).

Series 6 Assessing the mechanisms of action of cortisol on Na^+ uptake stimulation

When the cortisol-treated larvae were briefly exposed to bafilomycin A1, a specific inhibitor of H^+-ATPase, the cortisol-induced stimulation in Na^+ uptake was prevented (N = 6; Fig. 4.7; one-way ANOVA).

Ammonia excretion was significantly elevated in larvae treated with cortisol (N = 6; Fig. 4.8A; Student's t-test). In agreement with a previous study (Kumai and Perry, 2011), the

ammonia excretion in acid-exposed larvae was significantly elevated (N = 6; Fig. 4.8B; one-way ANOVA). However, the stimulation of ammonia excretion was inhibited by pre-treating the larvae with RU-486 (N = 6; Fig. 4.8B; one-way ANOVA). Although knockdown of Rhcg1 or NHE3b did not affect basal rates of Na^+ uptake (N = 5 - 7; Figs. 4.9A, B; two-way ANOVA), which also agrees with a previous study (Kumai and Perry, 2011), the morphants were no longer responsive to cortisol treatment (N = 5 - 7; Figs. 4.9A, B; two-way ANOVA). In agreement with previous studies (Braun et al., 2009a; Shih et al., 2008), ammonia excretion by Rhcg1 morphants was significantly lower than sham-injected larvae, suggesting the effective knockdown of Rhcg1 (data not shown).

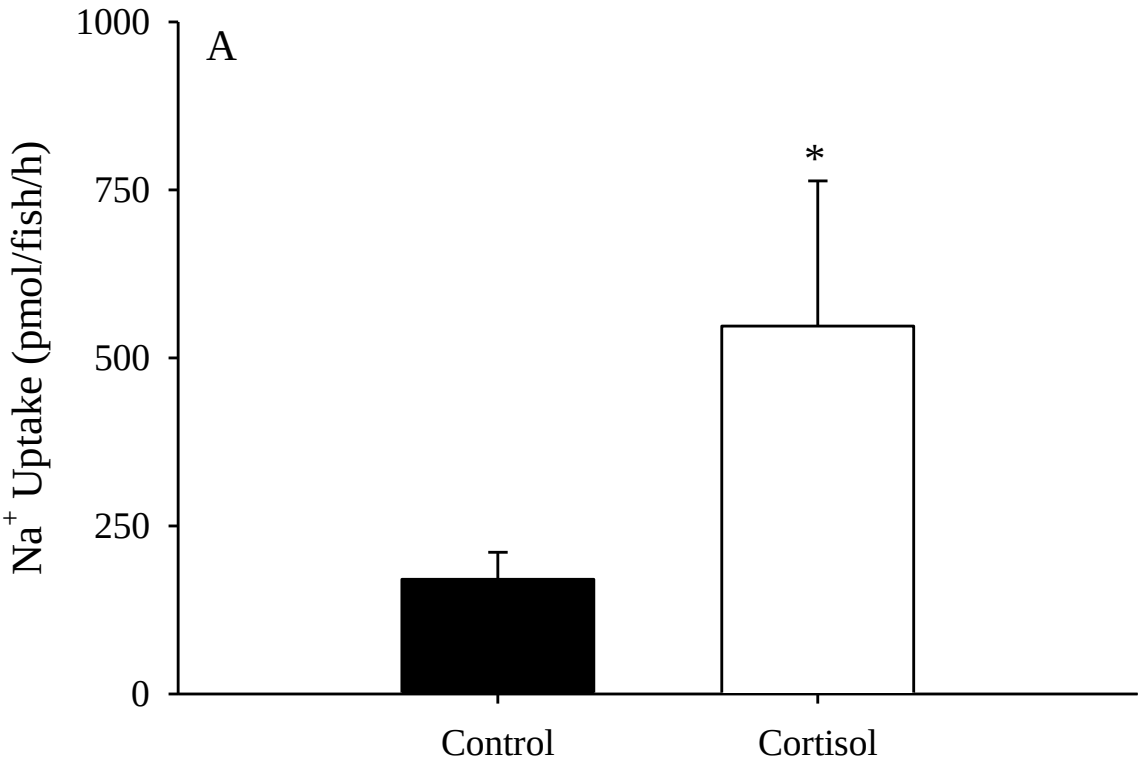
Series 7. Cortisol effect on gene expression of Rhcg1 and NHEb.

Treatment with 500nM cortisol did not affect the gene expression of Rhcg1 nor NHE3b (N = 8 - 10; Fig. 4.10; Student's t-test).

Series 8. Assessing the effects of cortisol in larvae lacking HR ionocytes.

As reported previously (Chang et al. 2009; Shono et al., 2011), *gcm-2* knockdown led to a considerable reduction in the total number of conA positive HRCs (Figs. 4.11A, B; representative images from morpholino injection experiments performed on two separate occasions). The mRNA expression of GR was significantly reduced in *gcm-2* morphants (N = 4 - 6; Fig. 4.11C; Student's t-test). Na^+ uptake was significantly stimulated in *gcm-2* morphants (see also Shono et al. 2011), however cortisol treatment of *gcm-2* morphants failed to induce further stimulation of Na^+ uptake (N = 5 - 6; Fig. 4.11D; two-way ANOVA).

Figure 4.1. The effect of waterborne cortisol treatment on Na^+ balance. Treating 2 dpf larvae for 2 days with 500 nM cortisol significantly increased whole body Na^+ uptake (N = 6; Fig 4.1A) and whole body Na^+ content (N = 7; Fig 4.1B) at 4 dpf. An asterisk denotes significant difference from the control group. Data are presented as means ± SEM.


A
1000
750
500
250
0
Na^+ Uptake (pmol/fish/h)
*
Control
Cortisol

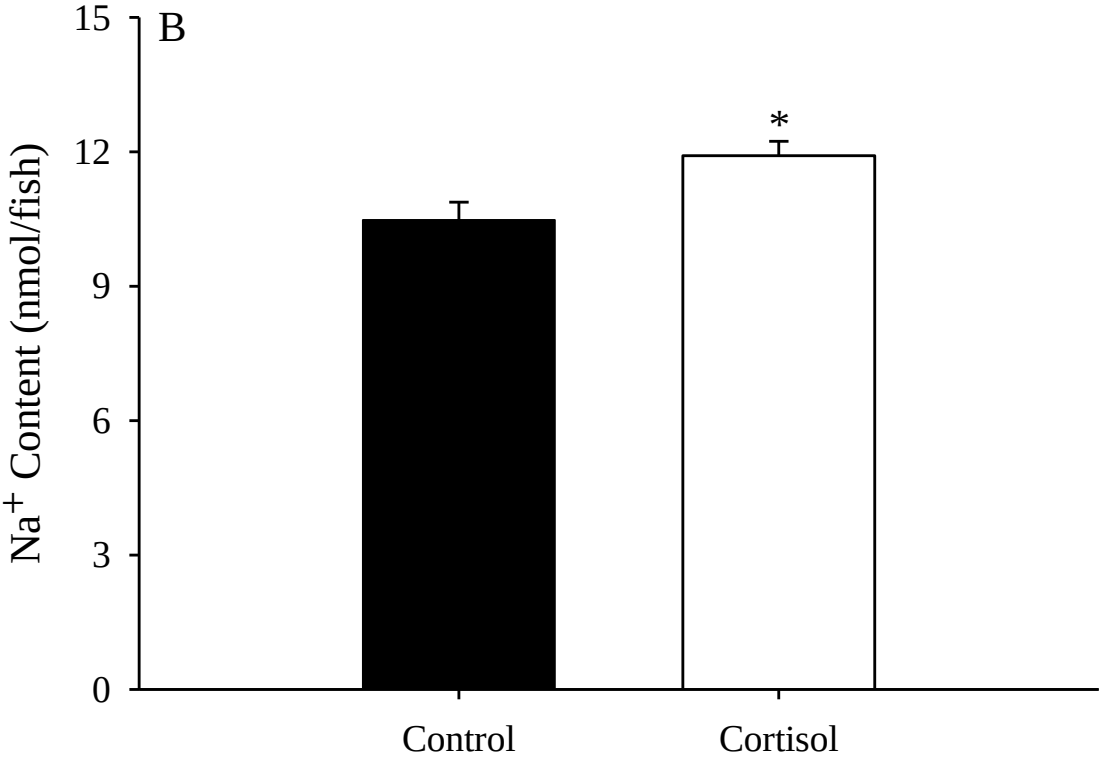

B
15
12
9
6
3
0
Na^+ Content (nmol/fish)
*
Control
Cortisol

Figure 4.2. The effects of GR and MR agonists and antagonists on Na^+ uptake. Treating 2 dpf larvae for 2 days with 1 μM dexamethasone (dex) significantly increased Na^+ uptake; co-treating the larvae with RU-486 (RU) prevented the stimulatory effect of dexamethasone (N = 6; Fig. 4.2A). RU-486 also prevented the stimulatory effect of cortisol treatment on Na^+ uptake (N = 6; Fig. 4.2B). On the other hand, treating larvae with 500 nM aldosterone had no effect on Na^+ uptake (N = 5 - 6; Fig. 4.2C). Data are presented as means ± SEM. Different letters denote significant difference between treatment groups.

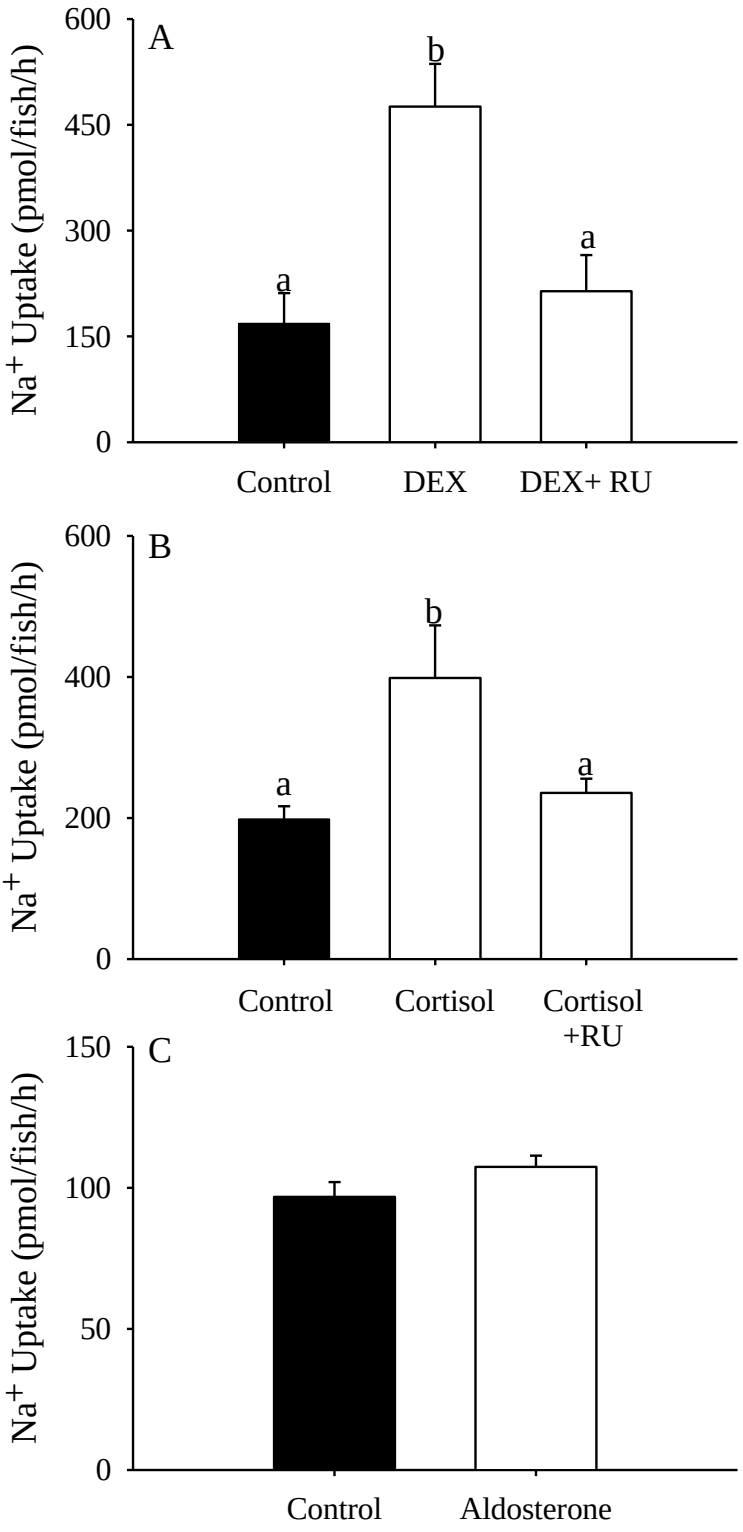

A
600
450
300
150
0
Na+ Uptake (pmol/fish/h)
a
b
a
Control
DEX
DEX+ RU
B
600
400
200
0
Na+ Uptake (pmol/fish/h)
a
b
a
Control
Cortisol
Cortisol +RU
C
150
100
50
0
Na+ Uptake (pmol/fish/h)
Control
Aldosterone

Figure 4.3. Effect of low pH-exposure on Na^+ uptake in the presence of GR and MR antagonists. Prior exposure of zebrafish larvae to low pH water (pH 4.0) for 24 h significantly increased whole Na^+ uptake at 4 dpf (N = 6 - 12; Figs. 4.3A, B). When the larvae were exposed to acidic water in the presence of 1 µM RU-486 (RU) , the induction of Na^+ uptake was prevented (N = 6 - 12; Fig. 4.3A). On the other hand, when zebrafish larvae were exposed to acidic water in the presence of 10 µM eplerenone (EP), the usual stimulation of Na^+ uptake was observed (N = 6 - 12; Fig. 4.3B). Data are presented as means ± SEM. Different letters denote significant difference between treatment groups.

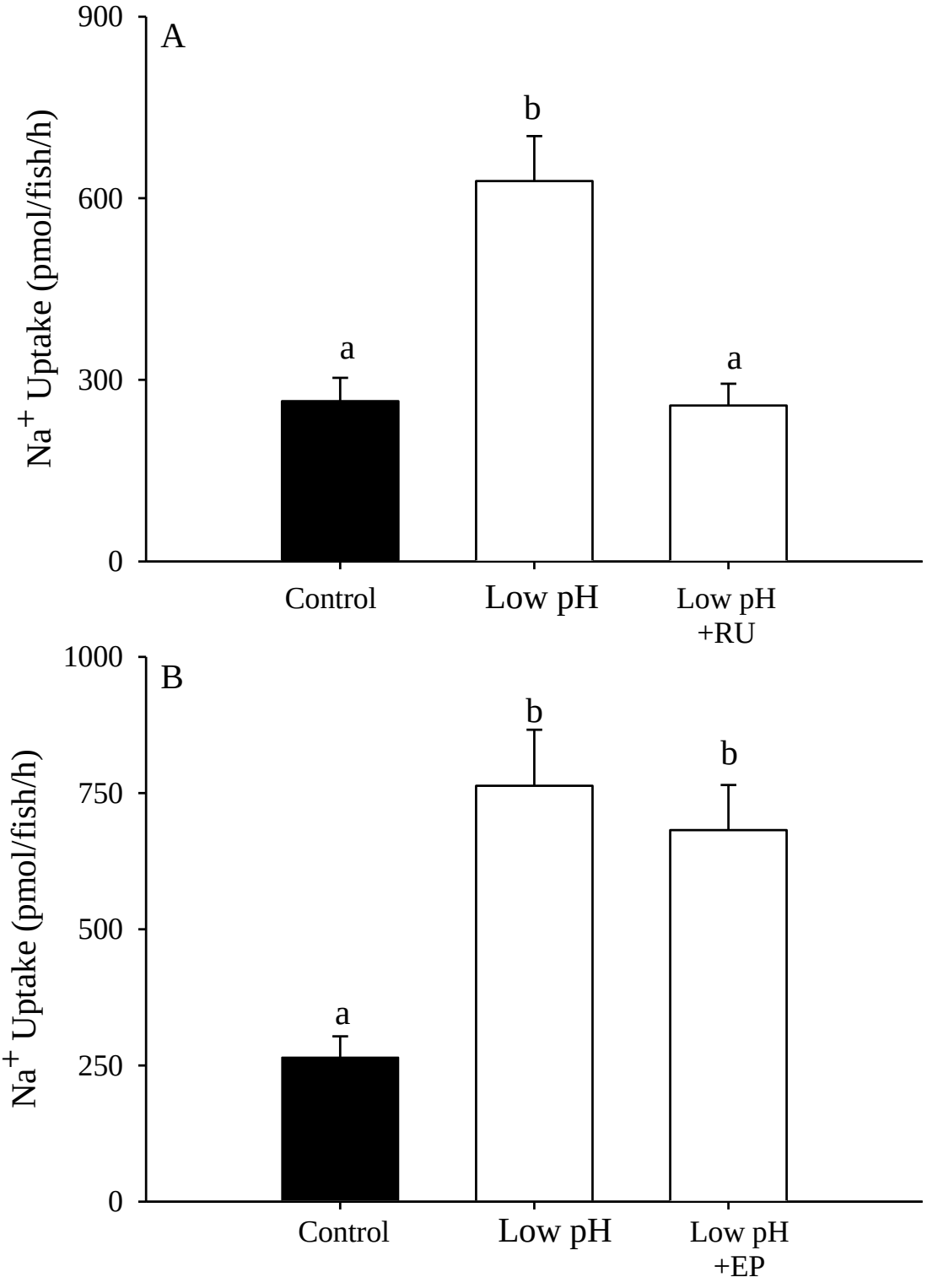

A
900
600
300
0
Na^+ Uptake (pmol/fish/h)
a
b
a
Control
Low pH
Low pH
+RU
B
1000
750
500
250
0
Na^+ Uptake (pmol/fish/h)
a
b
b
Control
Low pH
Low pH
+EP

Figure 4.4. The effects of exposure to low pH water on Na^+ uptake in GR morphants. GR knockdown did not have any effect on Na^+ uptake by zebrafish larvae maintained under normal pH conditions (N = 6; Fig. 4.4A). However, while not eliminated entirely, the stimulation of Na^+ uptake following 24-h exposure to low pH water was significantly attenuated in the GR morphants (N = 6; Fig. 4.4A). Data are presented as means ± SEM. Different letters denote significant difference between treatment groups. The effectiveness of GR knockdown was confirmed by demonstrating via immunocytochemistry a marked reduction in the number of GR-positive cells on the yolk sac in the GR-MO's (Fig. 4.4C) in comparison to the control MO's (Fig 4.4C). Scale bars = 200 µm (Figs 4.4B, C).

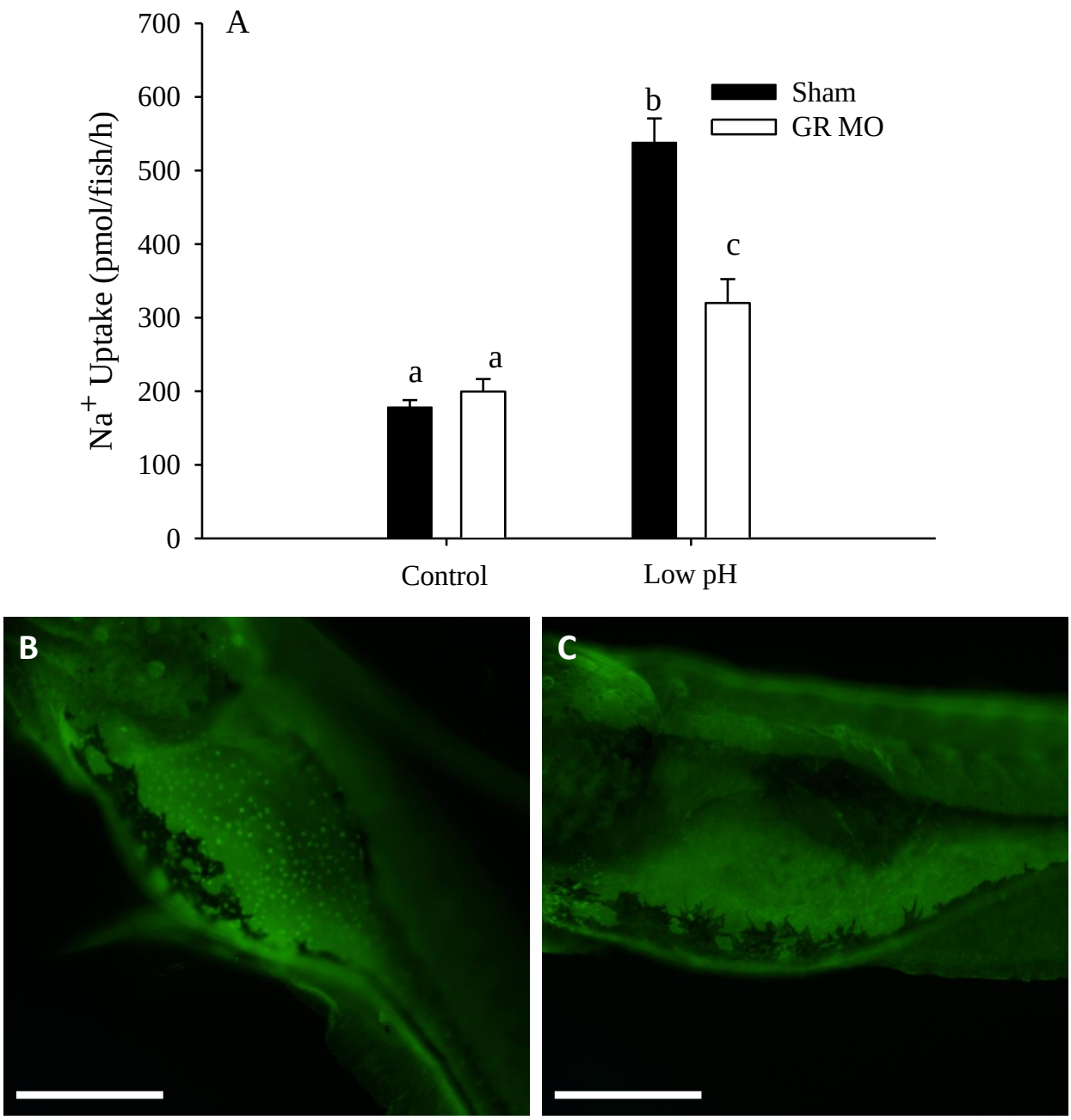

A
700
600
500
400
300
200
100
0
Na+ Uptake (pmol/fish/h)
Sham
GR MO
a
a
b
c
Control
Low pH
B
C

Figure 4.5. The effect of exposure to low pH water on whole body cortisol levels. Whole body cortisol levels at 4 dpf were significantly higher in larvae exposed to low pH water (pH 4.0) for 24 h. An asterisk indicates a statistically significant difference from the control group. N = 6 - 8. Data are presented as means ± SEM.

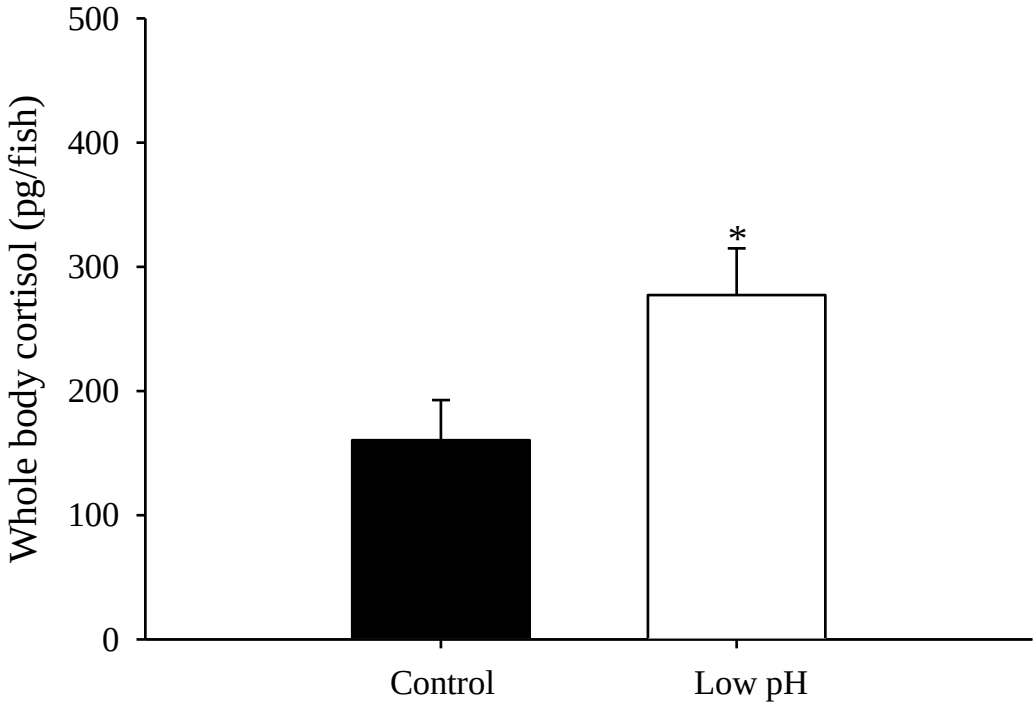

500
400
300
200
100
0
Whole body cortisol (pg/fish)
*
Control
Low pH

Figure 4.6. Localization of GR on yolk sack ionocytes in larval zebrafish. 4 dpf zebrafish larvae were stained with conA (green, a vital dye for HRCs) and GR (red, denoted with asterisks). The majority of GR-positive cells were also stained with conA, suggesting that HRCs are enriched with GR (Figs. 4.6A, B). On the other hand, no obvious expression of GR (red, denoted with asterisks) was observed in NaR cells (green; stained with Na^+/K^+-ATPase antibody, alpha 5, denoted with arrow; (Figs. 4.6C, D). This commercial antibody successfully recognized a corresponding sized band (~ 90 kDa) on western blotting (Fig. 4.6E). Scale bars: 200 μm for Figs. 4.6A, C and 50 μm for Figs. 4.6B, D.

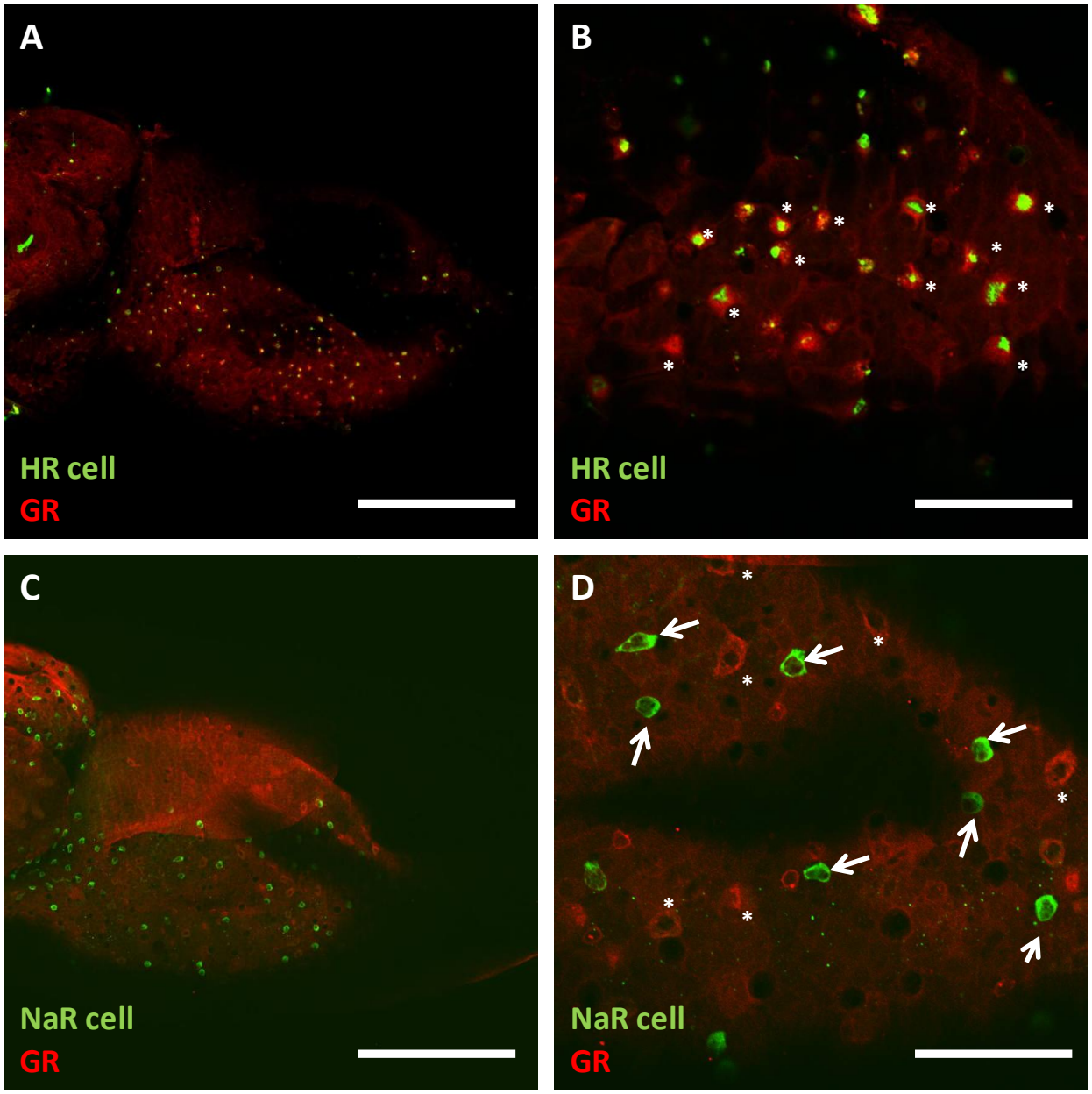

A
HR cell
GR
B
HR cell
GR
C
NaR cell
GR
D
NaR cell
GR
E
3dpf
4dpf
~90 kDa

Figure 4.7. The effect of cortisol treatment on H^+-ATPase mediated Na^+ uptake. Treatment of 4dpf zebrafish larvae with bafilomycin (baf) following 2-day exposure to cortisol significantly reduced Na^+ uptake. Different letters denote significant difference between treatment groups. N = 6. Data are presented as means ± SEM

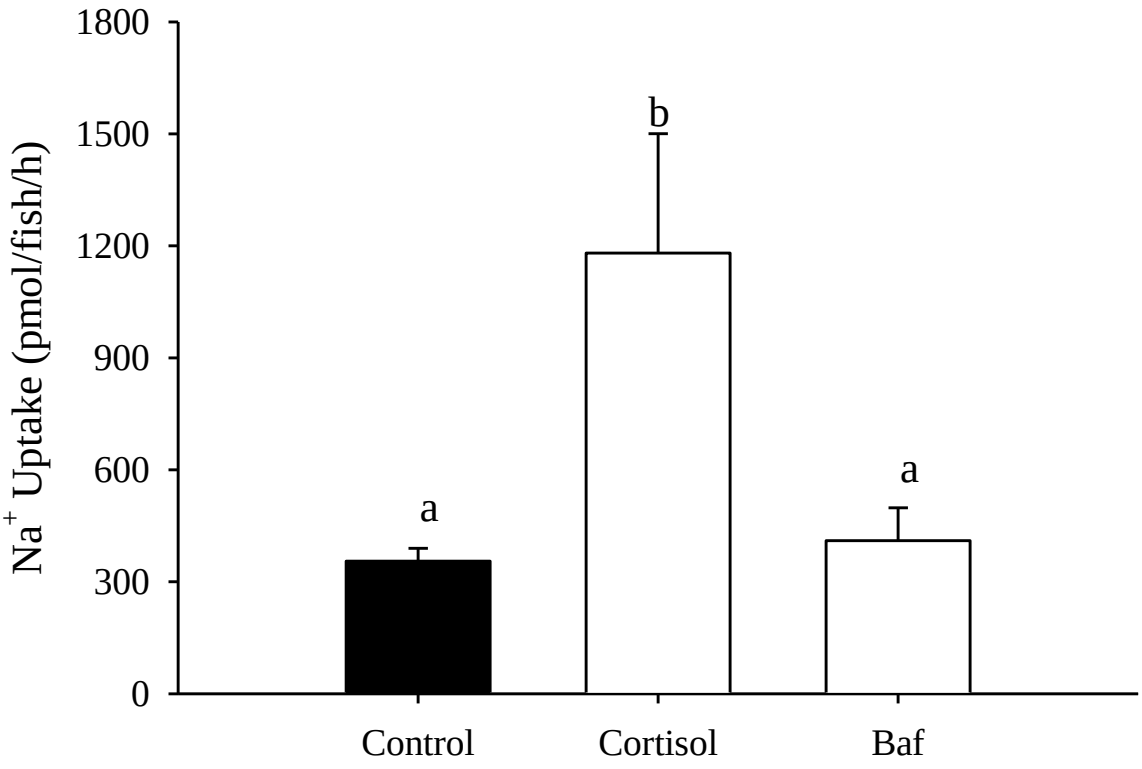
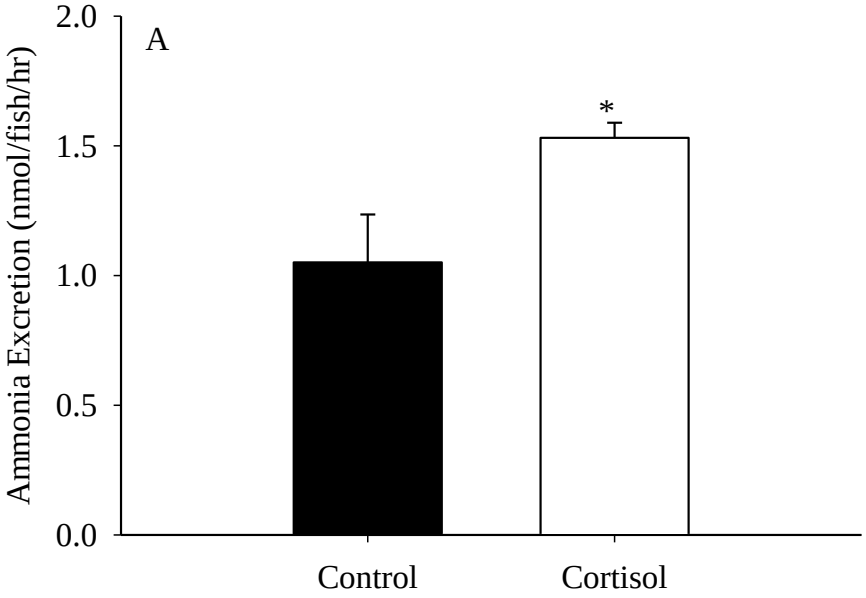

1800
1500
1200
900
600
300
0
Na+ Uptake (pmol/fish/h)
a
b
a
Control
Cortisol
Baf

Figure 4.8. The effect of cortisol treatment and exposure to low pH water on ammonia excretion. Cortisol treatment significantly increased ammonia excretion (N = 6; Fig. 4.8A). In addition, a 24-h acid exposure also stimulated ammonia excretion (N = 6; Fig. 4.8B). This stimulatory effect of low pH-exposure on ammonia excretion was inhibited by pre-treating the larvae with 1 μM RU-486 (RU), a potent antagonist for GR (N = 6; Fig. 4.8B). An asterisk in Fig. 4.8A indicates significant difference from the control group. Different letters in Fig. 4.8B denote significant difference between groups. Data are presented as means ± SEM


A
Ammonia Excretion (nmol/fish/hr)
2.0
1.5
1.0
0.5
0.0
*
Control
Cortisol

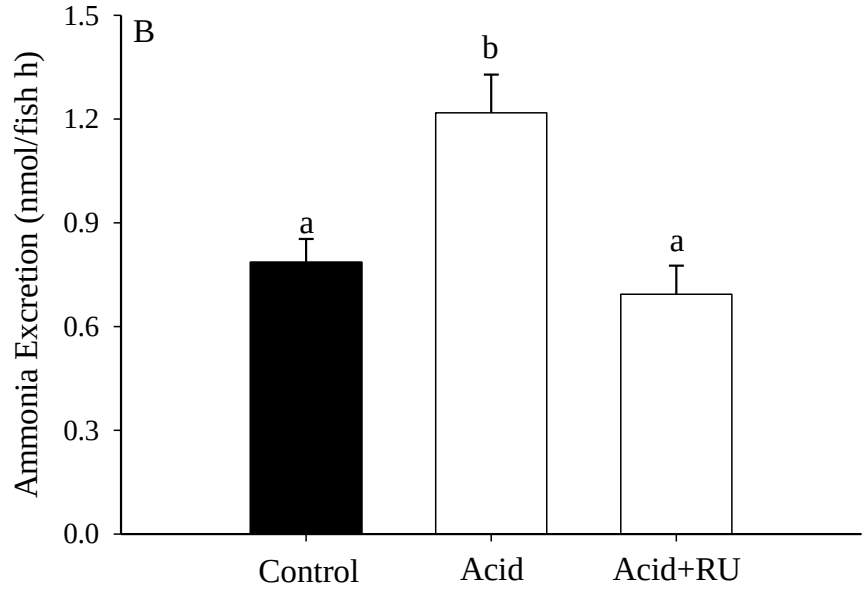
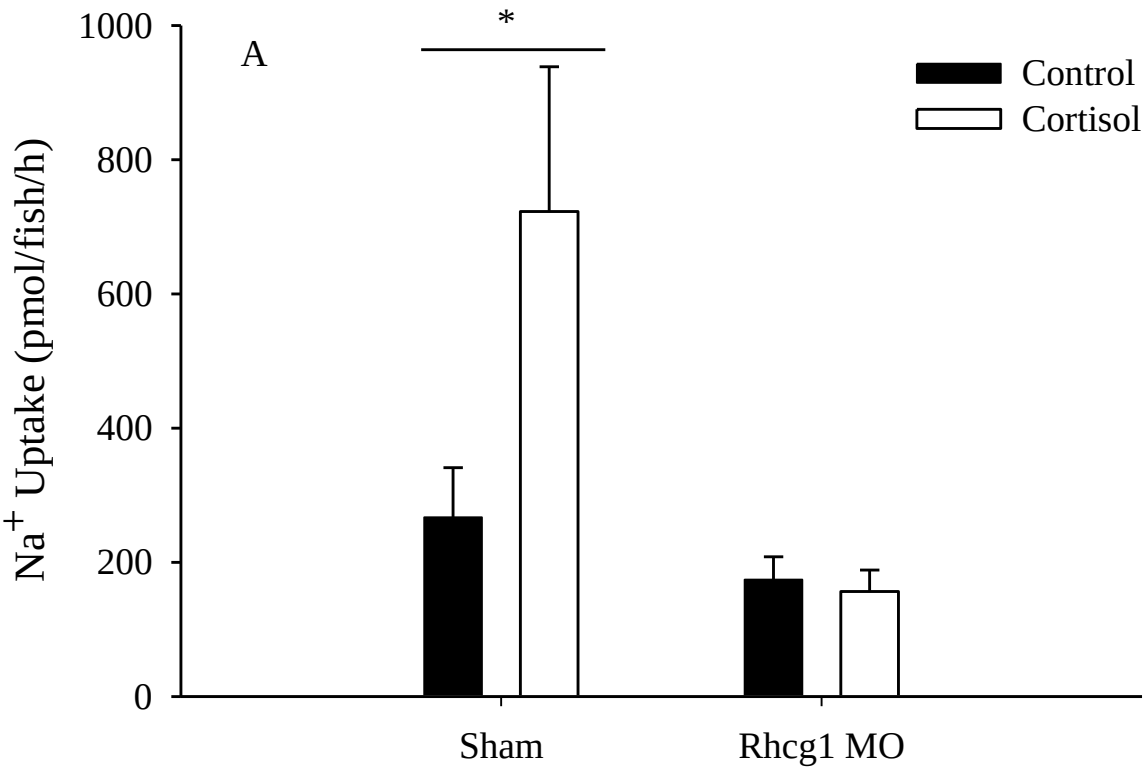

B
Ammonia Excretion (nmol/fish h)
1.5
1.2
0.9
0.6
0.3
0.0
a
b
a
Control
Acid
Acid+RU

Figure 4.9. The effect of cortisol treatment on Na^+ uptake in Rhcg1 and NHE3b morphants. Treating Rhcg1 morphants (N = 5 - 6; Fig. 4.9A) or NHE3b morphants (N = 6 - 7; Fig. 4.9B) with 500 nM cortisol for 48 h had no effect on their Na^+ uptake at 4 dpf. Asterisks denote significant differences between control and cortisol-treatment groups within sham and morphants. Data are presented as means ± SEM.


A
*
Control
Cortisol
1000
800
600
400
200
0
Na+ Uptake (pmol/fish/h)
Sham
Rhcg1 MO

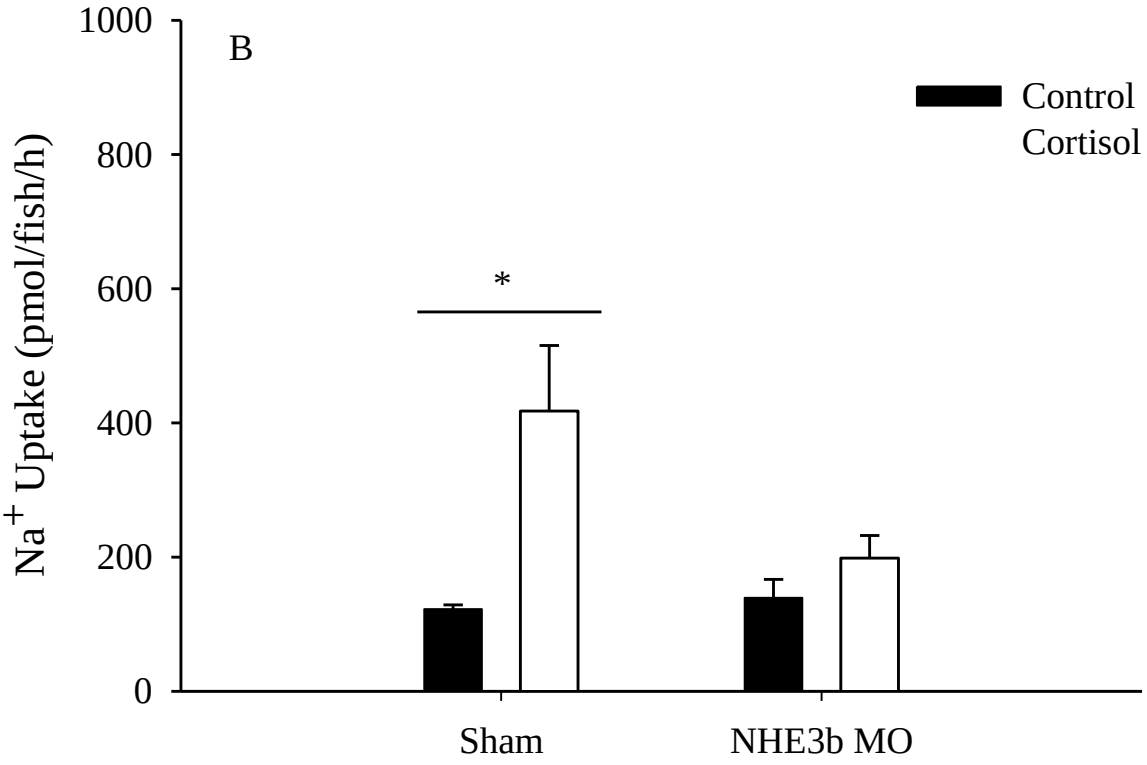

B
Control
Cortisol
*
1000
800
600
400
200
0
Na+ Uptake (pmol/fish/h)
Sham
NHE3b MO

Figure 4.10. The effect of cortisol treatment on Rhcg1 and NHE3b mRNA expression.

Treating larvae with 500 nM cortisol for 2 days (starting at 2dpf) did not significantly affect the mRNA expression level of Rhcg1 (N = 8 - 10; Fig 4.10A) or NHE3b (N = 8 - 10; Fig 4.10B). The expression level of genes of interest was normalized to that of 18S. Data are presented as means ± SEM.

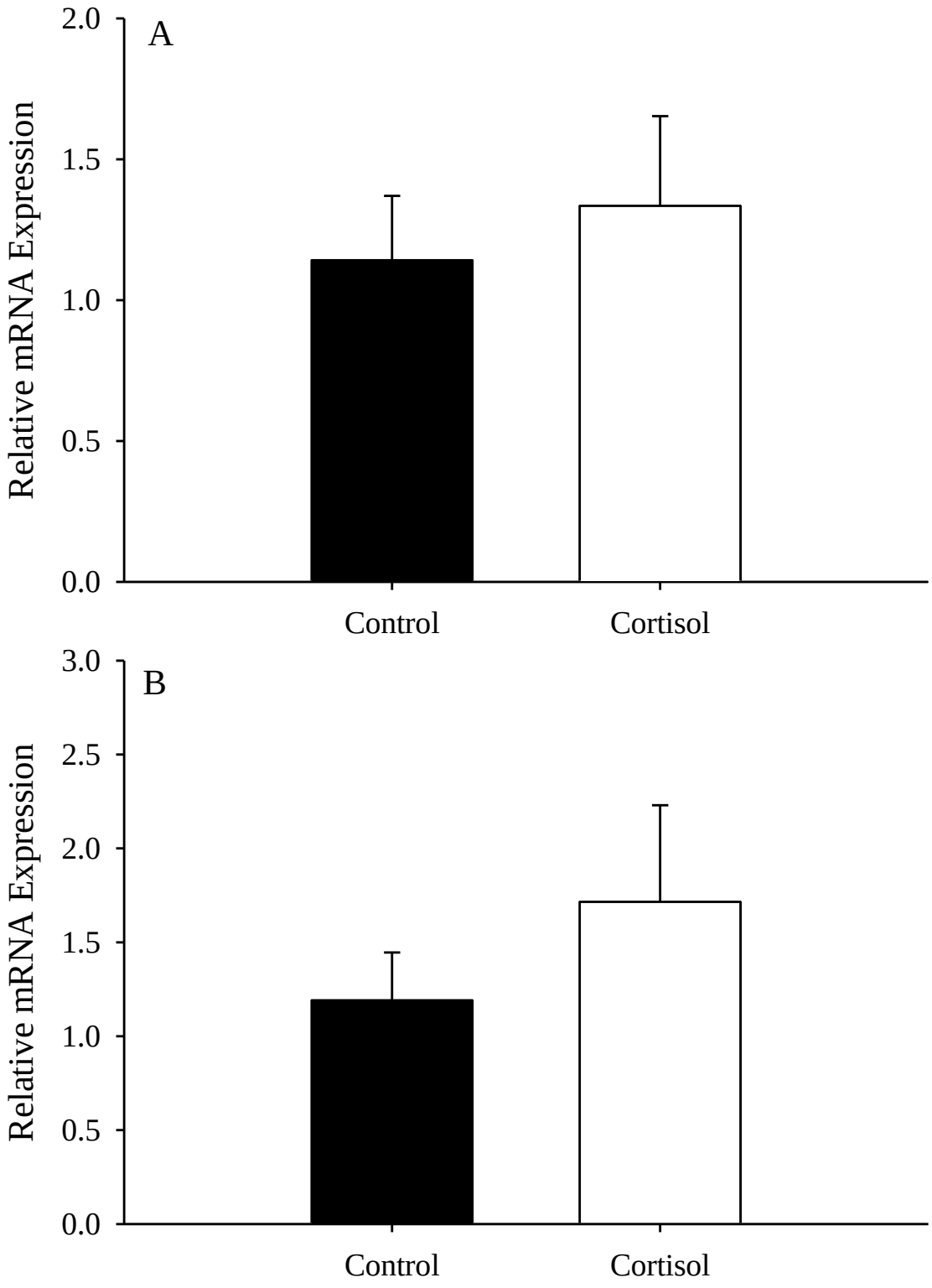
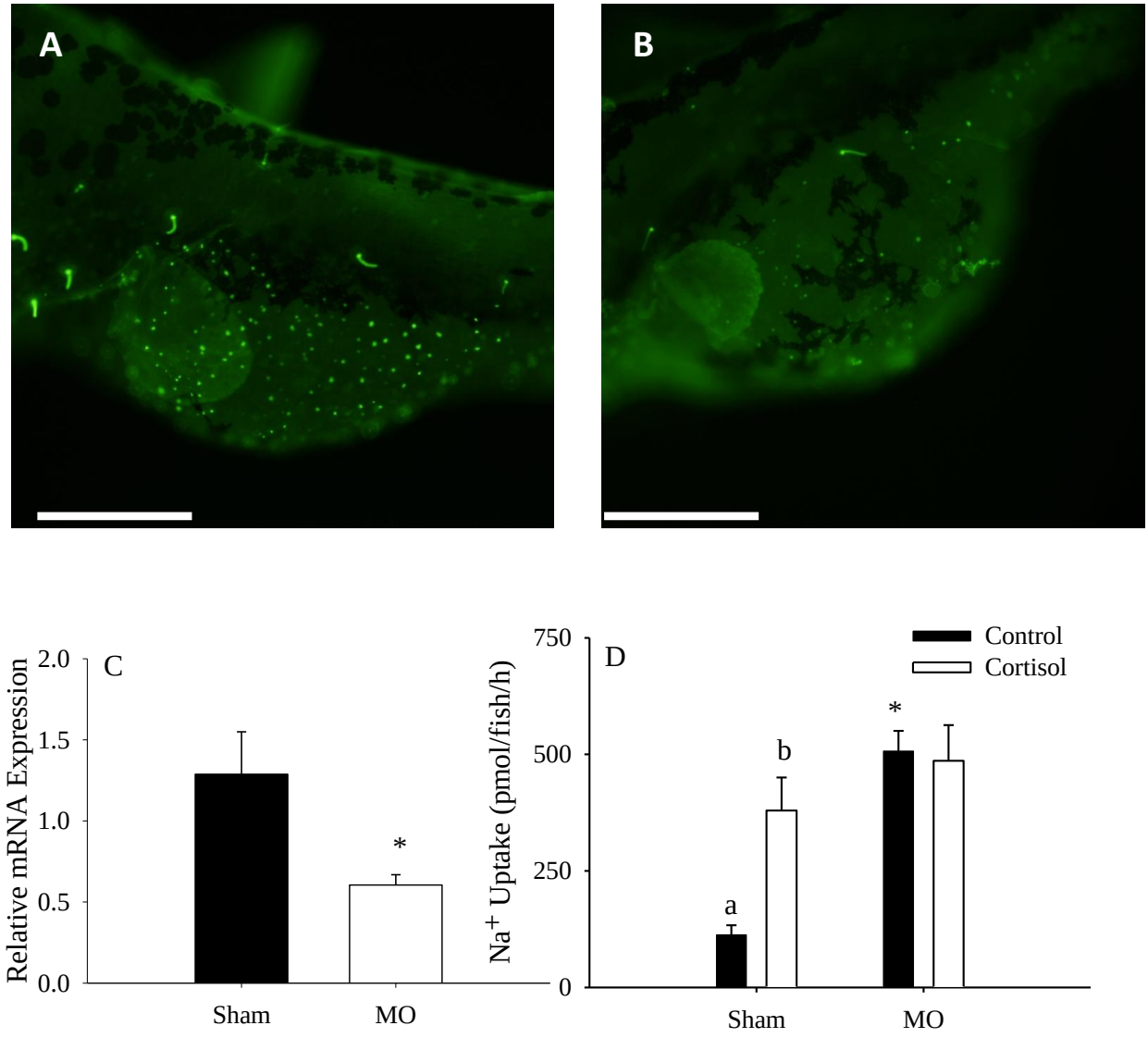

A
Relative mRNA Expression
2.0
1.5
1.0
0.5
0.0
Control
Cortisol
B
Relative mRNA Expression
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Control
Cortisol

Fig. 4.11. The lack of an effect of cortisol on Na^+ uptake via NCC cells. The microinjection of morpholino against a transcription factor *glial cell missing 2* led to a significant reduction in conA positive HRCs (Figs. 4.11A, B). mRNA expression of GR was significantly lower in *gcm-2* morphants than in sham injected larvae (N = 4 – 6; Fig. 4.11C). The Na^+ uptake was significantly elevated in *gcm-2* morphants, but no further stimulation was observed following the 48-h cortisol treatment (N = 5 – 6; Fig. 4.11D). An asterisk in Figs. 4.11C,D indicates significant difference from the sham group (within vehicle control treatment for Fig. 4.11D). Different letters in Fig. 4.11D denotes significant difference between DMSO and cortisol-treated groups. Data are presented as means ± SEM. Scale bars = 200 µm.


A
B
C
Relative mRNA Expression
2.0
1.5
1.0
0.5
0.0
*
Sham
MO
D
Na+ Uptake (pmol/fish/h)
750
500
250
0
Control
Cortisol
a
b
*
Sham
MO

Discussion

The present study tested the hypothesis that cortisol regulates Na^+ uptake in zebrafish larvae exposed to acidic water. The data clearly demonstrated that cortisol 1) indeed stimulates Na^+ uptake in zebrafish larvae, 2) likely plays an important role in stimulating Na^+ uptake under acidic conditions through its interaction with GR and 3) exerts its stimulatory effects on Na^+ uptake, at least in part, by affecting a recently-proposed Rhcg1-NHE3b metabolon (Kumai and Perry, 2011; Shih et al., 2012).

Cortisol regulates Na^+ uptake in zebrafish

The role of cortisol as a major endocrine factor regulating hydromineral balance in both SW and FW is well-established (for review see McCormick, 2001). In agreement with previous studies that demonstrated a role for cortisol in inducing ion absorption in several fish species (Laurent and Perry, 1990; Perry et al., 1992; Scott et al., 2005a; Lin et al., 2011), the present study extends these findings by demonstrating a stimulatory effect of cortisol on Na^+ uptake in zebrafish larvae (Fig. 4.1A) and consequent increase, although relatively small, in whole body Na^+ content (Fig. 4.1B), and its involvement in stimulating Na^+ uptake during exposure to acidic water (Figs. 4.3, 4.4).

Previous studies have shown that both GR (Kelly and Chasiotis, 2011; Kiilerich et al., 2011a; McCormick et al., 2008) and MR (Scott et al., 2005a; Sloman et al., 2001) could potentially mediate cortisol signalling and affect teleost osmoregulation. Thus, in the current study, it was attempted to determine whether the effect of cortisol on Na^+ uptake in zebrafish larvae was mediated by GR or MR (or both). The results of selective pharmacological blockade of GR and MR (Fig. 4.2) provided strong evidence that at least GR is responsible for the cortisol-mediated stimulation of Na^+ uptake in zebrafish larvae. Recently, Lin et al. (2011) demonstrated

that GR is involved in inducing Ca^{2+} uptake in zebrafish larvae exposed to low [Ca^{2+}] water, suggesting that GR-mediated cortisol signalling might regulate uptake of multiple ions in zebrafish.

The effects of GR/MR inhibitors in acid-exposed larvae (Fig.4.3) as well as translational knockdown of GR (Fig. 4.4) provided further evidence that GR-mediated activation of Na^+ uptake contributes to acid-tolerance in zebrafish. Although zebrafish are not typically found in acidic habitats in nature (Engeszer et al., 2007), their level of acid-tolerance is comparable to species indigenous to chronically acidic environments such as Osorezan dace, *Tribolodon hakonensis* (Hirata et al., 2003) and species from the Amazonian Rio Negro water system (for review see Gonzalez et al., 2005). Exposure of these species to low pH water under laboratory conditions does not result in a strong stimulation of Na^+ uptake as observed in zebrafish, suggesting that cortisol-mediated stimulation of Na^+ uptake in response to acid exposure is not a common feature of acid-tolerant species. However, it is possible that cortisol might still play a role in defending the ion balance of those species native to acidic environments, possibly by regulating gill permeability and minimizing ion losses to the environment. It would be of interest to investigate the potential role of cortisol in osmoregulation in fish species native to acidic environments.

In contrast to the role of GR, the potential role played by MR in regulation of Na^+ uptake was less obvious. In the present study, it was showed that 1) treatment with 500 nM aldosterone (a more MR-selective agonist) did not affect Na^+ uptake (Fig. 4.2C), and that 2) exposure to acidic water in the presence of 10 µM eplerenone (Fig. 4.3B) as well as 10 µM spironolactone (data not shown) did not prevent the stimulation in Na^+ uptake. Taken together, these data appear to suggest the limited, if any, role of MR in mediating the effect of cortisol. However,

such negative results should be interpreted with caution. For example, although 10 µM eplerenone has been shown to effectively inhibit the activity of zebrafish MR (Pippal et al., 2010), it might be possible that 10 µM was not sufficiently high to be effective in the *in vivo* system, unlike previously reported in the *in vitro* system. Furthermore, it is worth mentioning that when zebrafish larvae were treated with sufficiently high dose of aldosterone (10 µM as opposed to 500 nM), such treatment did increase Na^+ uptake in zebrafish larvae. Therefore, the potential role played by MR in regulating Na^+ uptake still remains an open question and ultimately it could be addressed by assessing the effect of cortisol on MR morphants. Assessing the relative contribution of GR and MR in regulation of Na^+ uptake might provide an interesting future research direction.

Cortisol affects a NHE3b-Rhcg1 functional metabolon

Previous research has provided compelling evidence that both H^+-ATPase and NHE are involved in Na^+ uptake in acid-exposed zebrafish (the latter in association with Rhcg1, an apically distributed ammonia conducting channel; Horng et al., 2009b; Kumai and Perry, 2011). Because both H^+-ATPase (Lin and Randall, 1993) and isoforms of NHE (Ivanis et al., 2008a; Ivanis et al., 2008b) are known targets of cortisol in tissues related to osmoregulation in FW fish, the effect of cortisol treatment on these transporters were investigated through a combination of pharmacological inhibition and morpholino knockdown.

Consistent with previous studies that demonstrated a significant role of H^+-ATPase in Na^+ uptake by zebrafish larvae (Boisen et al., 2003; Esaki et al., 2007; Horng et al., 2007), treatment with bafilomycin, a H^+-ATPase inhibitor, significantly reduced Na^+ uptake in cortisol-treated fish in the current study (Fig. 4.7). In addition, subjecting larvae to translational knockdown of either Rhcg1 or NHE3b using previously validated protocols (Braun et al., 2009a;

Kumai and Perry, 2011), rendered them unresponsive to cortisol treatment (Figs. 4.9A,B). As discussed in a previous study (Kumai and Perry, 2011), the observation that inhibition of H^+-ATPase and Rhcg1/NHE3b knockdown both suppress cortisol-mediated Na^+ uptake may be related to the dependence of Na^+ uptake on H^+-ATPase owing to its 1) linkage to a yet identified electrically coupled epithelial Na^+-conducting channel, and 2) facilitation of ammonia excretion, which in turn promotes the uptake of Na^+ through NHE3b (the metabolon model). In the metabolon model, excretion of ammonium via Rhcg is thought to create an alkalized microenvironment, which is exploited by the NHE3 to absorb Na^+ from the environment (Kumai and Perry, 2011; Wright and Wood, 2009; Wu et al., 2009). Although the relative contribution of H^+-ATPase in affecting these two pathways remains unknown, the results of the Rhcg1 and NHE3b knockdown experiments suggest that cortisol is acting chiefly by facilitating the recently proposed "Rhcg1-NHE" functional metabolon (Kumai and Perry, 2011; Wright and Wood, 2009). Further circumstantial support for this hypothesis comes from the observed stimulatory effect of cortisol on ammonia excretion, which also appears to be under the control by GR (Fig. 4.8A, B). Although FW animals can excrete ammonia via multiple pathways (for recent review see (Weihrauch et al., 2009), it is possible that the overall increase, partially mediated by Rhcg1, in ammonia excretion in cortisol-treated larvae favours the ammonia-dependent Na^+ uptake mechanism in zebrafish.

Previous reports on the effect of cortisol on the mRNA expression of Rhcg in fish gill showed conflicting results. Tsui et al. (2009) reported 2-day exposure to 1000 ng/ml (~ 2.7 μM) cortisol had no effect on mRNA expression level of Rhcg1 in gill cell culture derived from rainbow trout. On the other hand, Rodela et al. (2012) reported that cortisol could reduce the mRNA expression level of Rh proteins in *O. beta.* Such results clearly suggest that sample

preparations (eg, method of cortisol delivery and dose) as well as study system (gill cell culture vs. whole gill tissue) could significantly affect the observed effect of cortisol on Rhcg gene expressions. When the effect of cortisol on mRNA expression of Rhcg1 in zebrafish larvae was assessed, cortisol treatment did not have any effect on the mRNA expression level of Rhcg1 (Fig. 4.10A). One possible interpretation is that cortisol truly does not affect Rhcg1 gene expression. Indeed, when promoter analysis for Rhcg1 was performed using TESS software (http://www.cbil.upenn.edu/cgi-bin/tess/tess), no full canonical glucocorticoid response element (GRE) sequence was identified. However, the lack of full canonical GRE does not mean cortisol could not regulate the mRNA expression of Rhcg. Indeed, Rodela et al. (2011) reported that, using luciferase reporter assay, the expression of toadfish urea transporter could be under cortisol regulation, even though they could not detect a full canonical GRE in the upstream of tUT. Similarly, the expression of NHE3b mRNA also did not change following cortisol treatment. Although the lack of any effect on the mRNA expression level of these genes appears inconsistent with the proposed idea that cortisol affects Na^+ uptake through stimulation of the metabolon model, it is also important to note that the ammonia excretion rate did significantly increase following the cortisol treatment (Fig. 4.8A), most likely due to the increased internal ammonia production (and subsequent steeper PNH_3 gradient) from increased protein catabolism (Andersen et al., 1991). Therefore, it is possible that an increased PNH_3 gradient is sufficient to increase the Na^+ uptake mechanism facilitated by ammonia excretion even when there was no change in Rhcg1 transcript level. Alternatively, it is possible that Rhcg1 and NHE3b mRNA expressions were altered (presumably increased) within ionocytes, but as RNA was extracted from a whole larval homogenate, such regional changes in gene expression was obscured. Collecting and analyzing only tissues of interest, harvested using techniques such as laser capture

microscopy (LCM) would provide better insight whether such cell-specific regulation of gene expression are indeed occurring in zebrafish larvae.

Although knockdown provided strong support for the stimulatory effect of cortisol on Na^+ uptake being linked to a "Rhcg1-NHE3b" metabolon, it is interesting to note that external application of 100 µM 5-(N-ethyl-N-isopropyl) amiloride (EIPA), a pharmacological agent which was previously reported to inhibit Na^+ uptake in zebrafish under both control (Esaki et al., 2007) and acidic conditions (Kumai and Perry, 2011), had no inhibitory effect on Na^+ uptake by cortisol-treated larvae (data not shown). However, treating larvae with 100 µM 5-(N,N)-hexamethylene amiloride (HMA), another amiloride derivative selective for NHE, which has been used previously in fish (Preest et al., 2005; Wood et al., 2002b), caused a robust, 50-60 % inhibition of Na^+ uptake in cortisol-treated larvae (data not shown). The inconsistency in the results between the experiments using different amiloride analogs, each with apparent high selectivity for NHE, implies that pharmacological agents designed for mammalian research may not always be appropriate in research on lower vertebrates and reinforces the need to confirm experimental results based on pharmacology using alternative means when possible (e.g. translational knockdown).

Does cortisol target other types of ionocytes in zebrafish larvae?

Through immunostaining and in situ hybridization, it has been firmly established that there are at least three types of ionocytes (HRC, NaRC and Na^+-Cl^- co-transporter expressing NCCC) in zebrafish larvae, with each performing distinctive functions in osmoregulation (Hwang, 2009; Hwang et al., 2011). Although the results of the present study strongly establish a role of GR in regulating Na^+ uptake through HRCs, it is also possible that cortisol is targeting other ionocyte subtypes. Indeed, a recent study by Lin et al. (2011) demonstrated that cortisol,

acting through GR, stimulates Ca^{2+} uptake and also modulates the expression of Ca^{2+} uptake related genes in zebrafish larvae. In the light of their finding, it was particularly surprising that no obvious co-localization between GR and NaRC was observed in this study, given that NaRC is thought to be primarily responsible for Ca^{2+} uptake in zebrafish (Pan et al., 2005). It is possible that the observed effect of cortisol on Ca^{2+} uptake is not direct, but rather mediated by some other GR-expressing cells. It is also worth noting that the dose of cortisol used by Lin et al. (2011) was considerably higher (60-120 μM) than in the present study (500 nM). Therefore, the level of expression of GR in NaR cells might have been too low to be detected by standard immunohistochemistry. Pre-treating the zebrafish larvae with low ambient [Ca^{2+}], which was shown to increase GR mRNA expression (Lin et al., 2011), might help to visualize GR in NaR cells, if present.

NCCCs are thought to play an important role in Na^+ and Cl^- uptake in zebrafish (Wang et al., 2009). To determine if cortisol was also affecting Na^+ uptake through NCC, a cortisol-treatment experiment was performed on a morphants injected with morpholino against *glial cell missing 2*, a transcription factor recently shown to play an important role in the differentiation of HRCs (Chang et al., 2009; Shono et al., 2011). As expected from these previous studies, a number of con-A positive HRCs was greatly reduced in *gcm-2* morphants (Fig. 4.11, A, B), thus providing a condition that facilitates investigation on physiological regulations on NCC. When *gcm-2* morphants were treated with 500 nM cortisol for 48 h, they showed no significant increase in Na^+ uptake (Fig.4. 11D). Although the knockdown of *gcm-2* itself significantly increased Na^+ uptake, presumably through the compensatory mechanism via NCC (Shono et al., 2011), it was unlikely that Na^+ uptake mechanisms were operating at their highest capacity in *gcm-2* morphants because when they were subjected to other treatments, such as isoproterenol (a

generic β adrenergic receptor agonist), a robust stimulation of Na^+ uptake was observed (Y. Kumai and S.F. Perry, unpublished observation). Therefore, the simplest interpretation on the lack of stimulation is that NCCCs are unresponsive to cortisol, at least at the dose of 500 nM. The mRNA expression of GR was also significantly reduced in *gcm2* morphants (Fig. 4.11C). Whereas this is certainly in agreement with the high expression level of GR in HRCs (Fig. 4.6), which were eliminated in *gcm-2* morphants, it is also surprising that whole body mRNA was also reduced given the relatively high mRNA expression of GR in somatic tissues in developing larval zebrafish (Nesan et al., 2012). Thus, it is also possible that *gcm-2* knockdown affected general GR distribution patterns, though no such effect has been reported previously in zebrafish, and such global disruption in GR distribution secondarily affected the responsiveness of NCCCs to cortisol treatment. Therefore, while data in the present study clearly demonstrate the role of cortisol in Na^+ uptake via HRCs (and most likely through NHR3b-Rhcg1 metabolon), the potential regulation of NCC by cortisol remains an open question and thus would merit from further investigations.

In conclusion, results of the present study provide strong evidence for the involvement of cortisol in regulating Na^+ uptake in zebrafish larvae, that its effect is mediated at least by GR, and that it promotes Na^+ uptake during exposure of larvae to acidic conditions. Currently, it is unknown whether cortisol is also influencing other pathways contributing to Na^+ homeostasis. For example, cortisol could also be affecting Na^+ efflux in acid-exposed zebrafish larvae. Given that previous research has shown that the permeability and mRNA/protein expression of tight junction associated genes are under sophisticated regulation by cortisol in other fish species (Bui et al., 2010; Chasiotis et al., 2010), it is possible that increased whole body cortisol levels affects the function of tight junctions in acid-exposed zebrafish larvae. Although Na^+ efflux was

significantly increased in zebrafish larvae during exposure to acidic water (Kumai and Perry, unpublished observation), this observation does not rule out the possibility that cortisol is preventing an even more severe Na^+ loss in acid-exposed larvae. In addition, more detailed analyses on the potential role of MR in conveying the cortisol signal on Na^+ uptake might provide better insights in multiple regulation of ion homeostasis by cortisol.

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CHAPTER 5
Angiotensin II promotes Na^+ uptake in larval zebrafish, *Danio rerio*, in acidic and ion-poor environments

Notes on Chapter:

The present chapter has been submitted to *Endocrinol* as follows:

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Author contributions: NJB performed RIA for ANG-II. This study was conceived by YK and all remaining experiments were performed by YK under the supervision of SFP.

Abstract

The contribution of the renin-angiotensin system (RAS) to Na^+ uptake was investigated in larval zebrafish (*Danio rerio*). At 4 days post fertilization (dpf), the whole body angiotensin-II (ANG-II) level was significantly increased after 1- or 3-h exposure to acidic (pH = 4.0) or ion-poor water (20-fold dilution of Ottawa tap water), suggesting rapid activation of the RAS. Longer (24 h) treatment of 3 dpf larvae with ANG-I or ANG-II significantly increased Na^+ uptake which was accompanied by an increase in mRNA expression of the Na^+-Cl^- co-transporter (*zslc12a10.2*). Induction of Na^+ uptake by exposure to ANG-I was blocked by simultaneously treating larvae with lisinopril (an angiotensin converting enzyme inhibitor). Acute (2 h) exposure to acidic water or artificial water depleted of only Na^+ or Cl^- led to significant increases in Na^+ uptake which were partially blocked by the ANG-II receptor antagonist, telmisartan. Consistent with these data, translational gene knockdown of renin prevented the stimulation of Na^+ uptake following exposure to acidic or ion-poor water. The lack of any effects of pharmacological inhibition (using RU-486) or gene knockdown of glucocorticoid receptors on the stimulation of Na^+ uptake during acute exposure to acidic or ion-poor environments, suggest that the acute effects of RAS occur independently of cortisol signalling. The results of this study demonstrate that the RAS is involved in the regulation of Na^+ homeostasis in larval zebrafish.

Introduction

To maintain their body fluids hypertonic to the dilute environment, freshwater (FW) teleosts actively absorb ions through specialized epithelial cells termed ionocytes. The molecular mechanisms underlying the active absorption of Na^+, Cl^- and Ca^{2+} have been investigated extensively over the past 80 years (for recent reviews see Dymowska et al., 2012; Evans, 2011; Hwang and Perry, 2010; Hwang et al., 2011; Kumai and Perry, 2012). Based on these previous studies, it is clear that ion uptake is tightly regulated by several hormones, including prolactin (Breves et al., 2013; Breves et al., 2010; Pickford and Phillips, 1959; Pisam et al., 1993), cortisol (Cruz et al., 2013; Kumai et al., 2012a; Laurent and Perry, 1990; Lin et al., 2011; Sloman et al., 2001), stanniocalcin (Tseng et al., 2009), vitamin D (Lin et al., 2012) and isotocin (Chou et al., 2011), as well as being under neurohumoral control (Kumai et al., 2012b; Perry et al., 1984; Vermette and Perry, 1987). The existence of multiple ionoregulatory mechanisms highlights the importance of body fluid ionic homeostasis.

In mammalian kidney, which shares several similar functions as the fish gill, angiotensin-II (ANG-II) is recognized as a major regulatory hormone controlling salt reabsorption (Crowley and Coffman, 2012). ANG-II is derived in the presence of angiotensin converting enzyme 1 (ACE1) from biologically inactive ANG-I which, in turn, is synthesized from angiotensinogen by the enzyme renin. Although recent studies have identified additional proteins interacting with ANG-II and renin, such as prorenin, renin receptors and ACE-2, the enzymatic reactions resulting in the synthesis of ANG-II constitute the renin-angiotensin system (RAS; Passos-Silva et al., 2013; Santos et al., 2013; Zimmerman and Burns, 2012). Based on *in vivo* and *in vitro* studies, ANG-II is known to increase the expression and/or activities of all major transporters involved with Na^+ and acid transport in the various segments of the nephron, including Na^+/H^+

exchanger 3 (NHE3; Cano et al., 1994; Du Cheyron et al., 2003; Geibel et al., 1990), H^+-ATPase (Pech et al., 2008; Rothenberger et al., 2007; Wagner et al., 2011), epithelial Na^+ channel (ENaC; Peti-Peterdi et al., 2002) and thiazide sensitive Na^+-Cl^- co-transporter (Castaneda-Bueno et al., 2012; Castaneda-Bueno and Gamba, 2012; San-Cristobal et al., 2009; Sandberg et al., 2007; Talati et al., 2010). Although there are two distinct types of ANG-II receptors, referred to as type-I and -II (AT_1 and AT_2) receptors, expression of AT_2 is much lower than AT_1 in adult mammalian tissues, and consequently, the majority of the physiological effects of ANG-II, including the above-mentioned transporter activation, are attributed to AT_1-mediated signalling (Stegbauer and Coffman, 2011).

Similar to mammals, the RAS is physiologically relevant in fish, where it is known to participate in fluid volume control and blood pressure regulation (Bernier et al., 1999b; Nishimura, 2001; Russell et al., 2001; Smith et al., 1991; Takei and Tsuchida, 2000; Tierney et al., 1995). While significant inter-species differences exist with respect to the mechanisms for blood pressure regulation by ANG-II (Bernier et al., 1999b), plasma [ANG-II] and renin activity have often been reported to be transiently or chronically elevated following transfer to seawater in several euryhaline fish species (Anderson et al., 2006; Tierney et al., 1995) (Smith et al., 1991). Consequently, despite the extensive research related to ANG-II and renal salt reabsorption, surprisingly little is known about its potential role in FW fish ionic regulation. (Smith et al., 1991) reported a decline in plasma renin activity in FW acclimated rainbow trout (*Oncorhynchus* mykiss) fed a salt-enriched diet, suggesting a possible role for ANG-II in regulating salt balance in FW. Subsequently, Hoshijima and Hirose (2007) reported an increase in renin mRNA expression during acclimation of zebrafish to ion-poor water (20-fold dilution of the control water) suggesting a role of the RAS in promoting salt absorption. Despite these

studies, the question of whether the RAS promotes salt uptake in FW fish remains unexplored. Thus, the main objective of the present study was to test the hypothesis that ANG-II contributes to ionic homeostasis in a FW teleost. More specifically, I hypothesized that ANG-II stimulates the absorption of Na^+ when zebrafish are exposed to conditions that challenge Na^+ uptake (e.g. low environmental pH and ion-poor water; Kumai and Perry, 2011; Kumai et al., 2012b). By monitoring whole body ANG-II levels), treating larvae with inhibitors for various steps of RAS signalling and knocking down the expression of renin, I revealed an important role for ANG-II in acutely inducing Na^+ uptake in zebrafish larvae. The results of the present study introduce the RAS as an additional and important endocrine mechanism regulating ionic homeostasis in FW fish.

Materials and Methods

Experimental animals and water preparation

Adult zebrafish (*Danio rerio* Hamilton-Buchanan 1822) were purchased from Big Al's Aquarium Services (Ottawa, ON, Canada) and kept in the University of Ottawa Aquatic Care Facility where they were maintained in plastic tanks supplied with aerated, dechloraminated City of Ottawa tap water at 28° C. Fish were subjected to a constant 14 h L:10 h D photoperiod and fed daily until satiation with No. 1 crumble-Zeigler^{TM} (Aquatic Habitats, Apopka, FL, USA). Embryos were collected and reared in 50 ml Petri dishes with dechloraminated City of Ottawa tap water (pH 7.3-7.5) supplemented with 0.05% methylene blue. The Petri dishes were kept in incubators set at 28.5° C. Dead embryos were removed and water was changed daily. The experiments were conducted in compliance with guidelines of the Canadian Council of Animal Care (CCAC) and after the approval of the University of Ottawa Animal Care Committee (Protocol BL-226). Unless stated otherwise, all chemicals were purchased from Sigma.

To determine the effects of ANG on Na^+ uptake in developing zebrafish, the following series of experiments were performed.

Series 1. Effect of acute exposure to acidic/ion-poor environments on whole body ANG-II levels

Zebrafish larvae (4 dpf) were flash frozen in liquid nitrogen after 1- or 3h exposure to acidic or ion-poor water (50 larvae were pooled to generate one sample) and stored at -80° C until extraction and analysis of whole body ANG-II levels by radioimmunoassay (RIA) according to the protocol of (Bernier et al., 1999a). Ion-poor water was prepared by diluting Ottawa tap water 20-fold using distilled and deionised water. Acidic water (pH ~ 4.0) was prepared by adding H_2SO_4 to Ottawa tap water. Larval zebrafish were homogenized in 350 µl acidic acetone (a mixed solution of acetone, H_2O and 1M HCl at 40:5:1) and the homogenate

was centrifuged for 10 min at 4° C at 10,000 *g*. The supernatant was transferred to a 1.5-ml micro-centrifuge tube, and the extraction process was repeated on the remaining pellet. The supernatant collected from the two rounds of extractions was combined and lyophilized. The lyophilized samples were reconstituted in 350 µl RIA buffer (10 mM PBS, pH 7.4; 140 mM NaCl; 0.1% w/v NaN_3; 40 mM Na_2EDTA; 10 mM 6-aminogexanoic acid; 0.25% (v/v) Triton X-100 and 0.25% RIA-grade BSA fraction V).

For the RIA, samples for the standard curve were prepared by mixing serially diluted 0.1 ml standard [Asn^1,Val^5]-ANG-II ligand, 0.1 ml antiserum raised against [Asp^1,Ile^5]-ANG-II (final concentration was ~ 0.7 µg antibody/mixture; cat# T-4005, Bachem, Torrance, CA, USA) and 0.1 ml normal rabbit serum (1:250 dilution; cat# 869019; Calbiochem, Gibbstown, NJ, USA). After incubation for 20 h at 4° C, 0.05 ml ^{125}I-labelled [Asp^1, Ile^5]-ANG-II (~ 7500 CPM; specific activity = 2200 Ci $mmol^{-1}$; Perkin Elmer, Woodbridge, ON, Canada) was added to the mixture and incubated for another 24 h at 4° C. Antigen bound to ^{125}I ANG-II was precipitated by adding 0.1 ml PANSORBIN® Cells (0.25%; Calbiochem) and incubating for 5 h at 4° C. Subsequently, samples were centrifuged at 2,000 *g* for 1 h at 5° C and radioactivity of the precipitates was determined using a WIZARD2 gamma counter (Perkin Elmer). For measurement of ANG-II in extracted samples, 0.1 ml ANG-II standard was replaced with larval extract. To confirm that the extraction protocol originally developed for plasma also was appropriate for larval tissue, tissue samples were "spiked" with [Asn^1, Val^5]-ANG-II. ANG-II was extracted from the "spiked" sample and was serially diluted, to confirm that the dilution curve ran parallel to the standard curve. All samples were measured in triplicate within a single assay**.**

Series 2. Consequences of treatment with waterborne ANG

2.1. Na^+ uptake

To determine the effect of extended (24 h) elevation of ANG on Na^+ uptake, larvae were treated with 100, 500 or 1000 nM ANG-I ([Asn^1, Val^5, Asn^9]-ANG-I) or ANG-II ([Asn^1, Val^5]-ANG-II) between 3 and 4 dpf. After the 24-h exposure, Na^+ uptake was measured in the control water. To measure Na^+ uptake, 12 larvae were placed in a 2-ml micro-centrifuge tube and 0.25 µCi ^{22}Na in the form of NaCl (Perkin Elmer, Woodbridge, ON, Canada) was added to each tube to a final activity of 0.15 µCi/ml. Water samples (50 µl) were collected at 5 min and 2 h after the addition of isotope. At the end of the 2 h flux period, larvae were killed by overdose with ethyl 3-aminobenzoate methanesulfonate (MS-222) and briefly washed in isotope-free water containing high levels of Na^+ (>200 mM) to displace residual isotope attached to the surface of the fish. The remaining water in the tube was stored separately for later measurement of the total [Na^+].

To determine the effectiveness of lisinopril as an ACE1 inhibitor, another group of larvae exposed to 500 nM ANG-I was co-treated with 100 µM lisinopril kept in the control water. The Na^+ uptake in these groups of larvae was measured on 4 dpf as described above.

2.2. RNA extraction and RT-qPCR for Na^+-transporting genes

To determine the effects of ANG-II on the expression of Na^+ transporting genes, mRNA levels of NHE3b (*zslc9a3b*), Na^+-Cl^- co-transporter (*zslc12a10.2*) and H^+-ATPase (*zatp6v1a)* were analyzed in 4 dpf zebrafish larvae after 24 h treatment with 500 nM ANG-II. This dose of ANG-II was chosen as it was the lowest dose shown to induce uptake of Na^+ following chronic exposure (see results for series 2.1). After treatment, larvae were killed by MS-222 overdose, flash frozen and stored at -80^o C until RNA extraction. Total RNA (10 larvae were pooled for N = 1) was extracted with TRIzol® (Invitrogen) according to manufacturer's instructions. cDNA

was synthesized by treating 1 μg of extracted RNA with DNase (Invitrogen) and RevertAid^{TM} M-MNuLV reverse transcriptase (Fermentas, Burlington, ON, Canada) according to the manufacturer's instructions. RT-qPCR was performed using a Bio-Rad CFX96 qPCR system with Brilliant III SYBR Green Master Mix (Agilent Technologies, USA). PCR conditions for all primer sets were as follows; 95° C for 3 min, 40 cycles of 95° C for 20 s and 58° C for 20 s, with final extension for 5 min at 72° C. Data were normalized to the expression of 18S, and were presented relative to the control group. For the list of primers, see Table 5.1.

Series 3 Effects of acute exposure to ion-poor or acidic water

To determine whether short (~ 2 h) exposure to ion-poor or acidic water could stimulate Na^+ uptake, the following groups were prepared; 1) fish raised in control water until 4 dpf and transferred to control water (handling control), 2) fish raised in control water until 4 dpf and directly transferred to water deficient in either Na^+, Cl^- or Ca^{2+}, or low pH water for 2 h. Artificial water containing low Na^+, Cl^- and Ca^{2+} concentrations was prepared by adding appropriate amounts of NaCl, $CaCl_2$, Na_2SO_4, K_2HPO_4, KH_2PO_4, $MgSO_4$ and $CaSO_4$ to make the final levels of individual target ions roughly 1/10 of Ottawa tap water, where [Na^+], [Cl^-] and [Ca^{2+}] are approximately 700, 400 and 200 μM, respectively. A low Ca^{2+} exposure group was prepared to serve as the negative control, as no mechanism of Na^+ uptake coupled with Ca^{2+} uptake has been proposed for fish (Hwang et al., 2011). Following the 2-h exposure, all treatment groups were transferred back to the control water and their Na^+ uptake was measured in the control water as described above.

Series 4 Consequences of pharmacological inhibition of RAS during acute exposure to acidic or ion-poor water.

To assess the potential role of the RAS in regulating Na^+ uptake, zebrafish larvae (4 dpf) raised in control water were exposed for 30 min to the following commonly used RAS inhibitors; 1) 10 µM telmisartan (Santa Cruz Biotech; a selective AT_1 blocker) and 2) 1 or 10 µM PD123319 (Santa Cruz Biotech; a selective AT_2 receptor blocker). Following the 30-min pre-incubation, larvae were exposed to either low pH or ion-poor water for 2 h in the continued presence of the antagonists/inhibitors. After the acid-exposures, larvae were transferred back to the control water to measure Na^+ uptake.

Series 5. Consequences of renin knockdown on Na^+ uptake

To more directly determine the role of RAS in rapidly modulating Na^+ uptake, a translation blocking morpholino targeting renin (5'- AGTCAAGCAGTGGATTTTCATTCTC-3') was designed by Gene Tools; the 3' end was conjugated with carboxyfluorescein. Larvae were injected with renin MO at a dose of 4 ng/embryo at 1- to 2- cell stages and after 24 hpf were screened microscopically for the widespread presence of fluorescein (SMZ1500 microscope; Nikon Instruments, Melville, NY). Only fluorescein-positive embryos were used for subsequent experiments. To control for the effect of microinjection, a separate group of larvae were injected with control MO (5'-CCTCTTACCTCAGTTACAATTTATA-3') and handled as the renin MO injected group. The Na^+ uptake in sham and renin morphants following acute exposure to acidic and ion-poor water was determined as described above using 4 dpf larvae. No morphological abnormalities were observed in the renin morphants.

Effectiveness of knockdown was confirmed with western blotting using a renin antibody (ARP41409_T100; Aviva Systems Biology, San Diego, USA) whose epitope shares 78% identity with zebrafish renin precursor amino acids 348-394; accession number NP_998025.1). Total protein was extracted from MO- and sham-injected larvae using Tris buffer (10 mM Tris-

HCl with 2% Triton X-100; pH adjusted to 7.4) supplemented with protease inhibitor tablet (Complete Mini, Roche). Ten larvae were pooled to prepare one (N = 1) sample. Extracted samples were loaded onto a 10% SDS-PAGE, size-fractionated at 200 V and transferred onto a polyvinylidene difluoride (PVDF) membrane (Bio Rad). After transfer, membranes were blocked with 5% BSA in 0.2% Tween 20 in Tris-buffered saline (TBST) for 2 h at room temperature. Membranes were incubated overnight with gentle shaking with anti-renin antibody (1:1000 in 2% BSA in TBST) at 4° C. Subsequently, membranes were washed (3 x 5 min) with TBST and incubated with horseradish peroxidase-conjugated secondary antibody against rabbit IgG (Invitrogen; 1:15000 in 2% BSA in TBST) for 2 h at room temperature. The membranes were then washed (4 x 10 min) and the immunoreactive bands were detected using enhanced chemi-luminescence (Millipore) with a ChemiDoc system (Bio-Rad). Subsequently, the membrane was re-probed with β-actin antibody (1:4000; Sigma) after stripping with Re-Blot Plus solution (Millipore).

Series 6. Potential involvement of cortisol in mediating the effects of the RAS on Na^+ uptake

It was recently demonstrated that chronic waterborne treatment with cortisol can induce Na^+ uptake in zebrafish larvae through signalling via the glucocorticoid receptor (GR) (Kumai et al., 2012a). Because of the well-known interaction between angiotensin and aldosterone, I investigated whether cortisol might be involved in regulating Na^+ uptake, potentially by interacting with ANG-II. The function of GR was inhibited either by pharmacological blockade of the receptor using 1 μM RU-486 (Kumai et al., 2012a) or translational gene knockdown of GR using a morpholino antisense oligonucleotide against zebrafish GR (5'-CTCCAGTCCTCCTTGATCCATTTTG-3') (Kumai et al., 2012a). For RU-486 treatment,

larvae were exposed to 1 µM of RU-486 dissolved in DMSO for 30 min before being transferred to either acidic or ion-poor water for 2 h in the continued presence of inhibitor/DMSO. Final concentration of DMSO did not exceed 0.1%. Fish were subsequently transferred back to the control media (without inhibitor/DMSO) and their Na^+ uptake was measured. In addition, a vehicle/handling group was evaluated where larvae were exposed to the same concentration of DMSO but kept in the control water. In addition, to confirm the lack of involvement of mineralocorticoid receptor (MR) in acute Na^+ uptake regulation, larvae were pre-treated with 10 µM eplerenone (dissolved in DMSO) for 30 min and acutely challenged with acidic and ion-poor water before their Na^+ uptake was measured.

GR morpholino- and sham-injected larvae were raised in control water until 4 dpf and then directly challenged with acidic or ion-poor water for 2 h. Subsequently, they were transferred back to the control water and their Na^+ uptake was measured. To verify the effectiveness of knockdown, separate groups of sham and GR morphants were treated with either DMSO or 500 nM cortisol for 2 days (between 2 and 4 dpf); Na^+ uptake was measured in the control water at 4 dpf.

Analytical tools and calculations

To determine Na^+ uptake, all collected water samples were supplemented with 5 ml of scintillation cocktail (Biosafe-II, RPI Corp., Mt. Prospect, IL, USA) and their radioactivity was measured with a liquid scintillation counter (model LS-6500 Beckman Coulter, Co. Mississauga ON, Canada). After being rinsed in an isotope-free medium, larvae were digested in a tissue solubilizer (Solvable^{TM}, Perkin Elmer) for 4 h at 65° C. After digestion, samples were supplemented with 5-ml of the same scintillation cocktail. Samples were then neutralized by adding 500 µl of glacial acetic acid before measuring their radioactivity. The concentration of

total Na^+ in the water was measured using flame emission spectrophotometry (model AA260, Varian, Palo Alto, CA, USA). Owing to the limited volume of water, [Na^+] was measured, and hence external specific activity was determined, only at the end of the flux period. It was assumed that, given the typical [Na^+] of the experimental water (700 - 1000 μM), Na^+ influx rate (on the scale of 1 nmol/fish/ or less) and even smaller expected net flux of Na^+ (difference between influx and efflux), changes in total [Na^+] during the flux period would be negligible. The rate of Na^+ uptake (J_{in}^{Na} , pmol/fish/h) was calculated as follows:

$$J_{in}^{Na} = \frac{F}{SA \cdot n \cdot t}$$

, where F = total incorporated radioactivity (DPM, disintegration per minute), SA = specific activity of the medium (DPM/pmol), n = number of larvae (typically 1) and t = the duration of the incubation (h). DPM was calculated by the liquid scintillation counting program after taking quenching and counting efficiency into consideration.

Statistical analysis

All statistical analyses were performed with SigmaPlot (v. 11, Systat Inc. Chicago, IL, USA). Student's *t*-test was used to analyze data from Series 2.2. One-way ANOVA followed by Tukey post hoc test was used to analyze data from Series 1, 3, 4, 2.1 and 6 (consequences of RU-486 treatment). Data in Series 1 were analyzed by comparing all treatment groups to the control group, with critical values adjusted to account for the multiple comparisons. Two-way ANOVA followed by Tukey post hoc test was used to analyze data from Section 6 (all data involving GR knockdown) and 5). When assumptions of normality or equal variance were violated, data were transformed using natural log- or square-root transformation. For all analyses, the level of statistical significance was set at $p<0.05$.

Results

Series 1 Effect of exposure to ion-poor or acidic water on whole body ANG-II levels

Acute (1 or 3 h) exposure to either acidic or ion-poor water led to significant increases in whole body ANG-II levels compared to the control group (N = 7 - 8; Fig. 5.1; one-way ANOVA).

Series 2 Effect of chronic exposure to ANG-I and -II on Na^+ uptake

Exposure to either ANG-I or ANG-II led to significant increases in Na^+ uptake by zebrafish larvae (N = 6 - 12; Figs. 5.2A, B; one-way ANOVA). The stimulatory effect of ANG-I was inhibited by co-treating the larvae with 100 µM lisinopril (N = 7; Fig.5.2C; one-way ANOVA). When the mRNA expression of three major Na^+ transporting genes was analyzed after 24-h treatment with ANG-II, the expression of only NCC (*zslc12a10.2*) was found to be significantly elevated, suggesting that the increase in Na^+ uptake after ANG-II treatment is at least partially mediated by NCC (N = 6; Fig. 5.2D; Student's t-test).

Series 3 Effect of acute variations in water chemistry on Na^+ uptake

Acute exposure to acidic water, or to water where [Na^+] or [Cl^-] was selectively lowered, significantly increased Na^+ uptake in zebrafish larvae (N = 5 - 11; Fig. 5.3; one-way ANOVA). As predicted, exposure to low Ca^{2+} water had no effect on Na^+ uptake (N = 6 - 11; Fig 5.3; one-way ANOVA).

Series 4 Pharmacological inhibition of RAS during exposure to acidic or ion-poor water

Acute exposure to acidic water caused a significant increase in Na^+ uptake (Figs 5.4A, B). When larvae were pre-treated with telmisartan (N = 5 - 12; Fig 5.4A; one way ANOVA) the stimulation of Na^+ uptake following the acid-exposure was attenuated. Pre-treatment with PD123319, however, was without effect (N = 6; Fig. 5.4B; one-way ANOVA).

Prior treatment with telmisartan attenuated the stimulation of Na^+ uptake after exposure to ion-poor water (N = 6 - 12; Fig. 5.5A; one-way ANOVA), whereas PD123319 treatment had no effect (N = 6; Fig. 5.5B; one-way ANOVA).

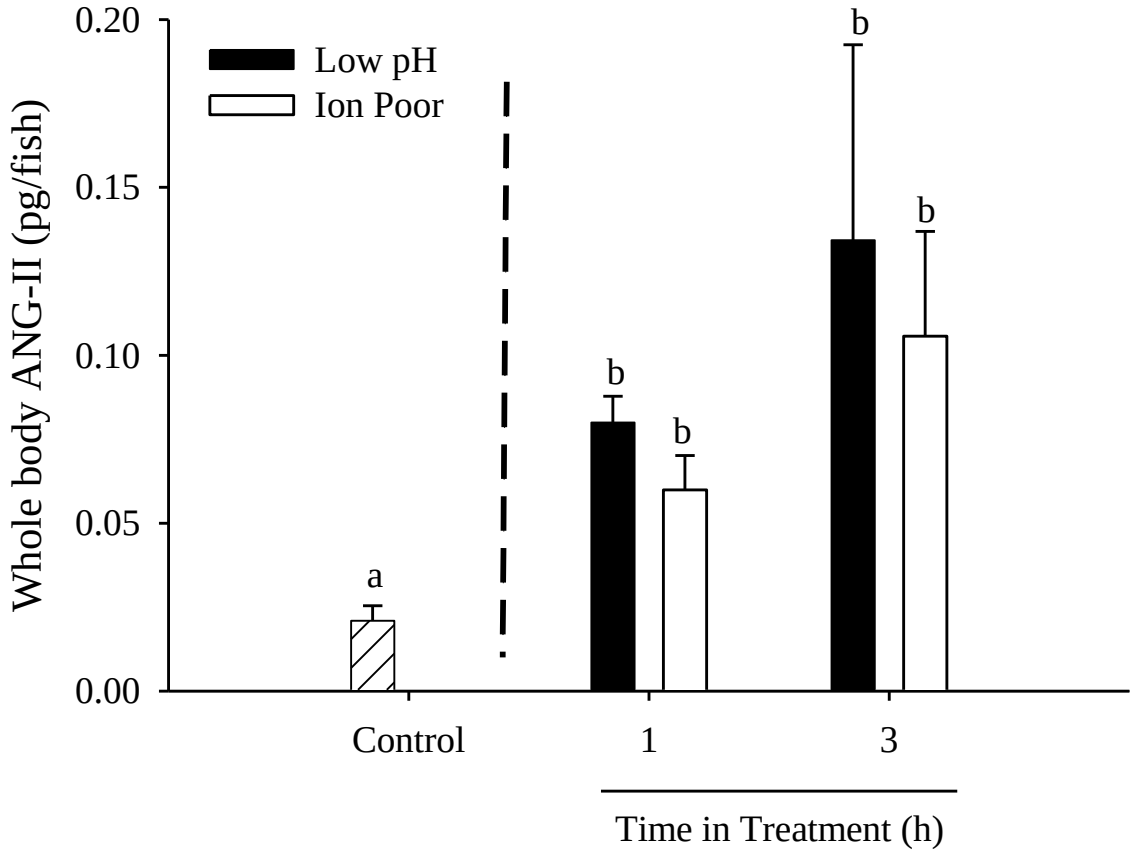
Series 5. Consequences of renin knockdown on Na^+ uptake in response to acute challenges

Western blotting using commercial antibodies against renin (Fig. 5.6A) and β-actin (Fig. 5.6B) demonstrated effective knockdown of renin following morpholino injection. The usual stimulatory effects of acid- or ion-poor water exposure on Na^+ uptake were absent in the renin morphants (N = 6 - 13; Fig. 5.6C, D; two-way ANOVA).

Series 6. Lack of an effect of cortisol on Na^+ uptake during acute exposure to acidic or ion-poor water.

Pre-treating larvae with 1 μM RU-486 before and during *acute* exposure to acidic or ion-poor water did not influence the usual stimulation of Na^+ uptake (N = 5 - 6; Figs. 5.7A, B; one-way ANOVA). A similar result was observed in GR morphants; acid- or ion-poor water-exposure increased Na^+ uptake equally in both sham and GR morphants (N = 6; Fig 5.7D, E; two-way ANOVA). However, the previously reported prominent role of GR in the regulation of Na^+ uptake following *chronic* elevation of cortisol was re-confirmed in the present study; unlike shams, the GR morphants did not respond to treatment with 500 nM cortisol (N = 6; Fig. 5.7C, two-way ANOVA).

Figure 5.1. The effect of acute exposure to low pH or ion-poor water on whole body ANG-II content. Acute (1 and 3 h) exposure to low pH or ion-poor water significantly increased whole body ANG-II content in 4 dpf zebrafish larvae. Different letters denote significant difference between the treatment and control groups. N = 7 - 8. Data are presented as means ± SEM.


0.20
0.15
0.10
0.05
0.00
Whole body ANG-II (pg/fish)
Low pH
Ion Poor
a
b
b
b
b
Control
1
3
Time in Treatment (h)

.

Figure 5.2. The effect of chronic exposure to ANG on Na^+ uptake. Treatment of 3 dpf larvae for 24-h with 100 - 1000 nM ANG-I (N = 12; Fig. 5.2A) or ANG-II (N = 6; Fig. 5.2B) significantly increased Na^+ uptake at doses above 500 nM. The induction of Na^+ uptake by 500 nM ANG-I was abolished when larvae were co-treated with 100 μM lisinopril (N = 7; Fig. 5.2C). The mRNA expression level of NCC was significantly elevated (N = 6; Fig. 5.2D); NHE3b and H^+-ATPase expression levels were unchanged. Different letters in Figs. 5.2A - C denotes significant difference among treatment groups, and asterisks in Fig. 5.2D denotes significant differences in treatment groups from the control group. Data are presented as means ± SEM.

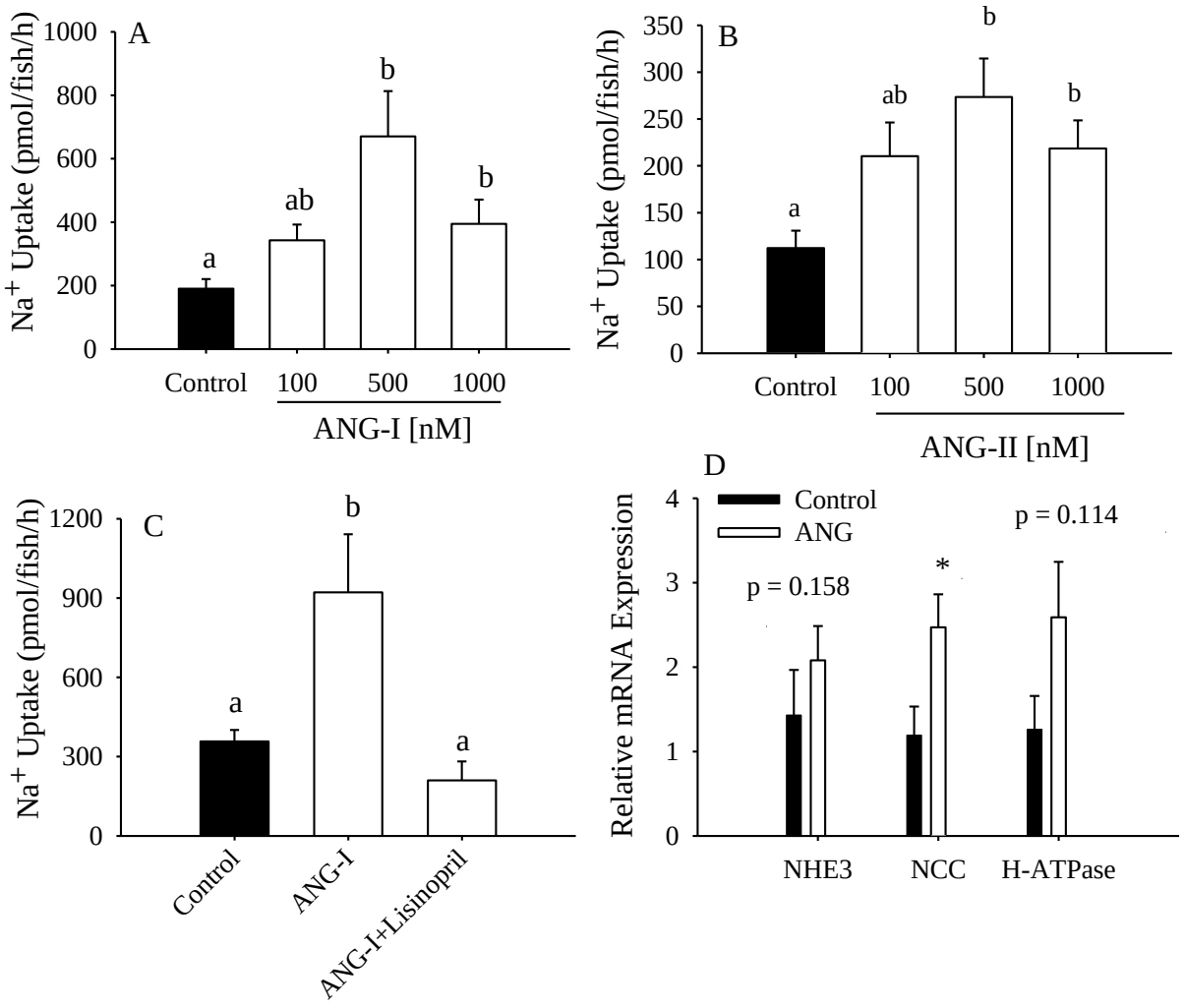

A
Na^+ Uptake (pmol/fish/h)
1000
800
600
400
200
0
a
ab
b
b
Control
100
500
1000
ANG-I [nM]
B
Na^+ Uptake (pmol/fish/h)
350
300
250
200
150
100
50
0
a
ab
b
b
Control
100
500
1000
ANG-II [nM]
C
Na^+ Uptake (pmol/fish/h)
1200
900
600
300
0
a
b
a
Control
ANG-I
ANG-I+Lisinopril
D
Relative mRNA Expression
4
3
2
1
0
Control
ANG
p = 0.158
*
p = 0.114
NHE3
NCC
H-ATPase

Figure 5.3. The effects of acute exposure to stressful water chemistry on Na^+ uptake.

Uptake of Na^+ was induced in larvae after acute (2 h) exposure to low pH, low Na^+ and low Cl^- water, whereas no significant effect was observed in larvae exposed to low Ca^{2+} water. An asterisk denotes significant difference from the control group. N = 5 - 11. Data are presented as means ± SEM.

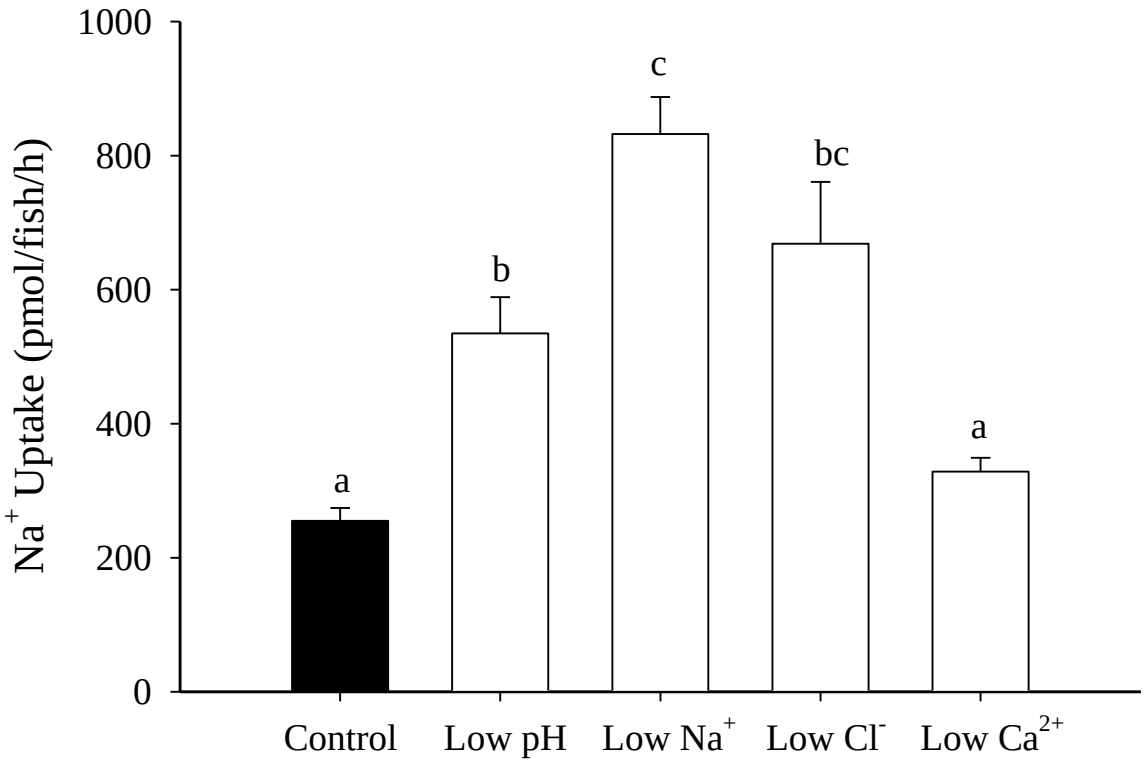

1000
800
600
400
200
0
Na+ Uptake (pmol/fish/h)
a
b
c
bc
a
Control
Low pH
Low Na+
Low Cl-
Low Ca2+

Figure 5.4. Effect of RAS inhibition during acute exposure to low pH water. Exposure to low pH water in the presence of 10 µM telmisartan (an AT_1 selective inhibitor; Tel) significantly reduced Na^+ uptake (N = 5 - 12; Fig. 5.4A), whereas treatment with PD123319 (an AT_2 selective inhibitor) had no effect on Na^+ uptake (N = 6; Fig. 5.4B). Different letters in Fig. 5.4A denote significant differences between treatment groups and an asterisk in Fig. 5.4B denotes significant difference from the control group. Data are presented as means ± SEM.

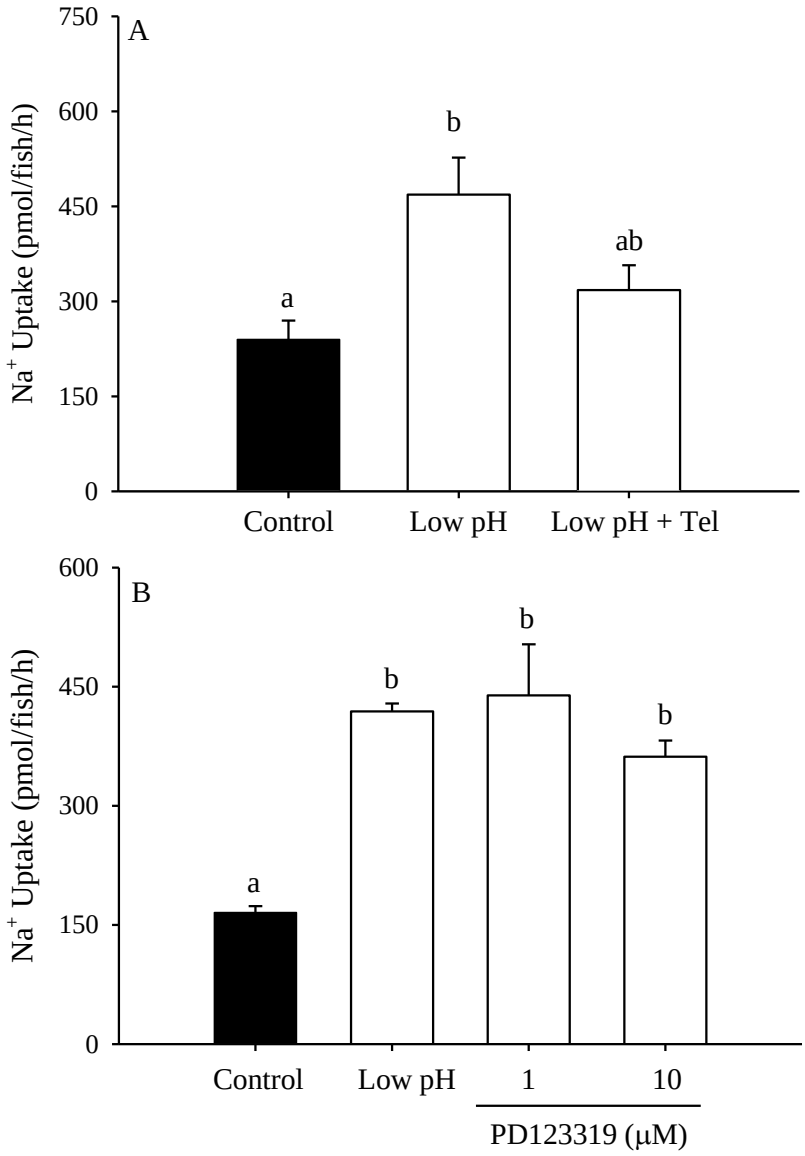

A
750
600
450
300
150
0
Na+ Uptake (pmol/fish/h)
a
b
ab
Control
Low pH
Low pH + Tel
B
600
450
300
150
0
Na+ Uptake (pmol/fish/h)
a
b
b
b
Control
Low pH
1
10
PD123319 (μM)

Figure 5.5. The effect of RAS inhibition during acute exposure to ion-poor water. Exposure to ion-poor water in the presence of 10 μM telmisartan (an AT_1 selective inhibitor; Tel) significantly reduced Na^+ uptake (N = 6 - 12; Fig. 5.5A), whereas treatment with PD123319 was without effect on Na^+ uptake (N = 6; Fig. 5.5B). Different letters in Fig. 5.5A denote significant differences among treatment groups and an asterisk in Fig. 5.5B denotes significant difference from the control group. Data are presented as means ± SEM.

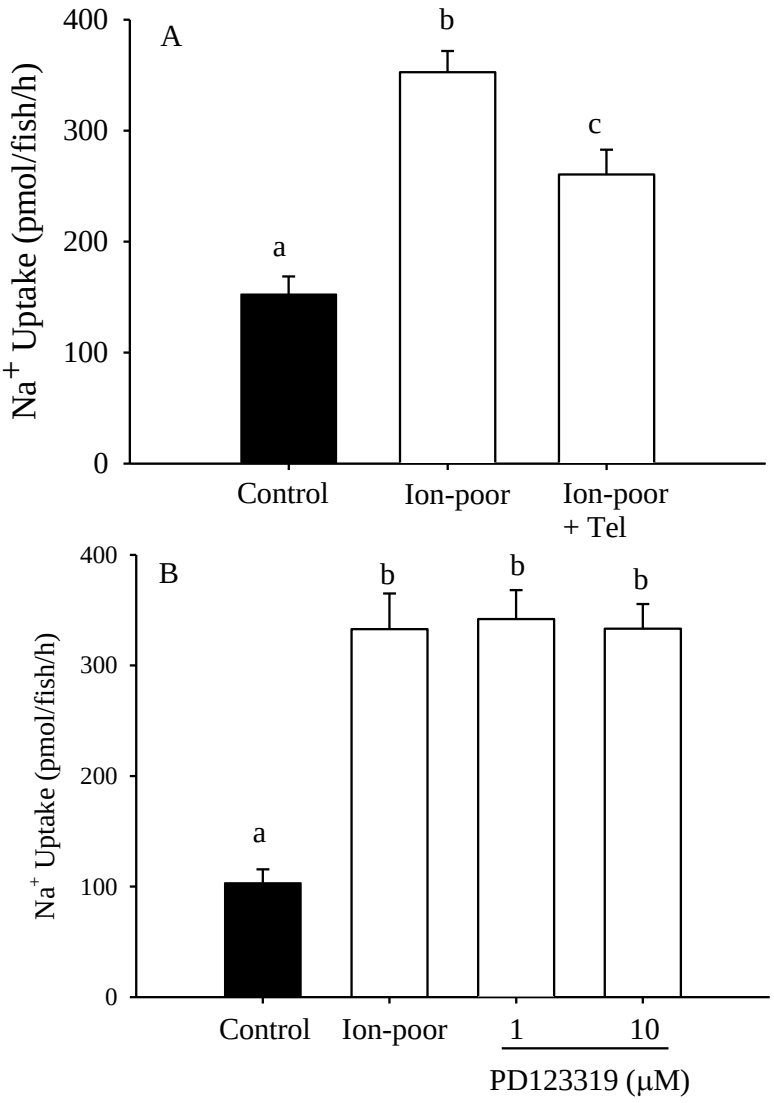

A
400
300
200
100
0
Na+ Uptake (pmol/fish/h)
a
b
c
Control
Ion-poor
Ion-poor
+ Tel
B
400
300
200
100
0
Na+ Uptake (pmol/fish/h)
a
b
b
b
Control
Ion-poor
1
10
PD123319 (μM)

Figure 5.6. The effect of renin knockdown on Na^+ uptake during acute low pH and ion-poor water exposure. Western blotting with a renin antibody detected a band corresponding to the expected size of renin in a protein derived from sham-injected 4dpf larvae (~35 kDa; Fig. 5.6A; lane "S"). This band was not observed in protein derived from the renin morphants, confirming the successful knockdown of renin (Fig. 5.6A; lane "M"). Blotting the same membrane with an antibody against β-actin demonstrated the equal loading of the protein between lanes (Fig. 5.6B). Na^+ uptake in renin morphants was not significantly induced following acute exposure to low pH- or ion-poor water (N = 6 - 13; Figs. 5.6C, D). Different letters denotes significant differences among treatment groups. An asterisk in Fig 5.6D indicates significant difference between Na^+ uptake in sham and renin morphants following ion-poor water exposure. Data are presented as means ± SEM.

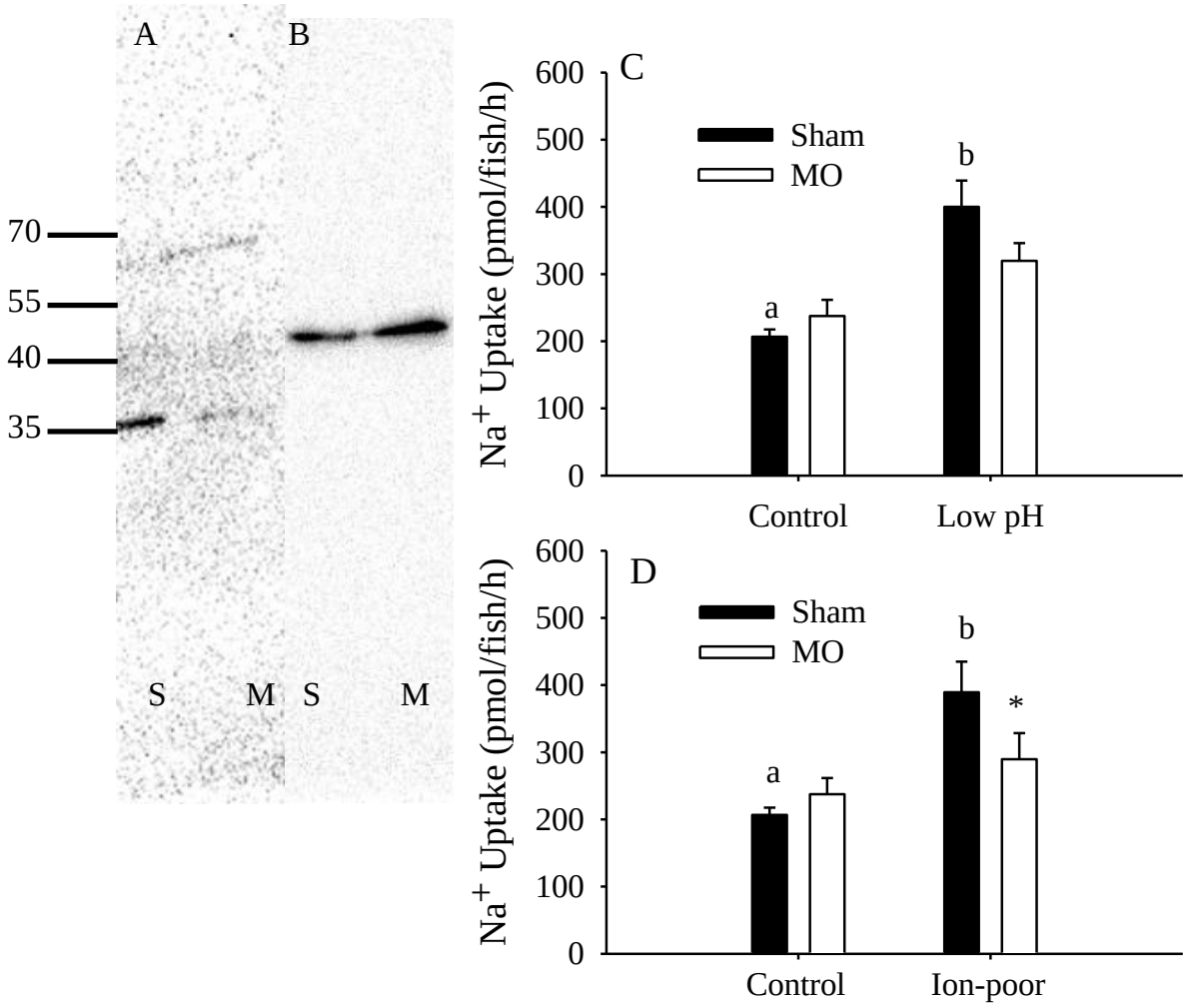

A
B
70
55
40
35
S
M
S
M
C
Sham
MO
Na^+ Uptake (pmol/fish/h)
600
500
400
300
200
100
0
a
b
Control
Low pH
D
Sham
MO
Na^+ Uptake (pmol/fish/h)
600
500
400
300
200
100
0
a
b
*
Control
Ion-poor

Figure 5.7. Cortisol does not contribute to the stimulation of Na^+ uptake during acute exposure to low pH- or ion-poor water. Treatment of larvae with 1 µM RU-486 (a GR antagonist) did not affect Na^+ uptake stimulation during acute exposure to low pH (N = 5 - 6; Fig. 5.7A) or ion-poor water (N = 5 - 6; Fig. 5.7B). Following GR knockdown, Na^+ uptake was not affected by 500 nM cortisol treatment (N = 6; Fig. 5.7C), thus confirming the effectiveness of the knockdown. GR knockdown did not impair the ability of larvae to stimulate their Na^+ uptake in response to acute acid or ion-poor water exposure (N = 6; Fig. 5.7D, E). Different letters denotes significant differences among treatment groups. Data are presented as means ± SEM.

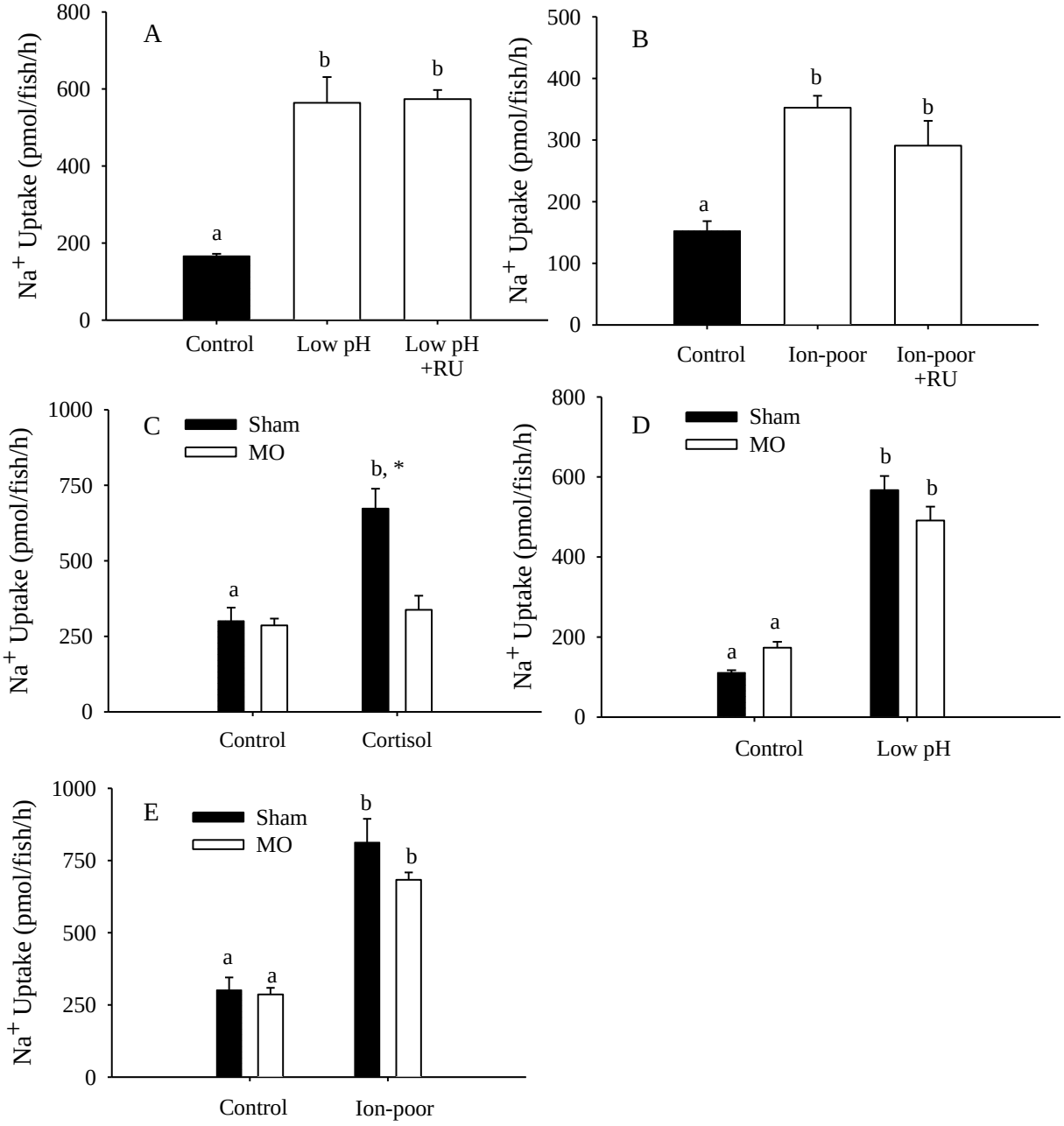

A
B
C
D
E
Na+ Uptake (pmol/fish/h)
Control
Low pH
Low pH +RU
Ion-poor
Ion-poor +RU
Cortisol
Sham
MO
a
b
b, *

Table 5.1. List of primers for RT-qPCR.

Gene	Sequence	Reference
NHE3 (*zslc9a3b*)	FWD: 5′-TGC AGA CAG CGC CTC TAG C-3′ REV: 5′-TGT GGC CTG TCT CTG TTT GC-3′	(Yan et al., 2007)
H^+-ATPase (*zatp6v1a*)	FWD: 5′-GAG GAA CCA CTG CCA TTC CA-3′ REV: 5′-CAA CCC ACA TAA ATG ATG ACA TCG-3′	(Chang et al., 2009)
NCC (*zslc12a10.2*)	FWD: 5′-GCC CCC AAA GTT TTC CAG TT-3′ REV: 5′-TAA GCA CGA AGA GGC TCC TTG-3′	(Wang et al., 2009)
18S	FWD: 5’-GGC GGC GTT ATT CCC ATG ACC-3’ REV: 5’-GGT GGT GCC CTT CCG TCA ATT C-3’	(Kumai et al., 2011)

Discussion

Based on the increase in whole body levels of ANG-II during exposure of larvae to acidic or ion-poor water and the marked attenuating effects of RAS inhibition on Na^+ uptake, the present study provides the direct evidence that the RAS is involved in stimulating Na^+ uptake in FW fish. Additionally, chronic (24 h) waterborne treatment of larvae with ANG-I or ANG-II significantly elevated Na^+ uptake, suggesting that the RAS may affect Na^+ uptake in FW fish during both acute and chronic environmental stress. Because cortisol does not appear to acutely stimulate Na^+ uptake, the mechanism of action of the RAS, at least during acute exposure to acidic or ion-poor water, apparently is unrelated to cortisol signalling.

The RAS promotes Na^+ uptake in zebrafish larvae

The physiological significance of the RAS in the regulation of salt reabsorption in the mammalian kidney has been firmly established (see Introduction). Although previous studies (e.g. Hoshijima and Hirose, 2007) suggested a role for the RAS in activating ion uptake in FW fish, no convincing physiological data have been published to support this idea. The rapid increase in whole body ANG-II content in response to two experimental treatments known to induce uptake of Na^+ (Fig. 5.1) as well as the elevation of Na^+ uptake following chronic treatment with either ANG-I or ANG-II (Fig. 5.2) demonstrate the potential of the RAS to stimulate Na^+ uptake in zebrafish.

ANG-II exerts its physiological effects by interacting with two distinctive receptor sub-types, AT_1 and AT_2. Because the expression of AT_2 is relatively low compared to AT_1, previous studies on the signalling pathways of ANG-II have focused largely on AT_1 (Stegbauer and Coffman, 2011). In fish, AT_1 receptors have been identified in several species, including zebrafish (Tucker et al., 2007), eel, *Anguilla anguilla*, toadfish, *Opsanus beta*, and rainbow trout, *Oncorhynchus mykiss*. Based on immunohistochemistry staining with a mouse monoclonal

antibody, the AT_1 receptor was localised to osmoregulatory tissues, including ionocytes from eel gill (for reviews see Nishimura, 2001; (Russell et al., 2001). The results of the present study revealed an inhibitory effect of AT_1 blockade (using telmisartan) on Na^+ uptake but the lack of an effect of AT_2 inhibition (using PD123319; Figs. 5.4, 5.5), suggesting the likely involvement of AT_1 in mediating the stimulatory effect of ANG-II on Na^+ uptake in zebrafish. While Tucker et al. (2007) detected a widespread expression of AT_1-like a (*zagtrl1a*) during embryogenesis of zebrafish, distribution patterns of ANG receptors in zebrafish larvae are unknown. In an attempt to visualize ANG-II binding sites in zebrafish larvae, fish were incubated with fluorescently conjugated ANG-II, which was used previously to visualize ANG-II receptors in rainbow trout posterior cardinal vein (Bernier and Perry, 1997). However, no clear staining was observed (Y. Kumai and S.F. Perry, unpublished observations) and there is no commercial antibody able to recognize the zebrafish ANG-II receptor. It should also be noted that, while the treatment with PD123319 did not have any effect on Na^+ uptake by zebrafish larvae, this negative result should be treated with caution, especially given that the efficacy of PD123319 as an antagonist of the zebrafish AT_2 receptor has not yet been established. Recently, Wong and Takei (2013) reported the sequence of AT_2 receptor from *Anguilla japonica* and observed its high expression in spleen and gill; no particularly strong staining was observed in branchial ionocytes based on *in situ* hybridization. Although it remains to be seen whether AT_2 is also expressed in zebrafish gill (or larval yolk sac skin), a role for AT_2 in mediating the observed effects of ANG-II in zebrafish cannot be excluded. The development of homologous antibodies directed against zebrafish AT_1 and AT_2, combined with knockdown of these receptors, would provide additional insight into the relative importance of these receptors in regulating Na^+ uptake.

As discussed in the Introduction, in mammalian kidney ANG-II activates H^+-ATPase, NHE3 and NCC, all of which have been proposed to play a role in Na^+ uptake by zebrafish (for recent reviews see Kumai and Perry, 2012; Hwang and Chou, 2013; Hwang et al., 2011). As an initial attempt to determine the transporter(s) activated by ANG-II treatment, I assessed the mRNA expression level of NCC, NHE3b and H^+-ATPase in 4 dpf zebrafish larvae following 24-h treatment with ANG-II. Interestingly, only the expression level of NCC was significantly elevated following chronic ANG-II treatment (Fig. 5.2D), suggesting that at least part of the increase in Na^+ uptake following chronic ANG-II treatment is mediated by NCC. It is interesting to note that stimulation of Na^+ uptake following acute (2h) exposure to low Cl^- water was attenuated in the presence of telmisartan (data not shown), suggesting that NCC might also be activated by ANG-II within hours. Clearly, the exact identity of the Na^+ transporters being regulated by ANG-II warrants further investigation.

Does RAS interact with cortisol in zebrafish?

Although recent studies have demonstrated independent effects of aldosterone and the RAS on salt reabsorption in the mammalian kidney (van der Lubbe et al., 2011; van der Lubbe et al., 2012), the two endocrine systems are intricately linked and indeed the RAS is sometimes referred to as the RA"A(aldosterone)"S. Although fish possess mineralocorticoid receptors (MR; Sturm et al., 2005), they lack the capacity to synthesize significant amounts of aldosterone (Colombo et al., 1972). Thus, in fish, cortisol binds to both MR and GR to activate their downstream signalling; Takahashi and Sakamoto, 2013). The physiological role of cortisol in promoting ionic uptake is extensively documented in FW fish (Cruz et al., 2013; Kumai et al., 2012a; Laurent and Perry, 1990; Lin et al., 2011; Shahsavarani and Perry, 2006), and it was recently demonstrated that chronic (48 h) waterborne treatment with cortisol promotes Na^+

uptake in zebrafish larvae through signalling via GR (Kumai et al., 2012a). To test whether the stimulation of Na^+ uptake by ANG-II is mediated by secondary induction of cortisol, the function of GR was inhibited by treatment with 1 μM RU-486 (a dose that was shown to abolish the stimulatory effect of cortisol) and injection of a GR-morpholino (Kumai et al., 2012a). Inhibition of GR function using either approach did not impede the increase in Na^+ uptake in response to acute acidic or ion-poor water exposure. These observations indicate that, during *acute* (2 h) experimental challenges, signalling via GR does not play a major role in inducing Na^+ uptake. Although (Kumai et al., 2012a) suggested that MR does not play a significant role in regulating Na^+ uptake in zebrafish during *chronic* exposure to acidic water, in the present study the hypothesis was tested that cortisol might interact with MR during *acute* challenge with acidic or ion-poor water. Treating larvae with 10 μM eplerenone, a recently developed MR inhibitor known to be effective in zebrafish (Pippal et al., 2010), did not hinder the capacity of fish to increase Na^+ uptake during acute exposure to acidic or ion-poor water (data not shown). Thus, the effect of ANG-II on Na^+ uptake reported in the present study is likely to be cortisol-independent. Whether the acute physiological responses mediated by ANG-II eventually trigger, over a longer term, cortisol-mediated increase in Na^+ uptake is an interesting area for further research.

Interactive effects of adrenergic receptor and RAS during acute osmotic challenge?

In fish (Bernier and Perry, 1997) and mammals (DiBona, 2000), activation of the RAS is known to trigger the secretion of catecholamines. This interaction between the RAS and catecholamines has been suggested to play an important role in regulating cardiovascular function in response to hypotension (Bernier et al., 1999a; 1999b) and hypoxia (Bernier and Perry, 1997; Lapner and Perry, 2001). A recent study demonstrated that, while knockdown of β_1

and β_{2B} adrenergic receptors blunted the stimulation of Na^+ uptake following chronic (24 h) exposure to acidic water, knockdown of only β_{2A} receptor prevented the stimulation of Na^+ uptake in zebrafish larvae acutely (~ 3 h) exposed to ion-poor water (Kumai et al., 2012b). Given the interaction between RAS and catecholamine, results from the present study raise an interesting possibility that at least during acute osmoregulatory challenge, RAS and catecholamines (and subsequent activation of at least β_{2A}-adrenergic receptors) might be interacting to achieve stimulation of Na^+ uptake. While whole body catecholamine levels in zebrafish larvae are known to be elevated following chronic exposure (4 days) to hypoxia (Steele et al., 2011a), it is unknown whether whole body catecholamine levels in larval zebrafish could be modulated through activation of RAS within only a few hours, the time course over which Na^+ uptake was activated by the RAS in the present study.

Based on the colocalization of fluorescent propranolol (a generic β-receptor antagonist) and concanavalin-A, a vital marker of H^+-ATPase rich cells (HRCs), Kumai et al. (2012b) suggested that stimulation of Na^+ uptake by catecholamines is predominantly mediated by HRCs, where NHE3b and H^+-ATPase enable Na^+ uptake linked to acid-secretion (Dymowska et al., 2012; Horng et al., 2007; Hwang, 2009; Hwang et al., 2011). Although the staining was weaker, there was an additional population of cells stained with propranolol that were concanavalin-A negative. Determining whether this additional population of propranolol-positive cells are NCC-expressing cells remains a difficult task, because of the lack of an antibody that reliably stains NCC. However, given the results of the current study, the potential expression of β-adrenergic receptors on non-HRCs (more specifically, NCC-expressing cells) warrants investigation.

Perspectives

Despite the well-documented role of the RAS in promoting salt reabsorption in the mammalian kidney, its role in promoting ionic uptake in FW fish has largely been unexplored. With the current study providing the first evidence implicating the RAS in the regulation of Na^+ uptake in zebrafish, a number of issues emerge including i) whether ANG-II receptors are expressed in osmoregulatory tissues (adult gill, kidney, larval skin) and the relative contribution of AT_1 and AT_2 receptors, ii) how ANG-II interacts with other known regulatory mechanism of ion uptake in FW fish, iii) what are the downstream signalling cascades activated by ANG-II receptors, that ultimately lead to the increase in Na^+ uptake, and iv) what are the mechanisms that detect rapid alterations in water chemistry and trigger the synthesis of ANG-II. Development of more specific inhibitors for AT_1 and AT_2 receptors, as well as homologous antibodies for these receptors, would allow some of these interesting issues to be addressed in the future.

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CHAPTER 6
β-adrenergic regulation of Na^+ uptake by larval zebrafish *Danio rerio* in acidic and ion-poor environments

Notes on Chapter

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SFP conceived the project; YK and MARW performed the experiments. The manuscript was

written by YK and edited by SFP.

Abstract

The potential role of adrenergic systems in regulating Na^+ uptake in zebrafish (*Danio rerio*) larvae was investigated. Treatment with isoproterenol (a generic β–adrenergic receptor agonist) stimulated Na^+ uptake, whereas treatment with phenylephrine (an α_1-adrenergic receptor agonist) as well as clonidine (an α_2-adrenergic receptor agonist) significantly reduced Na^+ uptake, suggesting opposing roles of α- and β-adrenergic receptors in Na^+ uptake regulation. The increase in Na^+ uptake associated with exposure to acidic water (pH = 4.0), was attenuated in the presence of the non-selective β-receptor blocker propranolol or the β_1-receptor blocker atenolol; the β_2-receotor antagonist ICI-118551 was without effect. The stimulation of Na^+ uptake associated with ion-poor water (32-fold dilution of Ottawa tap water) was unaffected by β-receptor blockade. Translational gene knockdown of β-receptors using antisense oligonucleotide morpholinos was used as a second method to assess the role of adrenergic systems in the regulation of Na^+ uptake. Whereas β_1- or β_{2B}-receptor knockdown led to significant decreases in Na^+ uptake during exposure to acidic water, only β_{2A}-receptor morphants failed to increase Na^+ uptake in response to ion-poor water. In support of the pharmacology and knockdown experiments that demonstrated an involvement of β-adrenergic systems in the control of Na^+ uptake, it was showed that the H^+-ATPase rich cells (HRCs), a subtype of ionocyte known to be a site of Na^+ uptake, is innervated and appears to express β-adrenergic receptors (propranolol binding sites) at 4 days post fertilization (dpf). These data indicate an important role of adrenergic systems in regulating Na^+ uptake in developing zebrafish.

Introduction

In freshwater (FW) teleosts, ionic constancy of the body fluids in the face of continuous passive ion loss is achieved by the active absorption of ions from the environment. Ion uptake occurs via a specialized subset of epithelial cells, termed ionocytes or mitochondrion rich cells (MRCs) distributed in the adult gill and on the skin (predominantly on the yolk sac) of larvae. The molecular mechanisms underlying the active uptake of the major ions, Na^+, Cl^- and Ca^{2+}, have received considerable attention over the past 80 years. However, there are still several unresolved questions regarding the nature of ion uptake by FW fish (for recent reviews on FW fish osmoregulation, see: Hwang, 2009; Hwang and Perry, 2010; Hwang et al., 2011).

In response to changes in the environment, FW teleosts modulate their ion transport mechanisms to maintain overall ion homeostasis. In particular, endocrine factors, including cortisol and prolactin, have received significant attention in the modulation of branchial ion transport mechanisms. Cortisol, though initially identified as a "seawater (SW)" acclimation hormone, is now recognized to play an important role in regulating ion balance in FW fish. For example, (Lin et al., 2011) and (Kumai et al., 2012a) reported that waterborne exposure to cortisol significantly increased Ca^{2+} and Na^+ uptake by zebrafish larvae, and a similar stimulation of ion uptake following cortisol treatment was also reported for rainbow trout (Flik and Perry, 1989; Laurent and Perry, 1990). In addition, application of cortisol to a cell culture derived from rainbow trout gill led to a significant reduction in paracellular permeability, presumably serving to reduce the passive loss of ions to the environment (Chasiotis et al., 2010). Similarly, prolactin is known to reduce membrane permeability (for review see Manzon, 2002) as well as inducing "FW" type ionocytes in Mozambique tilapia, *Oreochromis mossambicus*, gill (Breves et al., 2010). The results of these studies clearly indicate the presence of multiple endocrine factors regulating salt balance in FW fish.

In addition to these endocrine factors, neurohumoral agents, such as catecholamines, could contribute to ion homeostasis. In mammals, stimulation of β-adrenergic receptors leads to Na^+ and Cl^- retention by the kidney owing to the activation of various channels and/or transporters including Na^+-Cl^- co-transporter (NCC; Mu et al., 2011) and pendrin (SLC26A4; Azroyan et al., 2012; for review see DiBona, 2005). Similarly, a role for adrenergic receptors in ion homeostasis has been proposed for FW fish. For example, Perry et al. (1984) observed an inhibitory effect of β-adrenergic receptor stimulation and an opposing, stimulatory effect of α-adrenergic receptor stimulation on branchial Cl^- uptake. Donald (1989) reported a reduction in Ca^{2+} uptake following the stimulation of branchial nerves or addition of adrenaline, suggesting an inhibitory role of adrenergic nerves in Ca^{2+} uptake. Na^+ uptake also appears to be under adrenergic regulation. For example, Girard and Payan (1977) demonstrated a stimulation of Na^+ uptake by perfused rainbow trout gills following adrenaline treatment. Subsequently, Morgan and Potts (1995) reported a significant increase in $[Na^+]_i$ and $[Cl^-]_i$ of brown trout (*Salmo trutta*) gill pavement cells following treatment with isoproterenol, a non-selective β-adrenergic receptor agonist. Although the latter results should be interpreted cautiously (see discussion in Morgan and Potts, 1995), these two studies suggest that adrenergic systems, in particular those leading to activation of β-receptors, are playing a stimulatory role in Na^+ uptake. On the other hand, Vermette and Perry (1987) reported that continuous intra-arterial infusion of rainbow trout with adrenaline significantly inhibited the branchial uptake of both Na^+ and Cl^-. Although the results of all of these studies support a role of adrenergic systems in the control of Na^+ uptake, the use of different experimental approaches (gill perfusion versus whole animal studies, isotopic fluxes versus intracellular concentration measurement) makes generalization difficult.

In addition, there are few data concerning the innervation of ionocytes, especially during larval stages prior to maturation of an endocrine adrenergic response. Although circulating catecholamine could be sufficient to regulate ion uptake, the direct innervations of ionocytes, if observed, would strongly support the neurohumoral regulation of ion uptake in larval fish. To date, only a single study (Jonz and Nurse, 2006) has provided evidence that ionocytes in FW fish are innervated. Specifically, it was reported that Na^+-K^+-ATPase rich cells (NaRCs) in zebrafish are innervated both in the adult gill and in the skin of larvae at 3 days post fertilization (dpf) (for a recent review on gill innervation, see Jonz and Nurse, 2008) . Importantly, however, recent studies have demonstrated that Na^+ uptake by zebrafish larvae is carried out by H^+-ATPase rich cells (HRCs), not NaRCs (for a review of zebrafish ionocyte subtypes and their functions, see (Hwang et al., 2011). However, there are no data concerning the innervation status of HRCs or whether they express adrenergic receptors.

With this background, the overall objective of the present study was to investigate the potential regulation of Na^+ uptake by neurohumoral factors in zebrafish larvae. Specifically, the present study addressed whether 1) catecholamines and other neurotransmitters including serotonin and dopamine, could affect Na^+ uptake; 2) whether adrenergic signalling is involved in maintaining Na^+ balance during exposure of fish to environmental conditions known to challenge Na^+ homeostasis (exposure to acidic or ion-poor water) and 3) whether HR cells are innervated and express adrenergic receptors. The data clearly demonstrate that HRCs in larvae at 4 dpf are innervated, express β-adrenergic receptors and that adrenergic signalling via these β-receptors contribute significantly to the regulation of Na^+ uptake in zebrafish larvae.

Materials and Methods

Experimental animals and husbandry

Adult zebrafish (*Danio rerio* Hamilton-Buchanan 1822) were purchased from Big Al's Aquarium Services (Ottawa, ON, Canada) and kept in the University of Ottawa Aquatic Care Facility where they were maintained in plastic tanks supplied with aerated, dechloraminated City of Ottawa tap water at 28° C. Fish were subjected to a constant 14 h L:10 h D photoperiod and fed daily until satiation with No. 1 crumble-Zeigler^{TM} (Aquatic Habitats, Apopka, FL, USA). Embryos were collected following the standard method (Westerfield, 2000). Briefly, male and female pairs (1 male and 2 female) were placed in breeding cage and collected embryos were reared in 50 ml Petri dishes supplemented with dechloraminated City of Ottawa tap water (pH 7.3-7.5) with 0.05% ethylene blue unless otherwise stated. The Petri dishes were kept in incubators set at 28.5° C. Dead embryos were removed and water was changed daily. As all experiments were performed on 4 dpf (days post fertilization) fish, they were not fed for the duration of the experiment. The experiments were conducted in compliance with guidelines of the Canadian Council of Animal Care (CCAC) and after the approval of the University of Ottawa Animal Care Committee (Protocol BL-226). Unless stated otherwise, all chemicals used for the experiments were purchased from Sigma.

Experimental protocols

To test the potential adrenergic regulation on Na^+ uptake by larval zebrafish, the following series of experiments were performed.

Series 1. Effect of receptor agonist/antagonist treatment on Na^+ uptake

Na^+ uptake was assessed in 4 dpf larvae that were acutely treated with selected pharmacological agents (agonists and/or antagonists) for adrenergic (α and β), dopaminergic and

serotonergic receptors (for the list of pharmacological agents and their targets, see Table 6.1). All pharmacological agents were dissolved in water and fish were exposed to final external concentrations of 100 µM.

Immediately after the addition of pharmacological agents, the rate of Na^+ uptake was measured using ^{22}Na. For the measurement, 0.25 µCi ^{22}Na in the form of NaCl (Perkin Elmer, Woodbridge, ON, Canada) was added to each tube containing larvae to a final activity of 0.15 µCi/ml. Water samples (50 µl) were collected at 5 min and 2 h for the later determination of radioactivity. At the end of the 2 h flux period, larvae were killed by overdose with ethyl 3-aminobenzoate methanesulfonate (MS-222; 4 mg/ml, pH = 7.4) and briefly washed in isotope-free water containing high levels of Na^+ (>200 mM) to remove any residual radioisotope attached to the surface of the fish. The remaining water in the tube was stored separately for later measurement of the total [Na^+]. For the processing of samples and calculation of influx rate, see "analytical methods and calculation" section below.

For phenylephrine and clonidine (α_1 and α_2 agonist, respectively), dobutamine and procaterol (β_1 and β_2 agonist respectively) and epinephrine and norepinephrine ("natural" adrenergic agonists), in addition to testing the effect of these pharmacological reagents at one dose (100 µM), larvae were also treated with these reagents at 1 and 10 µM to assess the relative contribution of different adrenergic receptors on Na^+ uptake regulation. Na^+ uptake was measured as described above.

Series 2. Consequence of β-adrenergic receptor inhibition on Na^+ uptake in ion-poor and low pH water.

Previous studies demonstrated zebrafish increase their Na^+ uptake capacity in response to soft water (Boisen et al., 2003) or low pH (Kumai and Perry, 2011). To assess the potential role

of β-adrenergic receptors in the response to low pH, larvae were raised in control water until 3 dpf. They were then given one of the treatments for 24 h: 1) transfer to control water; 2) exposure to acidic water in the absence of any pharmacological reagents; 3) exposure to acidic water in the presence of propranolol, atenolol or ICI-118551 (β-receptor antagonists). Na^+ uptake was measured as described above using 4 dpf larvae.

To assess the possible role of β-receptors in stimulating Na^+ uptake in response to ion-poor water, 4 dpf zebrafish were subjected to the following treatments: 1) transfer to control water; 2) transfer to ion-poor water for 2 h followed by acute return to the control ($[Na^+]$ ~700 μM) water; and 3) exposure to ion-poor water as above but in the presence of propranolol, atenolol or ICI-118551 (the concentration of these blockers was kept at 100 μM during dilution of control water by appropriate adjustment of drug quantities). In all cases, Na^+ uptake was measured in water containing normal levels of Na^+ (~700 μM) as described above. Acidic water (pH = 4; 3.95-4.01) was prepared by adding H_2SO_4 to Ottawa tap water. Ion-poor water was prepared by 2-fold dilution of the control water with deionised water at 15 min intervals until the target $[Na^+]$ (~ 30 μM) was reached (see Discussion for the rationale for using this $[Na^+]$). Larvae were then left in ion-poor condition for 2 h before transferred back to the control water, in which Na^+ uptake was measured. No mortality was observed.

Series 3. Consequences of β-receptor knockdown on Na^+ uptake in ion-poor or acidic water.

Fluorescein isothiocyanate (FITC)-tagged antisense oligonucleotide morpholinos targeting the translation start site of zebrafish β_1, β_{2A} or β_{2B} receptor mRNA were designed by GeneTools (http://www.gene-tools.com/), whose target specificity was validated in a previous study (for morpholino sequence, see Steele et al., 2011b). The morpholino was prepared to a

final concentration of 4 ng/nl in 1x Danieau buffer [58 mM NaCl, 0.7 mM KCl, 0.4 mM $MgSO_4$, 0.6 mM $Ca(NO_3)_2$, and 5.0 mM HEPES (pH 7.6)] and 0.05 % phenol red. Injections were performed using a microinjector system (model IM 300, Narishige, Long Island, NY). For the dose of injected morpholino, see (Steele et al., 2011b). Control groups were injected with a standard control morpholino (5-CCTCTTACCTCAGTTACAATTTATA-3) that was prepared and injected as the other morpholinos. No significant mortality or deformities were observed in injected larvae. At 24 hpf, injected larvae were screened for fluorescence and only FITC-positive fish were raised further and used for the subsequent experiments.

To assess the consequences of β-receptor knockdown on Na^+ uptake, β-receptor morphants as well as sham injected larvae were exposed to acidic or ion-poor water as described in Series 2. The rate of Na^+ uptake was measured using 4 dpf larvae; for calculation of Na^+ influx rate, see "analytical tools and calculations" section.

Series 4. Immunohistochemistry and visualization of β-receptors

To determine whether HRCs are innervated by 4 dpf, larvae were killed with an overdose of MS-222 and fixed overnight at 4° C in a 4% paraformaldehyde solution prepared in PBS. After fixation, larvae were briefly rinsed in PBS and permeabilized in 100% ethanol at – 20° C for 20 min. Subsequently, larvae were rehydrated in 50 and 100% PBS and incubated overnight at room temperature with 5% Triton-X mixed in PBS (PBST) with gentle shaking. Larvae were incubated with a rabbit polyclonal antibody against a highly conserved region of α-subunit of H^+-ATPase [peptide sequence AEMPADSGYPAYLGAR (Hayashi et al., 2000)] with 100% identity to the corresponding region of the zebrafish α-subunit H^+-ATPase. The antibody (a generous gift from Dr. M. Uchiyama, University of Toyama) was used at a dilution of 1:2000. A monoclonal antibody against a generic zebrafish neuronal marker (zn-12; Developmental Studies

Hybridoma Bank, University of Iowa) was used at a dilution of 1:250. After several washes in PBS, larvae were incubated with Alexa Fluor-488 conjugated anti-rabbit or alexa-546 conjugated anti-mouse secondary antibodies (1:500, Invitrogen, Burlington, ON) for 1 h at 37 ° C and mounted onto a glass slide. Samples were observed using confocal microscopy (Leica ZM-510) and images were acquired using commercial software (Zen, Zeiss).

To visualize the distribution of β-receptors, 4 dpf larvae were exposed to fluorescently labelled propranolol (Abcam, 100 nM) for 30 min at room temperature. Larvae were killed (MS-222 overdose), washed in PBS and mounted on a glass slide. To determine the ionocyte subtype expressing β-receptors, a separate group of larvae were exposed to Alexa Fluor-488 conjugated concanavalin-A (conA), a vital dye commonly used to identify HRCs in zebrafish (Esaki et al., 2007; Lin et al., 2006); 5 µg/ml, room temperature for 1 h), MitoTracker® Red (Invitrogen; 100 nM, room temperature for 1 h) and propranolol (500 nM, room temperature for 30 min). After staining, larvae were processed as described above. In all cases, samples were observed using confocal microscopy (Leica ZM-510) and images acquired using Zen software (Zeiss). For both staining, protocol was repeated on at least three different occasions with 4 or more larvae each time.

Analytical methods and calculations

To determine Na^+ uptake, all collected water samples were supplemented with 5 ml of scintillation cocktail (Biosafe-II, RPI Corp., Mt. Prospect, IL, USA) prior to measuring radioactivity using a liquid scintillation counter (model LS-6500 Beckman Coulter, Co. Mississauga ON, Canada). After being rinsed in an isotope-free medium, larvae were digested in a tissue solubilizer (Solvable^{TM}, Perkin Elmer) overnight at 65° C. After complete digestion, samples were supplemented with 5 ml of the same scintillation cocktail. Samples were then

neutralized by adding 400 µl of glacial acetic acid before measuring their radioactivity. The concentration of total Na^+ in the water was measured using flame emission spectrophotometry (model AA260, Varian, Palo Alto, CA, USA). Owing to the limited volume of water, [Na^+] was measured, and hence external specific activity was determined, only at the end of the flux period. It was assumed that, given the typical [Na^+] of the experimental water (700 - 1000 µM), Na^+ influx rate (on the scale of 1 nmol/fish/h or less) and an even smaller expected net flux of Na^+ (difference between influx and efflux), changes in total [Na^+] during the flux period would be negligible. The rate of Na^+ uptake (J_{in}^{Na} , pmol/fish/h) was calculated as follows:

$$J_{in}^{Na} = \frac{F}{SA \cdot n \cdot t}$$

, where F = total incorporated radioactivity (DPM, disintegration per minute), SA = specific activity of the medium (DPM/pmol), n = number of larvae per digest (1) and t = the duration of the incubation (h). DPM was calculated by the liquid scintillation counting program after taking quenching and counting efficiency into consideration.

Statistical analysis

All statistical analyses were performed and data were graphed with SigmaPlot (v. 11, Systat Inc. Chicago, IL, USA). The effect of pharmacological treatment under control conditions (Series 1) was analyzed using Student's t-test. The consequences of pharmacological inhibition of β-receptor in ion-poor and acidic water (Series 2) were analyzed using one-way ANOVA. The consequences of β-receptor knockdown on Na^+ uptake in ion-poor and acidic water (Series 3) were analyzed using two-way ANOVA. When normality or equal variance assumptions were violated (determined automatically by software), data were transformed by calculating log or square root. In all statistical analysis, the significance level was set at $p < 0.05$.

Results

Series 1. Effect of receptor agonist/antagonist treatment on Na^+ uptake in normal water

The effects of various receptor agonists/antagonists are summarized in Table 6.1. Na^+ uptake was stimulated by isoproterenol (ß+) or procaterol $ß_2$+); there was a strong trend for an increase in Na^+ uptake following dobutamine ($ß_1$+) treatment ($p = 0.06$). On the other hand, treatment with phenylephrine (α_1+) and clonidine (α_2+) significantly reduced Na^+ uptake. Treatment with propranolol (ß-), phentolamine (α-), 5-HT or dopamine was without effect on Na^+ uptake (Table 1). Co-treatment of fish with isoproterenol and propranolol prevented the increase in Na^+ uptake normally associated with isoproterenol treatment (control: 253 ± 19 pmol/fish/h, propranolol: 238 ± 14 pmol/fish/h and propranolol/isoproterenol: 282 ± 15 pmol/fish/h, N = 12 for all groups).

To better characterize the relative contribution of α- and β- adrenergic receptors on Na^+ uptake, dose responses were determined on selected pharmacology. Phenylephrine significantly reduced Na^+ uptake only at 10 and 100 µM dose, whereas clonidine reduced Na^+ uptake with as low as 1 µM (N = 5 - 6; Figs. 6.1A,B ; one-way ANOVA). On the other hand, both dobutamine and procaterol increased Na^+ uptake only at 100 µM (N = 5 - 13; Figs. 6.1C,D; one-way ANOVA). When larvae were treated with epinephrine, there was an overall inhibitory trend on Na^+ uptake ($p = 0.028$), although post-hoc test did not reveal significant difference among different groups (N = 5 - 6; Fig. 6.1E; one-way ANOVA). Treatment with norepinephrine significantly increased Na^+ uptake at 10 µM (N = 5 - 6; Fig. 6.1F; one-way ANOVA).

Series 2. Effect of β-adrenergic receptor inhibition on Na^+ uptake in acidic or ion-poor water

When larvae were exposed to low pH water in the presence of propranolol or atenolol, Na^+ uptake was significantly reduced (N = 5 - 7; Figs. 6.2 A,B; one-way ANOVA). On the other

hand, Na^+ uptake was unaffected when larvae were treated with the ß$_2$-receptor antagonist ICI-118551, (N = 5 - 7; Fig. 6.2 C; one-way ANOVA). In ion-poor water, Na^+ uptake was unaffected by treatment of larvae with propranolol, atenolol or ICI-118551 (N = 5 - 6; Figs. 6.3 A-C; one-way ANOVA).

In addition, the treatment of zebrafish larvae with α-receptor agonists (phenylephrine or clonidine) after 24-h exposure to low pH water or brief exposure to ion-poor water, markedly reduced Na^+ uptake, further implying an inhibitory role of the α-adrenergic receptor on Na^+ uptake (data not shown).

Series 3. Consequences of β-receptor knockdown on Na^+ uptake in acidic and ion-poor waters

All morphant fish (β_1, β_{2A}, β_{2B}, and β_{2A}/β_{2B} dual morphants) exhibited control rates of Na^+ uptake (N = 4 - 7; Figs. 6.4, 6.5; two-way ANOVA). However, when the morphants were exposed to acidic or ion-poor waters, an isoform (subtype)-dependent effect on Na^+ uptake was observed. Specifically, Na^+ uptake was reduced in acidic water by β_1, β_{2B}, and dual β_{2A}/β_{2B} knockdown (N = 5 - 7; Fig. 6.4; two-way ANOVA), whereas in the fish exposed to ion-poor water, only the β_{2A} and dual morphants exhibited decreases in Na^+ uptake (N = 4 - 7; Fig.6.5; two-way ANOVA). It is worth noting that inhibitory effect of β-adrenergic receptor knockdown was only marginal (though still significant) in acidic water, whereas β_{2A} and dual morphants exhibited no increase in Na^+ following their exposure to ion-poor water (Figs. 6.4,6.5).

Series 4. Visualization of HRC innervation and β-receptor expression

Numerous HRCs and an extensive network of nerves were detected on the yolk sack of 4 dpf larvae (Fig. 6.6A). Upon close inspection, nerve terminals appeared to be making a contact with HR cells (Fig. 6.6B), suggesting the innervation of HRCs by 4 dpf.

Similar to H^+-ATPase immuno-staining, numerous propranolol-bound cells were detected on the yolk sack of 4 dpf larvae. When the larvae were co-stained with conA and propranolol, the majority of propranolol-positive cells were also stained with conA, suggesting that HRCs indeed express β-receptors (Fig. 6.7A-D). In addition, there appeared to be a propranolol-positive, conA-negative population of cells, although in these cells the propranolol staining was relatively weak. These conA-negative cells were co-labelled with MitoTracker®, thereby hinting at the presence of another ionocyte group that also harbours β-receptors (Fig. 6.7E-H).

Figure 6.1. The dose response to selected adrenergic agonists. Whereas phenylephrine (α_1 agonist) significantly reduced Na^+ uptake at 10 and 100 µM (N = 5 - 6; Fig. 6.1A), 1 µM clonidine (α_2 agonist) was sufficient to reduce Na^+ uptake (N = 5 - 6; Fig. 6.1B). Both dobutamine (β_1 agonist) and procaterol (β_2 agonist; N = 5 - 13; Figs. 6.1C, D) increased Na^+ uptake only at 100 µM. Although there was an overall treatment effect in epinephrine-treated group ($p = 0.028$), post hoc test failed to detect no significant difference between any groups (N = 5 - 6; Fig. 6.1E). Unlike epinephrine treatment, norepinephrine treatment significantly increased Na^+ uptake, suggesting that Na^+ uptake could also be affected by treatment with natural adrenergic agonists (N = 5 - 6; Figs. 6.1E, F). Different letters denote significant difference among treatment groups. Data are presented as means ± SEM.

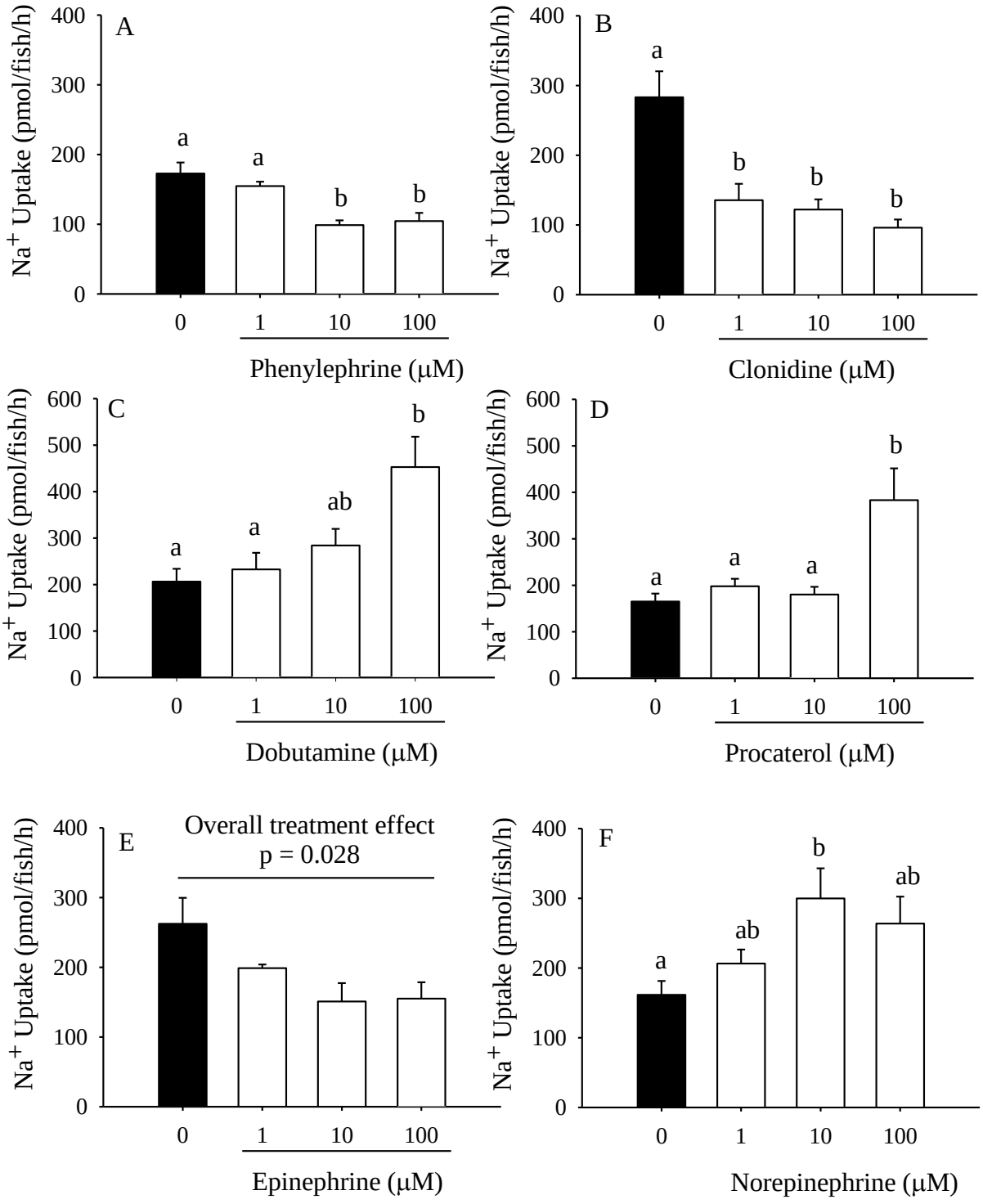
A
B
C
D
E
F
Na+ Uptake (pmol/fish/h)
Phenylephrine (μM)
Clonidine (μM)
Dobutamine (μM)
Procaterol (μM)
Epinephrine (μM)
Norepinephrine (μM)
Overall treatment effect
p = 0.028
0
1
10
100

Figure 6.2. The effect of β-receptor inhibition on Na^+ uptake in low pH. Zebrafish larvae (4 dpf) were treated with propranolol (N = 6 - 7; Fig. 6.2A), atenolol (N = 5 - 7; Fig. 6.2B), and ICI-118551 (N = 5 - 7; Fig. 6.2C; ICI) during the 24-h exposure to low pH water. Both propranolol and atenolol significantly reduced Na^+ uptake, whereas treatment with ICI-118551 did not affect Na^+ uptake. Different letters denote significant difference among treatment groups. Data are presented as means ± SEM.

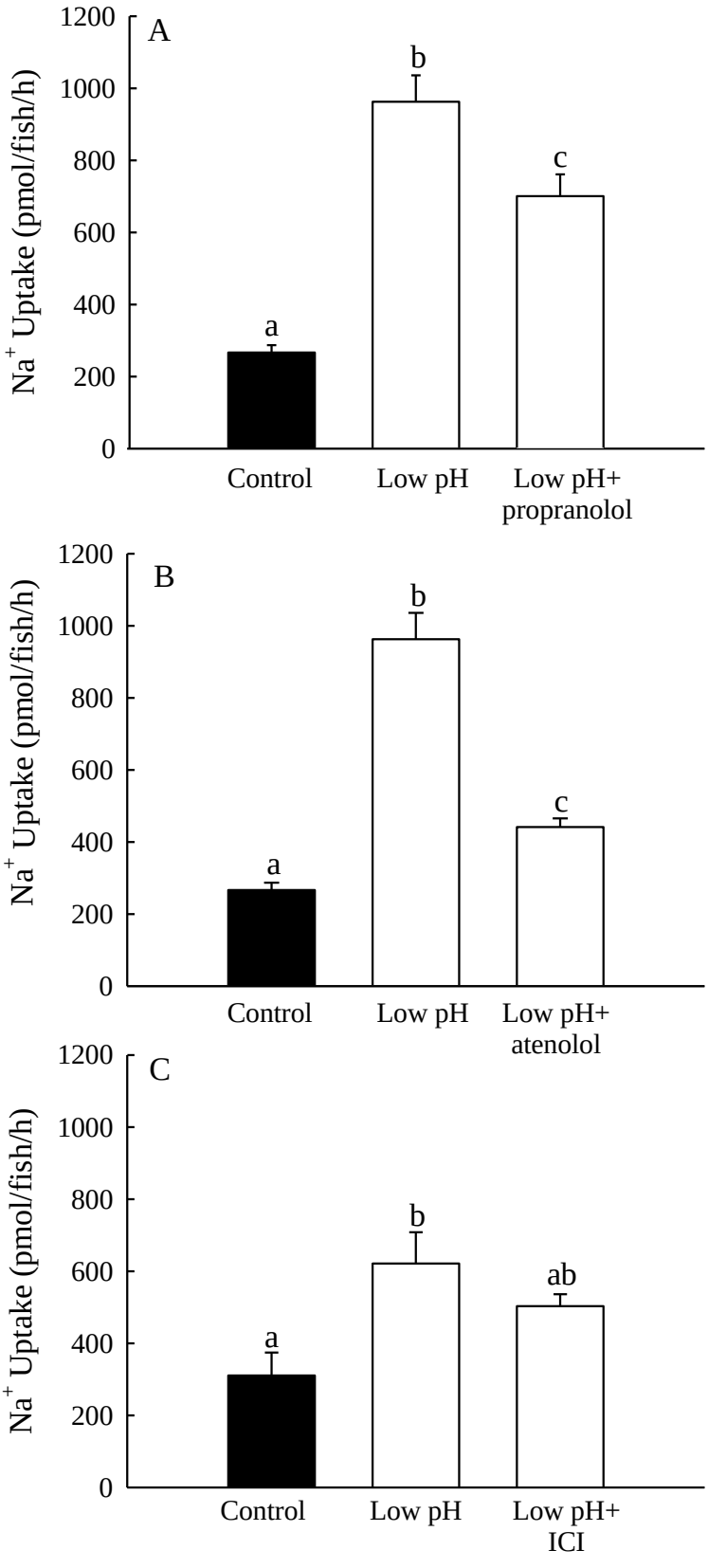
A
B
C
Na+ Uptake (pmol/fish/h)
1200
1000
800
600
400
200
0
a
b
c
ab
Control
Low pH
Low pH+ propranolol
Low pH+ atenolol
Low pH+ ICI

Figure 6.3. The effect of β-receptor inhibition on Na^+ uptake in ion-poor water. Zebrafish larvae (4 dpf) were treated with propranolol (N = 6; Fig. 6.3A), atenolol (N = 5 - 6; Fig. 6.3B), or ICI-118551 (N = 6; Fig. 6.3C; ICI) during the brief (~3 h) exposure to ion-poor water. In contrast to the data in Fig. 6.2, none of the treatment significantly affected Na^+ uptake. Different letters denote significant difference among treatment groups. Data are presented as means ± SEM.

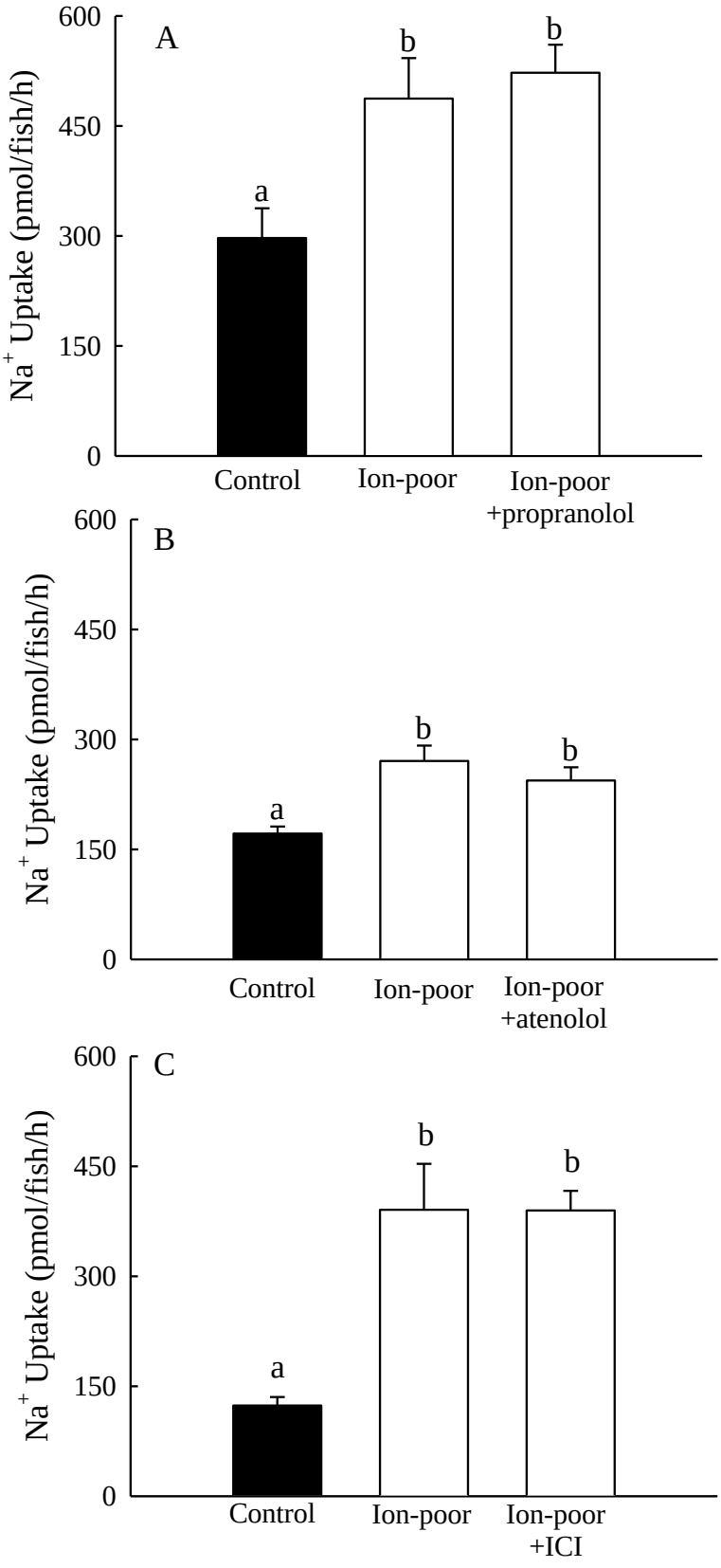
A
B
C
Na^+ Uptake (pmol/fish/h)
600
450
300
150
0
a
b
b
Control
Ion-poor
Ion-poor +propranolol
Ion-poor +atenolol
Ion-poor +ICI

Figure 6.4. The effect of β-receptor knockdown on Na^+ uptake in low pH. Knocking down selected isoforms of β receptors (β_1, β_{2A}, β_{2B} and both β_2) did not affect Na^+ uptake under control conditions. However, when these morphants were exposed to low pH water for 24 h, Na^+ uptake was significantly lower in β_1, β_{2B} and β_2 dual morphants (N = 5 – 7; Figs. 6.4A-D). Different letters denote significant difference among treatment groups. Data are presented as means ± SEM.

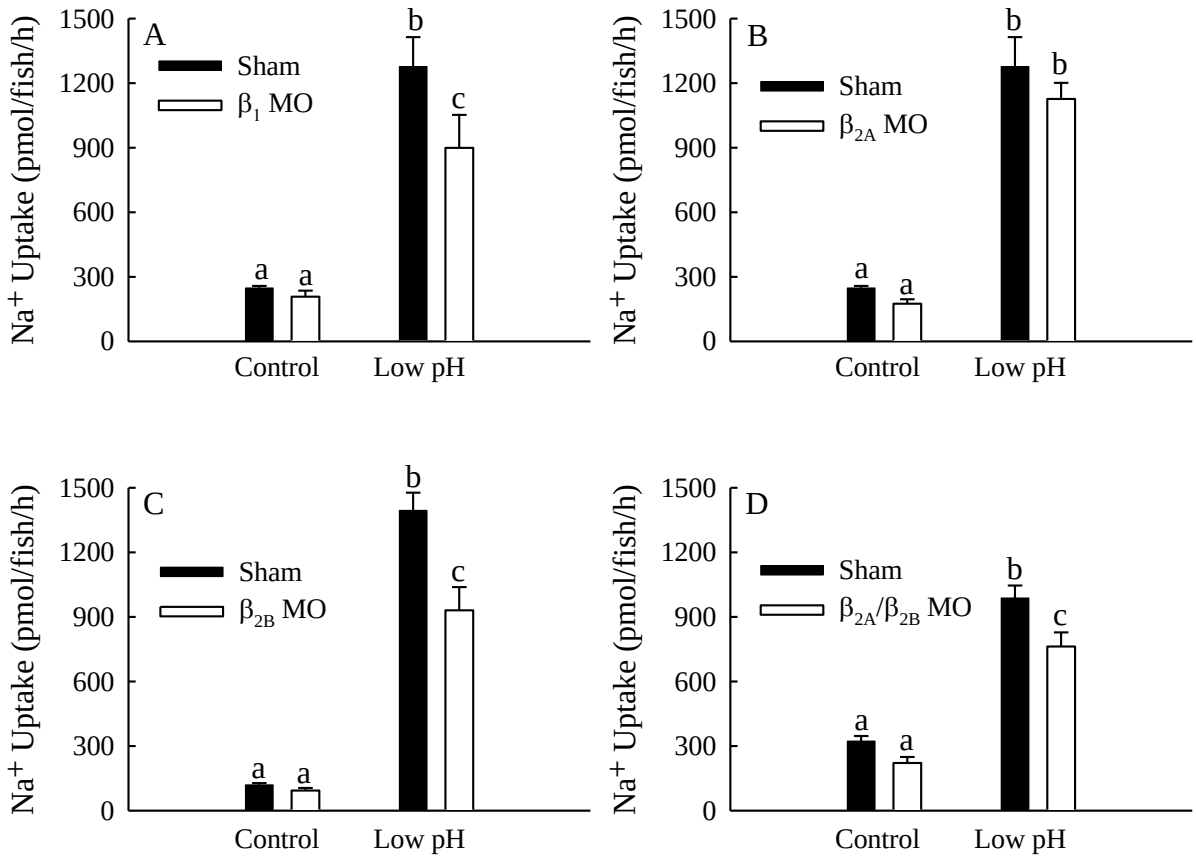
A
Sham
β1 MO
B
Sham
β2A MO
C
Sham
β2B MO
D
Sham
β2A/β2B MO
Na+ Uptake (pmol/fish/h)
1500
1200
900
600
300
0
a
a
b
c
Control
Low pH

Figure 6.5. The effect of β-receptor knockdown on Na^+ uptake in ion-poor water. Knocking down selected isoforms of β receptors (β_1, β_{2A}, β_{2B} and both β_2) did not affect Na^+ uptake under control conditions. However, when these morphants were briefly exposed to ion-poor water, Na^+ uptake was significantly lower in β_{2A} and β_2 dual morphants (N = 4 – 7; Figs. 6.5A-D). Different letters denote significant difference among treatment groups. Data are presented as means ± SEM.

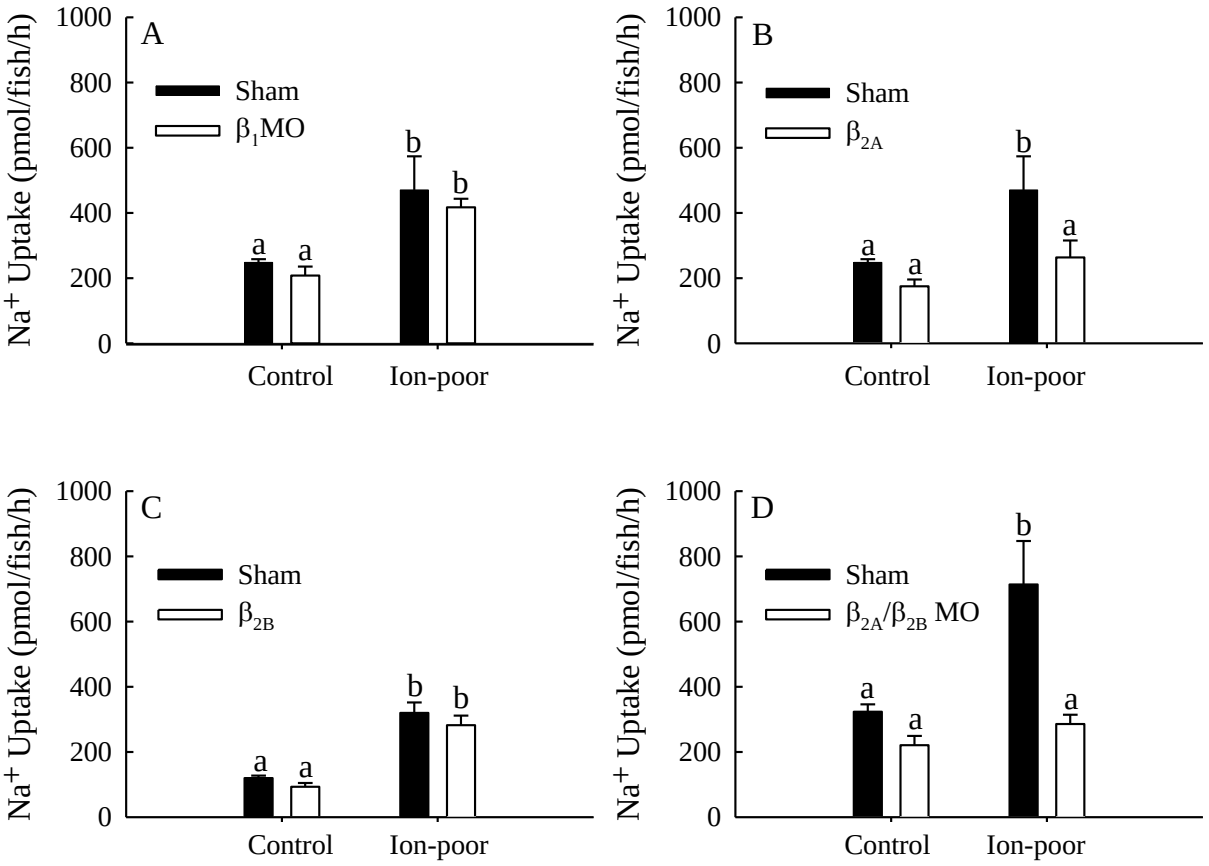
A
Sham
β1MO
B
Sham
β2A
C
Sham
β2B
D
Sham
β2A/β2B MO
Na+ Uptake (pmol/fish/h)
1000
800
600
400
200
0
a
a
b
b
Control
Ion-poor

Figure 6.6. Innervation of HRCs in developing zebrafish larvae. Larvae (4 dpf) were immuno-stained with zn-12 (a generic neuronal marker, red) and H^+-ATPase antibody (green). Numerous H^+-ATPase positive cells (HRCs) were observed, most of which appeared to be surrounded by zn-12 staining. Scale bars: 10 μm for Fig. 6.6A. A z-stack image (Fig. 6.6B) was acquired along the line indicated in Fig. 6.6A. The arrow indicates the point of contact between zn-12 and H-ATPase, suggesting the innervations of HRCs. The staining was repeated on three separate occasions with 4-5 larvae inspected each time. Only the representative images are shown.

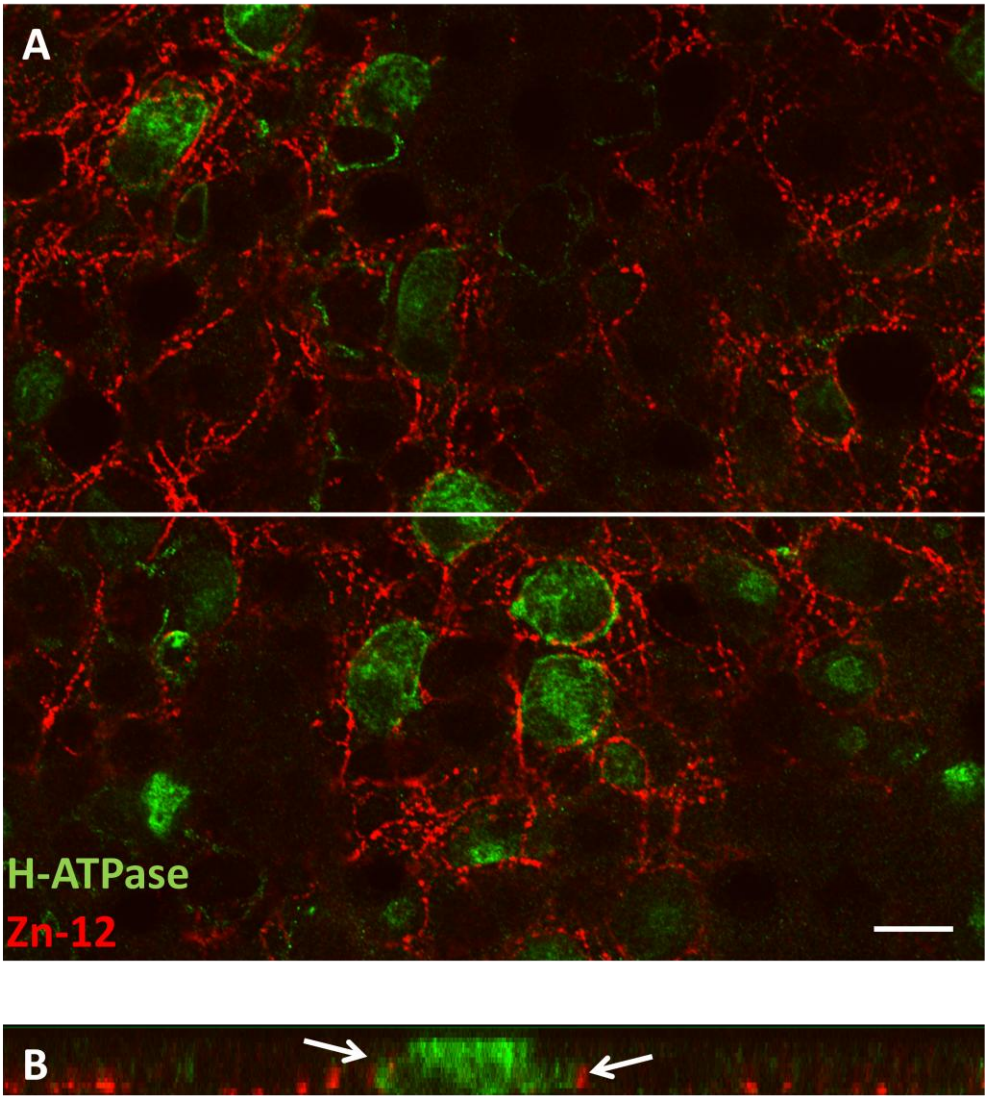
A
H-ATPase
Zn-12
B

Figure 6.7. Distribution of β receptors in developing zebrafish larvae. Larvae (4 dpf) were stained with (*S*)-Propranolol Fluorescent ligand (blue; Fig. 6.7A), MitoTracker® (red; Fig. 6.7B) and concanavalin A (green; Fig. 6.7C). All conA positive cells were colocalized with propranolol (Fig. 6.7D). Under higher magnification, additional propranolol-positive, conA negative cells were observed (indicated with an asterisk; Fig. 6.7E), which appear to colocalize with MitoTracker® (Figs. 6.7F, H). MitoTracker® and concanavalin A did not colocalize (Fig. 6.7H). Scale bars: 50 µm for Figs. 6.7A-D; 10 µm for Figs. 6.7E-H.

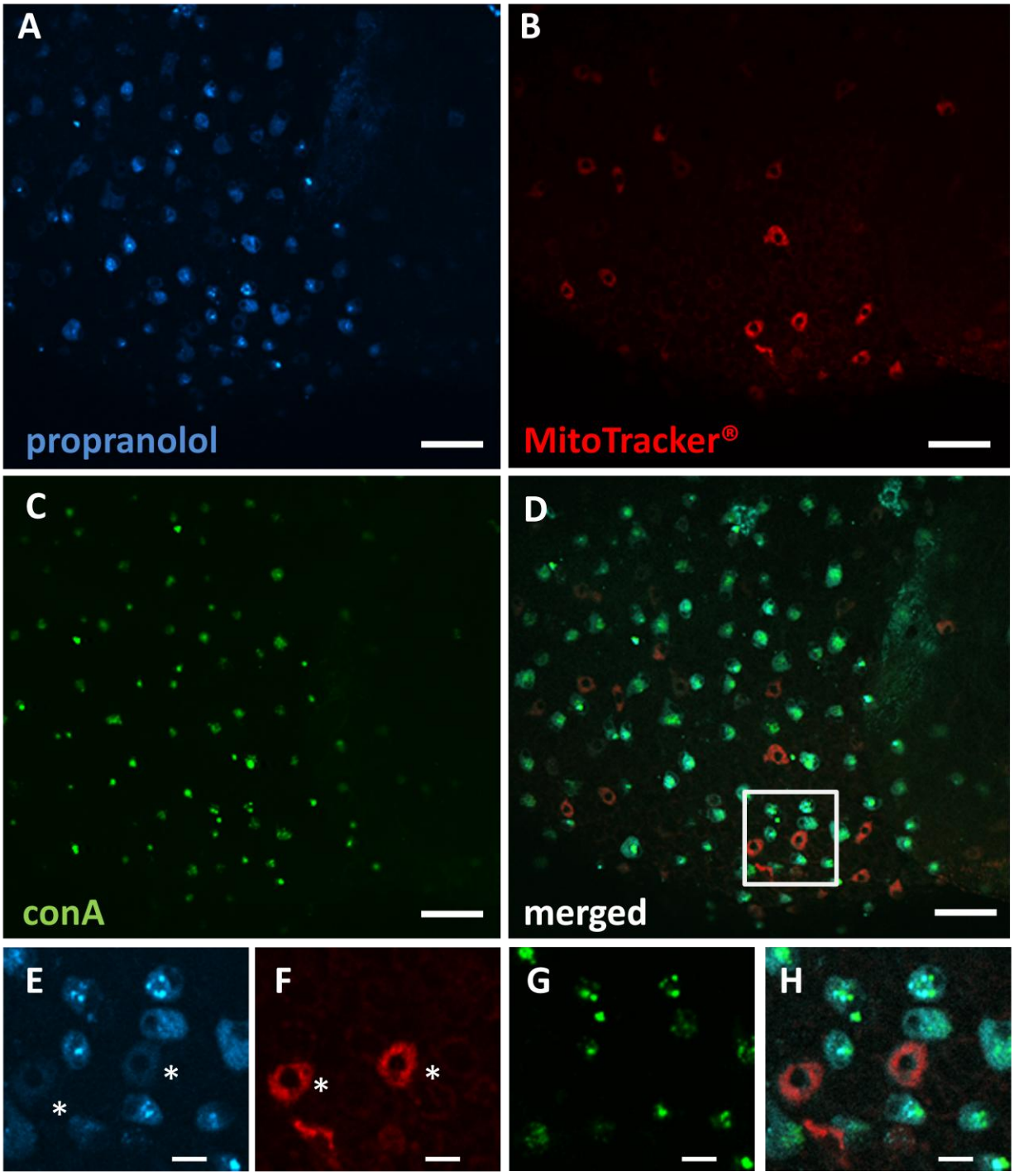
A
propranolol
B
MitoTracker®
C
conA
D
merged
E
F
G
H

Table 6.1. Effect of receptor agonist/antagonist treatment on Na^+ uptake in normal water. Data are presented as means ± 1 SEM. An asterisk indicates significant difference between the control and treatment groups and the number of replicates for each group is indicated in parentheses. "+" indicates an agonist and "-" indicates an antagonist. Note that when the effect of more than one drug was tested using larvae from a single batch, all treated groups were compared to the same control group.

Agonist/antagonist and target	Control (pmol/fish/h)	Treated (pmol/fish/h)
Isoproterenol (β+)	253 ± 19 (12)	657 ± 62* (12)
Dobutamine (β_1+)	220 ± 27 (12)	359 ± 76 (12)
Procaterol (β_2+)	220 ± 27 (12)	341 ± 37* (12)
Propranolol (β-)	253 ± 19 (12)	238 ± 14 (12)
Atenolol (β_1-)	224 ± 47 (6)	208 ± 48 (6)
ICI-118551 (β_2-)	124 ± 12 (6)	120 ±5 (6)
Phenylephrine (α_1+)	151 ± 9 (6)	70 ± 13* (6)
Clonidine (α_2+)	131 ± 36 (5)	67 ± 9* (6)
Phentolamine (α-)	323 ± 22 (18)	341 ± 27 (18)
Dopamine (dopamine, D+)	379 ± 55 (6)	276 ± 30 (6)
5-hydroxytryptamine (serotonin, 5-HT+)	379 ± 55 (6)	402 ± 44 (6)

Discussion

The present study tested the hypothesis that Na^+ uptake in zebrafish larvae is under neurohumoral regulation. The data clearly demonstrated stimulatory and inhibitory roles of β- and α-adrenergic receptors, respectively, on Na^+ uptake, as well as a functional role of β-receptor signalling in stimulating Na^+ uptake when zebrafish are challenged with conditions that promote ion loss (low pH water) or hinder ion uptake (ion-poor water). In keeping with a proposed model for ß-adrenergic activation of Na^+ uptake, the present study provided compelling evidence that a subtype of ionocyte implicated in Na^+ uptake, the HRCs, is innervated and express β-adrenergic receptors.

Neurohumoral regulation of Na^+ uptake in zebrafish.

Although the autonomic regulation of ion reabsorption in the mammalian kidney has received considerable attention (Azroyan et al., 2012; DiBona, 2005; Mu et al., 2011), a similar role in the fish gill has not yet been established. Thus, a first objective of the present study was to determine whether pharmacological manipulation of neurohumoral receptors (adrenergic, serotonergic and dopaminergic) affected Na^+ uptake by zebrafish larvae under control conditions.

Zebrafish did not respond to 5-HT (serotonergic receptor agonist) or dopamine (dopaminergic receptor agonist; Table 6.1). Although the lack of an effect suggests that signalling mediated by these receptors may not be involved in controlling Na^+ uptake in developing zebrafish, it is conceivable that longer exposure periods or higher doses might have initiated an effect on Na^+ uptake. For example, pharmacologically-induced synthesis of 5-HT was shown to cause a significant reduction in NaCl intake in rats (Rouah-Rosilio et al., 1994), thus suggesting a possible link between the serotonergic system and overall regulation of salt balance. It is worth noting that because dopamine could act as an agonist for serotonergic as

well as adrenergic system when used at sufficiently high concentration, the dopamine treatment was repeated with lower (1 and 10 μM) doses. Similar to the treatment with 100 μM, lower dose of dopamine was without effect on Na^+ uptake (data not shown).

Treating zebrafish larvae with agonists/antagonists for adrenergic receptors significantly affected Na^+ uptake. Specifically, α- and β-adrenergic receptors would appear to play inhibitory and stimulatory roles, respectively, in the control of Na^+ uptake (Table 6.1). Perry et al. (1984) reported a similar opposing role of α- and β-receptors in ionic uptake in rainbow trout, although in their study, activation of the α-adrenergic receptor was found to stimulate Cl^- uptake (as opposed to its inhibitory role on Na^+ uptake reported in the present study). Given these observations, it is likely that role of α and β adrenergic receptors in ionic regulation depends on ions as well as species in question, and that simple generalization should be avoided. It would be of interest to assess the consequences of α-receptor knockdowns on ion balance in larvae exposed to high [Na^+] conditions, where the fish presumably would attempt to reduce their Na^+ uptake capacity.

An additional series of experiments was performed where larvae were treated with multiple doses (1, 10 and 100 μM) of adrenergic receptor agonists/antagonists to better characterize the relative contribution of different receptor subtypes. In this series of experiment, clonidine appeared to be more effective than phenylephrine in reducing Na^+ uptake (Fig, 6.1A, B). Although this result could be interpreted that α_2 receptors are more effective in inhibiting Na^+ uptake in zebrafish larvae, it remains to be tested more rigorously as the permeability of these reagents through larval skin, as well as relative affinity to zebrafish adrenergic receptors might be different between clonidine and phenylephrine. On the other hand, both dobutamine and procaterol affected Na^+ uptake only at the highest tested dose (100 μM; Figs. 6.1C, D). In

addition, it was also tested whether treatment with natural adrenergic agonist (epinephrine and norepinephrine) could affect Na^+ uptake in zebrafish larvae. Interestingly, epinephrine and norepinephrine appeared to have an opposing effect on Na^+ uptake by zebrafish larvae (Figs. 6.1E, F), although no significant difference was detected in post hoc test among epinephrine-treated larvae. While such results are difficult to interpret, as both α- and β-receptors could be stimulated, one possibility is that epinephrine has higher affinity to α_2 receptor than norepinephrine. If the greater inhibitory effect seen with clonidine is the result of greater inhibitory effect exerted by α_2 receptor, the overall inhibitory effect could be sufficient to overcome the stimulatory effect as seen in norepinephrine-treated larvae (Figs. 6.1E, F). Of course, it remains highly speculative and thus future studies utilizing more specific systems (eg. cell culture expressing zebrafish adrenergic receptor as used in Steele et al, 2011b) would be required to draw significant conclusions.

Stimulation of Na^+ uptake via β-adrenergic receptors in ion-poor and acidic water

As summarized in Table 6.1, activation of β-receptors stimulates Na^+ uptake under control conditions. The present study investigated the physiological significance of this phenomenon as a potential regulatory mechanism during exposure of zebrafish to ion-poor or acidic water, two experimental conditions known to increase Na^+ transport capacity of this species (Boisen et al., 2003; Kumai and Perry, 2011). Boisen et al. (2003) reported that, following a 40-day acclimation to ion-poor water, the affinity constant (K_M) and maximal transport capacity (J_{MAX}) for Na^+ uptake were halved (from 160 to 74 μM) and doubled (525 to 1160 nmol/g/h), respectively. Because zebrafish in the present study were exposed to ~30 μM [Na^+], which is considerably lower than the K_M for Na^+ uptake post-acclimation (74 μM), it is likely that they experienced significant challenges to maintain their Na^+ balance. Surprisingly,

despite the short duration of exposure to ion-poor water (~4 h including the serial dilution phase), it was sufficient to increase their Na^+ uptake capacity, as indicated by a significantly greater rate of Na^+ uptake when those fish were transferred back to control water. Given the short time frame, it is unlikely that the increase in Na^+ uptake was related to an increase in the synthesis of new Na^+ transporters. Rather, it seemed more likely that the mechanism underlying the stimulation of Na^+ uptake could have been caused by a redistribution of an existing pool of proteins and/or an alteration in HRC apical surface area. In support of the latter idea, Shen et al. (2011) observed a rapid (~5 h) change in ionocyte morphology in euryhaline medaka, *Oryzias latipes*, transferred from SW to FW.

As predicted, pharmacological blockade of β-adrenergic receptors or their knockdown using morpholinos, impaired Na^+ uptake during exposure of larvae to acidic or ion-poor water. However, the extent of the impairment of Na^+ uptake caused by β-receptor loss-of-function varied according to the acclimation regimen (acidic water *versus* ion-poor water), the mode of inducing loss-of-function (knockdown *versus* pharmacological blockade) and the specific sub-type of β-receptor targeted (β_1, B_{2A} or β_{2B}). The lack of an effect of β-blockers in the fish exposed to ion-poor water possibly reflects the relatively short duration of inhibitor treatment (4 h as opposed to 24 h in the fish exposed to acidic water). Additionally, while the knockdown experiments implicated β_1- and β_{2B}-receptors in aiding Na^+ uptake in acid-exposed larvae, only the β_{2A} receptors appeared to be involved in stimulating Na^+ uptake in fish exposed to ion-poor water. Finally, the relative impact of β-receptor knockdown was much larger in the fish exposed to ion-poor water suggesting greater involvement of adrenergic control mechanisms in stimulating Na^+ uptake at such times in comparison to the acid-water water condition where β-receptor knockdown only partially prevented the full stimulation of Na^+ uptake. Devic et al.

(2001) suggested that differential signalling pathways could be activated by β_1 and β_2 pathway in cardiac myocytes, and thus it is possible that in zebrafish, each β-receptor might affect Na^+ uptake differently. Interestingly, Yan et al (2007) reported that exposure to acidic and ion-poor water increased mRNA expression of H^+-ATPase and NHE3b respectively, suggesting environment-dependent recruitment of the Na^+ uptake mechanism in zebrafish (but see also Kumai and Perry (2011) for potential involvement of NHE3b in Na^+ uptake in acidic water). Furthermore, recent studies on cAMP suggest the presence of multiple cAMP hotspots within a cell, where [cAMP] is regionally elevated and potentially could trigger specific localized downstream effects (for a recent review on cAMP signalling, see Tresguerres et al., 2011). Given these findings, it is conceivable that β_1 and β_2 receptors could be affecting different targets to stimulate Na^+ uptake. A detailed observation of receptor distributions using homologous isoform-specific antibodies (currently unavailable commercially), as well as their interactions with Na^+ transporters might provide interesting insight on this issue.

Ionocyte innervation, distribution of β-receptors and origin of catecholamines.

Although the innervation of the fish gill at a gross level has been documented extensively (for a recent review, see Hwang et al., 2011), there are surprisingly few data on the direct innervation of ionocytes in FW fish species. To date, only a single study (Jonz and Nurse, 2006) has described the direct innervations of ionocytes (NaRC) in zebrafish. The present study demonstrated using double immunohistochemistry that HRCs, another subtype of ionocyte primarily responsible for Na^+ uptake (Hwang et al., 2011), are also innervated at 4 dpf.

In fish, as in other vertebrates, the catecholamines (adrenaline and noradrenaline) are directed toward target tissues either by secretion directly into synapses as neurotransmitter or by release from chromaffin cells usually resulting in their more widespread distribution. In the

present study, it was attempted to determine whether the nerves innervating the HRCs are indeed adrenergic by immuno-staining the larvae with several commercially available antibodies against tyrosine hydroxylase (TH), a key enzyme for the synthesis of catecholamines. Although various antigen retrieval techniques were attempted, no convincing immuno-staining was observed in the yolk sack region or in the brain (which should serve as the positive control for the immunohistochemistry; data not shown). In addition, previous studies that visualized TH either with immunohistochemistry (Schweitzer et al., 2011) or a transgenic zebrafish line (Tay et al., 2011) did not report strong staining in the yolk sack region. When it was attempted to render the catecholaminergic (dopaminergic/adrenergic) nerves non-functional by treating zebrafish larvae with 6-hydroxydopamine (for 3 days at 500 μM, a dose previously shown to selectively destroy catecholaminergic nerve ending in zebrafish larvae; Coccimiglio and Jonz, 2012), and then to assess the impact on subsequent exposure to acidic or ion-poor water, no stimulation of Na^+ uptake was observed (data not shown). Although consistent with the idea that adrenergic nerves are involved in activating Na^+ uptake, the results should be interpreted with caution because the 6-hydroxydopamine treated group exhibited a higher rate of Na^+ uptake than the ion-poor water exposed group (thus, further increases may not have been necessary). Alternatively, β-receptors could be activated by catecholamines secreted from chromaffin cells. By 2 dpf, chromaffin cells (identified by *in situ* hybridization for dopamine β-hydroxylase) are present in the interrenal region (To et al., 2007; for review see Yi-Wen, 2007). It is possible therefore that by 4 dpf, catecholamines secreted from these chromaffin cells are able to activate β-adrenergic receptors on the HRCs. The use of the transgenic zebrafish line *cloche*, where development of endothelial as well as blood cells are completely prevented (Stainier et al., 1995) might yield interesting insight into the role of chromaffin-cell derived catecholamines in osmoregulation, although

passive diffusion of catecholamine throughout the body might be sufficient to activate β receptor on the target tissue (yolk sack) during the early development.

Although the exact origin of the catecholamines that activate adrenergic receptors remains unknown, to further confirm an involvement of β-adrenergic systems in Na^+ uptake, the distribution of β-receptors was visualized by vital staining of larvae with fluorescently labelled propranolol. As expected from the flux data, strong staining was observed on HRCs (stained with conA; Fig. 6.7C). Weak staining was also observed in some conA-negative cells, which appeared to colocalize with MitoTracker® (Fig. 6.7 E-H). Because MitoTracker® is known to stain both NaRCs and HRCs, but more prominently NaRCs (Lin et al., 2006; but also see Esaki et al., 2007), the sub-population of conA-negative, propranolol-positive ionocyte is likely to be composed of NaRCs. It should be noted, however, that isoproterenol treatment had no effect on Ca^{2+} uptake (a putative function of NaRCs) in 4 dpf zebrafish larvae (Y. Kumai and S.F. Perry, unpublished data).

It is also interesting to note that no strong staining in heart was observed (Fig. 6.7) despite the previous report that β-receptors, at least at the mRNA level, are highly expressed in zebrafish cardiac tissue (Steele et al., 2011b). When the fish were exposed to both fluorescent propranolol (100 nM) and non-fluorescent propranolol (100 μM), the propranolol staining was significantly reduced (data not shown), demonstrating that fluorescent propranolol is at least capable of binding to the same target as the "conventional" propranolol, which has been shown to bind to zebrafish β-receptors *in vitro* (Steele et al., 2011b). Although the discrepancy is puzzling, it is possible that the conjugation of fluorescent molecule to propranolol renders it less permeable and thus less likely to penetrate the heart tissue.

Integrating the regulatory mechanisms of Na^+ uptake; cortisol and catecholamines

It was recently observed that cortisol, acting chiefly through the glucocorticoid receptor (GR), is capable of increasing Na^+ uptake (Kumai et al., 2012a), most likely by inducing the recently proposed Rhcg-NHE functional metabolon (Kumai and Perry, 2011; Shih et al., 2012; Wright and Wood, 2009; Wu et al., 2010). Given the stimulation of Na^+ uptake via β-adrenergic receptors reported here, it would be interesting to determine whether these two mechanisms act synergistically or rather target independent proteins. One such interaction between GR-mediated signalling and β-adrenergic receptors was recently proposed by Mu et al (2011), where it was reported that β-adrenergic receptor activation could regulate the transcription of WNK4 via GR, which prevents the reduction in Na^+/Cl^- co-transporter (NCC) and ultimately caused the salt-sensitive hypertension in mice. Although NCC was shown to play a role in Na^+ uptake in zebrafish (Wang et al., 2009), this transporter is not highly expressed in HRCs (Hwang et al., 2011). Given the observation that β-receptors are predominantly expressed on HRCs (Fig. 6.7), it is unlikely that NCC is a primary target of the observed adrenergic regulation. On the other hand, cAMP, a downstream signalling messenger synthesized following the activation of β-receptors, is known to play a key role in regulating H^+-ATPase. In acid-secreting epithelial cells such as A-type kidney intercalated cells (Pastor-Soler et al., 2003; Păunescu et al., 2008) and epididymis clear cells (Pastor-Soler et al., 2008), cAMP is responsible for apically accumulating H^+-ATPase. Tresguerres et al. (2010c) reported a similar cAMP-mediated translocation of H^+-ATPase in dogfish shark gill, though in this case the H^+-ATPase was trafficked toward the basolateral membrane. Because previous research has demonstrated a key role for H^+-ATPase in Na^+ uptake in zebrafish (Boisen et al., 2003; Esaki et al., 2007; Kumai and Perry, 2011), it is not unreasonable to suggest that the observed effects of β-adrenergic receptor activation on Na^+ uptake are caused by an apical accumulation of H^+-ATPase via cAMP. Indeed, when zebrafish

larvae were treated with a membrane-permeable cAMP analog, Na^+ uptake was significantly increased (Y. Kumai and S.F. Perry, unpublished observation), suggesting that, as expected from the stimulatory role of β-adrenergic receptor, cAMP is involved in Na^+ uptake regulation. However, as discussed above, it is also possible that multiple Na^+ transporters are affected by β-receptors in an isoform-dependent manner. A detailed examination of how each β-receptor affects Na^+ uptake may shed light onto the potentially complex regulatory networks associated with adrenergic signalling in zebrafish ionocytes.

Conclusions and perspectives

This is the first study to investigate the significance of adrenergic regulation of Na^+ uptake in a developing fish. The finding that HR cells are innervated and express β-receptors by 4 dpf extends the previous report by Jonz and Nurse (2006) of innervated NaRCs and indicates that the uptake of several ions could be neuronally regulated in developing zebrafish. Pharmacological and functional knockdown experiments demonstrated that both α and β adrenergic systems play an important, but opposing roles in regulating Na^+ uptake. Potentially, different subtypes of β receptors could be involved in stimulating Na^+ uptake under different environmental conditions. The present study raises several interesting questions, such as: 1) whether different β-receptors are distributed differently among the various ionocytes, 2) how cAMP, a downstream signalling agent following activation of β- adrenergic receptors, ultimately affects Na^+ uptake and 3) how multiple regulatory mechanisms of Na^+ uptake, most notably involving catecholamines and cortisol, orchestrate their actions to maintain overall Na^+ homeostasis in fluctuating FW environment. Research is currently underway to elaborate on these complex regulatory mechanisms of Na^+ uptake in zebrafish.

Acknowledgements

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CHAPTER 7
The role of cAMP-mediated intracellular signalling in the acute stimulation of Na^+ uptake in larval zebrafish (*Danio rerio*) exposed to acidic water

Notes on Chapter:

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Kumai, Y. and Perry, SF. The role of cAMP-mediated intracellular signalling in the acute stimulation of Na^+ uptake in larval zebrafish (*Danio rerio*) exposed to acidic water

The study was conceived by YK; all experiments were performed by YK under the supervision of SFP.

Abstract

Zebrafish (*Danio rerio*) exposed to acidic water maintain Na^+ balance by a compensatory increase in Na^+ uptake. In the current study, the role of cAMP in stimulating Na^+ uptake in larval zebrafish exposed to acidic water was investigated. Treatment of larvae at 4 days post fertilization (dpf) with 10 μM forskolin or 1 μM 8-bromo cAMP significantly increased Na^+ uptake by 3- and 2-fold, respectively. The cAMP-dependent stimulation of Na^+ uptake was probably unrelated to protein trafficking via microtubules because pre-treatment with 200 μM colchicine or 30 μM nocodazole did not attenuate the magnitude of the response. The acid-induced increase in Na^+ uptake was accompanied by a 2-fold elevation in whole body cAMP levels and attenuated by inhibiting protein kinase A (PKA) with 10 μM H-89. These results suggest that cAMP signalling is critically involved in the stimulation of Na^+ uptake following acid-exposure. Forskolin-treatment significantly elevated acid-secretion suggesting activation of Na^+-H^+ exchange (NHE) but also stimulated Na^+ uptake in *glial cell missing 2* morphants in which the Na^+-Cl^- co-transporter (NCC) is the predominant route of Na^+ uptake. These data suggest that at least two Na^+ uptake mechanisms, NHE and NCC, are targeted by cAMP in zebrafish larvae. In agreement with this idea, staining of larvae with fluorescent forskolin and propranolol (a β-adrenergic receptor ligand) revealed the presence of tmAC (forskolin binding sites) within multiple subtypes of ionocytes expressing β-adrenergic receptors. Taken together, the present study suggests that cAMP-mediated intracellular signalling plays an important role in regulating Na^+ uptake in zebrafish larvae.

Introduction

Maintaining body fluid electrolyte balance is crucial for the survival of most vertebrates. Because fish living in freshwater (FW) suffer from the constant passive loss of ions by diffusion, they must actively absorb the major ions (Na^+, Cl^- and Ca^{2+}) from the environment. The molecular mechanisms for the absorption of these ions have been intensely investigated (for reviews see Dymowska et al., 2012; Hwang, 2009; Hwang and Chou, 2013; Hwang and Perry, 2010; Hwang et al., 2011; Kumai and Perry, 2012). The results of these studies revealed significant roles for several hormones in regulating ionic uptake, including cortisol (Cruz et al., 2013; Gilmour et al., 2011; Ivanis et al., 2008b; Kumai et al., 2012a; Kwong and Perry, 2013a; Lin et al., 2011), prolactin (Breves et al., 2013; Breves et al., 2010; Pickford and Phillips, 1959; Pisam et al., 1993), isotocin (Chou et al., 2011), stanniocalcin (Tseng et al., 2009), vitamin D (Lin et al., 2012), angiotensin-II (Hoshijima and Hirose, 2007) and endothelin (Hyndman and Evans, 2009), as well as neuroendocrine factors (e.g. catecholamines) (Isaia et al., 1978; Morgan and Potts, 1995; Perry et al., 1984; Vermette and Perry, 1987).

A series of recent studies (Kumai et al., 2011; Kumai et al., 2012a; Kumai and Perry, 2011; Kumai et al., 2012b) demonstrated that in both larval and adult zebrafish, Na^+ uptake is significantly elevated following exposure to extremely acidic (pH 4) water. In larvae, the stimulation of Na^+ uptake in acidic water largely reflects activation of Na^+/H^+ exchange (NHE3b; Yan et al., 2007) that is functionally linked to ammonia excretion via Rhcg1, an apically expressed ammonia channel (Braun et al., 2009a; Kumai and Perry, 2011; Nakada et al., 2007a; Shih et al., 2008; for review see Wright and Wood, 2009; Wright and Wood, 2012). These effects are mediated, at least in part, by cortisol (acting via glucocorticoid receptors) (Kumai et al., 2012a) and catecholamines (acting via β-adrenergic receptors) (Kumai et al., 2012b). Although the mode of action of cortisol in stimulating Na^+ uptake in acidic water was

recently investigated (Kumai et al.2012a), the specific downstream signalling pathways activated by catecholamines have not been identified.

Stimulation of β-adrenergic receptors, like other G-protein coupled receptors (GPCR) activates trans-membrane adenylyl cyclase (tmAC) which initiates downstream signalling pathways mediated by 3'-5'-cyclic adenosine monophosphate (cAMP ; for review see Pierce et al., 2002). Discovered almost 60 years ago (Sutherland and Rall, 1958), cAMP is known to act as an important secondary messenger in virtually all physiological systems (Beavo and Brunton, 2002). In mammalian tissues, cAMP inhibits Na^+-H^+ exchanger 3 activity through interaction with Na^+-H^+-exchanger regulatory factor 1 (NHERF1; Weinman et al., 1987) ;(Honegger et al., 2006); (Alexander and Grinstein, 2009; Weinman et al., 2000). cAMP can also be formed via activation of soluble adenylyl cyclase (sAC or AC10; Chen et al., 2000). Unlike other isoforms of AC, which are trans-membrane proteins, sAC is distributed in the cytosol and activated by a rise in [HCO_3^-] (Chen et al., 2000). The activation of sAC rapidly induces the apical accumulation of H^+-ATPase in epididymis clear cells (Pastor-Soler et al., 2003; Pastor-Soler et al., 2008) and A-type intercalated cells in renal collecting duct (Păunescu et al., 2008; Păunescu et al., 2010). In dogfish (*Squalus acanthias*) gill ionocytes, sAC-derived cAMP also evokes the translocation of H^+-ATPase, although in this case the direction of movement is toward the basolateral membrane (Tresguerres et al., 2010c). Additionally, sAC contributes to the regulation of intestinal Na^+ and Cl^- reabsorption in the marine teleost, *Opsanus beta* (Tresguerres et al., 2010b). The results of these studies clearly implicate cAMP (derived either from tmAC or sAC) as an important intracellular signal promoting body fluid electrolyte homeostasis across vertebrate taxa. However, surprisingly little is known about the cAMP-mediated regulation of ion, especially Na^+, uptake in FW fish.

Based on the role of β-adrenergic receptor activation in regulating Na^+ uptake in acid-exposed zebrafish larvae and the extensive involvement of cAMP (a product of β-adrenergic receptor activation) as a regulator of trans-epithelial ion (and acid/base) transport, the present study was designed to assess the potential for cAMP to modulate Na^+ uptake in zebrafish larvae. The specific objectives were to i) directly demonstrate that cAMP is capable of stimulating Na^+ uptake, ii) identify the Na^+ transporters being regulated by cAMP, and iii) implicate cAMP in acutely inducing Na^+ uptake during acid-exposure.

Materials and Methods

Experimental animals and water preparation

Ottawa

Adult zebrafish (*Danio rerio* Hamilton-Buchanan 1822) were purchased from Big Al's Aquarium Services (Ottawa, ON, Canada) and kept in the University of Ottawa Aquatic Care Facility where they were maintained in plastic tanks supplied with aerated, dechloraminated City of Ottawa tap water (the ionic composition of the water was (in mM) Na^+ = 0.78; Cl^- = 0.4; Ca^{2+} = 0.25; K^+ = 0.025; pH 7.0) at 28° C. Fish were subjected to a constant 14 h L:10 h D photoperiod and fed daily until satiation with No. 1 crumble-Zeigler™ (Aquatic Habitats, Apopka, FL, USA). Embryos were collected and reared in 50 ml Petri dishes with dechloraminated City of Ottawa tap water (pH 7.3-7.5) supplemented with 0.05% methylene blue. The Petri dishes were kept in incubators set at 28.5° C. Dead embryos were removed and water was changed daily. The experiments were conducted in compliance with guidelines of the Canadian Council of Animal Care (CCAC) and after the approval of the University of Ottawa Animal Care Committee (Protocol BL-226).

Vancouver

Experiments described in Series 4.1 were performed at University of British Columbia Department of Zoology. Zebrafish were purchased from Noah's Pet Arch and kept in dechlorinated city of Vancouver tap water (the ionic composition of the water was (in mM) Na^+ = 0.08; Cl^- = 0.06; Ca^{2+} = 0.03; K^+ = 0.004; pH 7.0; Metro Vancouver Water Quality Report, 2011). Fish were housed in a temperature controlled environmental room, subjected to constant 14 h L:10 h D photoperiod and fed twice daily until satiation. Embryos were collected and reared until 4 days post fertilization (dpf) in City of Vancouver tap water supplemented with

0.05% methylene blue. All protocols were approved by the Animal Care Committee at University of British Columbia.

To determine the effect of cAMP on Na^+ uptake in developing zebrafish, the following series of experiments were performed. Unless stated otherwise, all chemicals used for the experiments were purchased from Sigma.

Series 1. Consequences of increasing cAMP levels on Na^+ uptake

cAMP levels in larval zebrafish were elevated by treatment with 1) forskolin, a pan-tmAC activator and 2) 8-bromo cAMP, a membrane-permeable, phosphodiestrase-resistant cAMP analog. Zebrafish larvae were raised in control water until 4 dpf and exposed to either forskolin at 0.1, 1 or 10 µM or 1 µM 8-bromo cAMP (Santa Cruz Biotechnology Inc.). A forskolin stock solution was prepared in DMSO; the control group was treated with DMSO as a vehicle control. The final concentration of DMSO did not exceed 0.1%. Na^+ uptake measurements began five min after the addition of forskolin or 8-bromo cAMP. To measure Na^+ uptake, 12 larvae were placed in a 2-ml micro-centrifuge tube and 0.25 µCi ^{22}Na (in the form of NaCl; Perkin Elmer, Woodbridge, ON, Canada) was added to each tube to a final activity of 0.15 µCi/ml. Water samples (50 µl) were collected at 5 min and 2 h after the addition of isotope. At the end of the 2 h flux period, larvae were killed by overdose with ethyl 3-aminobenzoate methanesulfonate (MS-222) and briefly washed in isotope-free water containing high levels of Na^+ (>200 mM) to displace residual isotope attached to the surface of the fish. The remaining water in the tube was stored separately for later measurement of total [Na^+].

Series 2. Consequences of microtubule disruption on Na^+ uptake during acute exposure to acidic water

Previous studies demonstrated that activation of electrolyte transporters by cAMP may be associated with microtubule-dependent translocation of target proteins (Tresguerres et al., 2006; Tresguerres et al., 2010c). To test whether the increase in Na^+ uptake triggered by forskolin treatment also might be associated with physical re-location of target transporters, zebrafish were pre-treated with two microtubule disruptors, either colchicine (200 µM for 4 h; stock solution prepared in water; Tsai and Hwang, 1998) or nocodazole (10 - 30 µM for 30 min; stock solution prepared in DMSO; Santa Cruz Biotech Inc.; Yoo et al., 2012) before further treatment with 10 µM forskolin. Na^+ uptake measurements began 5 min after the addition of forskolin, as described above.

It was recently demonstrated that zebrafish larvae are able to increase Na^+ uptake after only brief (2 h) exposure to acidic or ion-poor environments. To test whether microtubule-dependent transporter trafficking is involved in such rapid activation of Na^+ uptake, larvae were pre-treated with colchicine or nocodazole, and then transferred to acidic water [prepared by adding H_2SO_4 to Ottawa tap water (pH ~ 4.0)] for 2 h in the continuing presence of these inhibitors. After the 2-h exposure to acidic water, larvae were transferred to the control water and Na^+ uptake was measured as described above.

Series 3. Consequences of protein kinase A inhibition during acute exposure to acidic water

To determine whether cAMP-dependent signalling mediated by protein kinase A (PKA) is involved in the induction of Na^+ uptake during acute challenge, 4 dpf larvae were pre-treated with 10 µM H-89 (LC Labs; MA, USA) for 30 min in control water before being transferred to acidic water in the continued presence of H-89. The H-89 stock solution and vehicle control

solution were prepared with DMSO. After the 2-h exposure, larvae were transferred to the control water without DMSO/H-89 and Na^+ uptake was measured as described above.

Series 4. Elucidation of target proteins activated by cAMP

4.1. Effect of cAMP on EIPA-sensitive acid secretion

To help identify the transporter(s) being activated by cAMP, whole body net acid excretion ($J_{NET}H^+$) was determined in fish treated with forskolin. Briefly, fifteen 4 dpf larvae (to generate N = 1) were placed in 4.5-ml of Vancouver tap water in a 15-ml tube. Water samples (1.5 ml) were collected at the beginning and end of a 1.5-h flux period and stored at 4° C until analysis (all samples were analyzed within 24 h of collection). To determine titratable alkalinity flux $J_{NET}TA$, 1.2 ml of the initial and final water samples from each flux period was titrated to pH 4.00 using 0.001 M HCl; the difference in titrant volume added between the samples was noted. Acid was delivered with a Gilmont microburette (GS-1200 GE 2.0 mL; Fisher) and pH was recorded (pHC3005-8 electrode and PHM201 pH meter; Radiometer Analytical) throughout the titration. Samples were bubbled with nitrogen continuously to ensure mixing. Ammonia excretion ($J_{NET}AMM$) was determined by measuring water total ammonia levels at the start and end of the flux period. Water ammonia concentrations were determined colorimetrically as reported by (Verdouw et al., 1978), with slight modifications for microplate reading. $J_{NET}H^+$ was calculated as the sum of $J_{NET}TA$ and $J_{NET}AMM$, signs considered (McDonald and Wood, 1981).

To determine whether forskolin treatment could stimulate Na^+ uptake/acid secretion via Na^+-H^+ exchanger 3 (NHE3), the effect of treatment with 5-(N-Ethyl-N-isopropyl) amiloride (EIPA; an inhibitor of NHE) on Na^+ uptake and $J_{NET}H^+$ was determined. For Na^+ uptake, larvae were treated with 50 or 100 µM EIPA (DMSO as vehicle) and influx was started 5 min after the addition of EIPA. Measurement was performed as described above. $J_{NET}H^+$ was measured in

larvae treated with DMSO (vehicle control) or 10 µM forskolin as described above. After the initial flux, larvae were treated with 50 µM EIPA and $J_{NET}H^+$ was determined again. This dose of EIPA was chosen as it was sufficient to significantly inhibit uptake of Na^+ (see results). To confirm that EIPA (or DMSO) did not alter the buffering capacity of the water samples, a known quantity of acid was added to a water sample with and without DMSO or EIPA/DMSO, and the samples were titrated to pH 4.00 as described above. The presence of these chemicals did not significantly alter the required titrant volumes.

4.2. Effect of forskolin treatment on *glial cell missing 2* (*gcm2*) morphants

The transcription factor, *glial cell missing 2* (*gcm2*) plays a critical role in the differentiation of H^+-ATPase rich cells (HRCs) and its knockdown prevents HRC formation (Chang et al., 2009). The loss of HRCs is accompanied by a compensatory increase in the numbers of Na^+-Cl^- co-transporter (NCC; *zslc12a10.2*) expressing cells (Chang et al., 2009; Wang et al., 2009; Shono et al., 2011). To test whether NCC is regulated by cAMP, embryos were injected with a morpholino against *gcm2* or a control morpholino (Kumai et al., 2012a). The larvae were raised in control water until 4 dpf and Na^+ uptake was measured in response to forskolin treatment as described above.

Series 5. Consequences of acute acid exposure on whole body cAMP levels

To demonstrate the effect of acute acid exposure on cAMP metabolism, whole body cAMP content was analyzed using a commercial ELISA kit (Cayman Chemicals). 4 dpf larvae were exposed to acidic water for 3 h or kept in control water. After the treatment, larvae were killed (30 larvae were pooled for N = 1), homogenized in 100 µL 0.1N HCl and centrifuged at 6000 g for 10 min at 4° C. Supernatant was collected and further diluted 10-fold using 0.1 N

HCl. The diluted extract was stored at - 80° C until analysis and directly used for the ELISA assay according to the manufacturer's instructions.

Series 6. Vital staining of larvae using fluorescently tagged forskolin or propranolol

To visualize forskolin-binding sites (presumed to be AC) in zebrafish, 4 dpf larvae were vitally stained with BODIPY®-forskolin (Invitrogen) at 15 nM for 40 min. Larvae were stained simultaneously with alexa-633 conjugated concanavalin A (conA; Invitrogen), a lectin that has been used extensively to visualize HRCs. Subsequently, larvae were killed with MS-222, briefly rinsed with PBS and observed using a confocal microscope (Olympus FV1000 BX-61 with Fluoview software).

It was previously shown that a subset of larval zebrafish ionocytes are enriched with β-adrenergic receptors based on their staining with fluorescently tagged propranolol (Kumai et al., 2012b). To demonstrate that these propranolol-positive ionocytes also express forskolin-binding AC, 4 dpf larvae were stained with BODIPY-propranolol and MitoTracker® (a pan-ionocyte marker) as described in (Kumai et al., 2012b) as well as BODIPY-forskolin as described above. Furthermore, to test the specificity of BODIPY-forskolin, a group of larvae were pre-treated with 10 µM non fluorescent forskolin for 10 min before vital staining with propranolol, MitoTracker® and forskolin in the continued presence of non-fluorescent forskolin.

Analytical tools and calculations

To determine Na^+ uptake, all collected water samples were supplemented with 5 ml of scintillation cocktail (Biosafe-II, RPI Corp., Mt. Prospect, IL, USA) and their radioactivity was measured with a liquid scintillation counter (model LS-6500 Beckman Coulter, Co. Mississauga ON, Canada). After being rinsed in an isotope-free medium, larvae were digested in a tissue solubilizer (Solvable^{TM}, Perkin Elmer) for 4 h at 65° C. After complete digestion, samples were

supplemented with 5 ml of the same scintillation cocktail. Samples were then neutralized by adding 500 µl of glacial acetic acid before measuring their radioactivity. The concentration of total Na^+ in the water was measured using flame emission spectrophotometry (model AA260, Varian, Palo Alto, CA, USA). For Na^+ uptake experiments in Series 4.1, samples were counted directly using a gamma counter (Wallac 1470 Wizard Gamma Counter, PerkinElmer, Turku, Finland), without tissue digestion/scintillation cocktail. Owing to the limited volume of water, $[Na^+]$ was measured, and hence external specific activity was determined, only at the end of the flux period. It was assumed that, given the typical $[Na^+]$ of the experimental water (700 - 1000 µM), Na^+ influx rate (less than 1 nmol/fish/h or less) and even smaller expected net flux of Na^+ (difference between influx and efflux), changes in total $[Na^+]$ during the flux period would be negligible. The rate of Na^+ uptake (J_{in}^{Na}, pmol/fish/h) was calculated as follows:

$$J_{in}^{Na} = \frac{F}{SA \cdot n \cdot t}$$

, where F = total incorporated radioactivity (DPM, disintegration per minute), SA = specific activity of the medium (DPM/pmol), n = number of larvae (typically 1) and t = the duration of the incubation (h). DPM was calculated by the liquid scintillation counting program after taking quenching and counting efficiency into consideration.

Statistical analysis

All statistical analyses were performed with SigmaPlot (v. 11, Systat Inc. Chicago, IL, USA). Student's t-test was used to analyze data from Series 1 (8-bromo cAMP treatment), 4.1 (changes in $J_{NET}H^+$ after EIPA treatment) and 5. One-way ANOVA followed by Tukey post hoc test was used to analyze data from Series 1 (forskolin treatment) and 2. Two-way repeated measures (RM) ANOVA was used to analyze flux data from Series 4.2. When assumptions of

normality or equal variance were violated, data were transformed using natural log- or square-root transformation. For all analyses, the level of statistical significance was set at $P<0.05$.

Results

Series 1. Consequences of increasing [cAMP] levels on Na^+ uptake

Acute treatment with forskolin between 0.1 and 10 µM significantly elevated Na^+ uptake in 4 dpf zebrafish larvae maintained in control water (N = 6 - 10; Fig. 1A; one-way ANOVA). Similarly, treatment with 1 µM 8-bromo cAMP led to a significant increase in Na^+ uptake (N = 5 - 6; Fig. 7.1B; Student's t-test).

Series 2. Consequences of microtubule disruption on Na^+ uptake during acute exposure to acidic water

Pre-treatment with 200 µM colchicine did not affect the stimulation of Na^+ uptake caused by forskolin treatment (N = 11 - 17; Fig. 7.2A; one-way ANOVA) or acute exposure to acidic water (N = 6; Fig. 7.2B; one-way ANOVA). Similarly, treatment with nocodazole also did not modify Na^+ uptake in response to forskolin or acidic water (data not shown).

Series 3. Consequences of protein kinase A inhibition during acute exposure to acidic water

The addition of 10 µM H-89 significantly attenuated the increase in Na^+ uptake associated with acute exposure to acidic water (N = 6; Fig. 7.3; one-way ANOVA).

Series 4. Effect of cAMP on EIPA-sensitive acid secretion or Na^+ uptake in gcm-2 morphants

Treatment with EIPA significantly reduced uptake of Na^+ (N = 5 - 6; Fig. 7.4A; one way ANOVA). Treatment with forskolin did not affect $J_{NET}H^+$, $J_{NET}TA$ or $J_{NET}AMM$ (N = 6; Fig. 7.4B-D; two-way RM ANOVA). However, $J_{NET}TA$ in forskolin-pre-treated, EIPA-treated fish was significantly lower than in EIPA-treated, DMSO-pre-treated fish (N = 6; Fig. 7.4B-D; two-way RM ANOVA). EIPA-sensitive inhibition of $J_{NET}H^+$ was significantly greater in forskolin-pre-treated groups (N = 6; Fig. 7.4E; Student's t-test).

As reported previously (Kumai et al., 2012a) knockdown of *gcm-2* led to a significant increase in Na^+ uptake (Fig. 7.5). Treatment of morphants with 10 µM forskolin further increased the uptake of Na^+, suggesting that Na^+ uptake via NCC is being stimulated by forskolin (N = 6; Fig 7.5; two-way ANOVA).

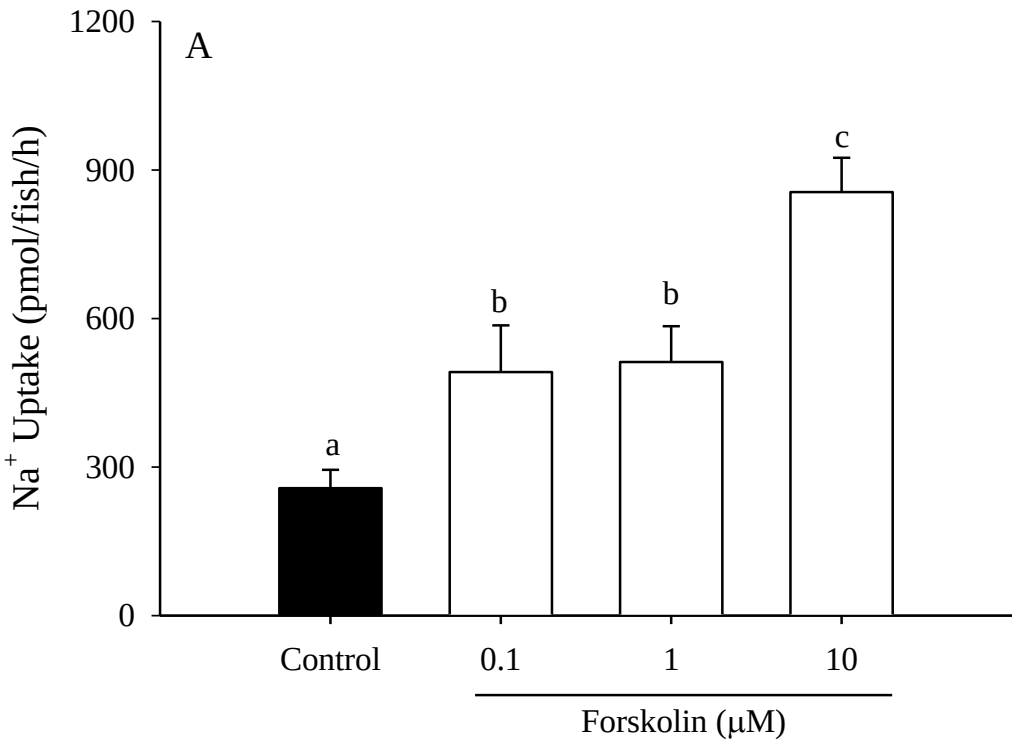
Series 5. Consequences of acute acid exposure on whole body cAMP levels

Whole body cAMP content was significantly increased following 3-h exposure to acidic water (N = 6 - 7; Fig. 7.6; Student's t-test).

Series 6. Vital staining of larvae using fluorescently tagged forskolin and propranolol

The conA-positive HRCs were also forskolin-positive (Figs. 7.7 A-C). . Forskolin also stained additional con-A negative cells that often were triangular in appearance. As expected, most of the forskolin-positive cells also expressed high levels of β-adrenergic receptors as visualized using fluorescently tagged propranolol (Figs. 7.8A-D). . As previously reported (Kumai e al., 2012b), intensity of MitoTracker® stain in forskolin/propranolol positive cells was relatively weak (Fig. 7.8). This observation is still consistent with the colocalization of conA and forskolin (Fig. 7.7); as HRCs tend to (though could still be stained) stain weakly with MitoTracker® (for general review on zebrafish ionocyte, see Hwang, 2009; Hwang and Perry, 2010; Hwang et al., 2011). The specificity of forskolin was confirmed as pre-treatment with "cold" forskolin greatly reduced staining intensity with BODIPY-forskolin (Figs. 7.8 E-H).

Figure 7.1. Effect of forskolin treatment on Na^+ uptake. Treatment with 0.1 - 10 µM forskolin increased Na^+ uptake in zebrafish larvae in a dose-dependent manner (N = 6 - 10; Fig. 7.1A). Treatment with 8-bromo cAMP also increased the uptake of Na^+ significantly (N = 5 - 6; Fig. 7.1B). Different letters denotes significant differences among the treatment groups; an asterisk in Fig. 7.1B denotes significant difference from the control group. Data are presented as means ± SEM.


A
1200
900
600
300
0
Na+ Uptake (pmol/fish/h)
a
b
b
c
Control
0.1
1
10
Forskolin (μM)

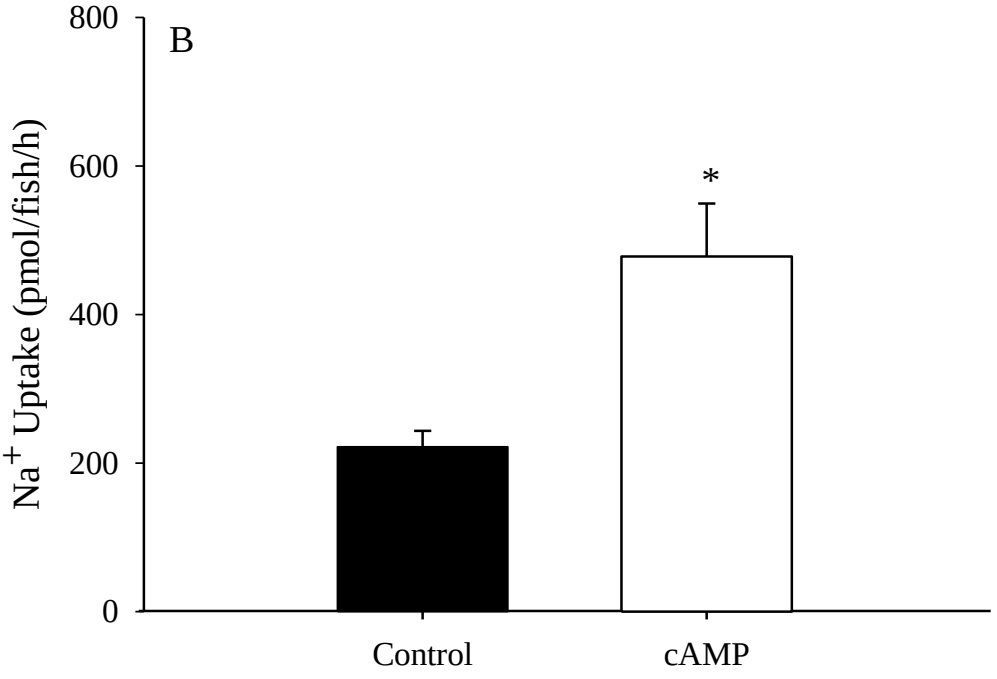
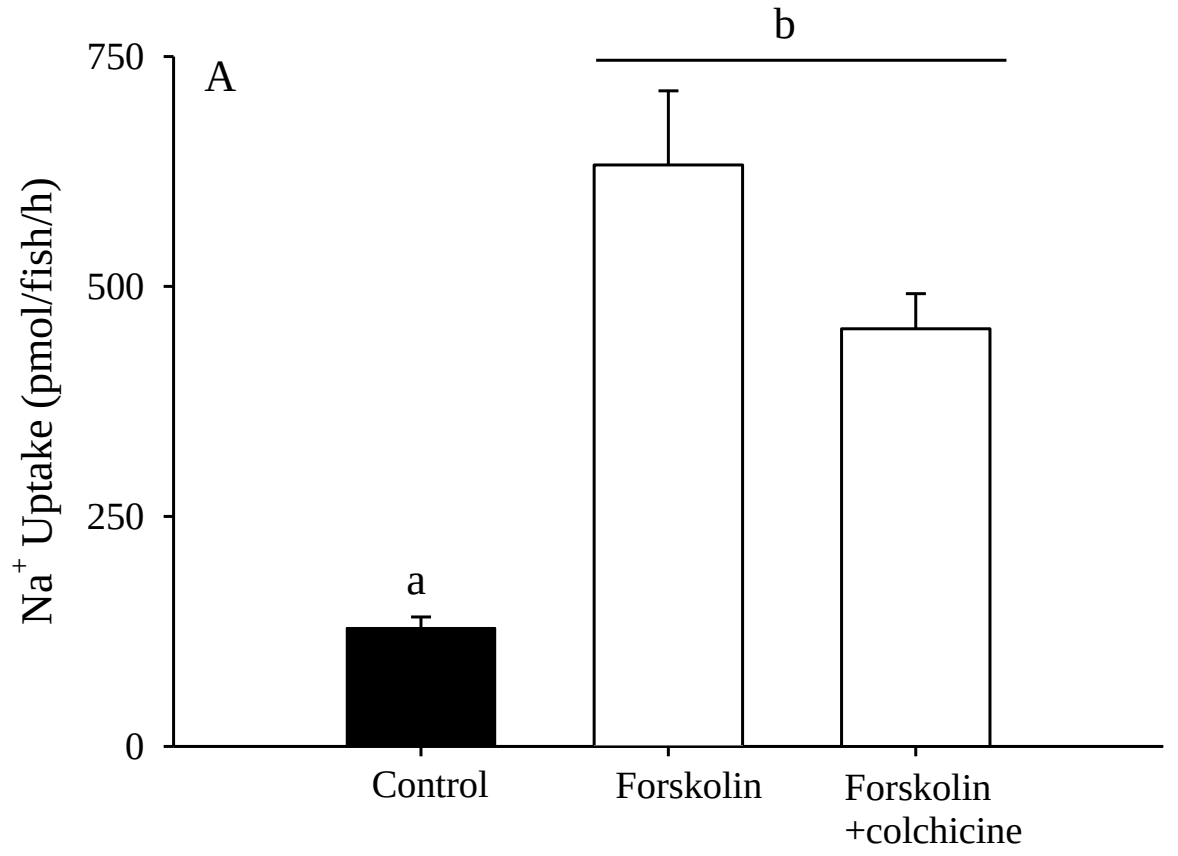

B
800
600
400
200
0
Na+ Uptake (pmol/fish/h)
*
Control
cAMP

Figure 7.2. Effect of colchicine pre-treatment on Na^+ uptake after forskolin or low pH-exposure. Pre-treating larvae with 200 µM colchicine for 4 h did not inhibit stimulation of Na^+ uptake following their treatment with forskolin (N = 11 - 17; Fig. 7.2A) or low pH water (N = 6; Fig. 7.2B). Different letters denotes significant differences between groups. Data are presented as means ± SEM.


A
b
a
750
500
250
0
Na+ Uptake (pmol/fish/h)
Control
Forskolin
Forskolin
+colchicine

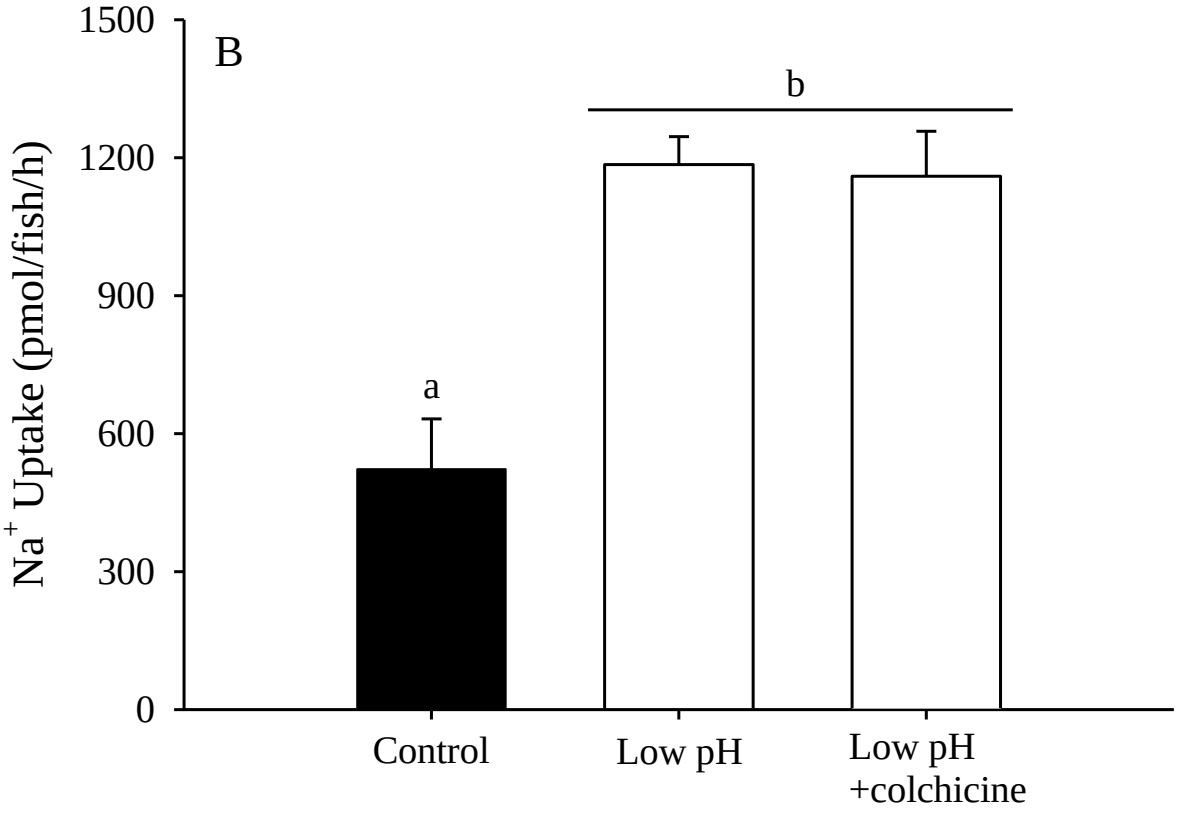

B
b
a
1500
1200
900
600
300
0
Na+ Uptake (pmol/fish/h)
Control
Low pH
Low pH
+colchicine

Figure 7.3. Effect of PKA inhibition during acute exposure to low pH water. Exposure to low pH water in the presence of 10 µM H-89 significantly reduced Na^+ uptake (N = 6; Fig. 7.3). Different letters denotes significant differences between groups. Data are presented as means ± SEM.

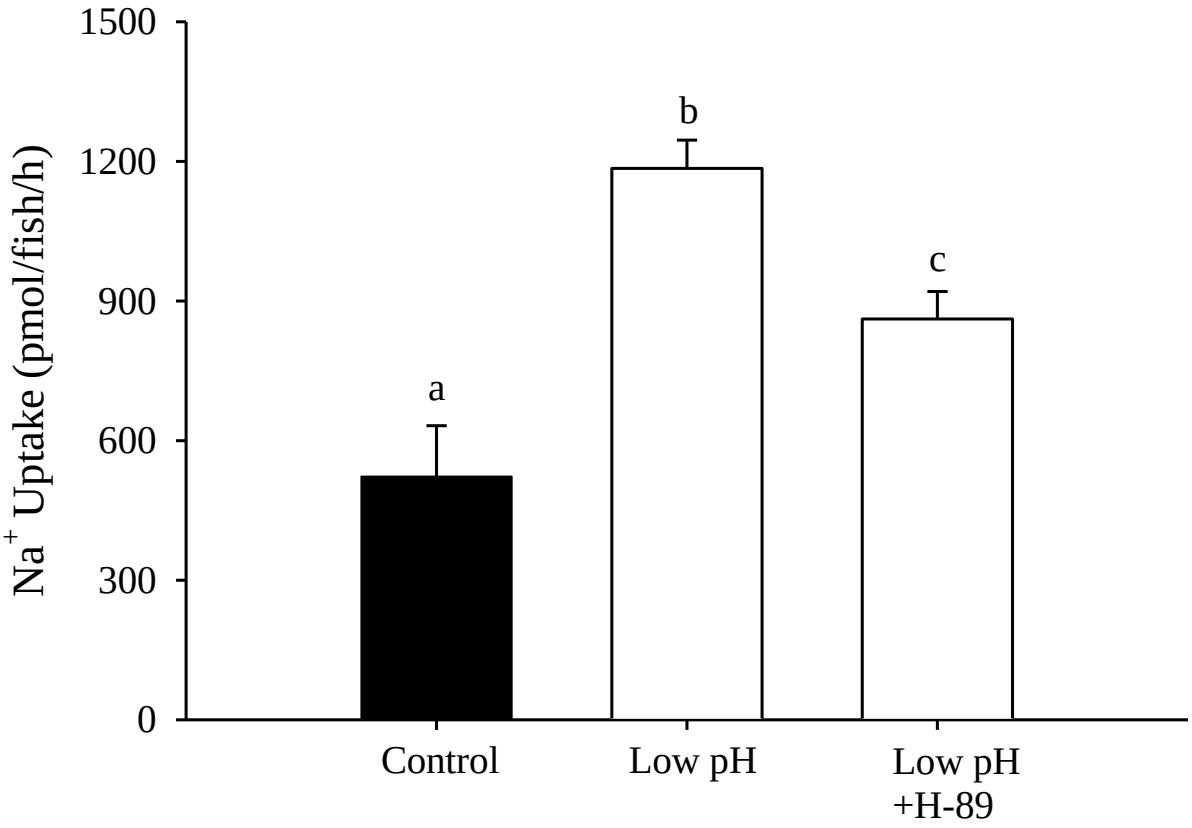

1500
1200
900
600
300
0
Na+ Uptake (pmol/fish/h)
a
b
c
Control
Low pH
Low pH
+H-89

Figure 7.4. Effect of forskolin treatment on EIPA-sensitive Na^+ and acid fluxes. Treatment of 4 dpf larvae with EIPA significantly reduced Na^+ uptake (N = 5 - 6; Fig. 7.4A). Treatment of 4 dpf larvae with forskolin had no significant effect on $J_{NET}TA$ (N = 6; Fig. 7.4B) $J_{NET}AMM$ (N = 6; Fig. 7.4C) or $J_{NET}H^+$ flux (N = 6; Fig. 7.4D). When larvae were treated with EIPA, $J_{NET}TA$ was significantly reduced in the forskolin pre-treated group only (N = 6; Fig. 7.4B). When the magnitude of $J_{NET}H^+$ flux was calculated, EIPA caused a significantly greater inhibition of $J_{NET}H^+$ flux than in the control group (N = 6; Fig. 7.4E). Different letters denotes significant differences between groups. Data are presented as means ± SEM.

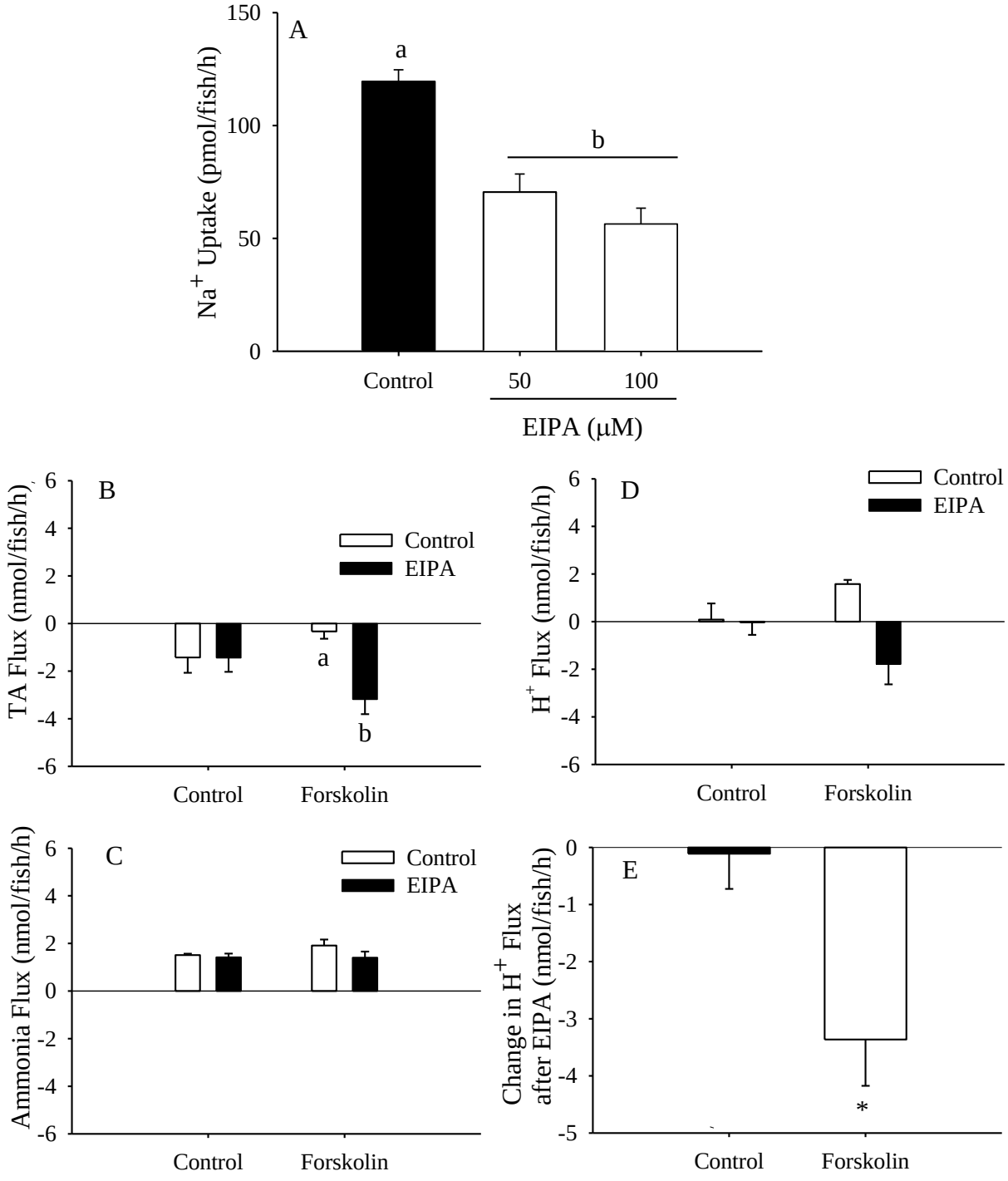

A
150
100
50
0
Na+ Uptake (pmol/fish/h)
a
b
Control
50
100
EIPA (μM)
B
6
4
2
0
-2
-4
-6
TA Flux (nmol/fish/h)
Control
EIPA
a
b
Control
Forskolin
D
H+ Flux (nmol/fish/h)
Control
EIPA
Control
Forskolin
C
Ammonia Flux (nmol/fish/h)
Control
EIPA
Control
Forskolin
E
0
-1
-2
-3
-4
-5
Change in H+ Flux after EIPA (nmol/fish/h)
*
Control
Forskolin

Figure 7.5. Effect of forskolin treatment on *gcm2* morphants. Exposure to 10 µM forskolin significantly increased Na^+ uptake in both sham and *gcm2* morphants. N = 6. Different letters denotes significant difference between sham and morphants, and asterisks denote difference between DMSO (vehicle) and forskolin treated groups. Data are presented as means ± SEM.

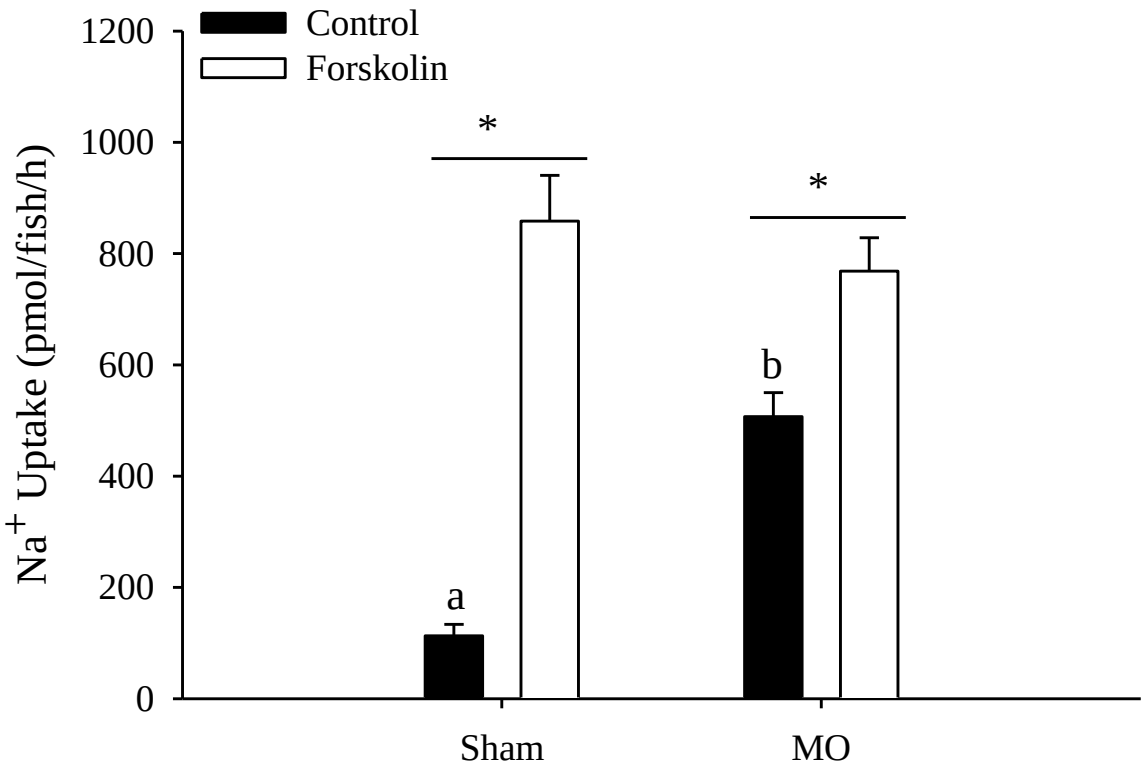

Control
Forskolin
1200
1000
800
600
400
200
0
Na^+ Uptake (pmol/fish/h)
*
*
a
b
Sham
MO

Figure 7.6. Effect of acid-exposure on whole body cAMP content. A brief (3 h) exposure to low pH water significantly elevated whole body cAMP content in 4 dpf zebrafish larvae. N = 6 - 7. An asterisk indicates significant difference between groups. Data are presented as means ± SEM.

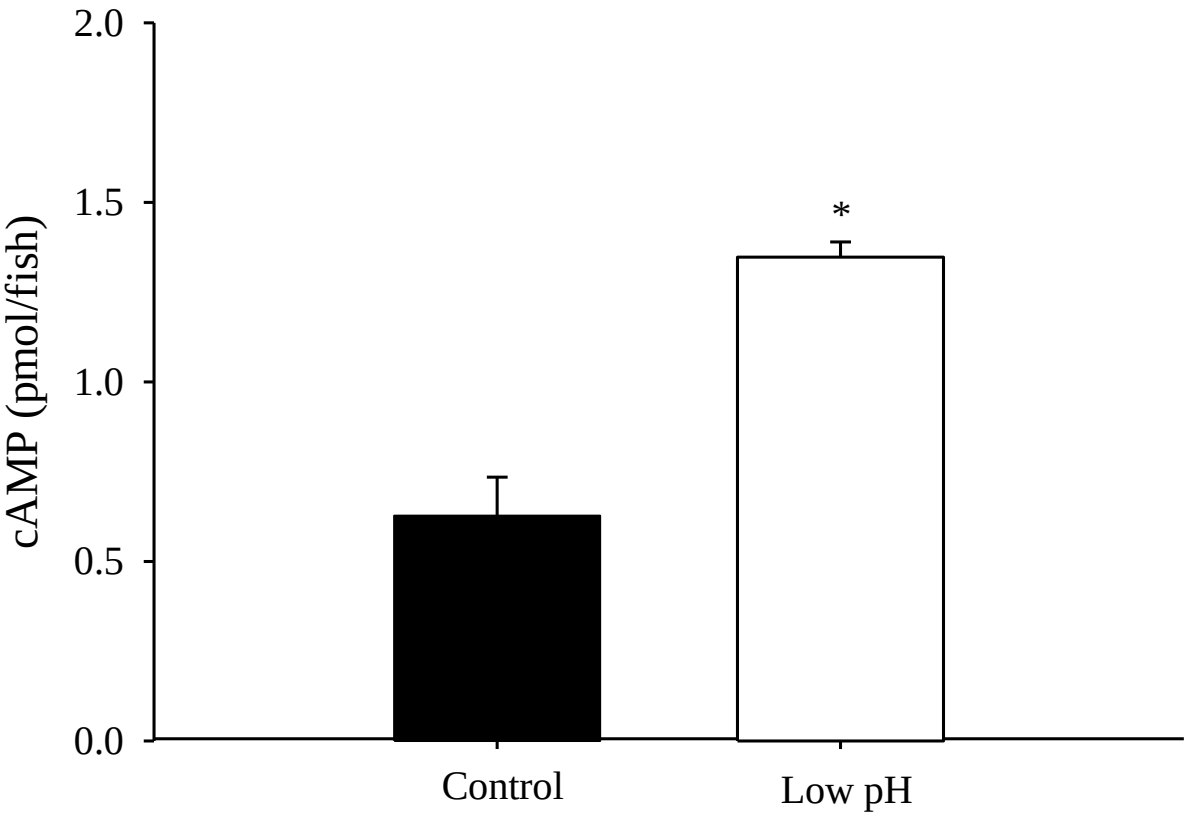

2.0
1.5
1.0
0.5
0.0
cAMP (pmol/fish)
*
Control
Low pH

Figure 7.7. Distribution of forskolin-binding sites in developing zebrafish larvae. Incubation of 4 dpf zebrafish larvae with BODIPY-conjugated forskolin revealed a subset of epithelial cells enriched with forskolin binding sites (presumed to be tmAC; Fig. 7.7A). A portion of tmAC-rich cells (indicated with an asterisk) were con-A positive HRCs (Figs. 7.7B, C). Scale bars = 50 µm.

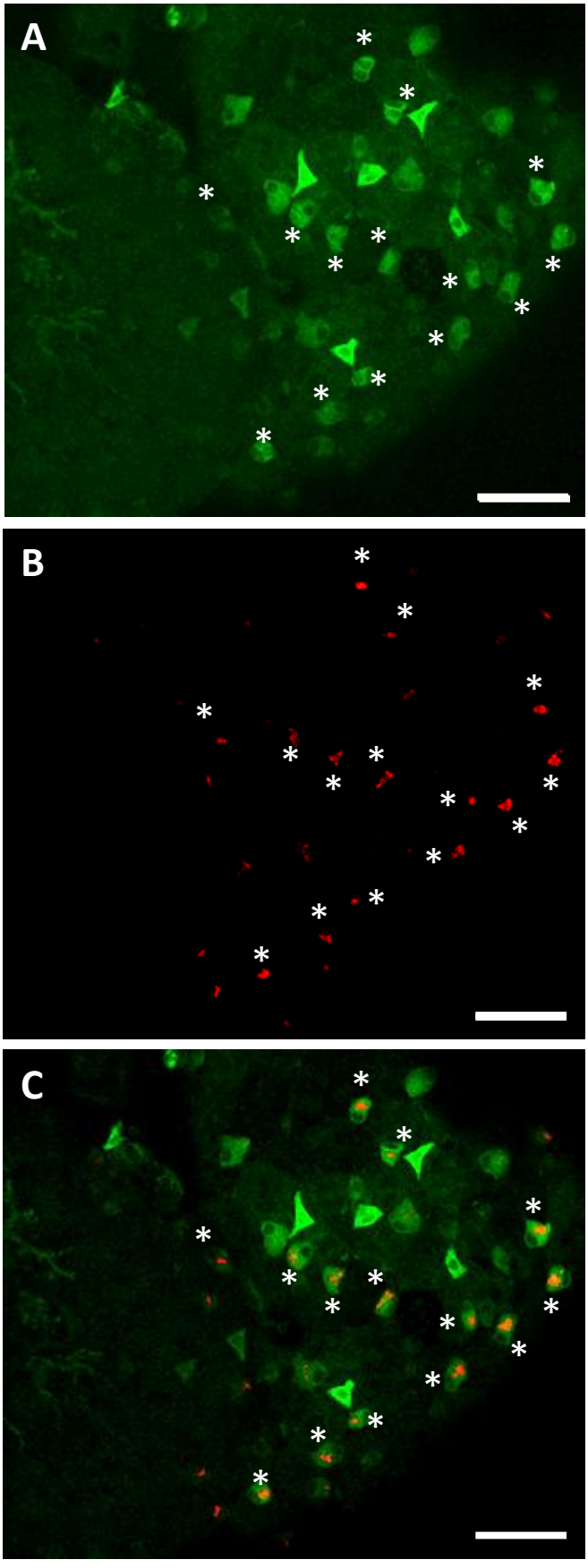
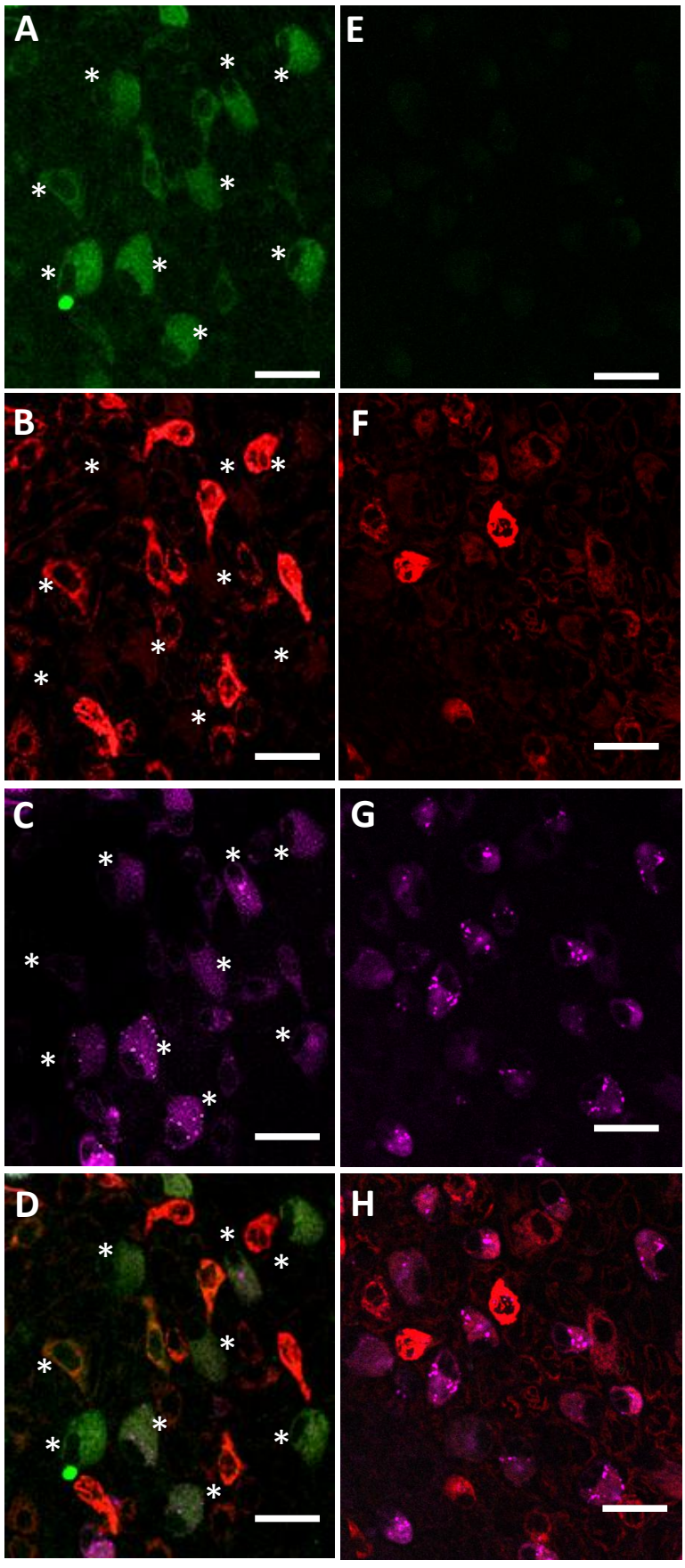

A
B
C

Figure 7.8. Co-localization of forskolin-binding sites with β-adrenergic receptors. Incubation of 4 dpf zebrafish larvae with BODIPY-conjugated forskolin (Figs. 7.8A, E), MitoTracker® (Figs. 7.8B, F) and BODIPY-conjugated propranolol (Figs. 7.8C, G) demonstrated that forskolin-biding tmAC enriched cells also express high level of β-adrenergic receptors (indicated with an asterisk), and that those cells are MitoTracker®-positive ionocytes, although the expression level of MitoTracker® was weaker than some other, forskolin-negative, MitoTracker®-positive cells. Co-incubation of larvae with non-fluorescent forskolin greatly reduced the staining intensity of BODIPY-forskolin (Fig. 7.8E), while staining by the two other vitals dyes was unaffected by the presence of non-fluorescent forskolin (Figs. 7.8F, G). Scale bars = 20 μm.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Discussion

The present study demonstrates that the rapid increase in Na^+ uptake following exposure of larval zebrafish to acidic water is caused, at least in part, by cAMP-mediated intracellular signalling. These data expand on the previous observation that in zebrafish, stimulation of Na^+ uptake is linked to activation of β-adrenergic receptors. The current study further reveals that NHE3 (and potentially NCC) are regulatory targets of cAMP signalling which, when activated, promote Na^+ uptake in acidic water.

cAMP stimulates Na^+ uptake

While the results of previous studies demonstrated a role for cAMP-mediated signalling in stimulating Cl^- secretion in isolated opercular epithelium of SW-acclimated killifish, *Fundulus heteroclitus* (Marshall et al., 1995) and decreasing Na^+-K^+-ATPase activity in gill cells isolated from brown trout, *Salmo trutta* (Tipsmark and Madsen, 2001), surprisingly little is known about the role of this universal secondary messenger in regulating ion fluxes in FW fish *in vivo*. Based on the stimulatory effects of forskolin and 8-bromo cAMP on Na^+ uptake, the present study clearly demonstrated the potential for cAMP to rapidly regulate Na^+ balance in zebrafish larvae. Because the activation of Na^+ uptake during exposure of zebrafish larvae to acidic water is linked to β-adrenergic stimulation uptake (Kumai et al., 2012b),these data are consistent with the previously reported stimulatory role of β-adrenergic receptor activation (which promotes downstream signalling mediated by cAMP) on Na^+ uptake.

cAMP is synthesized either by trans-membrane AC (tmAC or AC1 - 9) or by soluble AC (sAC or AC 10). Because sAC is activated by a rise in [HCO_3^-], its potential role in body fluid pH and electrolyte regulation has received considerable research interest in many animal and tissue models (Hallows et al., 2009; Pastor-Soler et al., 2003; Pastor-Soler et al., 2008; Păunescu et al., 2008; Tresguerres et al., 2010b; Tresguerres et al., 2010c). Surprisingly, despite the

widespread presence of sAC across phyla, phylogenetic analysis of vertebrate AC isoforms has not yet identified sAC (or a sAC-like isoform) in the zebrafish genome. In addition, treatment of zebrafish larvae with two frequently used sAC inhibitors, KH-7 and 4-catechol estradiol (4-CE) (Tresguerres et al., 2010c) was without effect on Na^+ uptake (Y. Kumai and S.F. Perry, unpublished results), further supporting the idea that there is no functional AC10 ortholog in zebrafish. However, it cannot be excluded that other AC isoforms have acquired sensitivity to $[HCO_3^-]$, and potentially assume functions similar to sAC as a HCO_3^- sensor in zebrafish. Measurement of cAMP levels in isolated adult gill tissue during exposure e to elevated $[HCO_3^-]$ might provide insight on this issue.

Because the stimulation of Na^+ uptake after forskolin treatment was apparent within 2 h, it was likely related to, at least initially, activation of existing proteins rather than to increased transcription and protein synthesis. Microtubule disruption using colchicine (previously used in larval tilapia *Oreochromis mossambicus*; Tsai and Hwang, 1998) was employed to determine whether the stimulation of Na^+ uptake was caused by trafficking of internal proteins toward the apical membrane (Tresguerres et al., 2006; Tresguerres et al., 2010c) or the activation of existing apical membrane proteins. Because pre-treatment with colchicine did not impede the stimulation of Na^+ uptake associated with forskolin treatment (Fig. 7.2A) or acid exposure (Fig. 7.2B), it seems unlikely that protein trafficking was involved. The same results (data not shown) were obtained using an alternate MT inhibitor, nocodazole, at a dose almost 5 times higher than previously used to inhibit MT-dependent cell migration in zebrafish larvae (Yoo et al., 2012). Thus, it is suggested that cAMP activates Na^+ uptake by activating Na^+ transporters (see below) already expressed on the apical membrane.

cAMP acts through PKA to activate Na^+ uptake

Although PKA initially was recognized as the sole mediator of downstream cAMP signalling, it was recently demonstrated that a second protein, "exchange protein directly activated by cAMP" (Epac), can initiate PKA-independent downstream signalling (Cheng et al., 2008; Gloerich and Bos, 2010). Previous studies showed that Epac1 is expressed in the mouse proximal tubule and that its activation inhibited NHE3 activity similarly to PKA activation (Honegger et al., 2006) by interacting with NHERF1 (Murtazina et al., 2007). Interestingly, even for a common physiological endpoint, the mechanisms of cAMP signalling may be tissue (and likely species) dependent. For example, (Murtazina et al., 2007) showed that in ileum, the inhibitory effect of cAMP on NHE3 activity was abolished by pre-treating the tissue with H-89, a commonly used PKA inhibitor, despite the presence of Epac1 in this tissue.

Because of the lack of commercially available Epac inhibitors, the present study focused on the consequences of PKA inhibition on cAMP-mediated stimulation of Na^+ uptake. When larvae were exposed to acidic water in the presence of 10 µM H-89, the magnitude of the increase in Na^+ uptake was diminished (Fig. 7.3A), suggesting that a portion of Na^+ uptake stimulation during acute exposure to acidic water is mediated by cAMP signalling acting via PKA. Because the specificity of H-89 as an inhibitor of PKA has been questioned (Lochner and Moolman, 2006), the role of PKA in stimulating Na^+ uptake was confirmed using another PKA inhibitor, Rp-cAMP (20 µM; Enzo Life sciences), which resulted in a similar reduction in Na^+ uptake (data not shown). While these data imply a role for PKA, further experiments should be performed using alternative methods to lower PKA activity, such as morpholino knockdown. While the global knockdown of PKA, a kinase with an extensive list of functions, would likely result in death or deformity, a recently introduced new class of photo-activatable morpholinos (Shestopalov et al., 2007) might provide an alternate strategy to evaluate the role of PKA.

The effects of PKA inhibition, when combined with the stimulation of Na^+ uptake after treatment with forskolin or 8-bromo cAMP, strongly suggest an important role for cAMP-PKA signalling in mediating the stimulation of Na^+ uptake during acid exposure. In agreement with this idea, whole body cAMP content significantly increased in larvae exposed for 3 h to acidic water (Fig. 7.6). It is important to note, however, that the observed rise in whole body cAMP content likely reflects the rapid induction of multiple physiological signalling pathways in response to acid-exposure, many of which would be occurring outside of ionocytes and thus not be directly related to ionic regulation. To better define the role of cAMP-mediated signalling in Na^+ uptake, live imaging of cAMP at the level of specific ionocytes, using techniques such as Fluorescence Resonance Energy Transfer (FRET)-based cAMP measurement (Borner et al., 2011; Willoughby and Cooper, 2008) would be helpful.

cAMP may target multiple Na^+ transporters

Based on mammalian studies, cAMP-dependent signalling is known to affect at least three ion transport mechanisms linked to Na^+ absorption; NHE3, epithelial Na^+ channel and H^+-ATPase (see Introduction). The results of the present study suggest that cAMP may stimulate NHE3 and NCC activities in larval zebrafish (in contrast to its inhibitory effect in mammals). An involvement of NHE3 was supported by the observation that forskolin-pre-treatment significantly increased the EIPA-sensitive H^+ flux in 4 dpf zebrafish larvae (Fig. 7.4). It is important to note, however, that no change in H^+ flux was observed in the control group after forskolin treatment, which should have activated NHE3 (and increased H^+ secretion). One possible explanation for this observation is that cAMP treatment also activated one or more isoforms of slc26 anion exchanger family, which has been implicated in Cl^- uptake in zebrafish (Bayaa et al., 2009; Perry et al., 2009). Because the titration protocol used in the current study

measures net movement of H^+-equivalents, the increase in acid-secretion (from NHE3 activation) might have been masked by a concurrent increase in base secretion (from slc26 activation). Indeed, in mouse cortical collecting duct, pendrin (SLC26A4) is stimulated by cAMP (Azroyan et al., 2012), and treatment with 10 µM forskolin increased Cl^- uptake by 1.5-fold in zebrafish larvae (Y Kumai and SF Perry, unpublished data). Although the observed increase in Cl^- uptake might also be partially mediated by NCC (see below), the potential role of cAMP in regulation of slc26 in zebrafish warrants further investigation.

The effectiveness of EIPA treatment on inhibiting Na^+ uptake in zebrafish has been inconsistent. Despite similar doses and water chemistry in which experiments were performed, Esaki et al. (2007) reported strong inhibition of Na^+ uptake by EIPA, whereas Boisen et al., (2003) and Kwong and Perry, (2013b) observed no inhibition. Kumai and Perry (2011) observed inhibition of Na^+ uptake in EIPA-treated larvae, but only after they were pre-exposed to acidic water. In the present study, EIPA-treatment did indeed inhibit Na^+ uptake (Fig. 7.5A). This effect was likely related to the low ion content of Vancouver water (e.g. $[Na^+] = 0.05$ mM) in which this experiment was performed. The results of previous studies suggested that NHE3 contributes proportionally more to Na^+ uptake when zebrafish are exposed to acidic (Kumai and Perry, 2011) or ion-poor (Shih et al., 2012) water. Thus, performing these experiments in ion-poor Vancouver water might have made the effects of EIPA treatment on Na^+ and acid fluxes more readily detectable. In agreement with the idea that cAMP stimulates NHE (presumably in association with β-adrenergic receptors), conA-positive HRCs appear to co-express tmAC (Fig. 7.7) and β-adrenergic receptors (Fig. 7.8). However, it should be noted that HRCs also express H^+-ATPase and thus it is possible that H^+-ATPase also is regulated by cAMP.

In addition to NHE, cAMP may stimulate NCC given that forskolin continued to stimulate Na^+ uptake in *gcm2* morphants, which have reduced numbers of HRCs. The idea that cAMP could also target ion transporters expressed on non-HRCs is supported by the observation of an additional population of forskolin-positive cells that were conA-negative (Fig. 7.7).

Perspectives

Despite its prominent role as a universal secondary messenger, the role of cAMP-dependent signalling on osmoregulation in FW fish is not well established. While the present study provides insight into the presence of cAMP-mediated signalling mechanism(s) capable of activating several Na^+ transporters, it also raises a number of issues that need to be addressed such as; 1) the relative roles of PKA and Epac in mediating cAMP-signalling, 2) whether specific isoform(s) of tmAC are involved in mediating cAMP-dependent uptake of Na^+, 3) whether the uptake of other ions (e.g. Ca^{2+} and Cl^-) is controlled by cAMP and 4) whether there is one or more functional analogs of sAC in zebrafish (or in other FW fish) playing a role similar to that in dogfish gill and mammalian kidney.

Acknowledgements

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CHAPTER 8
General Discussion

In this thesis, I used zebrafish, an increasingly powerful model organism, to investigate the mechanisms underlying their tolerance to an extremely acidic environment (pH 4). Results in Chapters 2-3 demonstrated that following chronic (> 24 h) exposure to acidic water, both adult and larval zebrafish are capable of increasing their rate of Na^+ uptake even when measured in acidic water. This response is strikingly different from that reported for other FW species that were previously investigated. Typically, these other species maintain Na^+ balance (if they can) by either keeping the basal, relatively high rate of Na^+ uptake (to minimize the impact from the increased efflux) or by limiting Na^+ losses by means of "tight" gill epithelia that are insensitive to acid-exposure (see Introduction for details). This initial observation of increased Na^+ uptake in acid-exposed zebrafish was the driving force for the remainder of the thesis. Chapter 3 also showed that the increase in Na^+ uptake associated with chronic acid-exposure is mediated, at least in part, by apically-expressed NHE3b, similar to the previous observation in Osorezan dace, *Troborodon hakonensis*, another acid-tolerant species (Hirata et al., 2003), and in Chapter 4, I showed that this response is at least partially mediated by cortisol, acting through glucocorticoid receptors. Subsequently, in Chapters 5-7, I demonstrated that even with acute (~3 h) exposure to acidic or ion-poor water, zebrafish are able to increase Na^+ absorption. As predicted, the results in Chapters 4-7 showed that the responses to acidic or ion-poor water are regulated differently depending on the duration of exposure; acute (3 h) versus chronic (24 h). These key findings from Chapters 4-7 are summarized in Figs. 8.1 and 8.2.

Given these major findings, in this chapter I will separately discuss osmoregulation by zebrafish during acute (~ 3 h) and chronic (~ 24 h) exposures to acidic and ion-poor environments. The discussion will focus largely on three key points raised in the Introduction, namely; i) sensing - what are the mechanisms whereby zebrafish sense the changes in their

environment to trigger the appropriate response (e.g. the rise in Na^+ uptake)? ; ii) cell proliferation - what are the mechanisms underlying cell proliferation and what is the accompanying mechanism?; and iii) molecular mechanisms of ion transporters - what are the molecular mechanisms of Na^+ uptake and how do they operate in the thermodynamically challenging FW environment?

Responses during the acute (~3 h) phase of exposure to acidic or ion-poor water

As stated in the Introduction, an important but challenging task is to identify the cellular signalling mechanisms activated in response to acid-exposure and to determine which initiate downstream events to trigger appropriate physiological responses. In the renal proximal tubule and collecting duct, several proteins, including soluble adenylyl cyclase (sAC), receptor-type tyrosine kinase gamma (RPTP-gamma) and G-protein coupled receptor 4 (GPCR4), have been suggested to be critically involved in acid-base sensing (for review see (Skelton et al., 2010); (Brown and Wagner, 2012). In fish, sAC has been implicated in acid-base sensing based on the observation that inhibition of sAC with KH-7 or 4 catechol estradiol (4CE) prevented the translocation of H^+-ATPase to the basolateral membrane in dogfish gill ionocytes during metabolic alkalosis (Tresguerres et al., 2010c). However, as discussed in Chapter 7, it is likely that sAC (AC10) does not exist in zebrafish, although other adenylyl cyclase isoforms may have acquired sAC-like function and, like sAC, are inducible by alterations in $[HCO_3^-]$. Whether other suggested candidates for acid-base sensors are expressed in zebrafish larval skin and/or adult gill, and whether they are involved in orchestrating the rapid induction of Na^+ uptake following acid-exposure remain interesting (and important) questions.

However, it is also possible that the physiological responses accompanying exposure to acidic water, at least during the initial phase, are cued to a reduction in ion content. Indeed, the data in Chapter 2 clearly demonstrated that one of the immediate effects of acid exposure in adult zebrafish is a large increase in Na^+ efflux and a transient reduction in whole body Na^+ content. A similar increase in ionic efflux was observed in larval zebrafish (Kwong and Perry, 2013a) as well as other FW fish and amphibians (e.g. (Freda et al., 1991); (McDonald, 1983a); (Meyer et al., 2010). This effect, as discussed in Chapter 2, is likely to arise from disruptions of tight junction triggered by the leaching of TJ-bound Ca^{2+}. The idea that a disruption in ionic balance (rather than changes in acid-base status) triggers the compensatory responses to acidic water is an attractive one, because two hormones capable of increasing Na^+ uptake rapidly, angiotensin-II (ANG-II; Chapter 5) and catecholamines (via β-adrenergic receptors; Chapter 6), are activated by exposure to acidic and ion-poor waters. One potential mechanism for such "osmosensing" is a signalling cascade triggered by a family of kinases “With No lysine (K) kinase” (WNK) (for recent reviews, see (Hoorn and Ellison, 2012; Hoorn et al., 2011; Park et al., 2012). WNKs are known to regulate the epithelial Na^+ channel (ENaC), renal outer medullary potassium channel (ROMK; a zebrafish orthoolog, *Kir 1.1*, is expressed in a subtype of ionocytes) (Abbas et al., 2011), and most notably, NCC (for review see) (Hoorn et al., 2011). The regulation of NCC by WNK-1 and -4 has received considerable research interest recently and it is now known that its effects are mediated by phosphorylation of STE20/SPS1-related proline/alanine-rich kinase (SPAK)/oxidative stress-responsive kinase-1 (OSR1), which in turn phosphorylates NCC to alter its activity (Chiga et al., 2011; Chiga et al., 2008; Gagnon et al., 2006;Pacheco-avarez et al., 2006). Additionally, (Talati et al., 2010) reported that acute (30 min) treatment with ANG-II led to significant phosphorylation of OSR1 and NCC in mouse kidney, as well as in mpkCDT, a

mouse distal convoluted tubule cell line expressing those proteins. Furthermore, they observed no effect of eplerenone (a mineralocorticoid receptor blocker) pre-treatment on ANG-II mediated phosphorylation of NCC during **acute** exposure, whereas when exposure was extended to 10 days, eplerenone prevented the ANG-II mediated phosphorylation of NCC. The differing modes of action of ANG-II over different time frames is in agreement with the data in Chapter 5 that demonstrated ANG-II acted independently of cortisol in *acute* experiments (but also see below for a potential caveat).

(Marshall et al., 2005) hinted that a similar signalling cascade might exist in fish based on their finding of a significant increase in protein expression of SPAK in the gill of FW-acclimated killifish, *Fundulus heteroclitus*, and demonstrated the co-localization of SPAK with ionocytes expressing Na^+-K^+-$2Cl^-$ co-transporter (NKCC). Although similar research has not yet been conducted on zebrafish, preliminary results revealed WNK1a and b as well as OSR1a and b mRNA expression in adult gill. Furthermore, mRNA expression of OSR1b, but not 1a, was significantly elevated in 4 dpf larvae following acclimation to ion poor water (Y. Kumai and S.F. Perry, unpublished data). Using a commercial antibody against SPAK, I detected an immunoreactive band roughly corresponding to the expected size of OSR1 (the antibody cannot distinguish between OSR1a and 1b) in protein extracted from larval zebrafish, and successfully co-localized OSR1 in Na^+-pump rich cells (NaRC) of 4 dpf zebrafish larvae, although OSR1 appeared to be also expressed in an additional population of epithelial cells (Y. Kumai and S.F. Perry, unpublished data). In addition, I have recently used a previously validated morpholino against NCC (Wang et al., 2009); the preliminary results indicated that in such NCC morphants stimulation of Na^+ uptake is abolished following brief acid-exposure. However, because of the lack of an antibody able to reliably detect NCC expression specifically, convincing data

demonstrating the effective knockdown of NCC (and implicating the indispensable role of NCC during acute challenge) are still lacking. Although it is entirely speculative for the moment, the possibility that the WNK-OSR1-NCC signalling pathway exists in zebrafish, and whether it plays a role in Na^+ homeostasis during acute exposure to acidic water, potentially in association with ANG-II, provides an attractive avenue for future research.

Unlike ANG-II and catecholamines, cortisol did not appear to modulate Na^+ uptake in zebrafish during acute exposure to acidic or ion-poor water (Chapter 5). This finding is particularly interesting given that during *chronic* exposure to acidic water, cortisol (signalling via glucocorticoid receptor; Chapter 4) plays an important role in stimulating Na^+ uptake. This conclusion is based on the lack of an effect of inhibiting glucocorticoid or mineralocorticoid receptors using pharmacological reagents and gene knockdown approaches (these treatments proved effective in blocking the stimulatory effects of acidic water on Na^+ uptake during *chronic* exposures). On the other hand, it is worth noting that a recent study on non-genomic function of cortisol demonstrated that cortisol could modulate the membrane fluidity itself, and this effect is not mediated by traditional corticosteroid receptors (Dindia et al., 2012). Thus, it is possible that cortisol is involved in regulating Na^+ uptake during acute exposure through this novel non-genomic mechanism. This possibility should be experimentally addressed before the role of cortisol in Na^+ balance regulation during the acute phase of acid exposure is dismissed.

Responses during the chronic phase of exposure to acidic or ion-poor water

When exposure to acidic water is extended (>24 h), one of the major physiological responses is proliferation of ionocytes. Indeed, (Horng et al., 2009b) observed an increase in the density of both HRCs and NaRCs. Proliferation of HRCs would help to rectify the acidosis that

appears to be induced by prolonged exposure to acidic water (Y. Kumai and S.F. Perry, unpublished results), and proliferation of NaRCs may facilitate the uptake of Ca^{2+} (or Cl^-), which might also be challenged in acid-exposed zebrafish. For example, I recently showed that in acidic water, the effective uptake of Ca^{2+} via epithelial Ca^{2+} channel (ECaC) is inhibited, to which fish respond by increasing the mRNA expression of ECaC and increasing the numbers of ECaC-expressing ionocytes (Y. Kumai, R.W.M. Kwong and S.F. Perry, unpublished results). The observed proliferation of NaRCs in acidic water highlights the fact that, while the present dissertation emphasized the regulation of Na^+ in acidic water, homeostasis for other ions (Cl^-, K^+ and Ca^{2+}) must be maintained, and the underlying regulatory mechanisms deserve further attention.

In zebrafish, at least two hormones, cortisol (Cruz et al., 2013) and isotocin (Chou et al., 2011) can induce the proliferation of ionocytes by modulating transcription factors responsible for ionocyte differentiation (Esaki et al., 2009; Hsiao et al., 2007; Jänicke et al., 2007). Because I demonstrated that acid-exposure causes an increase in whole body cortisol levels in zebrafish larvae (Chapter 4), it is likely that cortisol is at least partially responsible for the observed proliferation of ionocytes in acidic water. The potential role of isotocin in ionocyte proliferation in acidic water remains to be directly demonstrated. If isotocin is indeed involved, it will be an interesting challenge to determine whether these two hormones act independently of each other, or synergistically.

In Chapter 3, I determined that NHE3b plays a major role in the stimulation of Na^+ uptake in chronically acid-exposed zebrafish larvae. Furthermore, by combining pharmacological inhibition of NHE3b and knockdown of Rhcg1, I provided the first direct experimental evidence that conductance of ammonia via Rhcg1 enables the uptake of Na^+ via

NHE3 despite a "thermodynamic paradox" (Avella and Bornancin, 1989; Parks et al., 2008). Based on an *in situ* ligation proximity assay, (Ito et al., 2013) demonstrated that not only are NHE3b and Rhcg1 distributed within the same ionocytes (HRCs), they are also spatially near to each other. This observation further supports the proposed functional metabolon between Rhcg1 and NHE3b as discussed by (Wright and Wood, 2009; Wright and Wood, 2012). Although data reported in Chapter 3 significantly contributed to the long debate surrounding the functionality of NHE3 in FW fish, other aspects of the metabolon model (Wright and Wood, 2009; Wright and Wood, 2012) need further clarification, most notably the role of cytosolic carbonic anhydrase (CAc), which is also expressed in close proximity to NHE3b (Ito et al., 2013). While one might predict that CAc is generating H^+ to be secreted (and generate an acidic boundary layer), the knockdown of *CA2 like a*, an isoform expressed on HRC, actually stimulated the uptake of Na^+ and was accompanied by an increase in NHE3b mRNA expression (Lin et al., 2008). While (Lin et al., 2008) argued that reduced overall acid-secretion in *CA2 like a* morphants creates a thermodynamic gradient favourable for the uptake of Na^+ via NHE3b, such a scenario would also make the acid-trapping mechanism for ammonia secretion less effective and thus constrain the NHE3b-Rhcg1 metabolon model. Overall, the two recent studies on zebrafish (Kumai and Perry, 2011; Shih et al., 2012), that reported inhibition of Na^+ uptake by NHE3b knockdown support the view that NHE3b does indeed absorb Na^+ in FW. However, the full molecular details behind the functionality of NHE3 in FW fish as proposed by (Wright and Wood, 2009) require further study. It is likely that instrumentation which would permit the monitoring of changes in the concentrations of ions (Na^+, H^+, NH_4^+) in both the intracellular fluid and in the boundary layer water in the vicinity of the apical membrane of HRCs would be required to definitively answer some of the outstanding questions surrounding the functionality of NHE3.

Concluding remarks

By experimentally addressing the long-lasting debate on the functionality of NHE3, as well as investigating previously poorly explored physiological systems (ANG-II, cAMP), this thesis significantly contributes to the long history of research on osmoregulation by FW fish. Data from this thesis demonstrate that zebrafish exploit a unique osmoregulatory strategy when exposed to acidic water, and that multiple endocrine systems are involved in promoting the integrated responses. Two major challenges for future research are to identify the molecular mechanisms of osmo/acid-base sensing in FW fish, and to use a Systems Biology approach, including microarray and protein-chip, to determine how those multiple mechanisms are coordinated both at the cellular and systemic levels. Data from this thesis will provide a useful framework to initiate such an exciting new research direction.

Figure 8.1. Proposed model of Na+ uptake regulation during acute acid stress. Na+ uptake is primarily regulated by angiotensin-II (via AT1) and catecholamine (via β-adrenergic receptor) and likely mediated by activation of NHE (in association with Rhcg1) and NCC expressed on HRC and NCCC respectively. Solid lines indicates effects experimentally demonstrated in this thesis or previously, and dashed lines indicate proposed effects. Abbreviations: HRC: H+-ATPase rich cell; NCCC: Na+-Cl- co-transporter expressing cell; NHE: Na+-H+ exchanger; Rhcg1: rhesus c1 glycoprotein; PKA: protein kinase A; Epac: Exchange proteins directly activated by cAMP; AT1: type I angiotensin-II receptor, β-AR: beta-adrenergic receptor.

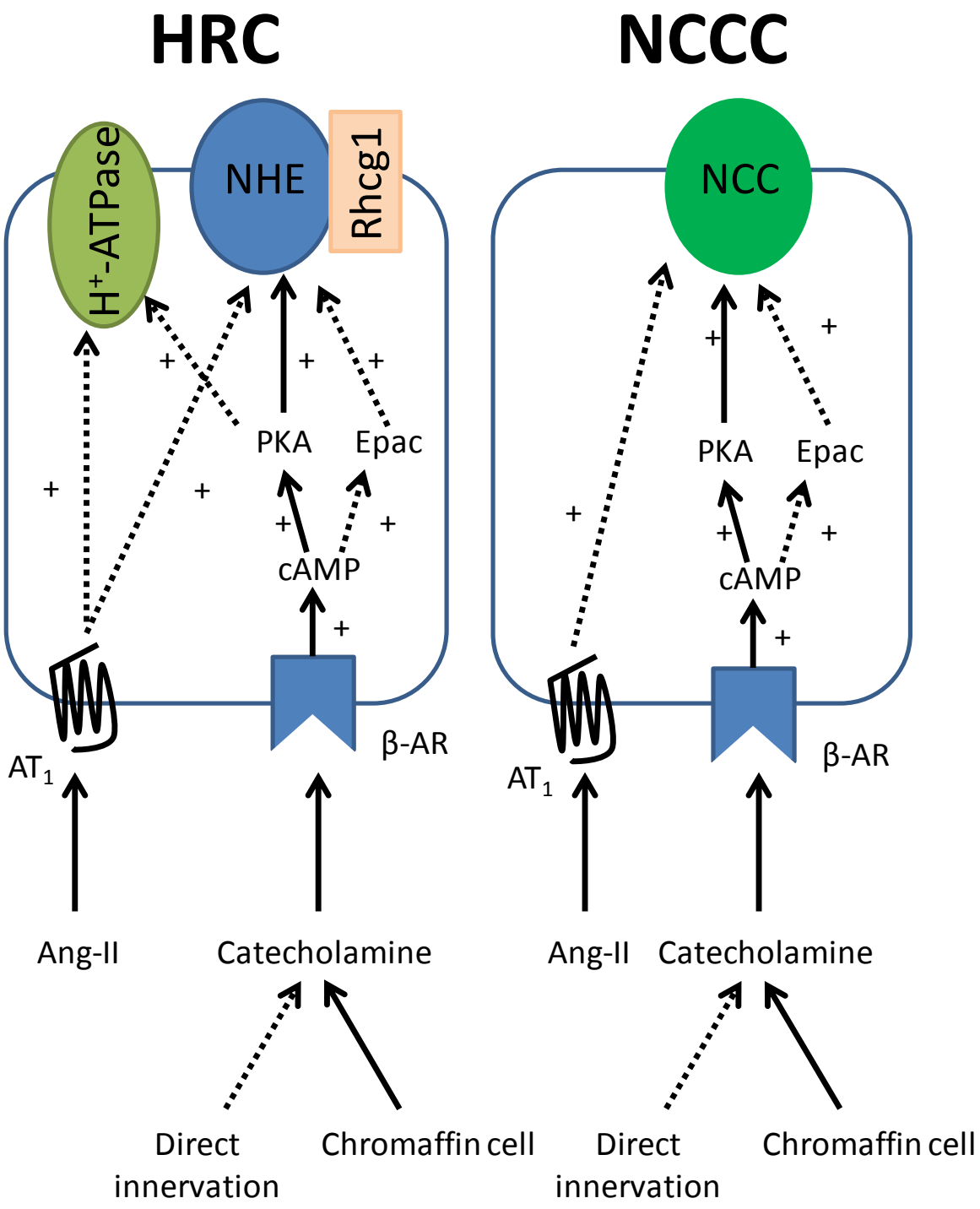
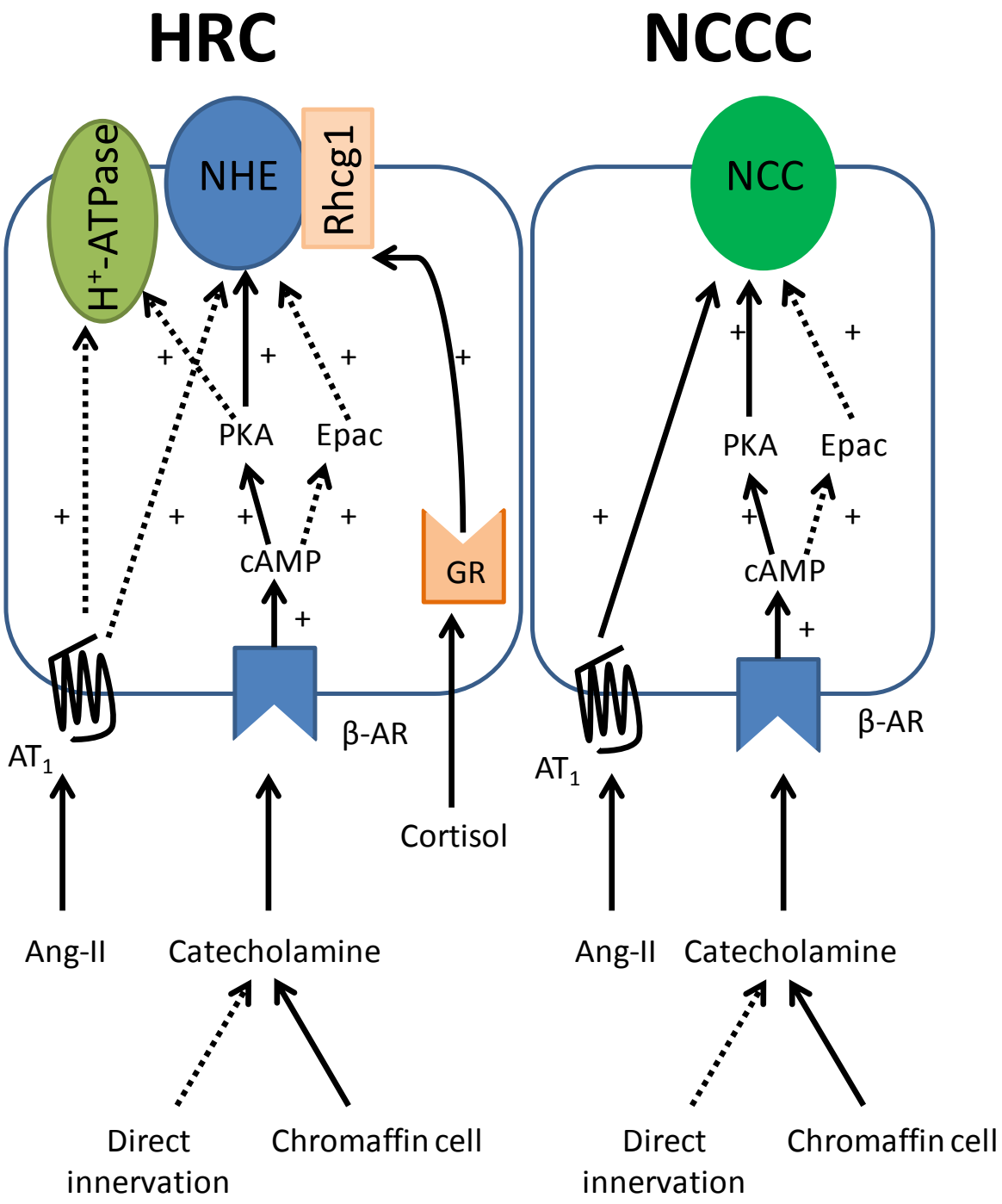

HRC
NCCC
H+-ATPase
NHE
Rhcg1
NCC
PKA
Epac
cAMP
β-AR
AT1
Ang-II
Catecholamine
Direct innervation
Chromaffin cell

Figure 8.2. Proposed model of Na^+ uptake regulation during chronic acid stress. Na^+ uptake is regulated by angiotensin-II (via AT_1), catecholamine (via β-adrenergic receptor) and cortisol (via GR) and likely mediated by activation of NHE (in association with Rhcg1). Solid lines indicate effects experimentally demonstrated in this thesis or previously, and dashed lines indicate proposed effects. Abbreviations: HRC: H^+-ATPase rich cell; NCCC: Na^+-Cl^- co-transporter expressing cell; NHE: Na^+-H^+ exchanger; Rhcg1: rhesus c1 glycoprotein; PKA: protein kinase A; Epac: Exchange proteins directly activated by cAMP; AT_1: type I angiotensin-II receptor, β-AR: beta-adrenergic receptor; GR: glucocorticoid receptor.


HRC
NCCC
H+-ATPase
NHE
Rhcg1
NCC
PKA
Epac
cAMP
GR
β-AR
AT1
Cortisol
Ang-II
Catecholamine
Direct innervation
Chromaffin cell

References

Abbas, L., Hajihashemi, S., Stead, L. F., Cooper, G. J., Ware, T. L., Munsey, T. S., Whitfield, T. T. and White, S. J. (2011). Functional and developmental expression of a zebrafish Kir1.1 (ROMK) potassium channel homologue Kcnj1. *The Journal of Physiology* **589**, 1489-1503.

Al-Awqati, Q. (2008). 2007 Homer W. Smith Award: Control of terminal differentiation in epithelia. *J Amer Soc Nephrol* **19**, 443-449.

Al-Awqati, Q. (2011). Terminal differentiation in epithelia: the role of integrins in hensin polymerization. *Annu Rev Physiol* **73**, 401-412.

Al-Awqati, Q. and Gao, X. B. (2011). Differentiation of intercalated cells in the kidney. *Physiology* **26**, 266-272.\

Alderman, S. L. and Bernier, N. J. (2009). Ontogeny of the corticotropin-releasing factor system in zebrafish. *Gen Comp Endocrinol* **164**, 61-69.

Alexander, R. T. and Grinstein, S. (2009). Tethering, recycling and activation of the epithelial sodium proton exchanger, NHE3. *J Exp Biol* **212**, 1630-1637.

Alexandre, M. D., Jeansonne, B. G., Renegar, R. H., Tatum, R. and Chen, Y.-H. (2007). The first extracellular domain of claudin-7 affects paracellular Cl^- permeability. *Bioche Biophys Res Comm* **357**, 87-91.

Alsop, D. and Vijayan, M. M. (2008). Development of the corticosteroid stress axis and receptor expression in zebrafish. *Amer J Physiol - Regul, Integr Comp Physiol* **294**, R711-R719.

Amasheh, S., Meiri, N., Gitter, A. H., Schoeberg, T., Mankertz, J., Schulzke, J. D. and Fromm, M. (2002). Claudin-2 expression induces cation-selective channels in tight junctions of epithelial cells. *J Cell Sci* **115**, 4969-4976.

Amasheh, S., Milatz, S., Krug, S. M., Bergs, M., Amasheh, M., Schulzke, J.-D. and Fromm, M. (2009). Na^+ absorption defends from paracellular back-leakage by claudin-8 upregulation. *Biochem Biophys Res Comm* **378**, 45-50.

Ambuhl, P. M., Yang, X., Peng, Y., Preisig, P. A., Moe, O. W. and Alpern, R. J. (1999). Glucocorticoids enhance acid activation of the Na^+/H exchanger 3 (NHE3). *J Clin Invest* **103**, 429-435.

Andersen, D. E., Reid, S. D., Moon, T. W. and Perry, S. F. (1991). Metabolic effects associated with chronically elevated cortisol in rainbow trout (*Oncorhynchus mykiss*). *Can. J. Fish. Aquat. Sci.* **48**, 1811-1817.

Anderson, W. G., Pillans, R. D., Hyodo, S., Tsukada, T., Good, J. P., Takei, Y., Franklin, C. E. and Hazon, N. (2006). The effects of freshwater to seawater transfer on circulating levels of angiotensin II, C-type natriuretic peptide and arginine vasotocin in the euryhaline elasmobranch, *Carcharhinus leucas*. *Gen Comp Endocrinol* **147**, 39-46.

Avella, M. and Bornancin, M. (1989). A new analysis of ammonia and sodium transport through the gills of the freshwater rainbow trout (*Salmo gairdneri*). *J Exp Biol* **142**, 155-175.

Azroyan, A., Morla, L., Crambert, G., Laghmani, K., Ramakrishnan, S., Edwards, A. and Doucet, A. (2012). Regulation of pendrin by cAMP: possible involvement in β adrenergic-dependent NaCl retention. *Amer J Physiol - Renal Physiol* **302**, 1180-1187.

Bagherie-Lachidan, M., Wright, S. and Kelly, S. (2009). Claudin-8 and -27 tight junction proteins in puffer fish *Tetraodon nigroviridis* acclimated to freshwater and seawater. *J Comp Physiol B* **179**, 419-431.

Bagherie-Lachidan, M., Wright, S. I. and Kelly, S. P. (2008). Claudin-3 tight junction proteins in *Tetraodon nigroviridis*: cloning, tissue-specific expression, and a role in hydromineral balance. *Amer J Physiol - Regul, Integr Comp Physiol* **294**, R1638-R1647.

Bagnat, M., Cheung, I. D., Mostov, K. E. and Stainier, D. Y. R. (2007). Genetic control of single lumen formation in the zebrafish gut. *Nature Cell Biol* **9**, 954-960.

Baum, M., Moe, O. W., Gentry, D. L. and Alpern, R. J. (1994). Effect of glucocorticoids on renal cortical NHE-3 and NHE-1 mRNA. *Am J Physiol* **267**, F437-42.

Bayaa, M., Vulesevic, B., Esbaugh, A., Braun, M., Ekker, M. E., Grosell, M. and Perry, S. F. (2009). The involvement of SLC26 anion transporters in chloride uptake in zebrafish (*Danio rerio*) larvae. *J Exp Biol* **212**, 3283-3295.

Beavo, J. A. and Brunton, L. L. (2002). Cyclic nucleotide research- still expanding after half a century. *Nat Rev Mol Cell Biol* **3**, 710-718.

Ben-Yosef, T., Belyantseva, I. A., Saunders, T. L., Hughes, E. D., Kawamoto, K., Van Itallie, C. M., Beyer, L. A., Halsey, K., Gardner, D. J., Wilcox, E. R. et al. (2003). Claudin 14 knockout mice, a model for autosomal recessive deafness DFNB29, are deaf due to cochlear hair cell degeneration. *Human Mol Genet* **12**, 2049-2061.

Bernier, N., Kaiya, H., Takei, Y. and Perry, S. (1999a). Mediation of humoral catecholamine secretion by the renin-angiotensin system in hypotensive rainbow trout (*Oncorhynchus mykiss*). *J Endocrinol* **160**, 351-363.

Bernier, N. J., Mckendry, J. E. and Perry, S. F. (1999b). Blood pressure regulation during hypotension in two teleost species: differential involvement of the renin-angiotensin and adrenergic systems. *J Exp Biol* **202**, 1677-1690.

Bernier, N. J. and Perry, S. F. (1997). Angiotensins stimulate catecholamine release from the chromaffin tissue of the rainbow trout. *Amer J Physiol - Regul Integr Comp Physiol* **273**, R49-R57.

Bill, B. R., Petzold, A. M., Clark, K. J., Schimmenti, L. A. and Ekker, S. C. (2009). A primer for morpholino use in zebrafish. *Zebrafish* **6**, 69-77.

Bindon, S., Gilmour, K., Fenwick, J. and Perry, S. (1994a). The effects of branchial chloride cell proliferation on respiratory function in the rainbow trout, *Onchorhynchus mykiss*. *J Exp Biol* **197**, 47-63.

Bindon, S. D., Fenwick, J. C. and Perry, S. F. (1994b). Branchial chloride cell proliferation in the rainbow trout, *Oncorhynchus mykiss*: implications for gas transfer. *Can J Zool* **72**, 1395-1402.

Bobulescu, I. A., Dwarakanath, V., Zou, L., Zhang, J., Baum, M. and Moe, O. W. (2005). Glucocorticoids acutely increase cell surface Na^+/H^+ exchanger-3 (NHE3) by activation of NHE3 exocytosis. *Amer J Physiol - Renal Physiol* **289**, F685-F691.

Boisen, A. M. Z., Amstrup, J., Novak, I. and Grosell, M. (2003). Sodium and chloride transport in soft water and hard water acclimated zebrafish (*Danio rerio*). *Biochimica et Biophysica Acta (BBA) - Biomembranes* **1618**, 207-218.

Borner, S., Schwede, F., Schlipp, A., Berisha, F., Calebiro, D., Lohse, M. J. and Nikolaev, V. O. (2011). FRET measurements of intracellular cAMP concentrations and cAMP analog permeability in intact cells. *Nature Protocols* **6**, 427-438.

Boron, W. F. (2006). Acid-base transport by the renal proximal tubule. *J Amer Soc Nephrol* **17**, 2368-2382.

Braun, M. H. and Perry, S. F. (2010). Ammonia and urea excretion in the Pacific hagfish *Eptatretus stoutii*: Evidence for the involvement of Rh and UT proteins. *Comp Biochem Physiol - A* **157**, 405-415.

Braun, M. H., Steele, S. L., Ekker, M. and Perry, S. F. (2009a). Nitrogen excretion in developing zebrafish (*Danio rerio*): a role for Rh proteins and urea transporters. *Amer J Physiol - Renal Physiol* **296**, F994-F1005.

Braun, M. H., Steele, S. L. and Perry, S. F. (2009b). The responses of zebrafish (*Danio rerio*) to high external ammonia and urea transporter inhibition: nitrogen excretion and expression of rhesus glycoproteins and urea transporter proteins. *J Exp Biol* **212**, 3846-3856.

Breves, J. P., Serizier, S. B., Goffin, V., McCormick, S. D. and Karlstrom, R. O. (2013). Prolactin regulates transcription of the ion uptake Na^+/Cl^- cotransporter (ncc) gene in zebrafish gill. *Mol Cell Endocrinol* **369**, 98-106.

Breves, J. P., Watanabe, S., Kaneko, T., Hirano, T. and Grau, E. G. (2010). Prolactin restores branchial mitochondrion-rich cells expressing Na^+/Cl^- cotransporter in hypophysectomized Mozambique tilapia. *Amer J Physiol - Regul Integr Comp Physiol* **299**, R702-R710.

Brown, D., Bouley, R., Paunescu, T. G., Breton, S. and Lu, H. A. J. (2012). New insights into the dynamic regulation of water and acid/base balance by renal epithelial cells. *Amer J Physiol - Cell Physiol* **302**, 1421-1433.

Brown, D. and Wagner, C. A. (2012). Molecular mechanisms of acid-base sensing by the kidney. *J Amer Soc Nephrol* **23**, 774-780.

Buck, J., Sinclair, M. L., Schapal, L., Cann, M. J. and Levin, L. R. (1999). Cytosolic adenylyl cyclase defines a unique signaling molecule in mammals. *Proc Nat Acad Sci USA* **96**, 79-84.

Bucking, C., Edwards, S., Tickle, P., Smith, C., McDonald, M. D. and Walsh, P. (2013). Immunohistochemical localization of urea and ammonia transporters in two confamilial fish species, the ureotelic gulf toadfish (Opsanus beta) and the ammoniotelic plainfin midshipman (Porichthys notatus). *Cell Tissue Res*, 1-15.

Bui, P., Bagherie-Lachidan, M. and Kelly, S. P. (2010). Cortisol differentially alters claudin isoforms in cultured puffer fish gill epithelia. *Mol Cell Endocrinol* **317**, 120-126.

Bury, N. R. and Sturm, A. (2007). Evolution of the corticosteroid receptor signalling pathway in fish. *Gen Comp Endocrinol* **153**, 47-56.

Cano, A., Miller, R. T., Alpern, R. J. and Preisig, P. A. (1994). Angiotensin II stimulation of Na-H antiporter activity is cAMP independent in OKP cells. *Amer J Physiol - Cell Physiol* **266**, C1603-C1608.

Castaneda-Bueno, M., Cervantes-Perez, L. G., Vazquez, N., Uribe, N., Kantesaria, S., Morla, L., Bobadilla, N. A., Doucet, A., Alessi, D. R. and Gamba, G. (2012). Activation of the renal Na^+:Cl^- cotransporter by angiotensin II is a WNK4-dependent process. *Proc Natl Acad Sci U S A* **109**, 7929-34.

Castaneda-Bueno, M. and Gamba, G. (2012). Mechanisms of sodium-chloride cotransporter modulation by angiotensin II. *Curr Opin Nephrol Hypertens* **21**, 516-22.

Chang, W.-J., Horng, J.-L., Yan, J.-J., Hsiao, C.-D. and Hwang, P.-P. (2009). The transcription factor, glial cell missing 2, is involved in differentiation and functional regulation of H^+-ATPase-rich cells in zebrafish (*Danio rerio*). *Amer J Physiol - Regul, Integr Comp Physiol* **296**, R1192-R1201.

Chasiotis, H., Effendi, J. and Kelly, S. (2009). Occludin expression in goldfish held in ion-poor water. *J Comp Physiol B* **179**, 145-154.

Chasiotis, H., Kolosov, D. and Kelly, S. P. (2012). Permeability properties of the teleost gill epithelium under ion-poor conditions. *Amer J Physiol - Regul, Integr Comp Physiol* **302**, R727-R739.
Chasiotis, H., Wood, C. M. and Kelly, S. P. (2010). Cortisol reduces paracellular permeability and increases occludin abundance in cultured trout gill epithelia. *Mol Cell Endocrinol* **323**, 232-238.

Chen, Y., Cann, M. J., Litvin, T. N., Iourgenko, V., Sinclair, M. L., Levin, L. R. and Buck, J. (2000). Soluble adenylyl cyclase as an evolutionarily conserved bicarbonate sensor. *Science* **289**, 625-628.

Cheng, X., Ji, Z., Tsalkova, T. and Mei, F. (2008). Epac and PKA: a tale of two intracellular cAMP receptors. *Acta Biochim Biophys Sinica* **40**, 651-662.

Chiga, M., Rafiqi, F. H., Alessi, D. R., Sohara, E., Ohta, A., Rai, T., Sasaki, S. and Uchida, S. (2011). Phenotypes of pseudohypoaldosteronism type II caused by the WNK4 D561A missense mutation are dependent on the WNK-OSR1/SPAK kinase cascade. *J Cell Sci* **124**, 1391-1395.

Chiga, M., Rai, T., Yang, S.-S., Ohta, A., Takizawa, T., Sasaki, S. and Uchida, S. (2008). Dietary salt regulates the phosphorylation of OSR1//SPAK kinases and the sodium chloride cotransporter through aldosterone. *Kidney Int* **74**, 1403-1409.

Chou, M.-Y., Hsiao, C.-D., Chen, S.-C., Chen, I. W., Liu, S.-T. and Hwang, P.-P. (2008). Effects of hypothermia on gene expression in zebrafish gills: upregulation in differentiation and function of ionocytes as compensatory responses. *J Exp Biol* **211**, 3077-3084.

Chou, M.-Y., Hung, J.-C., Wu, L.-C., Hwang, S.-P. and Hwang, P.-P. (2011). Isotocin controls ion regulation through regulating ionocyte progenitor differentiation and proliferation. *Cell Mol Life Sci* **68**, 2797-2809.

Chow, C.-W., Khurana, S., Woodside, M., Grinstein, S. and Orlowski, J. (1999). The epithelial Na^+/H^+ Exchanger, NHE3, is internalized through a clathrin-mediated pathway. *J Biol Chem* **274**, 37551-37558.

Clelland, E. S., Bui, P., Bagherie-Lachidan, M. and Kelly, S. P. (2010). Spatial and salinity-induced alterations in claudin-3 isoform mRNA along the gastrointestinal tract of the pufferfish *Tetraodon nigroviridis*. *Comp Biochem Physiol - A* **155**, 154-163.

Clelland, E. S. and Kelly, S. P. (2010). Tight junction proteins in zebrafish ovarian follicles: Stage specific mRNA abundance and response to 17β-estradiol, human chorionic gonadotropin, and maturation inducing hormone. *Gen Comp Endocrinol* **168**, 388-400.

Coccimiglio, M. L. and Jonz, M. G. (2012). Serotonergic neuroepithelial cells of the skin in developing zebrafish: morphology, innervation and oxygen-sensitive properties. *J Exp Biol* **215**, 3881-3894.

Colombe, L., Fostier, A., Bury, N., Pakdel, F. and Guiguen, Y. (2000). A mineralocorticoid-like receptor in the rainbow trout, *Oncorhynchus mykiss*: cloning and characterization of its steroid binding domain. *Steroids* **65**, 319-328.

Colombo, L., Bern, H. A., Pieprzyk, J. and Johnson, D. W. (1972). Corticosteroidogenesis in vitro by the head kidney of *Tilapia mossambica* (Cichlidae, Teleostei). *Endocrinology* **91**, 450-462.

Craig, P. M., Wood, C. M. and McClelland, G. B. (2007). Gill membrane remodeling with soft-water acclimation in zebrafish (*Danio rerio*). *Physiol Genom* **30**, 53-60.

Crowley, S. D. and Coffman, T. M. (2012). Recent advances involving the renin-angiotensin system. *Exper Cell Res* **318**, 1049-1056.

Cruz, S. A., Chao, P.-L. and Hwang, P.-P. (2013). Cortisol promotes differentiation of epidermal ionocytes through Foxi3 transcription factors in zebrafish (*Danio rerio*). *Comp Biochem Physiol A* **164**, 249-257.

Curtis, B. J. and Wood, C. M. (1991). The function of the urinary bladder in vivo in the freshwater rainbow trout. *J Exp Biol* **155**, 567-583.

Devic, E., Xiang, Y., Gould, D. and Kobilka, B. (2001). Beta-adrenergic receptor subtype-specific signaling in cardiac myocytes from beta 1 and beta 2 adrenoceptor knockout mice. *Mol Pharmacol* **60**, 577-583.

Deyev, I., Sohet, F., Vassilenko, K., Serova, O., Popova, N., Zozulya, S., Burova, E., Houillier, P., Rzhevsky, D., Berchatova, A. et al. (2011). Insulin Receptor-Related Receptor as an extracellular alkali sensor. *Cell Metabolism* **13**, 679-689.

DiBona, G. F. (2000). Nervous kidney: Interaction between renal sympathetic nerves and the renin-angiotensin system in the control of renal function. *Hypertension* **36**, 1083-1088.

DiBona, G. F. (2005). Physiology in perspective: The Wisdom of the Body. Neural control of the kidney. *Amer J Physiol - Regul Integr Comp Physiol* **289**, R633-R641.

Dickmeis, T., Lahiri, K., Nica, G., Vallone, D., Santoriello, C., Neumann, C. J., Hammerschmidt, M. and Foulkes, N. S. (2007). Glucocorticoids play a key role in circadian cell cycle rhythms. *PLoS biology* **5**.

Dindia, L., Murray, J., Faught, E., Davis, T. L., Leonenko, Z. and Vijayan, M. M. (2012). Novel nongenomic signaling by glucocorticoid may involve changes to liver membrane order in rainbow trout. *PLoS ONE* **7**, e46859.

Donald, J. A. (1989). Adrenaline and branchial nerve stimulation inhibit 45Ca influx into the gills of rainbow trout, *Salmo gairdneri*. *J Exp Biol* **141**, 441-445.
Dooley, R., Harvey, B. J. and Thomas, W. (2012). Non-genomic actions of aldosterone: From receptors and signals to membrane targets. *Mol Cell Endocrinol* **350**, 223-234.

Du Cheyron, D., Chalumeau, C., Defontaine, N., Klein, C., Kellermann, O., Paillard, M. and Poggioli, J. (2003). Angiotensin II stimulates NHE3 activity by exocytic insertion of the transporter: Role of PI 3-kinase. *Kidney Int* **64**, 939-949.

Dymowska, A. K., Hwang, P.-P. and Goss, G. G. (2012). Structure and function of ionocytes in the freshwater fish gill. *Resp Physiol Neurobiol* **184**, 282-292.

Eddy, F. B. (1975). The effect of calcium on gill potentials and on sodium and chloride fluxes in the goldfish, *Carassius auratus*. *J Comp Physiol B* **96**, 131-142.

Edwards, S. L., Wall, B. P., Morrison-Shetlar, A., Sligh, S., Weakley, J. C. and Claiborne, J. B. (2005). The effect of environmental hypercapnia and salinity on the expression of NHE-like isoforms in the gills of a euryhaline fish (*Fundulus heteroclitus*). *J Exp Zool A* **303**, 464-475.

Eisen, J. S. and Smith, J. C. (2008). Controlling morpholino experiments: don't stop making antisense. *Development* **135**, 1735-1743.

Ekker, M. and Akimenko, M.-A. (2010). Genetic tools. *Zebrafish* In *Fish Physiology*, vol 29 eds. S. F. Perry M. Ekker A. P. Farrell and C. J. Brauner), pp. 1-23. San Diego: Academic Press.

Eladari, D., Chambrey, R. g. and Peti-Peterdi, J. (2011). A new look at electrolyte transport in the distal tubule. *Annu Rev Physiol* **74**, 325-349.

Engeszer, R. E., Patterson, L. B., Rao, A. A. and Parichy, D. M. (2007). Zebrafish in the wild: a review of natural history and new notes from the field. *Zebrafish* **4**, 21-40.

Esaki, M., Hoshijima, K., Kobayashi, S., Fukuda, H., Kawakami, K. and Hirose, S. (2007). Visualization in zebrafish larvae of Na^+ uptake in mitochondria-rich cells whose differentiation is dependent on foxi3a. *Amer J Physiol - Regul Integr Comp Physiol* **292**, R470-R480.

Esaki, M., Hoshijima, K., Nakamura, N., Munakata, K., Tanaka, M., Ookata, K., Asakawa, K., Kawakami, K., Wang, W., Weinberg, E. S and Hirose, S. (2009). Mechanism of development of ionocytes rich in vacuolar-type H^+-ATPase in the skin of zebrafish larvae. *Dev Biol* **329**, 116-129.

Evans, D. H. (2008). Teleost fish osmoregulation: what have we learned since August Krogh, Homer Smith, and Ancel Keys. *Amer J Physiol - Regul Integr Comp Physiol* **295**, R704-R713.

Evans, D. H. (2011). Freshwater fish gill ion transport: August Krogh to morpholinos and microprobes. *Acta Physioloca*. **202**, 349-359.

Evans, D. H., Piermarini, P. M. and Choe, K. P. (2005). The multifunctional fish gill: Dominant site of gas exchange, osmoregulation, acid-base regulation, and excretion of nitrogenous waste. *Physiol Rev* **85**, 97-177.

Falkenstein, E., Tillmann, H.-C., Christ, M., Feuring, M. and Wehling, M. (2000). Multiple actions of steroid hormones—a focus on rapid, nongenomic effects. *Pharmacol Rev* **52**, 513-556.

Fenwick, J. C., Wendelaar Bonga, S. E. and Flik, G. (1999). In vivo bafilomycin-sensitive Na^+ uptake in young freshwater fish. *J Exp Biol* **202**, 3659-3666.

Flik, G. and Perry, S. F. (1989). Cortisol stimulates whole body calcium uptake and the branchial calcium pump in freshwater rainbow trout. *J Endocrinol* **120**, 75-82.

Flynt, A. S., Thatcher, E. J., Burkewitz, K., Li, N., Liu, Y. and Patton, J. G. (2009). miR-8 microRNAs regulate the response to osmotic stress in zebrafish embryos. *J Cell Biol* **185**, 115-127.

Freda, J. and McDonald, D. G. (1988). Physiological correlates of interspecific variation in acid tolerance in fish. *J. Exp. Biol.* **136**, 243-258.

Freda, J., Sanchez, D. A. and Bergman, H. L. (1991). Shortening of branchial tight junction acid-exposed rainbow trout (*Oncorhynchus mykiss*). *Canad J Fish Aquat Sci* **48**, 2028-2033.

Furuse, M., Furuse, K., Sasaki, H. and Tsukita, S. (2001). Conversion of zonulae occludentes from tight to leaky strand type by introducing claudin-2 into Madin-Darby canine kidney I cells. *J Cell Biol* **153**, 263-272.

Furuse, M., Sasaki, H. and Tsukita, S. (1999). Manner of interaction of heterogeneous claudin species within and between tight junction strands. *J Cell Biol* **147**, 891-903

Gagnon, K. B. E., England, R. and Delpire, E. (2006). Volume sensitivity of cation-Cl^- cotransporters is modulated by the interaction of two kinases: Ste20-related proline-alanine-rich kinase and WNK4. *Amer J Physiol - Cell Physiol* **290**, C134-C142.

Galvez, F., Reid, S. D., Hawkings, G. and Goss, G. G. (2002). Isolation and characterization of mitochondria-rich cell types from the gill of freshwater rainbow trout. *Amer J Physiol - Regul Integr Comp Physiol* **282**, R658-R668.

Gao, X., Eladari, D., Leviel, F., Tew, B. Y., Miro-Julia, C., Cheema, F., Miller, L., Nelson, R., Paunescu, T. G., McKee, M. et al. (2010). Deletion of hensin/DMBT1 blocks conversion of β- to α-intercalated cells and induces distal renal tubular acidosis. *Proc Nat Acad Sci USA* **107**, 21872-21877.

Garcia-Romeu, F. and Maetz, J. (1964). The mechanism of sodium and chloride uptake by the gills of a fresh-water fish, *Carassius auratus*. *J Gen Physiol* **47**, 1195-1207.

Garg, L. C. and Narang, N. (1988). Effects of aldosterone on NEM-sensitive ATPase in rabbit nephron segments. *Kidney Int* **34**, 13-17.

Geibel, J., Giebisch, G. and Boron, W. F. (1990). Angiotensin II stimulates both Na^+-H^+ exchange and Na^+/HCO_3^- cotransport in the rabbit proximal tubule. *Proc Nat Acad Sci USA* **87**, 7917-7920.

Gilmour, K., Collier, C., Dey, C. and Perry, S. (2011). Roles of cortisol and carbonic anhydrase in acid–base compensation in rainbow trout, *Oncorhynchus mykiss*. *J Comp Physiol B* **181**, 501-515.

Girard, J. P. and Payan, P. (1977). Kinetic analysis of sodium and chloride influxes across the gills of the trout in fresh water. *J Physiol* **273**, 195-209.

Gloerich, M. and Bos, J. L. (2010). Epac: Defining a new mechanism for cAMP action. *Ann Rev Pharmacol Toxicol* **50**, 355-375.

Gonzalez-Mariscal, L., Contreras, R. G., Bolivar, J. J., Ponce, A., Chavez De Ramirez, B. and Cereijido, M. (1990). Role of calcium in tight junction formation between epithelial cells. *Amer J Physiol - Cell Physiol* **259**, C978-C986.

Gonzalez, R. J. and Dunson, W. A. (1987). Adaptations of sodium balance to low pH in a sunfish (*Enneacanthus obesus*) from naturally acidic waters. *J Comp Physiol B* **157**, 555-566.

Gonzalez, R. J. and Dunson, W. A. (1989). Acclimation of sodium regulation to low pH and the role of calcium in the acid-tolerant sunfish *Enneacanthus obesus*. *Physiol Zool* **62**, 977-992.

Gonzalez, R. J. and Preest, M. R. (1999). Ionoregulatory specializations for exceptional tolerance of ion-poor, acidic waters in the neon tetra (*Paracheirodon innesi*). *Physiol Biochem Zool* **72**, 156-163.

Gonzalez, R. J. and Wilson, R. W. (2001). Patterns of ion regulation in acidophilic fish native to the ion-poor, acidic Rio Negro. *Journal of Fish Biology* **58**, 1680-1690.

Gonzalez, R. J., Wilson, R. W. and Wood, C. M. (2005). Ionoregulation in tropical fishes from ion poor, acidic blackwaters. In *Fish Physiology*, vol. Volume 21 eds. A. Val, L. V. M. De Almeida-Val and D. Randall, J.), pp. 397-442: Academic Press.

Gonzalez, R. J., Wilson, R. W., Wood, C. M., Patrick, M. L. and Val, A. L. (2002). Diverse strategies for ion regulation in fish collected from the ion-poor, acidic Rio Negro. *Physiol Biochem Zool* **75**, 37-47.

Goss, G. G., Adamia, S. and Galvez, F. (2001). Peanut lectin binds to a subpopulation of mitochondria-rich cells in the rainbow trout gill epithelium. *Amer J Physiol - Regul Integr Comp Physiol* **281**, R1718-R1725.

Hallows, K. R., Wang, H., Edinger, R. S., Butterworth, M. B., Oyster, N. M., Li, H., Buck, J., Levin, L. R., Johnson, J. P. and Pastor-Soler, N. r. M. (2009). Regulation of epithelial Na^+ transport by soluble adenylyl cyclase in kidney collecting duct cells. *J Biol Chem* **284**, 5774-5783.

Hardison, A. L., Lichten, L., Banerjee-Basu, S., Becker, T. S. and Burgess, S. M. (2005). The zebrafish gene claudin-J is essential for normal ear function and important for the formation of the otoliths. *Mech Dev* **122**, 949-958.

Hayashi, M., Yamada, H., Mitamura, T., Horii, T., Yamamoto, A. and Moriyama, Y. (2000). Vacuolar H^+-ATPase localized in plasma membranes of malaria parasite cells, *Plasmodium falciparum*, is involved in regional acidification of parasitized erythrocytes. *J Biol Chem* **275**, 34353-34358.

He, P., Lee, S.-J., Lin, S., Seidler, U., Lang, F., Fejes-Toth, G., Naray-Fejes-Toth, A. and Yun, C. C. (2011). Serum- and glucocorticoid-induced kinase 3 in recycling endosomes mediates acute activation of Na^+/H^+ exchanger NHE3 by glucocorticoids. *Mol Biol Cell* **22**, 3812-3825.

Hebert, S., Mount, D. and Gamba, G. (2004). Molecular physiology of cation-coupled Cl^- cotransport: the SLC12 family. *Pflugers Archiv Euro J Physiol* **447**, 580-593.

\

Hille, B. (1971). The permeability of the sodium channel to organic cations in myelinated nerve. *J Gen Physiol* **58**, 599-619.

Hirata, T., Kaneko, T., Ono, T., Nakazato, T., Furukawa, N., Hasegawa, S., Wakabayashi, S., Shigekawa, M., Chang, M.-H., Romero, M. F. Hirose, S. (2003). Mechanism of acid adaptation of a fish living in a pH 3.5 lake. *Amer J Physiol - Regul Integr Comp Physiol* **284**, R1199-R1212.

Hiroi, J., Miyazaki, H., Katoh, F., Ohtani-Kaneko, R. and Kaneko, T. (2005). Chloride turnover and ion-transporting activities of yolk-sac preparations (yolk balls) separated from Mozambique tilapia embryos and incubated in freshwater and seawater. *J Exp Biol* **208**, 3851-3858.

Hiroi, J., Yasumasu, S., McCormick, S. D., Hwang, P.-P. and Kaneko, T. (2008). Evidence for an apical Na^+ Cl^- cotransporter involved in ion uptake in a teleost fish. *J Exp Biol* **211**, 2584-2599.

Honegger, K. J., Capuano, P., Winter, C., Bacic, D., Stange, G., Wagner, C. A., Biber, J., Murer, H. and Hernando, N. (2006). Regulation of sodium-proton exchanger isoform 3 (NHE3) by PKA and exchange protein directly activated by cAMP (EPAC). *Proc Nat Acad Sci USA* **103**, 803-808.

Hoorn, E. J. and Ellison, D. H. (2012). WNK kinases and the kidney. *Exp Cell Res* **318**, 1020-1026.

Hoorn, E. J., Nelson, J. H., McCormick, J. A. and Ellison, D. H. (2011). The WNK kinase network regulating sodium, potassium, and blood pressure. *J Amer Soc Nephrol* **22**, 605-614.
Horng, J.-L., Hwang, P.-P., Shih, T.-H., Wen, Z.-H., Lin, C.-S. and Lin, L.-Y. (2009a). Chloride transport in mitochondrion-rich cells of euryhaline tilapia (*Oreochromis mossambicus*) larvae. *Amer J Physiol Cell Physiol* **297**, C845-854.

Horng, J.-L., Lin, L.-Y., Huang, C.-J., Katoh, F., Kaneko, T. and Hwang, P.-P. (2007). Knockdown of V-ATPase subunit A (atp6v1a) impairs acid secretion and ion balance in zebrafish (*Danio rerio*). *Amer J Physiol Regul Integr Comp Physiol* **292**, R2068-2076.

Horng, J.-L., Lin, L.-Y. and Hwang, P.-P. (2009b). Functional regulation of H^+-ATPase-rich cells in zebrafish embryos acclimated to an acidic environment. *Amer J Physiol - Cell Physiol* **296**, C682-C692.

Hoshijima, K. and Hirose, S. (2007). Expression of endocrine genes in zebrafish larvae in response to environmental salinity. *J Endocrinol* **193**, 481-491.

Houillier, P., Chambrey, R., Achard, J. M., Froissart, M., Poggioli, J. and Paillard, M. (1996). Signaling pathways in the biphasic effect of angiotensin II on apical Na//H antiport activity in proximal tubule. *Kidney Int* **50**, 1496-1505.

Hsiao, C.-D., You, M.-S., Guh, Y.-J., Ma, M., Jiang, Y.-J. and Hwang, P.-P. (2007). A positive regulatory loop between *foxi3a* and *foxi3b* Is essential for specification and differentiation of zebrafish epidermal ionocytes. *PLoS ONE* **2**, e302.

Hung, C. Y. C., Tsui, K. N. T., Wilson, J. M., Nawata, C. M., Wood, C. M. and Wright, P. A. (2007). Rhesus glycoprotein gene expression in the mangrove killifish *Kryptolebias marmoratus* exposed to elevated environmental ammonia levels and air. *J Exp Biol* **210**, 2419-2429.

Hwang, P.-P. (2009). Ion uptake and acid secretion in zebrafish (*Danio rerio*). *J Exp Biol* **212**, 1745-1752.

Hwang, P.-P. and Chou, M.-Y. (2013). Zebrafish as an animal model to study ion homeostasis. *Pflügers Archiv - Euro J Physiol*, 1-15.

Hwang, P.-P. and Lee, T.-H. (2007). New insights into fish ion regulation and mitochondrion-rich cells. *Comp Biochem Physiol A* **148**, 479-497.

Hwang, P.-P. and Perry, S. F. (2010). Ionic and acid-base regulation. *Zebrafish*. In *Fish Physiology*, vol. 29 eds. S. F. Perry M. Ekker A. P. Farrell and C. J. Brauner), pp. 311-344: San Diego, Academic Press.

Hwang, P. P., Lee, T. H. and Lin, L. Y. (2011). Ion regulation in fish gills: Recent progress in the cellular and molecular mechanisms. *Amer J Physiol - Regul, Integr Comp Physiol* **301**, R28-R47.

Hwang, P. P. and Wu, S. M. (1993). Role of cortisol in hypoosmoregulation in larvae of the tilapia (*Oreochromis mossambicus*). *Gen Comp Endocrinol* **92**, 318-324.

Hyndman, K. A. and Evans, D. H. (2009). Effects of environmental salinity on gill endothelin receptor expression in the killifish, Fundulus heteroclitus. *Comp Biochem Physiol A* **152**, 58-65.

Inokuchi, M., Hiroi, J., Watanabe, S., Hwang, P.-P. and Kaneko, T. (2009). Morphological and functional classification of ion-absorbing mitochondria-rich cells in the gills of Mozambique tilapia. *J Exp Biol* **212**, 1003-1010.

Inokuchi, M., Hiroi, J., Watanabe, S., Lee, K. M. and Kaneko, T. (2008). Gene expression and morphological localization of NHE3, NCC and NKCC1a in branchial mitochondria-rich cells of Mozambique tilapia (*Oreochromis mossambicus*) acclimated to a wide range of salinities. *Comp Biochem Physiol A* **151**, 151-158.

Isaia, J., Maetz, J. and Haywood, G. P. (1978). Effects of epinephrine on branchial non-electrolyte permeability in rainbow trout. *J Exp Biol* **74**, 227-237.

Ito, Y., Kobayashi, S., Nakamura, N., Esaki, M., Miyagi, H., Hoshijima, K. and Hirose, S. (2013). Close association of carbonic anhydrase (CA2a & CA15a), Na^+/H^+ exchanger (Nhe3b), and ammonia transporter Rhcg1 in zebrafish ionocytes responsible for Na^+ uptake. *Front Physiol* **4**.

Ivanis, G., Braun, M. and Perry, S. F. (2008a). Renal expression and localization of SLC9A3 sodium/hydrogen exchanger and its possible role in acid-base regulation in freshwater rainbow trout (*Oncorhynchus mykiss*). *Amer J Physiol - Regul Integr Comp Physiol* **295**, R971-R978.

Ivanis, G., Esbaugh, A. J. and Perry, S. F. (2008b). Branchial expression and localization of SLC9A2 and SLC9A3 sodium/hydrogen exchangers and their possible role in acid-base regulation in freshwater rainbow trout (*Oncorhynchus mykiss*). *J Exp Biol* **211**, 2467-2477.

Jänicke, M., Carney, T. J. and Hammerschmidt, M. (2007). Foxi3 transcription factors and notch signaling control the formation of skin ionocytes from epidermal precursors of the zebrafish embryo. *Dev Biol* **307**, 258-271.

Jonz, M. G. and Nurse, C. A. (2006). Epithelial mitochondria-rich cells and associated innervation in adult and developing zebrafish. *J Comp Neurol* **497**, 817-832.

Jonz, M. G. and Nurse, C. A. (2008). New developments on gill innervation: Insights from a model vertebrate. *J Exp Biol* **211**, 2371-2378.

Kaneko, T., Hasegawa, S., Uchida, K., Ogasawara, T., Oyagi, A. and Hirano, T. (1999). Acid tolerance of Japanese dace (a Cyprinid teleost) in Lake Osorezan, a remarkable acid lake. *Zool Sci* **16**, 871-877.

Kelly, S. P. and Chasiotis, H. (2011). Glucocorticoid and mineralocorticoid receptors regulate paracellular permeability in a primary cultured gill epithelium. *J Exp Biol* **214**, 2308-2318.

Kerstetter, T. H., Kirschner, L. B. and Rafuse, D. D. (1970). On the mechanisms of sodium ion transport by the irrigated gills of rainbow trout (*Salmo gairdneri*). *J Gen Physiol* **56**, 342-359.

Kiilerich, P., Kristiansen, K. and Madsen, S. S. (2007). Cortisol regulation of ion transporter mRNA in Atlantic salmon gill and the effect of salinity on the signaling pathway. *J Endocrinol* **194**, 417-427.

Kiilerich, P., Pedersen, S. H., Kristiansen, K. and Madsen, S. S. (2011a). Corticosteroid regulation of Na^+,K^+-ATPase alpha1-isoform expression in Atlantic salmon gill during smolt development. *Gen Comp Endocrinol* **170**, 283-289.

Kiilerich, P., Tipsmark, C. K., Borski, R. J. and Madsen, S. S. (2011b). Differential effects of cortisol and 11-deoxycorticosterone on ion transport protein mRNA levels in gills of two euryhaline teleosts, Mozambique tilapia (*Oreochromis mossambicus*) and striped bass (*Morone saxatilis*). *J Endocrinol* **209**, 115-126.

Kim, M. (1996). Measurement of ileal permeability with different-sized lyethylene glycols (PEG 400, 600, and 1000). *Arch Pharma Res* **19**, 100-105.

Kinsella, J., Cujdik, T. and Sacktor, B. (1984). Na^+-H^+ exchange activity in renal brush border membrane vesicles in response to metabolic acidosis: The role of glucocorticoids. *Proc Nat Acad Sci USA* **81**, 630-634.

Krogh, A. (1938). The active absorption of ions in some freshwater animals. *J Comp Physiol A* **25**, 335-350.

Kumai, Y., Bahubeshi, A., Steele, S. and Perry, S. F. (2011). Strategies for maintaining Na^+ balance in zebrafish (*Danio rerio*) during prolonged exposure to acidic water. *Comp Biochem Physiol A* **160**, 52-62.

Kumai, Y., Nesan, D., Vijayan, M. M. and Perry, S. F. (2012a). Cortisol regulates Na^+ uptake in zebrafish, *Danio rerio*, larvae via the glucocorticoid receptor. *Mol Cell Endocrinol* **364**, 113-125.

Kumai, Y. and Perry, S. F. (2011). Ammonia excretion via Rhcg1 facilitates Na^+ uptake in larval zebrafish, *Danio rerio*, in acidic water. *Amer J Physiol - Regul, Integr Comp Physiol* **301**, R1517-R1528.

Kumai, Y. and Perry, S. F. (2012). Mechanisms and regulation of Na^+ uptake by freshwater fish. *Resp Physiol Neurobiol* **184**, 249-256.

Kumai, Y., Ward, M. and Perry, S. F. (2012b). Beta-adrenergic regulation of Na^+ uptake by larval zebrafish, *Danio rerio*, in acidic and ion-poor environments. *Amer J Physiol - Regul Integr Comp Physiol* **303**, 1031-1041.

Kwong, R. W. M. and Perry, S. F. (2013a). Cortisol regulates epithelial permeability and sodium losses in zebrafish exposed to acidic water. *J Endocrinol* **217**, 253-264.

Kwong, R. W. M. and Perry, S. F. (2013b). The tight junction protein claudin-b regulates epithelial permeability and sodium handling in larval zebrafish, *Danio rerio*. *Amer J Physiol - Regul, Integr Comp Physiol.* **304,** R504-513.

Lapner, K. N. and Perry, S. F. (2001). The role of angiotensin II in regulating catecholamine secretion during hypoxia in rainbow trout *Oncorhynchus mykiss*. *J Exp Biol* **204**, 4169-4176.

Laurent, P. and Perry, S. F. (1990). Effects of cortisol on gill chloride cell morphology and ionic uptake in the freshwater trout, *Salmo gairdneri*. *Cell Tiss Res* **259**, 429-442.

Lee, Y.-C., Yan, J.-J., Cruz, S. A., Horng, J.-L. and Hwang, P.-P. (2011). Anion exchanger 1b, but not sodium-bicarbonate cotransporter 1b, plays a role in transport functions of zebrafish H^+-ATPase-rich cells. *Amer J Physiol - Cell Physiol* **300**, C295-C307.

Li, S., Sato, S., Yang, X., Preisig, P. A. and Alpern, R. J. (2004). Pyk2 activation is integral to acid stimulation of sodium/hydrogen exchanger 3. *J Clin Invest* **114**, 1782-1789.

Li, X. C., Hopfer, U. and Zhuo, J. L. (2012). Novel signaling mechanisms of intracellular angiotensin II-induced NHE3 expression and activation in mouse proximal tubule cells. *Amer J Physiol - Renal Physiol* **303**, F1617-F1628.

Li, X. C. and Zhuo, J. L. (2008). Intracellular ANG II directly induces in vitro transcription of TGF-β1, MCP-1, and NHE-3 mRNAs in isolated rat renal cortical nuclei via activation of nuclear AT1a receptors. *Amer J Physiol - Cell Physiol* **294**, C1034-C1045.

Liao, B.-K., Chen, R.-D. and Hwang, P.-P. (2009). Expression regulation of Na^+-K^+-ATPase alpha1-subunit subtypes in zebrafish gill ionocytes. *Amer J Physiol - Regul Integr Comp Physiol* **296**, R1897-R1906.

Liao, B.-K., Deng, A.-N., Chen, S.-C., Chou, M.-Y. and Hwang, P.-P. (2007). Expression and water calcium dependence of calcium transporter isoforms in zebrafish gill mitochondrion-rich cells. *BMC Genomics* **8**, 354.

Lin, C.-H., Su, C.-H., Tseng, D.-Y., Ding, F.-C. and Hwang, P.-P. (2012). Action of vitamin D and the receptor, VDRa, in calcium handling in zebrafish (*Danio rerio*). *PLoS ONE* **7**, e45650.

Lin, C.-H., Tsai, I. L., Su, C.-H., Tseng, D.-Y. and Hwang, P.-P. (2011). Reverse effect of mammalian hypocalcemic cortisol in fish: cortisol stimulates Ca^{2+} uptake via glucocorticoid receptor-mediated vitamin D_3 metabolism. *PLoS ONE* **6**, e23689.

Lin, H., Pfeiffer, D. C., Vogl, A. W., Pan, J. and Randall, D. J. (1994). Immunolocalization of H^+-ATPase in the gill epithelia of rainbow trout. *J Exp Biol* **195**, 169-183.

Lin, H. and Randall, D. J. (1991). Evidence for the presence of an electrogenic proton pump on the trout gill epithelium. *J. Exp. Biol.* **161**, 119-134.

Lin, H. and Randall, D. J. (1993). H^+-ATPase activity in crude homogenates of fish gill tissue: inhibitor sensitivity and environmental and hormonal regulation. *J Exp Biol* **180**, 163-174.

Lin, L. Y., Horng, J. L., Kunkel, J. G. and Hwang, P. P. (2006). Proton pump-rich cell secretes acid in skin of zebrafish larvae. *Amer J Physiol Cell Physiol* **290**, C371-8.

Lin, T.-Y., Liao, B.-K., Horng, J.-L., Yan, J.-J., Hsiao, C.-D. and Hwang, P.-P. (2008). Carbonic anhydrase 2-like a and 15a are involved in acid-base regulation and Na+ uptake in zebrafish H^+-ATPase-rich cells. *Am J Physiol Cell Physiol* **294**, C1250-1260.

Lochner, A. and Moolman, J. A. (2006). The many faces of H89: a review. *Cardiovascular Drug Reviews* **24**, 261-274.

Loh, Y. H., Christoffels, A., Brenner, S., Hunziker, W. and Venkatesh, B. (2004). Extensive expansion of the claudin gene family in the teleost fish, *Fugu rubripes*. *Genome Res* **14**, 1248-1257.

Maetz, J. and Garcia Romeu, F. (1964). The mechanism of sodium and chloride uptake by the gills of a fresh-water fish, *Carassius auratus*. *J Gen Physiol* **47**, 1209-1227.

Mak, D.-O. D., Dang, B., Weiner, I. D., Foskett, J. K. and Westhoff, C. M. (2006). Characterization of ammonia transport by the kidney Rh glycoproteins RhBG and RhCG. *Amer J Physiol - Renal Physiol* **290**, F297-F305.

Manzon, L. A. (2002). The role of prolactin in fish osmoregulation: a review. *Gen Comp Endocrinol* **125**, 291-310.

Marshall, W. S. (1985). Paracellular ion transport in trout opercular epithelium models osmoregulatory effects of acid precipitation. *Can J Zool* **63**, 1816-1822.

Marshall, W. S., Bryson, S. E., Midelfart, A. and Hamilton, W. F. (1995). Low-conductance anion channel activated by cAMP in teleost Cl^- secreting cells. *Amer J Physiol - Regul, Integr Comp Physiol* **268**, R963-R969.

Marshall, W. S. and Grosell, M. (2005). Ion transport and osmoregulation in fish. In *Physioogy of Fishes*, eds. D. H. Evans and J. B. Claiborne), pp. 177-230. Boca Raton: CRC Press.

Marshall, W. S., Ossum, C. G. and Hoffmann, E. K. (2005). Hypotonic shock mediation by p38 MAPK, JNK, PKC, FAK, OSR1 and SPAK in osmosensing chloride secreting cells of killifish opercular epithelium. *J Exp Biol* **208**, 1063-1077.

McCormick, S. D. (2001). Endocrine control of osmoregulation in teleost fish. *Amer Zool* **41**, 781-794.

McCormick, S. D. and Bradshaw, D. (2006). Hormonal control of salt and water balance in vertebrates. *Gen Comp Endocrinol* **147**, 3-8.

McCormick, S. D., Regish, A., O'Dea, M. F. and Shrimpton, J. M. (2008). Are we missing a mineralocorticoid in teleost fish? Effects of cortisol, deoxycorticosterone and aldosterone on osmoregulation, gill Na^+,K^+-ATPase activity and isoform mRNA levels in Atlantic salmon. *Gen Comp Endocrinol* **157**, 35-40.

McCurley, A. T. and Callard, G. V. (2008). Characterization of housekeeping genes in zebrafish: Male-female differences and effects of tissue type, developmental stage and chemical treatment. *BMC Molecular Biology* **9**.

McDonald, D. (1983a). The effects of H^+ upon the gills of freshwater fish. *Can. J. Zool.* **61**, 691-703.

McDonald, D. G. (1983b). The interaction of environmental calcium and low pH on the physiology of the Rainbow trout, *Salmo gairdneri*: I. Branchial and renal net ion and H+ fluxes. *J Exp Biol* **102**, 123-140.

McDonald, D. G., Hobe, H. and Wood, C. M. (1980). The influence of calcium on the physiological responses of the rainbow trout, *Salmo gairdneri*, to low environmental pH. *J Exp Biol* **88**, 109-132.

McDonald, D. G., Walker, R. L. and Wilkes, P. R. H. (1983). The interaction of environmental calcium and low pH on the physiology of the rainbow trout, *Salmo gairdneri*. II. Branchial ionoregulatory mechanisms. *J. Exp. Biol.* **102**, 141-155.

McDonald, D. G. and Wood, C. M. (1981). Branchial and renal acid and ion fluxes in the rainbow trout, *Salmo gairdneri*, at low environmental pH. *J. Exp. Biol.* **93**, 101-118.

McWilliams, P. G. (1982). The effects of calcium on sodium fluxes in the brown trout, *Salmo trutta*, in neutral and acid water. *J Exp Biol* **96**, 439-442.

McWilliams, P. G. and Potts, W. T. W. (1978). The effects of pH and calcium concentrations on gill potentials in the Brown Trout,*Salmo trutta*. *J Comp Physiol* **126**, 277-286.

Métrich, M., Berthouze, M., Morel, E., Crozatier, B., Gomez, A. and Lezoualc'h, F. (2010). Role of the cAMP-binding protein Epac in cardiovascular physiology and pathophysiology. *Pflugers Archiv Euro J Physiol* **459**, 535-546.

Meyer, E. A., Cramp, R. L. and Franklin, C. E. (2010). Damage to the gills and integument of *Litoria fallax* larvae (Amphibia: Anura) associated with ionoregulatory disturbance at low pH. *Comp Biochem Physiol A* **155**, 164-171.

Milligan, C. L. and Wood, C. M. (1982). Disturbances in haematology, fluid volume distribution and circulatory function associated with low environmental pH in the rainbow trout, *Salmo gairdneri*. *J Exp Biol* **99**, 397-415.

Mitrovic, D., Dymowska, A., Nilsson, G. E. and Perry, S. F. (2009). Physiological consequences of gill remodeling in goldfish (*Carassius auratus*) during exposure to long-term hypoxia. *Amer J Physiol Regul Integr Comp Physiol* **297**, R224-234.

Mitrovic, D. and Perry, S. F. (2009). The effects of thermally induced gill remodeling on ionocyte distribution and branchial chloride fluxes in goldfish (*Carassius auratus*). *J Exp Biol* **212**, 843-852.

Mommsen, T. P., Vijayan, M. M. and Moon, T. W. (1999). Cortisol in teleosts: dynamics, mechanisms of action, and metabolic regulation. *Rev Fish Biol Fisheries* **9**, 211-268.

Morgan, I. J. and Potts, W. T. W. (1995). The effects of the adrenoreceptor agonists phenylephrine and isoproterenol on the intracellular ion concentrations of branchial epithelial cells of brown trout (*Salmo trutta* L.). *J Comp Physiol B* **165**, 458-463.

Morgan, I. J., Potts, W. T. W. and Oates, K. (1994). Intracellular ion concentrations in branchial epithelial cells of brown trout (*Salmo trutta* L.) determined by X-ray microanalysis. *J Exp Biol* **194**, 139-151.

Motulsky, H. (1998). Comparing dose-response or kinetic curves with GraphPad Prism. *HMS Beagle* **34**.

Mu, S. Y., Shimosawa, T., Ogura, S., Wang, H., Uetake, Y., Kawakami-Mori, F., Marumo, T., Yatomi, Y., Geller, D. S., Tanaka, H. et al. (2011). Epigenetic modulation of the renal β-adrenergicg-WNK4 pathway in salt-sensitive hypertension. *Nat Med* **17**, 573-580.

Murtazina, R., Kovbasnjuk, O., Zachos, N. C., Li, X., Chen, Y., Hubbard, A., Hogema, B. M., Steplock, D., Seidler, U., Hoque, K. M. et al. (2007). Tissue-specific Regulation of Sodium/Proton Exchanger Isoform 3 Activity in Na^+/H^+ Exchanger Regulatory Factor 1 (NHERF1) Null Mice: cAMP inhibition is differentially dependent on NHERF1 and exchange protein directly activated by cAMP in ileum versus proximal tubule. *J Biol Chem* **282**, 25141-25151.

Nakada, T., Hoshijima, K., Esaki, M., Nagayoshi, S., Kawakami, K. and Hirose, S. (2007a). Localization of ammonia transporter Rhcg1 in mitochondrion-rich cells of yolk sac, gill, and kidney of zebrafish and its ionic strength-dependent expression. *Amer. J Physiol - Regul, Integr Comp Physiol* **293**, R1743-R1753.

Nakada, T., Westhoff, C. M., Kato, A. and Hirose, S. (2007b). Ammonia secretion from fish gill depends on a set of Rh glycoproteins. *The FASEB Journal* **21**, 1067-1074.

Nakhoul, N. L., DeJong, H., Abdulnour-Nakhoul, S. M., Boulpaep, E. L., Hering-Smith, K. and Hamm, L. L. (2005). Characteristics of renal Rhbg as an NH_4^+ transporter. *Amer J Physiol - Renal Physiol* **288**, F170-F181.

Nawata, C. M., Hung, C. C. Y., Tsui, T. K. N., Wilson, J. M., Wright, P. A. and Wood, C. M. (2007). Ammonia excretion in rainbow trout (*Oncorhynchus mykiss*): evidence for Rh glycoprotein and H^+-ATPase involvement. *Physiol Genom* **31**, 463-474.

Nawata, C. M. and Wood, C. M. (2008). The effects of CO_2 and external buffering on ammonia excretion and Rhesus glycoprotein mRNA expression in rainbow trout. *J Exp Biol* **211**, 3226-3236.

Nawata, M., Wood, C. M. and O'Donnell, M. J. (2010). Functional characterization of Rhesus glycoproteins from an ammoniotelic teleost, the rainbow trout, using oocyte expression and SIET analysis. *J Exp Biol* **213**, 1049-1059.

Nesan, D., Kamkar, M., Burrows, J., Scott, I. C., Marsden, M. and Vijayan, M. M. (2012). Glucocorticoid receptor signaling is essential for mesoderm formation and muscle development in zebrafish. *Endocrinology* **153**, 1288-1300.

Nishimura, H. (2001). Angiotensin receptors — evolutionary overview and perspectives. *Comp Biochem Physiol Part A: Mol & Integr Physiol* **128**, 11-30.

Orlowski, J. (1993). Heterologous expression and functional properties of amiloride high affinity (NHE-1) and low affinity (NHE-3) isoforms of the rat Na/H exchanger. *J Biol Chem* **268**, 16369-16377.

Pacheco-Alvarez, D., Cristóbal, P. S., Meade, P., Moreno, E., Vazquez, N., Muñoz, E., Díaz, A., Juárez, M. E., Giménez, I. and Gamba, G. (2006). The Na^+-Cl^- cotransporter is activated and phosphorylated at the amino-terminal domain upon intracellular chloride depletion. *J Biol Chem* **281**, 28755-28763.

Pan, T.-C., Liao, B.-K., Huang, C.-J., Lin, L.-Y. and Hwang, P.-P. (2005). Epithelial Ca^{2+} channel expression and Ca^{2+} uptake in developing zebrafish. *Amer J Physiol Regul Integr Comp Physiol* **289**, R1202-1211.

Park, S., Hong, J. H., Ohana, E. and Muallem, S. (2012). The WNK/SPAK and IRBIT/PP1 Pathways in Epithelial Fluid and Electrolyte Transport. *Physiology* **27**, 291-299.

Parks, S. K., Tresguerres, M. and Goss, G. G. (2008). Theoretical considerations underlying Na^+ uptake mechanisms in freshwater fishes. *Comp Biochem Physiol C* **148**, 411-418.

Passos-Silva, D. G., Verano-Braga, T. and Santos, R. A. S. (2013). Angiotensin-(1–7): beyond the cardio-renal actions. *Clinical Science* **124**, 443-456.

Pastor-Soler, N., Beaulieu, V., Litvin, T. N., Da Silva, N., Chen, Y., Brown, D., Buck, J., Levin, L. R. and Breton, S. (2003). Bicarbonate-regulated Adenylyl Cyclase (sAC) is a sensor that regulates pH-dependent V-ATPase recycling. *J. Biol. Chem.* **278**, 49523-49529.

Pastor-Soler, N. M., Hallows, K. R., Smolak, C., Gong, F., Brown, D. and Breton, S. (2008). Alkaline pH- and cAMP-induced V-ATPase membrane accumulation is mediated by protein kinase A in epididymal clear cells. *Amer J Physiol - Cell Physiol* **294**, C488-C494.

Păunescu, T. G., Da Silva, N., Russo, L. M., McKee, M., Lu, H. A. J., Breton, S. and Brown, D. (2008). Association of soluble adenylyl cyclase with the V-ATPase in renal epithelial cells. *Amer J Physiol - Renal Physiol* **294**, F130-F138.

Păunescu, T. G., Ljubojevic, M., Russo, L. M., Winter, C., McLaughlin, M. M., Wagner, C. A., Breton, S. and Brown, D. (2010). cAMP stimulates apical V-ATPase accumulation, microvillar elongation, and proton extrusion in kidney collecting duct A-intercalated cells. *Amer J Physiol - Renal Physiol* **298**, F643-F654.

Pech, V., Zheng, W., Pham, T. D., Verlander, J. W. and Wall, S. M. (2008). Angiotensin II activates H^+-ATPase in type A intercalated cells. *J Amer Soc Nephrol* **19**, 84-91.

Perry, S. F. (1997). The chloride cell:structure and function in the gills of freshwater fishes. *Annu Rev Physiol* **59**, 325-347.

Perry, S. F., Braun, M. H., Noland, M., Dawdy, J. and Walsh, P. J. (2010a). Do zebrafish Rh proteins act as dual ammonia–CO_2 channels? *J Exp Zool A* **313A**, 618-621.

Perry, S. F. and Gilmour, K. M. (2006). Acid-base balance and CO_2 excretion in fish: Unanswered questions and emerging models. *Resp Physiol Neurobiol* **154**, 199-215.

Perry, S. F., Goss, G. G. and Fenwick, J. C. (1992). Interrelationships between gill chloride cell morphology and calcium uptake in freshwater teleosts. *Fish Physiol Biochem* **10**, 327-337.

Perry, S. F., Payan, P. and Girard, J. P. (1984). Adrenergic control of branchial chloride transport in the isolated perfused head of the freshwater trout (*Salmo gairdneri*). *J Comp Physiol B* **154**, 269-274.

Perry, S. F., Schwaiger, T., Kumai, Y., Tzaneva, V. and Braun, M. H. (2010b). The consequences of reversible gill remodelling on ammonia excretion in goldfish (*Carassius auratus*). *J Exp Biol* **213**, 3656-3665.

Perry, S. F., Vulesevic, B., Grosell, M. and Bayaa, M. (2009). Evidence that SLC26 anion transporters mediate branchial chloride uptake in adult zebrafish (*Danio rerio*). *Amer J Physiol - Regul Integr Comp Physiol* **297**, R988-R997.

Peti-Peterdi, J., Warnock, D. G. and Bell, P. D. (2002). Angiotensin II directly stimulates ENaC activity in the cortical collecting duct via AT1 receptors. *J Amer Soc Nephrol* **13**, 1131-1135.

Petrenko, A. G., Zozulya, S. A., Deyev, I. E. and Eladari, D. Insulin receptor-related receptor as an extracellular pH sensor involved in the regulation of acid–base balance. *Biochim Biophys (BBA) - Proteins and Proteomics*. *In press.*

Pfaffl, M. W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* **29**, 2002-2007.

Pickford, G. E. and Phillips, J. G. (1959). Prolactin, a factor in promoting survival of hypophysectomized killifish in fresh water. *Science* **130**, 454-455.

Pierce, K. L., Premont, R. T. and Lefkowitz, R. J. (2002). Seven-transmembrane receptors. *Nat Rev Mol Cell Biol* **3**, 639-650.

Pippal, J. B., Cheung, C. M. I., Yao, Y.-Z., Brennan, F. E. and Fuller, P. J. (2010). Characterization of the zebrafish (*Danio rerio*) mineralocorticoid receptor. *Mol Cell Endocrinol* **332**, 58-66.

Pisam, M., Auperin, B., Prunet, P., Rentier-Delrue, F., Martial, J. and Rambourg, A. (1993). Effects of prolactin on α and β chloride cells in the gill epithelium of the saltwater adapted tilapia "*Oreochromis niloticus*". *Anat Rec* **235**, 275-284.

Preest, M. R., Gonzalez, R. J. and Wilson, R. W. (2005). A pharmacological examination of Na^+ and Cl transport in two species of freshwater fish. *Physiol Biochem Zool* **78**, 259-272.

Preisig, P. A. (2007). The acid-activated signaling pathway: Starting with Pyk2 and ending with increased NHE3 activity. *Kidney Int* **72**, 1324-1329.

Prunet, P., Sturm, A. and Milla, S. (2006). Multiple corticosteroid receptors in fish: From old ideas to new concepts. *Gen Comp Endocrinol* **147**, 17-23.

Randall, D. J. and Wright, P. A. (1989). The interaction between carbon dioxide and ammonia excretion and water pH in fish. *Can. J. Zool.* **67**, 2936-2942.

Reid, S. D., Hawkings, G. S., Galvez, F. and Goss, G. G. (2003). Localization and characterization of phenamil-sensitive Na^+ influx in isolated rainbow trout gill epithelial cells. *J Exp Biol* **206**, 551-559.

Rodela, T. M., Esbaugh, A. J., McDonald, M. D., Gilmour, K. M. and Walsh, P. J. (2011). Evidence for transcriptional regulation of the urea transporter in the gill of the Gulf toadfish, *Opsanus beta*. *Comp Biochem Physiol B* **160**, 72-80.

Rodela, T. M., McDonald, M. D., Walsh, P. J. and Gilmour, K. M. (2012). Interactions between cortisol and Rhesus glycoprotein expression in ureogenic toadfish, *Opsanus beta*. *J Exp Biol* **215**, 314-323.

Rothenberger, F., Velic, A., Stehberger, P. A., Kovacikova, J. and Wagner, C. A. (2007). Angiotensin II stimulates vacuolar H^+-ATPase activity in renal acid-secretory intercalated cells from the outer medullary collecting duct. *J Amer Soc Nephrol* **18**, 2085-2093.

Rouah-Rosilio, M., Orosco, M. and Nicolaidis, S. (1994). Serotoninergic modulation of sodium appetite in the rat. *Physiol Behav* **55**, 811-816.

Russell, M. J., Klemmer, A. M. and Olson, K. R. (2001). Angiotensin signaling and receptor types in teleost fish. *Comp Biochem Physiol A* **128**, 41-51.

Saitou, M., Furuse, M., Sasaki, H., Schulzke, J. D., Fromm, M., Takano, H., Noda, T. and Tsukita, S. (2000). Complex phenotype of mice lacking occludin, a component of tight junction strands. *Mol Biol Cell* **11**, 4131-4142.

Salama, A., Morgan, I. J. and Wood, C. M. (1999). The linkage between Na^+ uptake and ammonia excretion in rainbow trout: kinetic analysis, the effects of $(NH_4)_2SO_4$ and NH_4HCO_3 infusion and the influence of gill boundary layer pH. *J Exp Biol* **202**, 697-709.

San-Cristobal, P., Pacheco-Alvarez, D., Richardson, C., Ring, A. M., Vazquez, N., Rafiqi, F. H., Chari, D., Kahle, K. T., Leng, Q., Bobadilla, N. A. et al. (2009). Angiotensin II signaling increases activity of the renal Na-Cl cotransporter through a WNK4-SPAK-dependent pathway. *Proc Nat Acad Sci USA* **106**, 4384-4389.

Sandberg, M. B., Riquier, A. D. M., Pihakaski-Maunsbach, K., McDonough, A. A. and Maunsbach, A. B. (2007). ANG II provokes acute trafficking of distal tubule Na+-Cl− cotransporter to apical membrane. *Amer J Physiol - Renal Physiol* **293**, F662-F669.

Santos, R. A. S., Ferreira, A. J., Verano-Braga, T. and Bader, M. (2013). Angiotensin-converting enzyme 2, angiotensin-(1–7) and Mas: new players of the renin–angiotensin system. *J Endocrinol* **216**, R1-R17.

Schneeberger, E. E. and Lynch, R. D. (2004). The tight junction: A multifunctional complex. *Amer J Physiol - Cell Physiol* **286**, C1213-C1228.

Schweitzer, J., Löhr, H., Filippi, A. and Driever, W. (2011). Dopaminergic and noradrenergic circuit development in zebrafish. *Dev Neurobiol* **72**, 256-268.

Scott, G., Keir, K. and Schulte, P. (2005a). Effects of spironolactone and RU486 on gene expression and cell proliferation after freshwater transfer in the euryhaline killifish. *J Comp Physiol B* **175**, 499-510.

Scott, G. R., Claiborne, J. B., Edwards, S. L., Schulte, P. M. and Wood, C. M. (2005b). Gene expression after freshwater transfer in gills and opercular epithelia of killifish: insight into divergent mechanisms of ion transport. *J Exp Biol* **208**, 2719-2729.

Shahsavarani, A. and Perry, S. F. (2006). Hormonal and environmental regulation of epithelial calcium channel in gill of rainbow trout (*Oncorhynchus mykiss*). *Amer J Physiol - Regul Integr Comp Physiol* **291**, R1490-R1498.

Shen, L., Weber, C. R. and Turner, J. R. (2008). The tight junction protein complex undergoes rapid and continuous molecular remodeling at steady state. *J Cell Biol* **181**, 683-695.

Shen, W.-P., Horng, J.-L. and Lin, L.-Y. (2011). Functional plasticity of mitochondrion-rich cells in the skin of euryhaline medaka larvae (*Oryzias latipes*) subjected to salinity changes. *Amer J Physiol - Regul Integr Comp Physiol* **300**, 858-868.

Shestopalov, I. A., Sinha, S. and Chen, J. K. (2007). Light-controlled gene silencing in zebrafish embryos. *Nature Chem Biol* **3**, 650-651.

Shih, T.-H., Horng, J.-L., Hwang, P.-P. and Lin, L.-Y. (2008). Ammonia excretion by the skin of zebrafish (*Danio rerio*) larvae. *Amer J Physiol - Cell Physiol* **295**, C1625-C1632.

Shih, T.-H., Horng, J.-L., Liu, S.-T., Hwang, P.-P. and Lin, L.-Y. (2012). Rhcg1 and NHE3b are involved in ammonium-dependent sodium uptake by zebrafish larvae acclimated to low-sodium water. *Amer J Physiol - Regul Integr Comp Physiol* **302**, R84-R93.

Shono, T., Kurokawa, D., Miyake, T. and Okabe, M. (2011). Acquisition of *glial cells missing 2* enhancers contributes to a diversity of ionocytes in zebrafish. *PLoS ONE* **6**, e23746.

Siddiqui, M., Sheikh, H., Tran, C. and Bruce, A. E. (2010). The tight junction component Claudin E is required for zebrafish epiboly. *Dev Dynamic* **239**, 715-722.

Skelton, L. A., Boron, W. F. and Zhou, Y. (2010). Acid-base transport by the renal proximal tubule. *J Nephrol* **23 Suppl 16**, S4-18.

Sloman, K. A., Desforges, P. R. and Gilmour, K. M. (2001). Evidence for a mineralocorticoid-like receptor linked to branchial chloride cell proliferation in freshwater rainbow trout. *J Exp Biol* **204**, 3953-3961.

Smith, N. F., Eddy, F. B., Struthers, A. D. and Talbot, C. (1991). Renin, atrial natriuretic peptide and blood plasma ions in parr and smolts of Atlantic salmon *Salmo Salar L*. and rainbow trout *Oncorhynchus Mykiss* (Walbaum) in fresh water and after short-term exposure to sea water. *J Exp Biol* **157**, 63-74.

Soupene, E., Inwood, W. and Kustu, S. (2004). Lack of the Rhesus protein Rh1 impairs growth of the green alga *Chlamydomonas reinhardtii* at high CO_2. *Proc Nat Acad Sci USA* **101**, 7787-7792.

Stainier, D. Y. R., Weinstein, B. M., Detrich Iii, H. W., Zon, L. I. and Fishman, M. C. (1995). cloche, an early acting zebrafish gene, is required by both the endothelial and hematopoietic lineages. *Development* **121**, 3141-3150.

Steele, S., Ekker, M. and Perry, S. (2011a). Interactive effects of development and hypoxia on catecholamine synthesis and cardiac function in zebrafish (*Danio rerio*). *J Comp Physiol B* **181**, 527-538.

Steele, S. L., Yang, X., Debiais-Thibaud, M. l., Schwerte, T., Pelster, B., Ekker, M., Tiberi, M. and Perry, S. F. (2011b). In vivo and in vitro assessment of cardiac beta-adrenergic receptors in larval zebrafish (*Danio rerio*). *J Exp Biol* **214**, 1445-1457.

Stegbauer, J. and Coffman, T. M. (2011). New insights into angiotensin receptor actions: from blood pressure to aging. *Curr Opin Nephrol Hypertens* **20**, 84-8.

Stuart, R. O., Sun, A., Panichas, M., Hebert, S. C., Brenner, B. M. and Nigam, S. K. (1994). Critical role for intracellular calcium in tight junction biogenesis. *J Cell Physiol* **159**, 423-433.

Sturm, A., Bury, N., Dengreville, L., Fagart, J., Flouriot, G., Rafestin-Oblin, M. E. and Prunet, P. (2005). 11-Deoxycorticosterone is a potent agonist of the rainbow trout (*Oncorhynchus mykiss*) mineralocorticoid receptor. *Endocrinology* **146**, 47-55.

Sullivan, G., Fryer, J. and Perry, S. (1995). Immunolocalization of proton pumps (H^+-ATPase) in pavement cells of rainbow trout gill. *J Exp Biol* **198**, 2619-2629.

Sullivan, G. V., Fryer, J. N. and Perry, S. F. (1996). Localization of mRNA for the proton pump (H^+-ATPase) and Cl^-/HCO_3^- exchanger in the rainbow trout gill. *Canad J Zool* **74**, 2095-2103.

Sun, X., Yang, L. V., Tiegs, B. C., Arend, L. J., McGraw, D. W., Penn, R. B. and Petrovic, S. (2010). Deletion of the pH Sensor GPR4 decreases renal acid excretion. *J Amer Soc Nephrol* **21**, 1745-1755.

Suster, M., Kikuta, H., Urasaki, A., Asakawa, K. and Kawakami, K. (2009). Transgenesis in Zebrafish with the Tol2 Transposon System. In *Transgenesis Techniques*, vol. 561 (ed. E. J. Cartwright), pp. 41-63: Humana Press.

Sutherland, E. W. and Rall, T. W. (1958). Fractionation and characterization of a cyclic adenine ribonucleotide formed by tissue particles. *J Biol Chem* **232**, 1077-91.

Takahashi, H. and Sakamoto, T. (2013). The role of 'mineralocorticoids' in teleost fish: Relative importance of glucocorticoid signaling in the osmoregulation and 'central' actions of mineralocorticoid receptor. *Gen Comp Endocrinol* **181**, 223-228.

Takei, Y. and Tsuchida, T. (2000). Role of the renin-angiotensin system in drinking of seawater-adapted eels *Anguilla japonica*: a reevaluation. *Amer J Physiol - Regul Integr Comp Physiol* **279**, R1105-R1111.

Takito, J., Hikita, C. and Al-Awqati, Q. (1996). Hensin, a new collecting duct protein involved in the in vitro plasticity of intercalated cell polarity. *J Clin Invest* **98**, 2324-2331.

Talati, G., Ohta, A., Rai, T., Sohara, E., Naito, S., Vandewalle, A., Sasaki, S. and Uchida, S. (2010). Effect of angiotensin II on the WNK-OSR1/SPAK-NCC phosphorylation cascade in cultured mpkDCT cells and in vivo mouse kidney. *Biochem Biophys Res Comm* **393**, 844-848.

Tay, T. L., Ronneberger, O., Ryu, S., Nitschke, R. and Driever, W. (2011). Comprehensive catecholaminergic projectome analysis reveals single-neuron integration of zebrafish ascending and descending dopaminergic systems. *Nat Commun* **2**, 171.

Tierney, M. L., Luke, G., Cramb, G. and Hazon, N. (1995). The role of the renin-angiotensin system in the control of blood pressure and drinking in the European eel, *Anguilla anguilla*. *Gen Comp Endocrinol* **100**, 39-48.

Tipsmark, C. K., Baltzegar, D. A., Ozden, O., Grubb, B. J. and Borski, R. J. (2008a). Salinity regulates claudin mRNA and protein expression in the teleost gill. *Amer J Physiol - Regul Integr Comp Physiol* **294**, R1004-R1014.

Tipsmark, C. K., JÃ¸rgensen, C., Brande-Lavridsen, N., Engelund, M., Olesen, J. H. and Madsen, S. S. (2009). Effects of cortisol, growth hormone and prolactin on gill claudin expression in Atlantic salmon. *Gen Comp Endocrinol* **163**, 270-277.

Tipsmark, C. K., Kiilerich, P., Nilsen, T. O., Ebbesson, L. O. E., Stefansson, S. O. and Madsen, S. S. (2008b). Branchial expression patterns of claudin isoforms in Atlantic salmon during seawater acclimation and smoltification. *Amer J Physiol - Regul Integr Comp Physiol* **294**, R1563-R1574.

Tipsmark, C. K., Luckenbach, J. A., Madsen, S. S., Kiilerich, P. and Borski, R. J. (2008c). Osmoregulation and expression of ion transport proteins and putative claudins in the gill of Southern Flounder (*Paralichthys lethostigma*). *Comp Biochem Physiol A* **150**, 265-273.

Tipsmark, C. K. and Madsen, S. S. (2001). Rapid modulation of Na^+/K^+-ATPase activity in osmoregulatory tissues of a salmonid fish. *J Exp Biol* **204**, 701-709.

To, T. T., Hahner, S., Nica, G., Rohr, K. B., Hammerschmidt, M., Winkler, C. and Allolio, B. (2007). Pituitary-Interrenal interaction in zebrafish interrenal organ development. *Mol Endocrinol* **21**, 472-485.

Tresguerres, M., Buck, J. and Levin, L. (2010a). Physiological carbon dioxide, bicarbonate, and pH sensing. *Pflugers Archiv Euro J Physiol* **460**, 953-964.

Tresguerres, M., Levin, L. R. and Buck, J. (2011). Intracellular cAMP signaling by soluble adenylyl cyclase. *Kidney Int* **79**, 1277-1288.

Tresguerres, M., Levin, L. R., Buck, J. and Grosell, M. (2010b). Modulation of NaCl absorption by [HCO_3^-] in the marine teleost intestine is mediated by soluble adenylyl cyclase. *Amer J Physiol - Regul Integr Comp Physiol* **299**, R62-R71.

Tresguerres, M., Parks, S. K., Katoh, F. and Goss, G. G. (2006). Microtubule-dependent relocation of branchial V-H^+-ATPase to the basolateral membrane in the Pacific spiny dogfish (*Squalus acanthias*): a role in base secretion. *J Exp Biol* **209**, 599-609.

Tresguerres, M., Parks, S. K., Salazar, E., Levin, L. R., Goss, G. G. and Buck, J. (2010c). Bicarbonate-sensing soluble adenylyl cyclase is an essential sensor for acid/base homeostasis. *Proc Nat Acad Sci USA* **107**, 442-447.

Tsai, J. C. and Hwang, P. P. (1998). The wheat germ agglutinin binding sites and development of the mitochondria-rich cells in gills of tilapia (*Oreochromis mossambicus*). *Fish Physiol Biochem* **19**, 95-102.

Tseng, D.-Y., Chou, M.-Y., Tseng, Y.-C., Hsiao, C.-D., Huang, C.-J., Kaneko, T. and Hwang, P.-P. (2009). Effects of stanniocalcin 1 on calcium uptake in zebrafish (*Danio rerio*) embryo. *Amer J Physiol - Regul Integr Comp Physiol* **296**, R549-R557.

Tsuganezawa, H., Preisig, P. A. and Alpern, R. J. (1998). Dominant negative c-Src inhibits angiotensin II induced activation of NHE3 in OKP cells. *Kidney Int* **54**, 394-398.

Tsui, T. K. N., Hung, C. Y. C., Nawata, C. M., Wilson, J. M., Wright, P. A. and Wood, C. M. (2009). Ammonia transport in cultured gill epithelium of freshwater rainbow trout: the importance of Rhesus glycoproteins and the presence of an apical Na^+/NH_4^+ exchange complex. *J Exp Biol* **212**, 878-892.

Tsukita, S., Furuse, M. and Itoh, M. (2001). Multifunctional strands in tight junctions. *Nat Rev Mol Cell Biol* **2**, 285-293.

Tucker, B., Hepperle, C., Kortschak, D., Rainbird, B., Wells, S., Oates, A. C. and Lardelli, M. (2007). Zebrafish Angiotensin II Receptor-like 1a (agtrl1a) is expressed in migrating hypoblast, vasculature, and in multiple embryonic epithelia. *Gene Expression Patterns* **7**, 258-265.

van der Lubbe, N., Lim, C. H., Fenton, R. A., Meima, M. E., Jan Danser, A. H., Zietse, R. and Hoorn, E. J. (2011). Angiotensin II induces phosphorylation of the thiazide-sensitive sodium chloride cotransporter independent of aldosterone. *Kidney Int* **79**, 66-76.

van der Lubbe, N., Lim, C. H., Meima, M. E., van Veghel, R., Rosenbaek, L. L., Mutig, K., Danser, A. H., Fenton, R. A., Zietse, R. and Hoorn, E. J. (2012). Aldosterone does not require angiotensin II to activate NCC through a WNK4-SPAK-dependent pathway. *Pflugers Arch* **463**, 853-63.

Van Itallie, C., Rahner, C. and Anderson, J. M. (2001). Regulated expression of claudin-4 decreases paracellular conductance through a selective decrease in sodium permeability. *J Clin Invest* **107**, 1319-1327.

Van Itallie, C. M. and Anderson, J. M. (2006). Claudins and epithelial paracellular transport, *Annu Rev Physiol* **68**, 403-429.

Van Itallie, C. M., Fanning, A. S. and Anderson, J. M. (2003). Reversal of charge selectivity in cation or anion-selective epithelial lines by expression of different claudins. *Amer J Physiol - Renal Physiol* **285**, F1078-F1084.

Verdouw, H., Van Echteld, C. J. A. and Dekkers, E. M. J. (1978). Ammonia determination based on indophenol formation with sodium salicylate. *Water Research* **12**, 399-402.

Vermette, M. G. and Perry, S. F. (1987). The effects of prolonged epinephrine infusion on the physiology of the rainbow trout, *Salmo gairdneri*. II. Branchial solute fluxes. *J Exp Biol* **Vol. 128**, 255-267.

Vulesevic, B. and Perry, S. F. (2006). Developmental plasticity of ventilatory control in zebrafish, *Danio rerio*. *Resp Physiol Neurobiol* **154**, 396-405.

Wagner, C. A., Giebisch, G., Lang, F. and Geibel, J. P. (1998). Angiotensin II stimulates vesicular H^+-ATPase in rat proximal tubular cells. *Proc Nat Acad Sci USA* **95**, 9665-9668.

Wagner, C. A., Kovacikova, J., Stehberger, P. A., Winter, C., Benabbas, C. and Mohebbi, N. (2006). Renal acid-base transport: old and new players. *Nephron Physiology* **103**, 1-6.

Wagner, C. A., Mohebbi, N., Uhlig, U., Giebisch, G. H., Breton, S., Brown, D. and Geibel, J. P. (2011). Angiotensin II stimulates H-ATPase activity in intercalated cells from isolated mouse connecting tubules and cortical collecting ducts. *Cell Physiol Biochem* **28**, 513-520.

Wang, D., Sun, H., Lang, F. and Yun, C. C. (2005). Activation of NHE3 by dexamethasone requires phosphorylation of NHE3 at Ser663 by SGK1. *Amer J Physiol - Cell Physiol* **289**, C802-C810.

Wang, Y.-F., Tseng, Y.-C., Yan, J.-J., Hiroi, J. and Hwang, P.-P. (2009). Role of SLC12A10.2, a Na-Cl cotransporter-like protein, in a Cl uptake mechanism in zebrafish (*Danio rerio*). *Amer J Physiol - Regul Integr Comp Physiol* **296**, R1650-R1660.

Weihrauch, D., Wilkie, M. P. and Walsh, P. J. (2009). Ammonia and urea transporters in gills of fish and aquatic crustaceans. *J Exp Biol* **212**, 1716-1730.

Weinman, E. J., Minkoff, C. and Shenolikar, S. (2000). Signal complex regulation of renal transport proteins: NHERF and regulation of NHE3 by PKA. *Amer J Physiol - Renal Physiol* **279**, F393-F399.

Weinman, E. J., Shenolikar, S. and Kahn, A. M. (1987). cAMP-associated inhibition of Na^+-H^+ exchanger in rabbit kidney brush-border membranes. *Amer J Physiol - Renal Physiol* **252**, F19-F25.

Welsh-Bacic, D., Nowik, M., Kaissling, B. and Wagner, C. A. (2012). Proliferation of acid-secretory cells in the kidney during adaptive remodelling of the collecting duct. *PLoS ONE* **6**, e25240.

Wendelaar Bonga, S. E. (1997). The stress response in fish. *Physiol Rev* **77**, 591-625.

Westerfield, M. (2000). The zebrafish book. A guide for the laboratory use of zebrafish (*Danio rerio*). Eugene: Univ. of Oregon Press.

Wilkie, M. P. (2002). Ammonia excretion and urea handling by fish gills: present understanding and future research challenges. *J Exp Zool* **293**, 284-301.

Willoughby, D. and Cooper, D. M. F. (2008). Live-cell imaging of cAMP dynamics. *Nature Meth* **5**, 29-36.

Wilson, J. M., Randall, D. J., Donowitz, M., Vogl, A. W. and Ip, A. K. (2000). Immunolocalization of ion-transport proteins to branchial epithelium mitochondria-rich cells in the mudskipper (*Periophthalmodon schlosseri*). *J Exp Biol* **203**, 2297-2310.

Wilson, R. W. and Taylor, E. W. (1992). Transbranchial ammonia gradients and acid-base responses to high external ammonia concentration in rainbow trout (*Oncorhynchus mykiss*) acclimated to different salinities. *J Exp Biol* **166**, 95-112.

Winter, C., Kampik, N. B., Vedovelli, L., Rothenberger, F., Păunescu, T. G., Stehberger, P. A., Brown, D., John, H. and Wagner, C. A. (2011). Aldosterone stimulates vacuolar H^+-ATPase activity in renal acid-secretory intercalated cells mainly via a protein kinase C-dependent pathway. *Amer J Physiol - Cell Physiol* **301**, C1251-C1261.

Winter, C., Schulz, N., Giebisch, G., Geibel, J. P. and Wagner, C. A. (2004). Nongenomic stimulation of vacuolar H^+-ATPases in intercalated renal tubule cells by aldosterone. *Proc Natl Acad Sci U S A* **101**, 2636-41.

Wong, M. K.-S. and Takei, Y. (2013). Angiotensin AT2 receptor activates the cyclic-AMP signaling pathway in eel. *Mol Cell Endocrinol* **365**, 292-302.

Wood, C. M., Iftikar, F. I., Scott, G. R., De Boeck, G., Sloman, K. A., Matey, V., Valdez Domingos, F. X., Duarte, R. M., Almeida-Val, V. M. F. and Val, A. L. (2009). Regulation of gill transcellular permeability and renal function during acute hypoxia in the Amazonian oscar

(*Astronotus ocellatus*): new angles to the osmorespiratory compromise. *J Exp Biol* **212**, 1949-1964.

Wood, C. M., Kelly, S. P., Zhou, B., Fletcher, M., O'Donnell, M., Eletti, B. and Pärt, P. (2002a). Cultured gill epithelia as models for the freshwater fish gill. *Biochim Biophys Acta (BBA) - Biomembr* **1566**, 72-83.

Wood, C. M., Matsuo, A. Y. O., Gonzalez, R. J., Wilson, R. W., Patrick, M. L. and Val, A. L. (2002b). Mechanisms of ion transport in Potamotrygon, a stenohaline freshwater elasmobranch native to the ion-poor blackwaters of the Rio Negro. *J Exp Biol* **205**, 3039-3054.

Wood, C. M., Matsuo, A. Y. O., Wilson, R. W., Gonzalez, R. J., Patrick, M. L., Playle, R. C. and Val, A. L. (2003). Protection by natural blackwater against disturbances in ion fluxes caused by low pH exposure in freshwater stingrays endemic to the Rio Negro. *Physiol Biochem Zool* **76**, 12-27.

Wood, C. M. and Pärt, P. (1997). Cultured branchial epithelia from freshwater fish gills. *J Exp Biol* **200**, 1047-1059.

Wright, P. A., Randall, D. J. and Perry, S. F. (1989). Fish gill water boundary layer: a site of linkage between carbon dioxide and ammonia excretion. *J Comp Physiol B* **158**, 627-635.

Wright, P. A. and Wood, C. M. (1985). An analysis of branchial ammonia excretion in the freshwater rainbow trout: Effects of environmental pH change and sodium uptake blockade. *J Exp Biol***, 114**, 329-353.

Wright, P. A. and Wood, C. M. (2009). A new paradigm for ammonia excretion in aquatic animals: role of Rhesus (Rh) glycoproteins. *J Exp Biol* **212**, 2303-2312.

Wright, P. A. and Wood, C. M. (2012). 7 Things fish know about ammonia and we don't. *Resp Physiol & Neurobiol*.**184**, 231-240.

Wu, S.-C., Horng, J.-L., Liu, S.-T., Hwang, P.-P., Wen, Z.-H., Lin, C.-S. and Lin, L.-Y. (2010). Ammonium-dependent sodium uptake in mitochondrion-rich cells of medaka (*Oryzias latipes*) larvae. *Amer J Physiol - Cell Physiol* **298**, C237-C250.

Yan, J.-J., Chou, M.-Y., Kaneko, T. and Hwang, P.-P. (2007). Gene expression of Na^+/H^+ exchanger in zebrafish H^+-ATPase-rich cells during acclimation to low-Na^+ and acidic environments. *Amer J Physiol - Cell Physiol* **293**, C1814-1823.

Yao, M., Hu, F. and Denver, R. J. (2008). Distribution and corticosteroid regulation of glucocorticoid receptor in the brain of *Xenopus laevis*. *J Comp Neurol* **508**, 967-982.

Yi-Wen, L. (2007). Interrenal organogenesis in the zebrafish model. *Organogenesis* **3**, 44-48.

Yoo, S. K., Lam, P.-y., Eichelberg, M. R., Zasadil, L., Bement, W. M. and Huttenlocher, A. (2012). Role of microtubules in neutrophil polarity and migration in live zebrafish. *J Cell Sci*.

Yu, A. S. L. (2003). Claudins and epithelial paracellular transport: The end of the beginning. *Cur Op Nephrol Hyperten* **12**, 503-509.

Yun, C. C., Chen, Y. and Lang, F. (2002). Glucocorticoid activation of Na^+/H^+ exchanger isoform 3 revisited: the roles of SGK1 and NHERF2. *J Biol Chem* **277**, 7676-7683.

Zhou, Y., Zhao, J., Bouyer, P. and Boron, W. F. (2005). Evidence from renal proximal tubules that and solute reabsorption are acutely regulated not by pH but by basolateral and CO_2. *Proc Nat Acad Sci USA* **102**, 3875-3880.

Zhuo, J. L. and Li, X. C. (2007). Review: Novel roles of intracrine angiotensin II and signalling mechanisms in kidney cells. *Renin-Angioten-Aldost Sys* **8**, 23-33.

Zimmer, A., Nawata, C. and Wood, C. (2010). Physiological and molecular analysis of the interactive effects of feeding and high environmental ammonia on branchial ammonia excretion and Na^+ uptake in freshwater rainbow trout. *J Comp Physiol B* **180**, 1191-1204.

Zimmerman, D. and Burns, K. D. (2012). Angiotensin-(1–7) in kidney disease: a review of the controversies. *Clin Sci* **123**, 333-346.