Ion regulation under acidic conditions in zebrafish (*Danio rerio*) lacking functional corticosteroid receptors

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Abstract

Previous research suggests a role for cortisol in mediating ion balance in zebrafish (*Danio rerio*), an acid-tolerant freshwater fish that is used as a model species for understanding ionic, osmotic and acid-balance in teleost fishes. Exposure to acidic water (pH 4.0-4.5) increases diffusive ion loss and impairs ion uptake mechanisms. Cortisol, a corticosteroid hormone, exerts its effects through glucocorticoid (GR, encoded by the *nr3c1* gene) and mineralocorticoid (MR, encoded by the *nr3c2* gene) receptors. The objective of the present study was to generate *nr3c1*$^{-/-}$ and *nr3c2*$^{-/-}$ zebrafish lines to test the role of cortisol in eliciting compensatory responses to pH 4 water in adult zebrafish because most previous work has focused on larval stages. Whole-body Na^+ and Ca^{2+} levels, plasma cortisol concentrations, ionocyte abundances and branchial expression of genes previously implicated in responses to pH 4 water were measured in *nr3c1*$^{+/+}$*nr3c2*$^{+/+}$ (i.e. wildtype, WT), *nr3c1*$^{-/-}$ (GR-KO), and *nr3c2*$^{-/-}$ (MR-KO) zebrafish exposed for seven days to control or pH 4 water. Despite the absence of cortisol signaling via GR, GR-KO fish tolerated pH 4 water similarly to WT and MR-KO fish, in contrast to the literature on larval fish that suggested that GR is essential for these responses. Subtle differences were observed; GR-KO adults exposed to pH 4 water had slightly but significantly lower whole-body Na^+ content than WT fish, and higher abundance of H^{+}-ATPase-rich (HR) cells under control conditions that decreased in response to pH 4 water. These fish also exhibited significantly lower transcript abundance of *ncc*, the Na^+,Cl^--cotransporter. Together, the results of the present study highlight the complexity of endocrine regulation of ionic balance in freshwater fishes.

Résumé

Des recherches précédentes suggèrent un rôle du cortisol dans la médiation de l'équilibre ionique chez le poisson zèbre (*Danio rerio*), un poisson d'eau douce tolérant à l'acide, qui sert d'espèce modèle pour comprendre l'équilibre ionique, osmotique et acide chez les poissons téléostéens. L'exposition à l'eau acide (pH 4,0-4,5) augmente la perte d'ions par diffusion et interfère avec les mécanismes d'absorption des ions. Le cortisol, une hormone corticostéroïde, exerce ses effets par l'intermédiaire des récepteurs glucocorticoïdes (GR, codé par le gène *nr3c1*) et minéralocorticoïdes (MR, codé par le gène *nr3c2*). L'objectif de la principale étude était de générer des lignées de poissons zèbres *nr3c1*$^{-/-}$ et *nr3c2*$^{-/-}$ afin de tester le rôle du cortisol dans le déclenchement de réponses compensatoires à l'eau de pH 4 chez le poisson zèbre adulte, les travaux antérieurs s'étant concentrés sur les stades larvaires. Les niveaux de Na^+ et de Ca^{2+} dans le corps entier, les concentrations plasmatiques de cortisol, l'abondance des ionocytes et l'expression branchiale des gènes précédemment impliqués dans les réponses au pH 4 de l'eau ont été mesurés chez des poissons zèbres *nr3c1*$^{+/+}$*nr3c2*$^{+/+}$ (c'est-à-dire de type sauvage, WT), *nr3c1*$^{-/-}$ (GR-KO) et *nr3c2*$^{-/-}$ (MR-KO) exposés pendant sept jours à de l'eau de contrôle ou à du pH 4. Malgré l'absence de signalisation du cortisol par le GR, les poissons *nr3c1*$^{-/-}$ ont toléré l'eau à pH 4 de la même manière que les poissons WT et MR-KO, contrairement aux résultats obtenus sur les larves de poisson qui suggéraient que le GR était essentiel pour ces réponses. Des différences subtiles ont été observées ; les adultes GR-KO exposés à l'eau pH 4 avaient une teneur en Na^+ dans le corps entier légèrement mais significativement plus faible que les poissons WT, et une plus grande abondance de cellules riches en H^+-ATPase (HR) dans des conditions de contrôle qui diminuaient en réponse à l'eau pH 4. Ces poissons présentaient également une abondance de transcription significativement plus faible de *ncc*, le Na^+,Cl^--cotransporteur.

L'ensemble des résultats de cette étude met en évidence la complexité de la régulation endocrinienne de l'équilibre ionique chez les poissons d'eau douce.

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List of abbreviations

ACVS	Animal Care and Veterinary Services
ANOVA	Analysis of variance
bp	Base pair
BSA	Bovine serum albumin
CACC	Canadian Council of Animal Care
Cas9	CRISPR associated protein 9
cDNA	Complementary DNA
CRISPR	Clustered regularly interspaced short palindromic repeats
d	Day
DNA	Deoxyribonucleic acid
dpf	Days post fertilization
ECaC	Epithelial Ca^{2+} channel
EDTA	Ethylenediaminetetraacetic acid
EIA	Enzyme-linked immunosorbent assay
GR	Glucocorticoid receptor
HPI	Hypothalamic-pituitary-interrenal
HR	H^{+}-ATPase-rich
IHC	Immunohistochemistry
KO	Knockout
min	Minute
MR	Mineralocorticoid receptor
mRNA	Messenger RNA
MS-222	Ethyl 3-aminobenzoate methansulfonate salt
N	Sample size
NaR	Na^{+}-K^{+}-ATPase-rich
NCC	Na^{+},Cl^{-} cotransporter
NCX	Na^{+}/Ca^{2+} exchanger
p	P-value
PBS	Phosphate buffered saline
PBS-T	Phosphate buffered saline – Tween
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
RNA	Ribonucleic acid
SEM	Standard error of the mean
sgRNA	Single guide ribonucleic acid
TRIS	2-amino-2-(hydroxymethyl)propane-1,3-diol
V-type	Vacuolar type
Δ	Delta

Chapter 1: Introduction

Teleost fishes inhabit freshwater and/or marine environments that differ in osmotic and ionic concentrations from their body fluids. Consequently, they have evolved physiological mechanisms that allow them to maintain ionic and osmotic homeostasis and to respond to changes in the ion composition of their environment (Hwang and Lee, 2007; Lin et al., 2023). Unlike in mammals, where the kidney plays a key role in maintaining ionic and osmotic balance, fishes rely on the gill (in adults) and skin (in developing fishes) as the major organ of ionic and osmotic balance, with the kidney playing a supporting role (Takei and Hwang, 2016). The movement of ions across the gill epithelium is mediated by mitochondrion-rich cells, known as ionocytes, that actively pump ions, primarily, Na^+, Cl^- and Ca^{2+}, against the prevailing diffusion gradients. These gradients depend on whether the fish resides in freshwater or sea water. The concentrations of Na^+ and Cl^- in sea water are substantially higher than those in the body fluids of teleost fishes; thus, marine teleosts use NaCl-secreting "chloride cells" to actively excrete the excess NaCl that they gain from their environment by passive diffusion. Whereas freshwater teleosts face ion loss to their very dilute environment, which they counter by actively taking up Na^+, Cl^-, and Ca^{2+} via a range of branchial ionocyte cell types (Guh et al., 2015; Takei and Hwang, 2016).

In addition to maintaining body fluid ion levels in the face of strong diffusion gradients for ion loss, freshwater fishes must be able to respond to fluctuations in water ion concentrations (e.g. caused by run-off from the surrounding land) and to changes in water pH (e.g. caused by run-off, acid rain or elevated CO_2) (Hwang, 2011; Marshall and Grosell, 2006). Changes in water pH, particularly acidification, not only pose acid-base challenges for fishes, but also impact ion balance by increasing passive Na^+ loss, and inhibiting Na^+ uptake (Kwong et al., 2014). It is

important to note that the maintenance of acid-base balance in fishes is closely linked to ionic and osmotic regulation; responding to acid-base challenges typically requires net ion transport to re-establish homeostasis (Marshall and Grosell, 2006; Takei and Hwang, 2016).

The zebrafish (*Danio rerio*) is a freshwater teleost fish that has emerged as a model for research on ion regulation, ionocytes, and the hormonal control of ionic and osmotic balance (Guh et al., 2015; Hwang and Lee, 2007; Marshall and Grosell, 2006). Although ionocytes are located on the gill in adult fish, they are difficult to work on owing to the complex architecture of the gill. In contrast, during embryonic and larval stages, ionocytes are localized to the skin and the surface of the yolk sac where they are readily accessible. Consequently, most of the literature on ionic regulation in zebrafish focuses on larvae (Hwang and Chou, 2013; Hwang and Lee, 2007; Kwong et al., 2016, 2014). Zebrafish reproduce rapidly and regularly in a lab setting, providing the many embryos/larvae needed for experimentation (Hwang and Chou, 2013; Kwong et al., 2014). Their genome has been described, and they are amenable to the use of antisense morpholino oligonucleotides and gene editing technology, providing tools to probe the molecular and cellular mechanisms of ion transport (Hwang et al., 2011a). Thus, the zebrafish holds several advantages for investigating ion regulation in fishes. An additional advantage of this species for the present study, which focused on the role of the hormone cortisol in the regulation of ion balance, is that unlike most teleost fishes, zebrafish express only a single glucocorticoid receptor simplifying genetic manipulation and narrowing down possible glucocorticoid models of ion regulation (Alsop and Vijayan, 2008; Brunet et al., 2006). Below, I describe the ionocyte types found in zebrafish and outline the effects of acid exposure on ion regulation to establish the zebrafish as an acid-tolerant species. I also review the existing literature on the roles of cortisol in ion regulation in zebrafish, particularly with respect to the

responses to low pH 4 water. Finally, I identify the knowledge gap that the present thesis aims to address and present the hypothesis that was tested.

1.1 Ionocyte types in zebrafish

Multiple reviews have discussed the ionocyte types that have been identified in zebrafish together with their function in ion uptake and the ion transporters and associated proteins that they express for this purpose (e.g. Guh et al., 2015; Horng et al., 2009b; Hwang and Chou, 2013; Kumai et al., 2011; Liao et al., 2009; Perry et al., 2009; Yan and Hwang, 2019). At least five ionocyte types have been identified and named according to distinctive features that they express; vacuolar-type (V-type) H^+-ATPase rich (HR) cells (Horng et al., 2007; Lin et al., 2006), Na^+,K^+-ATPase rich (NaR) cells (Lin et al., 2006; Pan et al., 2005), Na^+,Cl^--cotransporter (NCC) cells (Chang et al., 2013; Kwong and Perry, 2016), K^+-secreting cells (Abbas et al., 2011; Liao et al., 2009) and solute carrier 26-expressing (SLC26) cells (Bayaa et al., 2009; Perry et al., 2009; Wang et al., 2009). The present study focused on three of these identified ionocytes, HR cells, NaR cells and NCC cells, which were chosen for study because of their important roles in Na^+ (HR and NCC cells) or Ca^{2+} (NaR cells) uptake. All three of these ionocyte types are mitochondrion-rich and can be identified using vital MitoTracker dyes for mitochondrial labelling (Esaki et al., 2007; Lin et al., 2006; Yan et al., 2007). In addition, however, these ionocytes express unique complements of ion transporters and associated proteins that allow them to be identified using immunohistochemical approaches (Fig. 1.1).

Arguably, the ionocyte that has been most studied and is best understood is the HR cell, which plays key roles in H^+ secretion, and Na^+ uptake (Esaki et al., 2009; Horng et al., 2009a; Hwang and Chou, 2013; Lin et al., 2006; Shih et al., 2023; Yan et al., 2007). Most models, supported by immunohistochemical, pharmacological and physiological evidence, propose that

HR cells are enriched with apically localized V-type H^+-ATPase, a transporter that actively secretes protons to the surrounding water (Esaki et al., 2007; Hwang, 2011; Hwang and Chou, 2013; Lin et al., 2006; Marshall and Grosell, 2006). A second commonly used marker for HR cells is the lectin concanavalin A (ConA) which was found to bind specifically to the apical membrane of HR cells and not to other ionocyte types, at least in larvae (Lin et al., 2006). Using these markers, HR cells have been localized to the yolk sac of zebrafish embryos, and to the gill of adult zebrafish (Horng et al., 2009a; Lin et al., 2006; Yan et al., 2007, Esaki et al., 2009; Guh and Hwang, 2017). Current models propose that Na^+ uptake by the HR cell is coupled to H^+ secretion through the actions of apical V-type H^+-ATPase and NHE3b, a Na^+/H^+ exchanger (Fig. 1.1). Through immunohistochemistry (IHC) and whole mount *in situ* hybridization, NHE3b was found to be co-localized with V-type H^+-ATPase in zebrafish HR cells (Yan et al., 2007) and the use of pharmacological inhibitors of NHE or V-type H^+-ATPase impaired Na^+ uptake in zebrafish larvae (Esaki et al., 2007). The roles of NHE3b (Shih et al., 2012) and V-type H^+-ATPase (Horng et al., 2007) in Na^+ uptake were further tested via antisense oligonucleotide morpholino knockdown (KD) resulting in decreased Na^+ influx along with decreased whole-body Na^+ content. Cytosolic carbonic anhydrase, specifically, Ca17a (formerly known as Ca2-like a) is also specifically localized to HR cells; this enzyme catalyzes the hydration of CO_2 into H^+ and HCO_3^- and is thought to help with H^+ production to power Na^+ uptake (Evans et al., 2005; Lin et al., 2008; Perry et al., 2003). Together, there is robust molecular and cellular evidence that HR cells are involved in Na^+ uptake and H^+ secretion and is moderated by NHE3b, V-type H^+-ATPase and Ca17a.

A second important contributor to Na^+ uptake is the NCC cell, which is thought to carry out Na^+ uptake coupled to Cl^- uptake (Guh et al., 2015; Wang et al., 2009). The identifying

feature of the NCC cell is its expression of Slc12a10.2, which is an NCC-like protein distinct from the mammalian orthologues of SLC12A3 and is expressed specifically in NCC cells (Kwong and Perry, 2016; Wang et al., 2009). Using NCC as a marker, NCC cells have been localized to the yolk sac and skin of zebrafish embryos and larvae and the gill of adult zebrafish (Chang et al., 2013; Kwong and Perry, 2016; Wang et al., 2009). Loss-of-function and pharmacological inhibitor approaches have been used to investigate the role of NCC cells (Wang et al., 2009). Following entry into the cell via apical NCC, it is thought that Na^+ and Cl^- travel across the basolateral membrane of the cell via Na^+,K^+-ATPase (NKA) α1 subunit (*atp1a1a.2*, NM_131687.1) and chloride channels, respectively (Chang et al., 2013; Kwong and Perry, 2016; Wang et al., 2009). Reductions in Cl^- and Na^+ influxes occurred when NCC was inhibited with metolazone, however, morpholino knockdown of NCC only impacted whole-body Cl^- content and influxes (Wang et al., 2009). Later, Chang et al. (2013) demonstrated that knockdown of NHE3b led to an increase in the abundance of NCC cells in accordance with an increase in Na^+ uptake. Together, these observations provide support for NCC cells having a supplementary role in facilitating Na^+ uptake (Fig. 1.1) but more evidence is required for a comprehensive understanding of this type of ionocyte.

The NaR cell mediates Ca^{2+} uptake from the aquatic environment to the body fluids of the zebrafish (Guh et al., 2015; Hwang, 2011; Hwang et al., 2011a; Marshall and Grosell, 2006). A monoclonal antibody against the α1 subunit of NKA is reliably used to identify NaR cells, as it has been shown to be co-expressed with the epithelial Ca^{2+} channel (ECaC) which is a key component of the Ca^+ uptake mechanism (Liao et al., 2009; T. C. Pan et al., 2005). Although other ionocytes express NKA, the NaR cells express a specific NKA α1 subunit, atp1a1a.1 (*atp1a1a.1*, NM_131686.1). Using NKA as a marker, NaR cells have been localized to the yolk

sac and the skin of zebrafish embryos and larvae as well as the gill of adult zebrafish (Horng et al., 2009a; Hwang and Lee, 2007; Kwong et al., 2014a; Liao et al., 2009; Pan et al., 2005). The current model of Ca^{2+} uptake (Fig. 1.1) proposes that Ca^{2+} enters the cell from the surrounding water through apical ECaC. The Na^+ gradient established by basolateral NKA drives the exchange of Na^+ for Ca^{2+} via the Na^+/Ca^{2+} exchanger (NCX) to result in Ca^{2+} movement out of the cell across the basolateral membrane; basolateral plasma membrane Ca^{2+}-ATPase (PMCA) also contributes to Ca^{2+} exit (Flik et al., 1995; Guh et al., 2015; Hwang and Chou, 2013; Liao et al., 2009; Pan et al., 2005). Key evidence to support this model has come from studies in which knockdown of stanniocalcin, a hormone that inhibits Ca^{2+} uptake, was carried out. These studies reported increased *ecac* mRNA levels and higher Ca^{2+} content and influx, providing evidence that ECaC mediates Ca^{2+} uptake (Tseng et al., 2009). The same group used morpholino knockdown to further support this model, demonstrating reduced Ca^{2+} uptake in zebrafish morphants experiencing ECaC knockdown (Tseng et al., 2009). Furthermore, low environmental Ca^{2+} stimulated increases in NaR cell abundance, Ca^{2+} influx and *ecac* expression, further validating the role of NaR cells in re-establishing Ca^{2+} homeostasis (Pan et al., 2005).

1.2 Zebrafish as an acid-tolerant model

The zebrafish is one of several teleost fishes that are known to be more tolerant of acid exposure than most teleosts (Kwong et al., 2014). Exposure of freshwater fishes to acidic conditions (pH 4.0-4.5) results in increased diffusive loss of Na^+ along with inhibition of Na^+ uptake mechanisms (Kwong et al., 2014). These effects can reduce plasma Na^+ levels to the point where a fluid shift from the extracellular to the intracellular environment occurs, which can increase blood viscosity, leading to mortality by cardiovascular failure (Milligan and Wood, 1982). The increase in passive Na^+ loss appears to be caused by weakened epithelial tight

junctions, likely as a result of Ca^{2+} leaching from tight junction proteins (Kwong et al., 2014). In freshwater fishes, Na^+ uptake is closely tied to H^+ secretion through the actions of the apical V-type H^+-ATPase and/or NHE (Horng et al., 2009; Kwong et al., 2014; Liao et al., 2009; Shih et al., 2023; Wu et al., 2010). The reduction in Na^+ uptake under low pH conditions is caused by the increase in environmental H^+ levels, which decreases the H^+ gradient necessary to drive Na^+ uptake (Cruz et al., 2013a; Evans, 2011; Guh and Hwang, 2017; Hwang, 2011; Hwang and Lee, 2007; Kumai et al., 2012; Kumai and Perry, 2012; Kwong et al., 2014; Lin et al., 2015).

Although zebrafish, like most other freshwater species, exhibit increased passive Na^+ loss resulting in the fall of whole-body Na^+ content when initially exposed to pH 4.0 water, they tolerate this low water pH well and recover whole-body Na^+ levels over several days (Kumai et al., 2011; Kwong and Perry, 2013). The increase in passive Na^+ loss appears to be caused by increased paracellular permeability, and increased expression of certain tight junction proteins (claudins b, c and 7) in response to low pH water may contribute to the ability of zebrafish to recover whole-body Na^+ content under acidic conditions (Kumai et al., 2011; Kwong and Perry, 2013). However, a key component of the recovery of whole-body Na^+ content during acid exposure is an increase in Na^+ uptake (Esaki et al., 2007; Lin et al., 2008). This response appears to be achieved through an increase in the abundance of HR cells (Cruz et al., 2013a; Horng et al., 2009a; Liao et al., 2009; Lin et al., 2015; Shir-Mohammadi and Perry, 2020); these cells secrete H^+ as a response to increase Na^+ uptake, as well as increasing transcript abundances of key proteins in H^+ secretion and Na^+ uptake, including *atp6v1aa* (H^+,ATPase), *slc9a3.2* (NHE3b), and the two carbonic anhydrase isoforms, *ca17a,* and *ca15a* (Cruz et al., 2013a; Esaki et al., 2007; Kumai and Perry, 2011; Liao et al., 2009; Lin et al., 2016, 2008; Shir-Mohammadi and Perry, 2020; Yan et al., 2007). The NCC cells also appear to play a role in Na^+ uptake during acid

exposure (Bayaa et al., 2009; Chang et al., 2013; Kwong and Perry, 2016). Exposure to pH 4 water also disrupts Ca^{2+} homeostasis in zebrafish lowering whole-body Ca^{2+} content owing to reduced Ca^{2+} uptake and increased Ca^{2+} efflux (Horng et al., 2009a; Kumai et al., 2015; Liao et al., 2009; Pan et al., 2005; Tseng et al., 2009). Compensatory responses include increased abundance of the NaR cells responsible for Ca^{2+} uptake as well as increased transcript abundance of *ecac*. Notably, however, these responses do not appear to be sufficient to allow whole-body Ca^{2+} content to recover to pre-exposure levels, at least in the short term (i.e. at 4 days post-fertilization: dpf) (Horng et al., 2009a; Kumai et al., 2015; Liao et al., 2009; Pan et al., 2005; Tseng et al., 2009).

1.3 The role of cortisol in regulating responses to low pH

Among other neuroendocrine control mechanisms, cortisol appears to play a key role in initiating responses to low pH water (Kumai et al., 2012; Kwong et al., 2016; Kwong and Perry, 2013). Cortisol is a corticosteroid hormone that is synthesized in response to activation of the hypothalamic-pituitary-interrenal (HPI) axis, which in fishes plays roles in regulating metabolism and initiating stress responses as well as regulating ionic and osmotic balance (Cruz et al., 2013a; Faught et al., 2016; Faught and Vijayan, 2022, 2021, 2019a, 2019b; Kumai et al., 2012; ; Lin et al., 2015 reviewed by: Hwang and Lee, 2007; Kwong and Perry, 2013; Yan and Hwang, 2019). Cortisol exerts its effects through two receptors, both of which serve as ligand-activated transcription factors, a glucocorticoid receptor (GR; encoded by the gene *nr3c1*) and a mineralocorticoid receptor (MR; encoded by *nr3c2*) (Faught and Vijayan, 2020, 2019a; Kwong et al., 2016; Yan and Hwang, 2019). Despite their names, which reflect the roles of these receptors in mammals, existing literature suggests that the effects of cortisol on ionic and

osmotic regulation in fishes are mediated by the GR (McCormick et al., 2008; Takahashi and Sakamoto, 2013) and this also appears to be the case for zebrafish (see below).

Several studies have provided support for a role for cortisol in the proliferation and differentiation of ionocytes in zebrafish (Cruz et al., 2013a, 2013b). Using *in situ* hybridization or immunohistochemical approaches, the GR was found to be expressed in HR (Kumai et al., 2012), NaR (Cruz et al., 2013a) and NCC cells (Lin et al., 2016) in larvae. Waterborne cortisol treatment of larvae increased the abundance of HR, NaR and NCC cells, as did cortisol treatment of cultured adult zebrafish gills (for HR and NaR cells) (Cruz et al., 2013a, 2013b; Lin et al., 2016). Additionally, Cruz et al. (2013b), found that the use of antisense morpholino oligonucleotides to knockdown GR lowered the abundance of HR and NaR cells in larvae. Ionocyte abundances were not altered in MR knockdown morphants, supporting the GR as the dominant receptor for mediating the effects of cortisol on the proliferation of ionocytes. Similar results were obtained for NCC cells (Lin et al., 2016).

Cortisol treatment also stimulated Na^+ and Ca^{2+} uptake and H^+ secretion in larval zebrafish and caused higher whole-body Na^+ and Ca^{2+} content (Kumai et al., 2012; Lin et al., 2016, 2015, 2011). Higher transcript abundances of several of the genes involved in ion uptake or H^+ secretion, including *atp6v1aa* (H^+,ATPase), *slc9a3.2* (NHE3b), *ca17a, ca15a, ncc* and *ecac* were detected in cortisol treated larvae (Lin et al., 2016, 2015, 2011). The extent to which these responses reflect changes in ionocyte abundances versus increases in gene expression that are independent of ionocyte abundance remains to be determined. Morpholino knockdown and pharmacological approaches supported GR rather than MR as the primary receptor involved in mediating the effects of cortisol on Na^+ uptake (Cruz et al., 2013a, 2013b; Kumai et al., 2012; Lin et al., 2015). The GR-selective antagonist, RU-486, blocked cortisol-induced increases in

Na^+ uptake. Further, exposure of larvae to dexamethasone, a known GR-selective agonist, caused a significant increase in Na^+ uptake whereas the MR-selective agonist aldosterone did not (Kumai et al., 2012). Complementary, results were obtained using GR or MR morphant larvae. Knockdown of GR but not MR blocked the effects of cortisol treatment on the mRNA expression of ion transporters and inhibited cortisol-induced increases in proton secretion, Na^+ uptake, and whole-body Na^+ and Ca^{2+} content (Kumai et al., 2012; Lin et al., 2016, 2015, 2011).

The parallels between the responses of zebrafish to low pH exposure and the effects of cortisol treatment suggest a role for cortisol in mediating the responses to low pH, and there is experimental support for this association in larvae. For example, whole-body cortisol levels in zebrafish larvae were elevated by exposure to pH 4 water (Kumai et al., 2015, 2012). In addition, the increases in Na^+ uptake elicited by exposure to pH 4 water were prevented in RU486-treated zebrafish but not in larvae treated with the MR-specific antagonist eplerenone, again pointing to the GR rather than the MR as the key mediator of cortisol-induced effects (Kumai et al., 2012). Similarly, increases in Na^+ uptake in pH 4-exposed fish were attenuated in zebrafish larvae experiencing GR knockdown (Kumai et al., 2012). GR morphants also failed to increase H^+ secretion in response to pH 4 water and displayed attenuated or no increases in the transcript abundances of key genes, including *atp6v1aa* and *ca15a* (Lin et al., 2015).

Comparable data for adult zebrafish remain sparse. Adult zebrafish exposed to pH 4 water experienced a significant reduction in whole-body Na^+ content that persisted for ~72 h before gradually recovering to control levels over 1-2 weeks (Kumai et al., 2011). Passive Na^+ efflux was elevated in acid-exposed fish, and recovery of whole-body Na^+ content appeared to be associated with a significant increase in Na^+ uptake that developed over several days (Kumai et al., 2011). A similar pattern of immediate inhibition of Na^+ uptake upon exposure to pH 4 water,

but with recovery over hours rather than days, has also been reported (Clifford et al., 2022). In addition, a higher capacity for H^+ secretion was detected in the gill of fish exposed to pH 4 water for 7 days (Shih et al., 2022). Adult zebrafish exposed to pH 4 water for 7 d had higher abundances of HR and NCC cells in the gill than did those exposed to control conditions (Chang et al., 2013, 2009; Shih et al., 2022). Significantly higher expression of *atp6v1aa* transcript abundance and H^+-ATPase protein abundance were detected in the gill of acid-exposed adult fish (Chang et al., 2009; Shih et al., 2022; Yan et al., 2007), but transcript abundance of *nhe3b* was lower in fish exposed to pH 4 water (Chang et al., 2013; Yan et al., 2007). Collectively, these data suggest that the responses of adult fish to pH 4 water are generally comparable to those of larvae, with the possible exception of *nhe3b* transcript abundance. To our knowledge, however, the only data pertaining to effects of cortisol are for gill arches incubated in vitro with cortisol, which had higher abundances of HR and NaR cells than those incubated under control conditions (Cruz et al., 2013a). Thus, a knowledge gap exists around the role of cortisol in eliciting responses to pH 4 water in adult zebrafish.

1.4 Goals and hypotheses of the present study

Many studies have examined the roles of ionocytes in larval zebrafish, but there is a need to characterize the responses of adult zebrafish to ionic and osmotic stressors. Although knockdown approaches have proven to be valuable in experiments on larvae, a knockout design has advantages for studies in adult fish. Zebrafish lines lacking functional expression of GR or MR were created by Faught and Vijayan (2019a) but their work to date has focused largely on assessing the roles of cortisol in metabolism and growth rather than ionic and osmotic regulation. Importantly, their work has shown that loss of GR elevates whole-body cortisol levels owing to loss of negative feedback regulation of the HPI axis. However, fish lacking GR are unable to

mount GR-mediated responses to the elevated cortisol (Faught et al., 2016; Faught and Vijayan, 2022, 2021, 2020, 2019b, 2019a, 2018). The objective of the present thesis was to generate GR-KO and MR-KO mutant lines and use them totest the hypothesis that corticosteroid receptors, with GR playing a dominant role, are involved in the modulation of ion balance in adult zebrafish. Specifically, I predicted that GR-KO fish would be unable to respond appropriately to exposure to pH 4 water, i.e. these fish would be unable to increase the abundance of HR, NCC or NaR cells or the expression of genes related to ion transport, and therefore would experience decreases in whole-body Na^+ and Ca^{2+} content.

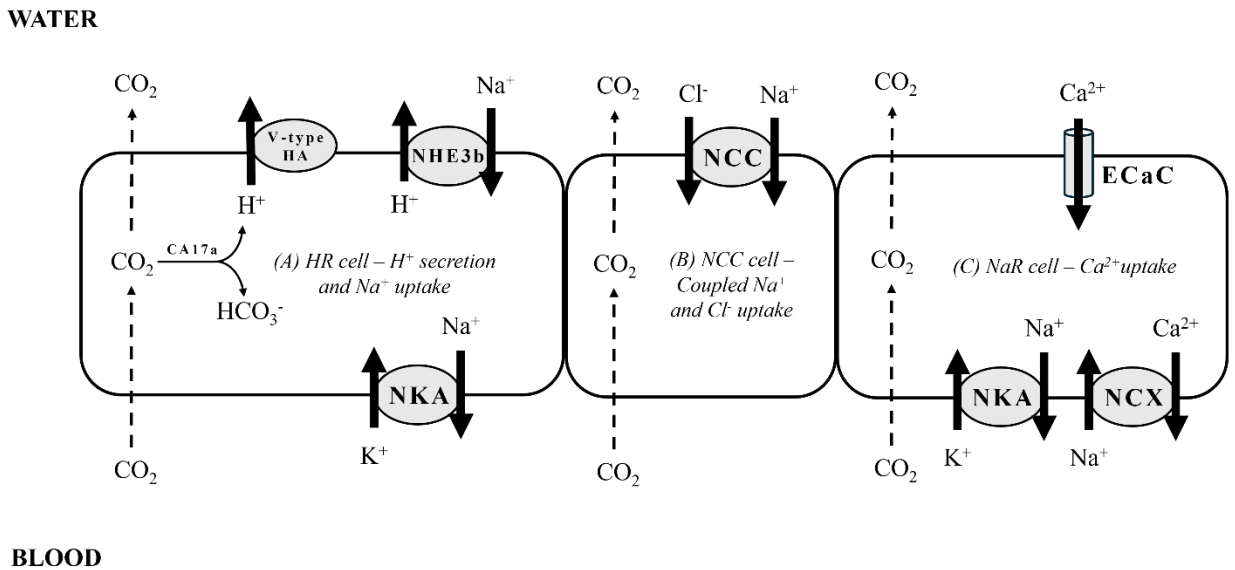


Figure 1.1 A schematic diagram of the three ionocytes types of zebrafish (*Danio rerio*) that were examined in the present study. (A) vacuolar-type (V-type) H^+-ATPase rich (HR) cells, (B) Na^+,Cl^--cotransporter (NCC) cells,and (C) Na^+,K^+-ATPase rich (NaR) cells. These ionocytes were chosen for study because of their importance in Na^+ (HR and NCC cells) or Ca^{2+} (NaR cells) uptake. Details of the ion uptake mechanisms are provided in the text (Section 1.1). *ca17a*, cytosolic carbonic anhydrase isoform; *ECaC*, epithelial Ca^{2+} channel; *HA*, H^+-ATPase; *NCC*, Na^+,Cl^--cotransporter; *NCX*, Na^+/Ca^{2+} exchanger; *NHE3b*, Na^+/H^+ exchanger; *NKA*, Na^+,K^+-ATPase.

Chapter 2: Materials and Methods

2.1 Experimental animals

Experiments were carried out on *nr3c1*$^{-/-}$ (GR-KO) and *nr3c2*$^{-/-}$ (MR-KO) zebrafish (*Danio* rerio) lines generated using CRISPR/Cas9 gene editing technology (see Chapter 2.2) and wildtype (WT) fish (*nr3c1*$^{+/+}$*;nr3c2*$^{+/+}$) that were bred from siblings of the knockout fish . These lines were generated from the in-house population of zebrafish at the University of Ottawa aquatics facility, with the initial fish obtained from the pet trade (Big Al's, Ottawa, ON, Canada). Adult zebrafish were housed in 4-L tanks supplied with circulating, dechloraminated city of Ottawa tap water ("system water", in mmol L^{-1}, 0.8 Na^+, 0.4 Cl^-, 0.25 Ca^{2+}; pH 7.6) and maintained at 28°C. The facility was maintained on a 14 h:10 h light:dark photoperiod. Embryos were obtained by placing three females and two males overnight in a 2-L breeding tank with an internal perforated insert. Embryos were collected in the morning and placed in 50 mL Petri dishes, at a density of 25-30 embryos per dish. Embryos were raised in system water containing 0.00003% methylene blue in an incubator held at 28.5°C. Dead embryos were removed daily. At 6 dpf, embryos were transferred to 3-L tanks for rearing to maturity. Larval fish were fed a combination of Gemma diet (Skretting, Vancouver, BC, Canada), rotifers (Skretting) or brine shrimp according to standard rearing practices. Past three months of age, zebrafish were provided with food pellets (Zeigler, Stewartstown, PA, USA) twice per day. All experiments using animals were in accordance with the guidelines of the Canadian Council on Animal Care (CCAC) for the use of animals in research and teaching and were approved by the University of Ottawa institutional animal care committee (protocol BLe-2118).

2.2 Generation of nr3c1$^{-/-}$ *(GR-KO) and nr3c2*$^{-/-}$ *(MR-KO) knockout lines*

Two distinct knockout lines targeting a full gene deletion of either the GR (*nr3c1;* NCBI accession number: NP_001018547), or the MR (*nr3c2*; NCBI accession number: NC_007112.7) were created using CRISPR/Cas9 technology. Designed using CHOPCHOP, four guide RNAs (gRNAs) and their corresponding target (T1-4) short-guide oligos (sgRNA) were developed to target the sequences upstream and downstream of each gene. The sgRNAs were synthesized using a cloning-free method (Talbot and Amacher, 2014) and microinjected into *roy*$^{-/-}$*;nacre*$^{-/-}$ (*casper*) embryos at the one-cell stage.

The injection solution included 40 ng µl^{-1} of each sgRNA, 20 ng µl^{-1} of Cas9 nuclease (New England Biolabs, Ipswich, MA, USA, catalogue # M0386S) and 0.1% phenol red in Danieau buffer. The use of *casper* embryos facilitated detection of founder fish carrying the *nr3c1* or *nr3c2* deletion. Founder fish raised to sexual maturity were crossed with wildtype adults from the in-house breeding program to yield heterozygous F1 populations, which were in-crossed to generate F2 populations of homozygous knockout fish (*nr3c1*$^{-/-}$ and *nr3c2*$^{-/-}$*)* and their wildtype siblings. Wildtype siblings from each of the *nr3c1*$^{-/-}$ and *nr3c2*$^{-/-}$ populations were crossed to yield the WT line.

Fish were screened using a multiplex PCR assay and genotypes were confirmed by Sanger sequencing (Genome Quebec, McGill University, Montreal, QC, Canada). At ~30 dpf, fish were anaesthetized in a solution of 0.012% (m/v) tricaine methanesulphonate (MS-222; Syndel, Nanaimo, BC, Canada) buffered to pH 7 with Tris. A small portion of the caudal fin was clipped and placed in 100 µL of 50 mM NaOH at 95°C for 10 min for DNA extraction. Samples were returned to room temperature and neutralized with 10 µL of 1 M Tris-HCl (pH 8) prior to PCR amplification. Each reaction contained 12.5 µL of goTaq Master Mix (catalogue # M7822; Promega Corporations, Madison, WI, USA), 1 µL of each primer (Table 2.1), 8.5 µL of nuclease

free water and 2 μL of DNA template. The thermal profile for PCR amplification for both genes was 95°C for 5 min, followed by 32 cycles of 94°C for 30 s, 57°C for 30 s, 72°C for 30 s, and then a final step of 72°C for 5 min. The PCR products were visualized on 2% agarose gels stained with Red Safe (Froggabio, North York, ON, Canada). For WT fish, a single band at 504 bp was observed, whereas GR-KO fish yielded a single band at 320 bp and MR-KO fish, a single band at 182 bp. For heterozygous fish, both the WT band and one of the mutant bands were observed.

Larval body lengths at 6 dpf and body mass of adults were recorded to assess size differences in knockout versus WT fish. To determine body length, 6 dpf larvae were placed in a Petri dish with millimeter (mm) paper underneath and viewed through a light microscope (Olympus CX41, Olympus, Richmond Hill, ON, Canada). Photos were taken on aniPhone XR mounted to the microscope and body lengths were determined from the photographs using Image J. For adult fish, body mass was recorded using an analytical digital balance (Sartorius ED124S, Gottingen, Germany). Fish were euthanized and patted try prior to weighing.

2.3 Experimental protocols

To assess their ionoregulatory responses, GR-KO, MR-KO and WT larvae and male adult fish were exposed to water adjusted to pH 4 by the addition of H_2SO_4, an ionoregulatory challenge that has been used in previous work on both larval (eg. Yan et al. 2007; Kumai et al, 2012) and adult zebrafish (eg. Chang et al. 2009, Kumai et al, 2011; reviewed by Kwong et al. 2014).

For larvae, embryos were reared to 4 dpf in system water (control) or system water adjusted to pH 4 (pH 4); in either case, the water contained 0.00003% methylene blue and was

changed daily. At the end of the exposure, larvae were euthanized by immersion in an ice-cold solution of Tris-buffered MS-222 (0.72 mg mL^{-1}) and fixed for IHC (see below) or flash frozen in liquid N_2 and stored at -80°C for later measurements of whole-body ion concentrations.

Male adult fish were subjected to a 7 d exposure to either system water (control) or system water adjusted to pH 4 (pH 4). Because females were limited in number in the GR-KO line, we used adult males to conserve the breeding population of GR-KO fish. Fish were held in static tanks placed in a temperature-controlled environment to maintain water temperature at 28-29°C. Daily water changes of approximately one-third of the water were used to maintain the desired pH and avoid accumulation of nitrogenous waste. Fish were fed on a daily basis. At the end of the exposure period, fish were euthanized by immersion in an ice-cold solution of Tris-buffered MS-222 (0.72 mg mL^{-1}), and gill tissue and blood plasma were collected for IHC and measurement of transcript abundances or measurement of plasma cortisol concentrations, respectively. Carcasses were flash frozen in liquid N_2 and stored at -80°C for later measurements of whole-body ion content.

2.4 Whole-body or carcass Na^+ and Ca^{2+} content measurements

Similar approaches were used to measure whole body ion content in larvae (Zimmer et al., 2021) or carcass ion content in adult fish (Kumai et al., 2011). Larvae were reared in control in pH 4 conditions to 4 dpf and were grouped into 15 larvae per sample. The choice of 4 dpf for measurements reflected the results of a pilot trial that followed whole-body ion content in WT larvae from 2 to 10 dpf, as well as previous literature. Samples were digested in 100 µL 2N HNO_3 at 65°C until complete digestion (generally <36h). Samples were diluted 15-fold with dH_2O prior to measurement of Na^+ and Ca^{2+} content by flame emission spectrophotometry

(model AA260, Varian, Palo Alto, CA, USA). The standards used for these measurements contained the same concentration of HNO_3 as the samples.

After a 7 d exposure to control or pH 4, adult carcasses (lacking the gill basket) were pulverized in liquid N_2 to a powder using a mortar and pestle and separated into pre-weighed centrifuge tubes, weighed, and digested in 2 mL 2N HNO_3 at 65°C until complete digestion (~48 h). Samples were diluted 15-fold dilution and measured as above.

2.5 Plasma cortisol levels

Blood plasma was collected from adult zebrafish by caudal severance according to the protocol of Babaei et al. (2013). Blood samples were centrifuged at 13,800 g for 5 min and plasma was collected and stored at -80°C until analysis. Prior to analysis, plasma samples were diluted ~30-fold using the extraction buffer with the commercial enzyme-linked immunosorbent assay kit (EIA; Neogen, Lexington, KY, USA) that was used to assay cortisol levels. Cortisol concentrations were assayed in duplicate according to the manufacturer's instructions. The intra-assay variation was 4.3% CV and inter-assay variation was 4.9% CV.

2.6 Whole-mount immunohistochemistry

Whole-mount IHC was used to quantify ionocyte abundances in 4 dpf larvae and adult gill tissue. Three types of ionocytes were assessed. The HR cells were labelled using the lectin concanavalin A (ConA) in larvae (Yan et al., 2007) or an antibody (see Table 2.2 for information on antibodies) raised against the α subunit of V-type H^+,ATPase in gill tissue (Chang et al., 2009; Kwong and Perry, 2013). The NaR cells were labelled using an antibody raised against the α subunit of NKA (Lin et al., 2006), and the NCC cells were labelled using an antibody raised against the zebrafish NCC (Kwong and Perry, 2016).

Immediately prior to being euthanized, larvae were incubated with ConA (50 ug L^{-1}) conjugated to Alexa Fluor 488 (Invitrogen, Waltham, MA, USA) for 30 min. Larvae were then euthanized, washed 5 x 5 min in phosphate-buffered saline containing 0.1% Tween 20 (PBST), and fixed in 4% paraformaldehyde (PFA) overnight at 4°C. Fixed larvae were washed 5 x 5 min in PBST and dehydrated in a stepwise fashion by incubating with 50% and 75% MeOH, each for 10 min, followed by 100% MeOH for 1h at -20°C. Samples were rehydrated by incubation in 75% and 50% MeOH in PBST in 10 min steps, followed by 5 x 5 min washes in 100% PBST. Antigen retrieval was carried out by incubating samples in Tris-HCl (150 mM) for 10 min at room temperature, then 15 min at 65°C. Following 5 x 5 min washes in PBST, larvae were immersed for 2 h at room temperature in a blocking solution containing 3% bovine serum albumin (BSA; Sigma-Aldrich, Oakville, ON, Canada) in PBST, 0.8% Triton X-100. Primary antibodies against NKA and NCC (Table 2.2) were added to the solution for an overnight incubation at 4°C. Following 5 x 5 min washes in PBST, samples were incubated in PBST plus 0.8% Triton X-100 containing secondary antibodies (Table 2.2) at a 1:500 dilution for 1 h in the dark at room temperature. After a final round of 5 x 5 mins washes in PBST, samples were mounted in depression slides using warm 1.5% agarose and VectaShield mounting medium containing 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA, USA).

Gill baskets from adult fish were fixed overnight in 4% PFA in phosphate-buffered saline (PBS) at 4°C. The fixed gill baskets were rinsed 5 x 5 min in PBST and the second and third gill arches were separated from the right and left side of the gill basket and placed together in separate 2 mL centrifuge tubes, for staining of NKA and V-type H^+-ATPase using one pair of arches and NCC using the other pair. Antigen retrieval was performed by incubating samples in Tris-HCl (150mM) at room temperature for 10 min followed by 15 min at 65°C. After another

round of washes 5 x 5 min with PBST, gill samples were incubated in blocking solution (as described above) with gentle agitation on a plate shaker overnight at 4°C. Primary antibodies (Table 2.2) were added to the solution and left to incubate overnight at 4°C. Following 5 x 5 min washes in PBST, samples were incubated with secondary antibodies (Table 2.2) in PBST plus 0.8% Triton X-100 overnight at 4°C on the plate shaker. Gill samples were washed 5 x 5 min in PBST and mounted as described above.

Samples were imaged using a Nikon A1RsiMP confocal microscope (Nikon Instruments Inc, Tokyo, Japan) with continuous wave lasers emitting at 488, 561, and 633 nm. Ionocyte densities were evaluated by counting labelled cells in maximum intensity projection Z-stack images with optical sections of 3 µm thick. Ionocyte abundances were counted by an observer who was blind to the genotype and treatment group of the fish from which the sample was obtained. The multi-point tool in Image J software (National Institute of Health, Bethesda, MD, USA) was used and the area (of the yolk sac in larvae or gill filament in adults) was measured (using Image J) to calculate ionocyte density (in cells mm^{-2})

2.7 Measurement of transcript abundances by semi-quantitative real-time RT-PCR

Real-time RT-PCR was used to evaluate the expression of genes that have been implicated in Na^+ and Ca^{2+} uptake of zebrafish and that have been reported to show changes in transcript abundance (or protein levels) following exposure to pH 4 water. These included *ncc* (*slc12a10.2*; Chang et al., 2013), *nhe3b* (*slc9a3.2;* Chang et al., 2013), *atp6v1aa* (Shih et al., 2022; Shir-Mohammadi and Perry, 2020), *ca17a* (Shir-Mohammadi and Perry, 2020), and *ecac* (Kumai et al., 2015).

Gill tissue was thawed on ice and sonicated (Sonic Dismembrator Model 100, Fisher Scientific, Ottawa, ON, Canada) on ice in 500µL of TRIzol reagent (catalogue # 15596026; Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The total RNA extracted using this protocol was suspended in nuclease-free water and quantified using a Nanodrop UV-vis spectrophotometer (ND 2000, ThermoFisher Scientific, Waltham, MA, USA). Following treatment with amplification grade DNaseI (catalogue # 18068015; Invitrogen) to remove genomic DNA, 1 µg of RNA was used to synthesize cDNA using the iScript reverse transcription kit (catalogue # 1708890; Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions.

Real-time RT-PCR was carried out using a CFX96 Touch Real-Time PCR system (Bio-Rad) in 20 µL duplicate reactions. Each reaction contained 1 µL of cDNA template, 500mM of gene-specific forward and reverse primers (Table 2.3), and 10 µL of SsoAdvanced Universal SYBR Green Supermix (catalogue # 1725271; Bio-Rad). Each assay included a no-template control (cDNA replaced with water), and a no-reverse transcriptase sample (to check for genomic DNA contamination; cDNA synthesis carried out without reverse transcriptase) Assays were carried out using the PCR cycling conditions suggested by the manufacturer, namely 95°C for 3 min, 40 cycles of 95°C for 10 s, 60°C for 10 s, and 72°C for 30 s.

Melting curve analyses were completed for each assay to confirm the absence of non-specific amplification. A standard curve was generated using a pooled sample of cDNA for each primer pair to confirm reaction efficiency (Table 2.3). Relative transcript abundances were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) with *rpl13a* (NM_212784.1) and *eef1a1* (NM_131263.1) as reference genes and expressed relative to the value for WT fish exposed to control conditions.

2.8 Statistical analyses

Data are presented as mean values ± 1 standard error of the mean (SEM). All statistical analyses were carried out using SigmaPlot 15.0 (Systat Software, Palto Alto, CA, USA). Grubb's test was used to remove outliers from each dataset before analysis. The Shapiro-Wilk test and Levene's test were used to check data for normality and equal variance, respectively. Data that did not meet these assumptions were transformed to meet the assumptions; if this could not be achieved, then an equivalent non-parametric test was used. In general, data were evaluated by two-way analysis of variance (ANOVA) using genotype (WT, *nr3c1*$^{-/-}$, *nr3c2*$^{-/-}$) and treatment group (control vs pH 4) as the two factors. This approach was used to analyse effects of genotype and exposure to pH 4 water on skin (larvae) or gill (adult) ionocyte abundances, whole-body (larvae) or carcass (adult) Na^+ and Ca^{2+} content, and plasma cortisol concentrations and gill transcript abundances in adult fish. For statistical analysis, gill transcript abundance data were $\log_2$ transformed. Body length and body mass were evaluated by one-way ANOVA. Because our focus was on the role of glucocorticoid receptors in mediating these responses, we did not make comparisons between larval and adult fish, across ionocyte types, or among transcript abundances of different genes.

Table 2.1 Primers used for multiplex PCR assessment of fish genotype

Primer	**Sequence (5'-3')**
NR3C1 F1	ATC CTA TCA AGG AGC TGT GTC
NR3C1 F2	CTG AGA ACG ATG CAC ACT TAC
NR3C1 R1	TAA TGA ATT AGT CTG ACC CCG
NR3C1 R3	GAC TGA ACC ATT AAC TGA CCG
NR3C2 F1	TGC AGC TTT TAC TGT GCT GAG
NR3C2 F2	TTA CAG TGA GTA CTG GTC ACG
NR3C2 R1	GCT GTA GAA CAT AAG TCC TCC

Table 2.2 Antibodies used in immunohistochemistry

Antibody	Target	Host	Dilution	Source
Primary				
anti-NCC	NCC (SLC2A10.2)	Rabbit, polyclonal	1:250	Custom antibody generated by Genscript (Kwong and Perry, 2016)
anti-H^+-ATPase	α-subunit of H^+-ATPase	Rabbit, polyclonal	1:250	Gift of Dr. M. Uchuyama, University of Toyama
α5	α-subunit of Na^+,K^+-ATPase	Mouse, monoclonal	1:250	Developmental Studies, Hybridoma Bank
Secondary				
Alexa fluor 633 conjugated anti-rabbit IgG	Used with anti-NCC primary	donkey	1:500	Invitrogen, Burlington, ON
Alexa fluor 488 conjugated anti-rabbit IgG	Used with α-subunit of H^+-ATPase primary	donkey	1:500	Invitrogen, Burlington, ON
Alexa fluor 564 conjugated anti-mouse IgG	Used with α-subunit of Na^+,K^+-ATPase primary	donkey	1:500	Invitrogen, Burlington, ON

Table 2.3 Primers used for real-time RT-PCR

Gene	Primer Pair (5'-3')	Amplicon Size (bp)	Annealing Temp (°C)	GenBank accession #	Reference	Efficiency (%)	R^2
ecac	FWD: TCC TTT CCC ATC ACC CTC T REV: GCA CTG TGG CAA CTT TCG T	261	53	NM_001001849	(Kumai et al., 2015)	104.5	0.985
*slc12a10.2 (*NCC*)*	FWD: GCC CCC AAA GTT TTC CAG TT REV: TAA GCA CGA AGA GGC TCC TTG	101	54	NM_001045001	(Shir-Mohammadi and Perry, 2020)	104.3	0.990
atpv6v1aa	FWD: GAG GAA CCA CTG CCA TTC CA REV: CAA CCC ACA TAA ATG ATG ACA TCG	103	51	NM_201135.2	(Shih et al., 2022)	106.5	0.980
ca17a	FWD: CAT CTG TGC CGA CCG TTG CT REV: TTT TGT GCG TGG TTT CCC G	151	55	NM_ BC057412	(Shir-Mohammadi and Perry, 2020)	98.1	0.987
*slc9a3.2 (*NHE3b*)*	FWD: TGC AGA CAG CGC CTC TAG C REV: TGT GGC CTG TCT CTG TTT GC	155	51	XM_021468124.1	(Chang et al., 2013)	106.9	0.986
rpl13a	FWD: TCTGGAGGACTGTAAGAGGTATGC REV: AGACGCACAATCTTGAGAGCAG	144	55	NM_212784.1	(Toms et al., 2020)	100.4	0.980
eefl1a	FWD - TACCTACCCTCCTCTTGGTCG REV - TTGGAACGGTGTGATTGAGGG	101	55	NM_131263.1	(Pan et al., 2021)	105.0	0.994

Chapter 3: Results

3.1 Generation of nr3c1$^{-/-}$ (GR-KO) and nr3c2$^{-/-}$ (MR-KO) lines

Using CRISPR/Cas9 gene editing techniques, either the *nr3c1* (GR-KO) or *nr3c2* (MR-KO) was deleted. GR knockout was confirmed at the genetic level using Sanger sequencing (Fig. 3.1A). Alignment with the wildtype *nr3c1* genomic sequence at Chromosome 14 and amplified GR-KO PCR products confirmed the deletion of 57,852 bp. Confirmation of gene deletion in MR-KO was completed prior to the generation of the GR-KO line and is not included in the present thesis. Knockout was also confirmed using a multiplex PCR approach that showed the absence of a WT amplicon in identified GR-KO and MR-KO fish (Fig 3.1B-C, respectively). No obvious differences in survival to 4 dpf or sexual maturity were noted among genotypes. However, we observed that adult GR-KO fish tended to be larger than WT or MR-KO fish. At 6 dpf, there was no significant effect of genotype on length (Fig 3.1D, one-way ANOVA, $p = 0.268$). However, the mass of adult fish (8-12 months) differed significantly among genotypes (Fig 3.1E, one-way ANOVA, $p <$ **0.001**) with GR-KO fish having larger masses than WT (Tukey HSD, $p <$ **0.001**) and tending to be larger than MR-KO fish (Tukey HSD, $p = 0.051$). In addition, it became apparent that female GR-KO adults were relatively rare, and this was not the case with WT or MR-KO fish. Assessment of female vs male numbers across three groups of GR-KO fish bred at different times revealed sex ratios of 5:22, 5:22 and 7:21 (female:male), suggesting a male-biased sex ratio in GR-KO fish. The GR-KO fish were also more difficult to breed, and there was a higher rate of fertilization when GR-KO fish were 4-6 m old than as they aged.

3.2 Whole-body ion content

After 7 d of exposure to pH 4 water, the whole-body Na^+ content of adult fish did not differ significantly as a result of genotype or treatment, although there was a trend towards an interaction between these factors (Fig.3.2A, two-way ANOVA, $p_{geno} = 0.727$, $p_{treat} = 0.693$, $p_{genoxtreat} = 0.087$). Given this trend, comparisons between control and pH 4-exposed fish were carried out within each genotype. No significant differences between treatment groups were detected within WT (Student's *t*-test, p = 0.726), or MR-KO (Student's *t*-test, p = 0.204) fish. In contrast, GR-KO fish exposed to pH 4 conditions had significantly lower whole-body Na^+ content than control fish (Student's *t*-test, p = **0.0449**).

Whole-body Ca^{2+} levels in adult fish did not differ significantly with genotype or 7 d exposure to pH 4 water, yet the interaction between these factors was close to statistical significance (Fig. 3.2B, two-way ANOVA, $p_{geno} = 0.557$, $p_{treat} = 0.147$, $p_{genoxtreat} = 0.081$). Given this trend, comparisons between control and pH 4-exposed fish were carried out within each genotype. However, no significant differences were detected (Student's *t*-tests, WT, p = 0.134, MR-KO, p = 0.0521, GR-KO, p = 0.352).

In 4 dpf larvae, genotype but not rearing in pH 4 water had a significant effect on whole-body Na^+ content (Fig.3.3A, two-way ANOVA**,** p_{geno} **= 0.003**, $p_{treat} = 0.619$, $p_{genoxtreat} = 0.350$). The GR-KO and MR-KO larvae had significantly higher whole-body Na^+ content relative to WT larvae (WT, 9.5 ± 0.4 nmol individual^{-1}, N = 15; GR-KO, 11.5 ± 0.2 nmol individual^{-1}, N = 11; MR-KO, 11.6 ± 0.2 nmol individual^{-1}, N = 10; values are presented as means ± SEM thereafter).

Whole-body Ca^{2+} content of 4 dpf larvae was significantly affected by both genotype and rearing in pH 4 water, but the interaction of these two factors was not significant (Fig.3.3B, two-way ANOVA**,** p_{geno} **< 0.001**, p_{treat} **= 0.041**, $p_{genoxtreat} = 0.227$). In terms of genotype, GR-KO larvae had significantly higher whole-body Ca^{2+} content than WT or MR-KO larvae (WT, 6.2 ±

0.3 nmol individual^{-1}, N = 15; GR-KO, 8.1 ± 0.6 nmol individual^{-1}, N = 11; MR-KO, 5.8 ± 0.3 nmol individual^{-1}, N = 10). For larvae reared in pH 4 water, whole-body Ca^{2+} content (6.2 ± 0.3 nmol individual^{-1}, N = 19) was significantly lower than that for larvae reared under control conditions (7.1 ± 0.4 nmol individual^{-1}, N = 18).

3.3 Plasma cortisol levels

After 7 d exposure to pH 4 water, plasma cortisol levels of adult fish differed significantly by genotype, but not exposure or the interaction of these factors (Fig.3.4, two-way ANOVA on log 10 transformed data**,** p_{geno} **< 0.001**, p_{treat} = 0.061, $p_{genoxtreat}$ = 0.173). Plasma cortisol levels were significantly higher in GR-KO fish (244.3 ± 41.3 ng mL^{-1}, N = 23) compared to WT (34.5 ± 8.41 ng mL^{-1}, N = 18) and MR-KO, (43.5 ± 11.09 ng mL^{-1}, N = 23) fish.

3.4 Ionocyte abundances

Exposure to pH 4 water for 7 d had significant effects on the abundance of HR cells (Fig. 3.5A, two-way ANOVA on log 10 transformed data, p_{geno} = 0.079, p_{treat} = 0.961, $p_{genoxtreat}$ = **0.005**) in gill tissue of adult fish. In WT fish exposed to pH 4, HR cell abundance was significantly higher than in fish held in system water (Tukey HSD, p = **0.045**). By contrast, HR cell abundance in GR-KO fish after 7 d in pH 4 water was significantly lower than values for fish held under control conditions. (Tukey HSD, p = **0.011**) No significant difference was detected in HR cell abundances in MR-KO fish between control and pH 4 treatments (Tukey HSD, p = 0.342). Under control conditions, HR cell densities in WT and MR-KO fish were comparable (Tukey HSD, p = 0.943), whereas GR-KO fish exhibited significantly higher HR cell abundance (Tukey HSD, p = **0.001**). However, no significant differences were detected when HR cell densities were compared across genotypes under pH 4 conditions (Tukey HSD, p = 0.519).

Similarly, NaR cell abundances in adult gill tissue were significantly affected by exposure to pH 4 water for 7 d (Fig. 3.5B, two-way ANOVA on log 10 transformed data, $p_{geno} = 0.018$, $p_{treatment} = 0.191$, $p_{genoxtreat}$ **= 0.018**) in adults. Under control conditions, GR-KO and MR-KO fish had lower NaR cell abundance than WT fish (Tukey HSD, p = **0.007**, p = **0.010**, respectively), but no significant differences were detected among genotypes in fish held in pH 4 water (Tukey HSD, p = 0.097). Additionally, in WT but not GR-KO or MR-KO fish, NaR cell abundance was significantly lower in fish exposed to pH 4 water than in fish held under control conditions (Tukey HSD, p = **0.007**).

No statistically significant differences in NCC cell densities in gill tissue of adult fish were detected with pH 4 water exposure, genotype or the interaction of these factors (Fig. 3.5C, two-way ANOVA, $p_{genotype} = 0.314$, $p_{treatment} = 0.848$, $p_{genotypextreatment} = 0.641$).

In larval fish, both genotype and rearing to 4 dpf in pH 4 water had significant effects on the abundance of HR cells, but the interaction of these factors did not reach statistical significance (Fig. 3.6A, two-way ANOVA on square transformed data, p_{geno} **= 0.032**, p_{treat} = **0.035**, $p_{genoxtreatt} = 0.525$). The GR-KO larvae exhibited significantly lower HR cell densities relative to WT and MR-KO larvae (WT, 182.0 ± 24.6 cell mm^{-2}, N = 13; GR-KO, 154.6 ± 19.1 cell mm^{-2}, N = 19; MR-KO, 231.2 ± 6.6 cell mm^{-2}, N = 15). The density of HR cells was significantly higher in larvae reared to 4 dpf in pH 4 water than in larvae reared in control water (control, 138.1 ± 22.5 cell mm^{-2}, N = 12; pH 4, 212.0 ± 13.9 cell mm^{-2}, N = 19). Notably, GR larvae appeared to have a stronger response to pH 4 water than WT or MR-KO larvae although this was not detected statistically.

The density of NaR cells in 4 dpf larvae was similarly significantly influenced by genotype and water conditions but without interaction between these two factors (Fig. 3.6B, two-

way ANOVA on log 10 transformed data, p_{geno} = **0.045**, p_{treat} = **0.005**, $p_{genoxtreat}$ = 0.30) The density of NaR cells was higher in WT larvae compared to GR-KO and MR-KO larvae (WT, 140.3 ± 19.5 cell mm^{-2}, N = 14; GR-KO, 96.7 ± 7.0 cell mm^{-2}, N = 20; MR-KO, 95.7 ± 10.6 cell mm^{-2}, N = 15). NaR cell density was lower in larvae raised in pH 4 than in larvae raised under control conditions (control, 131.5 ± 13.0 cell mm^{-2}, N = 22; pH 4, 90.4 ± 6.7 cell mm^{-2}, N = 27).

The density of NCC cells was significantly affected by the interaction of genotype and water pH (Fig. 3.6C, two-way ANOVA on reciprocal-transformed data, p_{geno} = 0.046, p_{treat} = 0.776, $p_{genoxtreat}$ = **0.047**). Fish raised in pH 4 water did not show significant differences in NCC cell density from fish raised under control conditions, nor was there a significant effect of genotype on NCC cell density for fish raised under control conditions. However, for fish raised in pH water, NCC cell density was significantly higher in GR-KO larvae than in WT larvae.

Representative images of each ionocyte type are presented in Figure 3.7.

3.5 Relative transcript abundance genes involved in ion uptake

Relative transcript abundances of genes implicated in Na^+ or Ca^{2+} uptake were measured in gill tissue of adult WT, GR-KO and MR-KO fish held under control conditions or following 7 d of exposure to pH 4 water. Gill tissue from fish exposed to pH 4 water showed significantly higher transcript abundances of *ca17a* (Fig. 3.8A, two-way ANOVA on rank transformed data, p_{geno} = 0.208, p_{treat} = **0.008**, $p_{genoxtreat}$ = 0.860) and *ecac* (Fig. 3.8B, two-way ANOVA on rank transformed data, p_{geno} = 0.073, p_{treat} = **0.039**, $p_{genoxtreat}$ = 0.983), with no effect of genotype. For *ca17a*, transcript abundance was 1.34 ± 0.21 (N = 18) under control conditions and 2.32 ± 0.24 (N = 18) following 7 d exposure to pH 4 water; the values for *ecac* were 1.11 ± 0.20 (N = 18) and 1.80 ± 0.26 (N = 17) for control and pH 4 conditions, respectively.

A significant effect of genotype but not exposure to pH 4 water was detected for mRNA expression of *ncc* (Fig. 3.8C, two-way ANOVA, $p_{geno} <$ **0.001**, $p_{treat} = 0.079$, $p_{genoxtreat} = 0.711$). Relative mRNA abundance of *ncc* was similar in gill tissue of WT (1.39 ± 0.17, N = 12) and MR-KO (1.21 ± 0.15, N = 10) fish and higher than that for gill tissue from GR-KO fish (0.31 ± 0.05, N = 12).

No significant differences were found in *nhe3b* transcript abundances with water pH, genotype, or the interaction of these two factors (Fig. 3.8D, two-way ANOVA, $p_{geno} = 0.266$, $p_{treat} = 0.481$, $p_{genoxtreat} = 0.214$). This was also the case for *atp6v1aa* transcript abundance (Fig. 3.8E, two-way ANOVA, $p_{geno} = 0.150$, $p_{treat} = 0.883$, $p_{genoxtreat} = 0.068$).

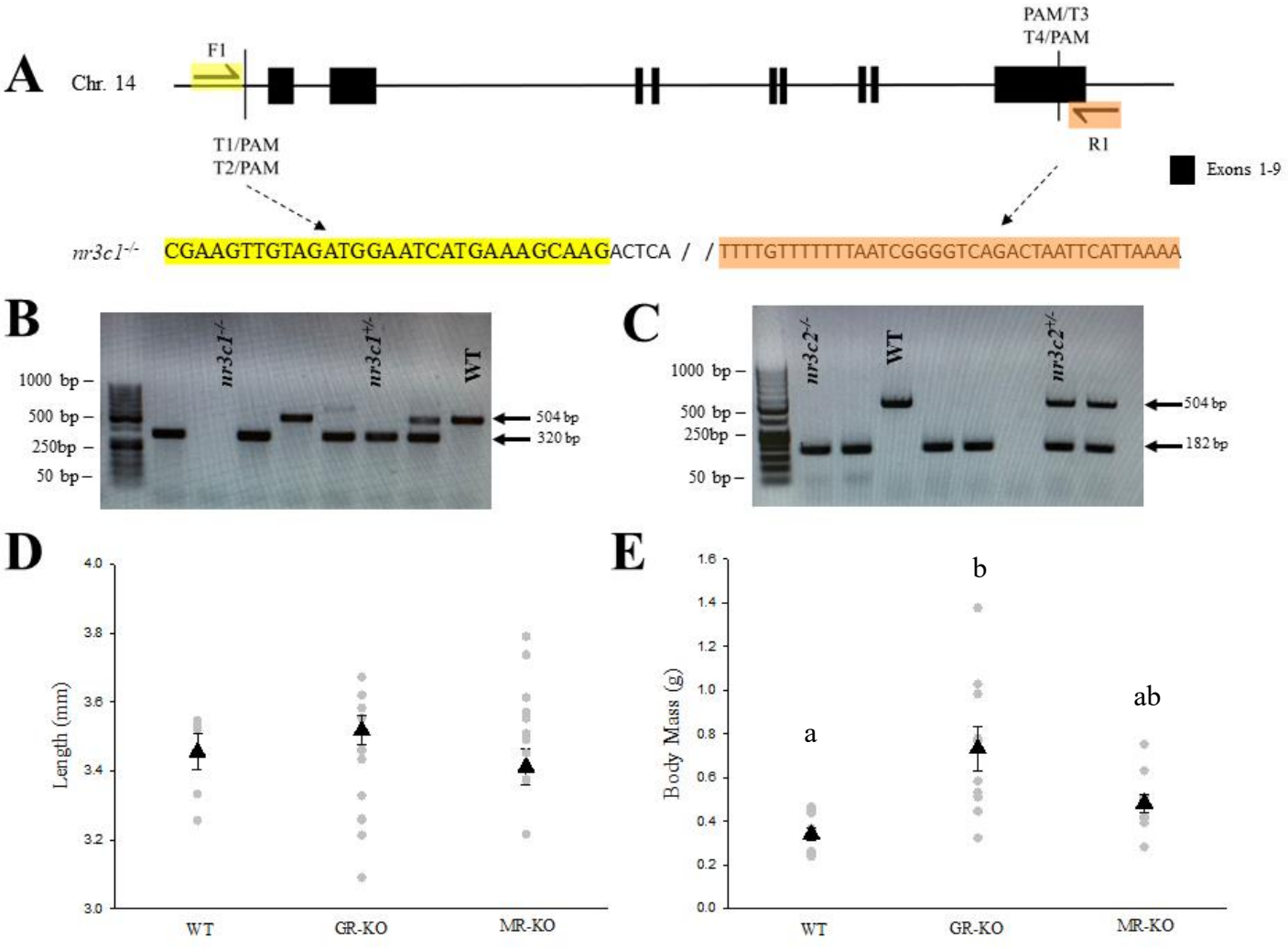
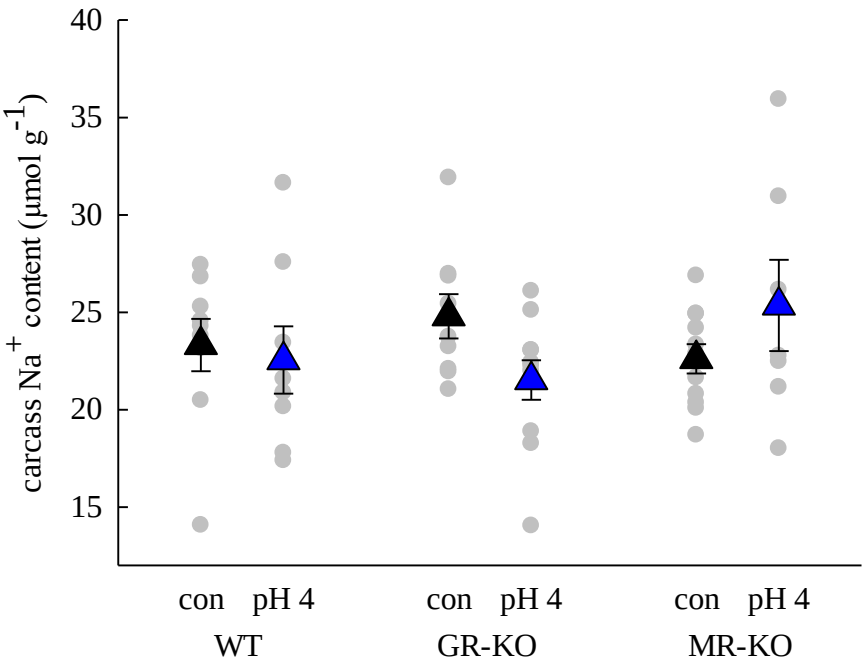

A
Chr. 14
F1
T1/PAM
T2/PAM
PAM/T3
T4/PAM
R1
Exons 1-9
nr3c1-/-
CGAAGTTGTAGATGGAATCATGAAAGCAAGACTCA / / TTTTGTTTTTTTAATCGGGGTCAGACTAATTCATTAAAA
B
1000 bp
500 bp
250bp
50 bp
nr3c1-/-
nr3c1+/-
WT
504 bp
320 bp
C
1000 bp
500 bp
250bp
50 bp
nr3c2-/-
WT
nr3c2+/-
504 bp
182 bp
D
Length (mm)
4.0
3.8
3.6
3.4
3.2
3.0
WT
GR-KO
MR-KO
E
Body Mass (g)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
a
b
ab
WT
GR-KO
MR-KO

Figure 3.1. Confirmation of *nr3c1*$^{-/-}$ (GR-KO) and *nr3c2*$^{-/-}$ (MR-KO) knockout in generating mutant zebrafish (*Danio rerio*) lines. (A) DNA sequencing of GR-KO fish aligned with *nr3c1* loci (Chromosome 14). (B-C) Visualized agarose gel electrophoresis of multiplex PCR products from caudal fins of F2 generation of *nr3c1*$^{+/-}$ *and nr3c2*$^{+/-}$ offspring, respectively. (C) Body lengths of 6 dpf larvae (D) Body mass of adult zebrafish. Triangles represent mean values (± SEM, N = 6-13) and grey circles represent values for individual fish. Data were evaluated by one-way ANOVA. Mean values that do not share a letter are significantly different from one another (see text for details).

A


40
35
30
25
20
15
carcass Na+ content (µmol g-1)
con
pH 4
con
pH 4
con
pH 4
WT
GR-KO
MR-KO

B

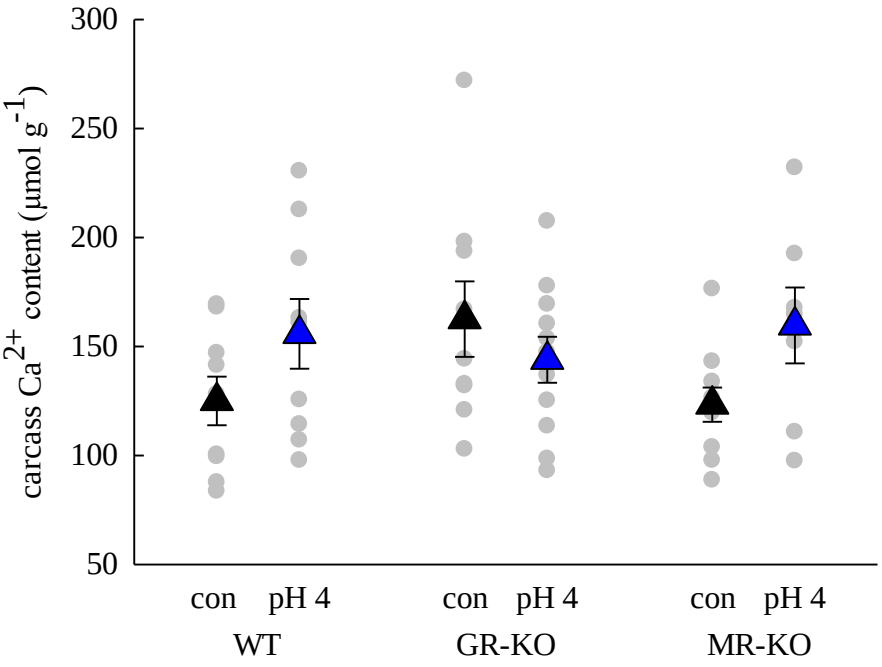
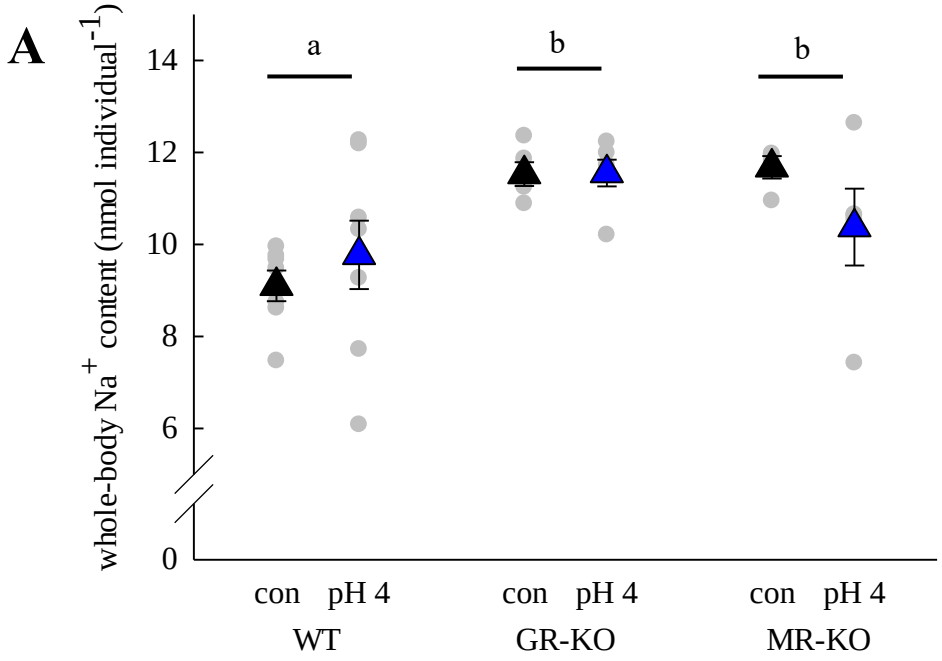

300
250
200
150
100
50
carcass Ca2+ content (µmol g-1)
con
pH 4
con
pH 4
con
pH 4
WT
GR-KO
MR-KO

Figure 3.2. Carcass (A) Na^+ and (B) Ca^{2+} content in adult wildtype (WT), GR-KO and MR-KO zebrafish (*Danio rerio*) exposed to control or pH 4 water for 7 d. Triangles represent mean values (± SEM, N = 7-12) and grey circles represent values for individual fish. Data were evaluated by two-way ANOVA and no significant differences were detected (see text for details).


A
whole-body Na+ content (nmol individual-1)
14
12
10
8
6
0
a
b
b
con
pH 4
con
pH 4
con
pH 4
WT
GR-KO
MR-KO

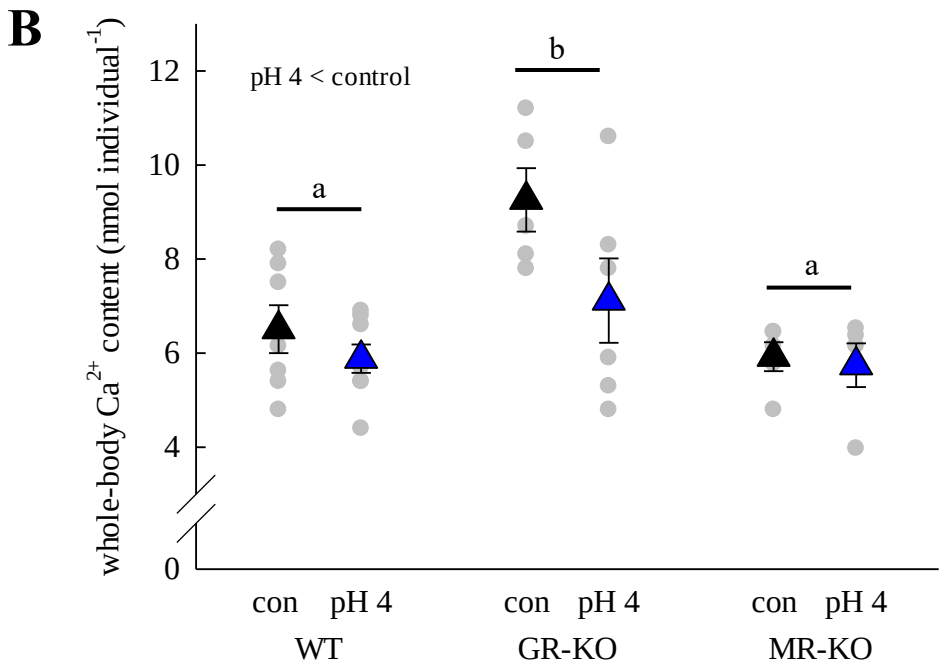

B
whole-body Ca2+ content (nmol individual-1)
12
10
8
6
4
0
pH 4 < control
a
b
a
con
pH 4
con
pH 4
con
pH 4
WT
GR-KO
MR-KO

Figure 3.3. Whole-body (A) Na^+ and (B) Ca^{2+} levels in 4 dpf wildtype (WT), GR-KO and MR-KO zebrafish (*Danio rerio*) reared in control or pH 4 water. Triangles represent mean values (± SEM, N = 5-7) and grey circles represent values for individual fish. Data were evaluated by two-way ANOVA. Mean values that do not share a letter are significantly different from one another. Significant effects of water pH are indicated on the relevant panel figures in text (i.e. pH 4 < control; see text for details).

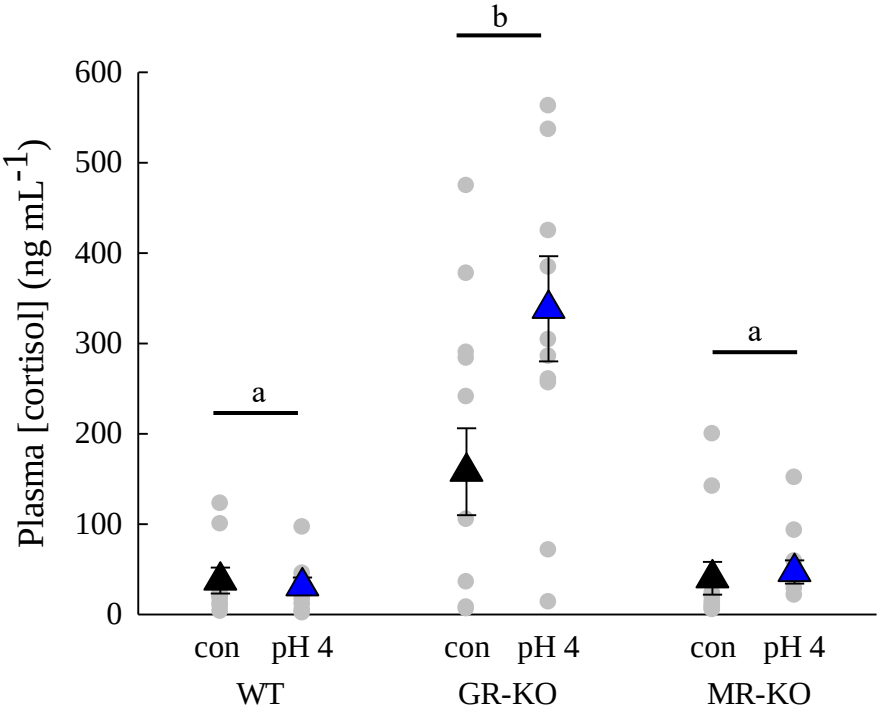


Figure 3.4. Plasma cortisol concentrations of adult wildtype (WT), GR-KO and MR-KO zebrafish (*Danio rerio*) exposed to control or pH 4 water for 7 d. Triangles represent mean values (± SEM, N = 9-15 per group) with grey circles representing values for individual fish. Mean values that do not share a letter are significantly different from one another (two-way ANOVA; see text for details).

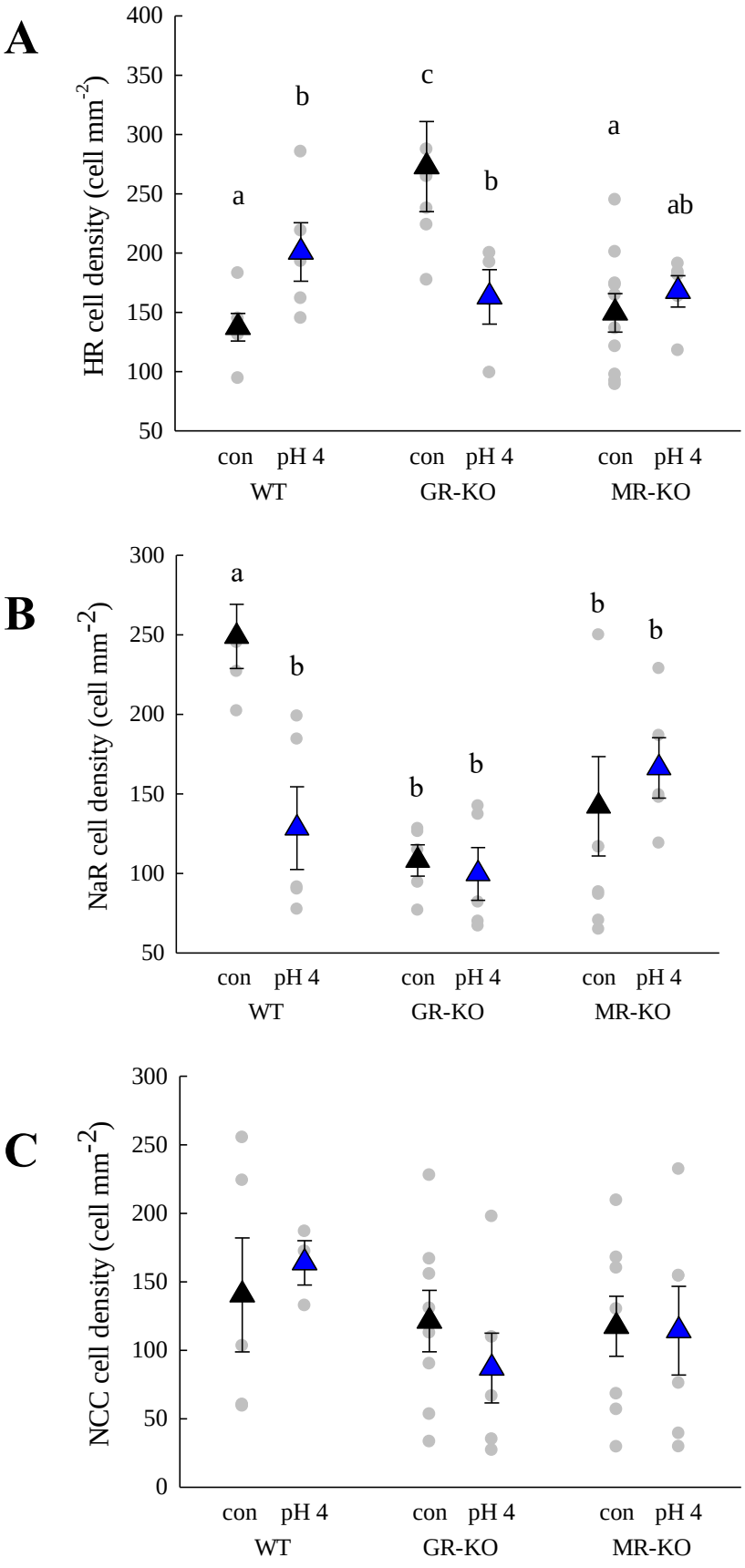
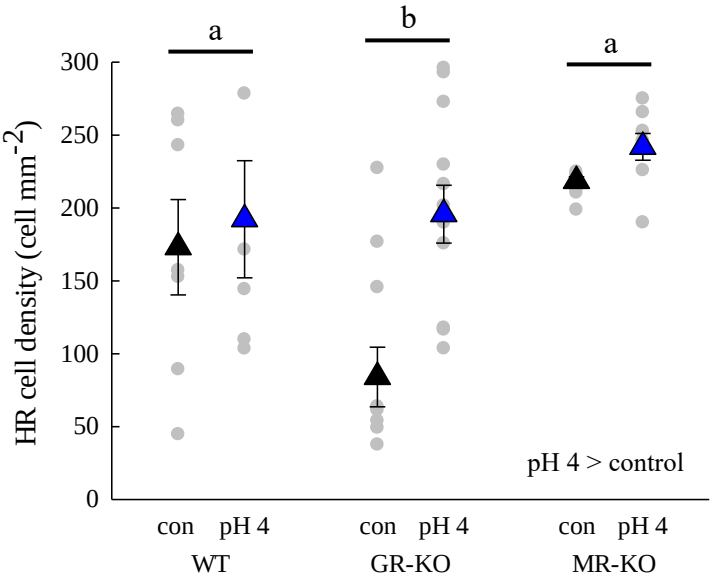
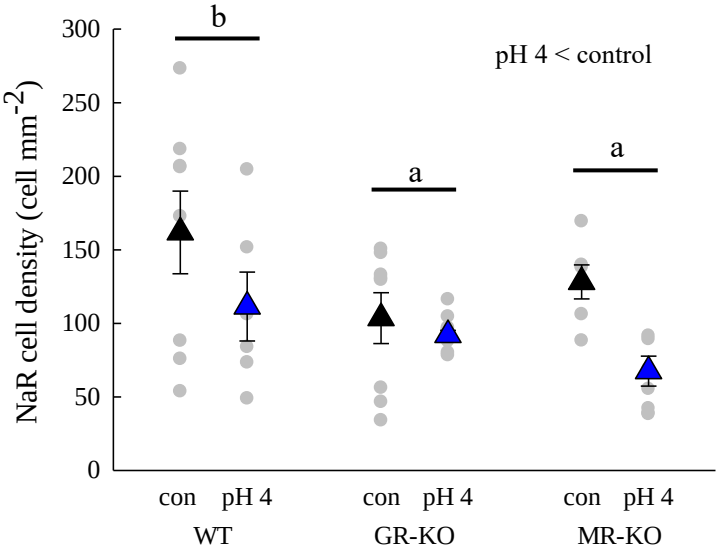

A
HR cell density (cell mm-2)
400
350
300
250
200
150
100
50
a
b
c
b
a
ab
con
pH 4
con
pH 4
con
pH 4
WT
GR-KO
MR-KO
B
NaR cell density (cell mm-2)
300
250
200
150
100
50
a
b
b
b
b
b
con
pH 4
con
pH 4
con
pH 4
WT
GR-KO
MR-KO
C
NCC cell density (cell mm-2)
300
250
200
150
100
50
0
con
pH 4
con
pH 4
con
pH 4
WT
GR-KO
MR-KO

Figure 3.5. Abundance of (A) H^+-ATPase rich (HR) cells, (B) Na^+,K^+-ATPase rich (NaR) cells, and (C) Na^+,Cl^--cotransporter (NCC) cells in gill tissue of adult wildtype (WT), GR-KO and MR-KO zebrafish (*Danio rerio*) exposed to control or pH 4 water for 7 d. Triangles represent mean values (± SEM, N = 3-10 per group) with grey circles representing values for individual fish. Mean values that do not share a letter are significantly different from one another (two-way ANOVA; see text for details).

A


a
b
a
HR cell density (cell mm-2)
300
250
200
150
100
50
0
pH 4 > control
con pH 4
WT
con pH 4
GR-KO
con pH 4
MR-KO

B


b
a
a
pH 4 < control
NaR cell density (cell mm-2)
300
250
200
150
100
50
0
con pH 4
WT
con pH 4
GR-KO
con pH 4
MR-KO

C

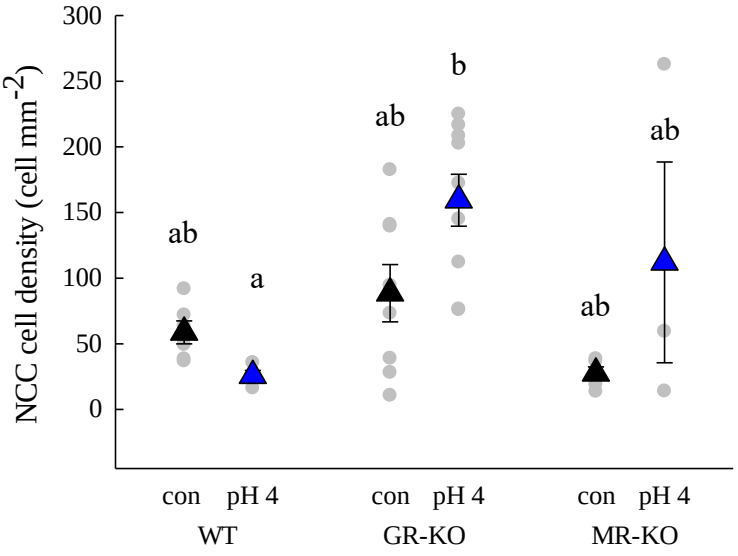

ab
a
ab
b
ab
ab
NCC cell density (cell mm-2)
300
250
200
150
100
50
0
con pH 4
WT
con pH 4
GR-KO
con pH 4
MR-KO

Figure 3.6. Abundance of (A) H^+-ATPase rich (HR) cells, (B) Na^+,K^+-ATPase rich (NaR) cells, and (C) Na^+,Cl^--cotransporter (NCC) cells in 4 dpf wildtype (WT), GR-KO and MR-KO zebrafish (*Danio rerio*) reared in control or pH 4 water. Triangles represent mean values (± SEM, N = 3-10 per group) with grey circles representing values for individual fish. Mean values that do not share a letter are significantly different from one another. (two-way ANOVA; see text for details). Significant effects of water pH are indicated on the relevant panel figures in text (i.e. pH 4 < control; see text for details).

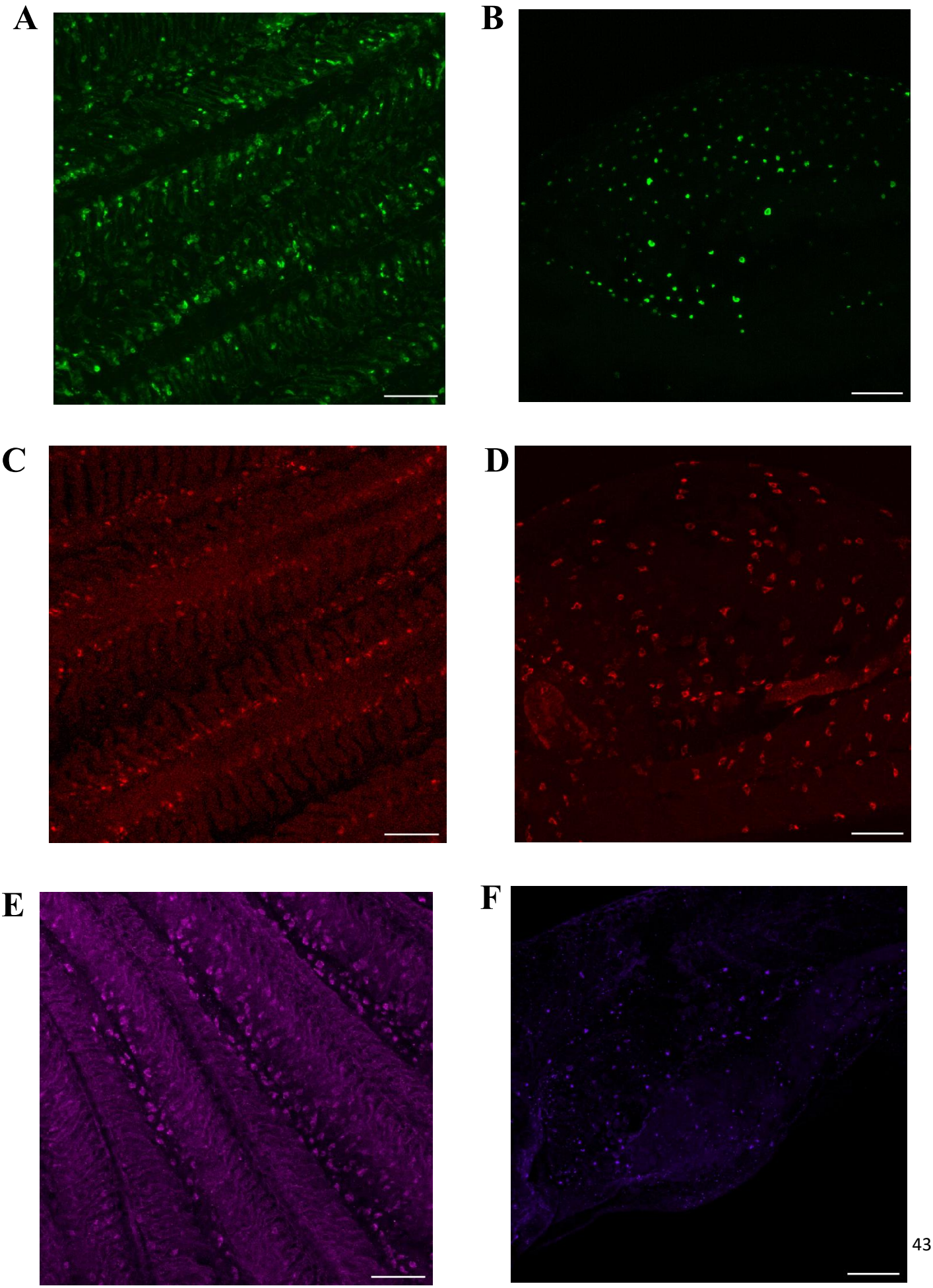
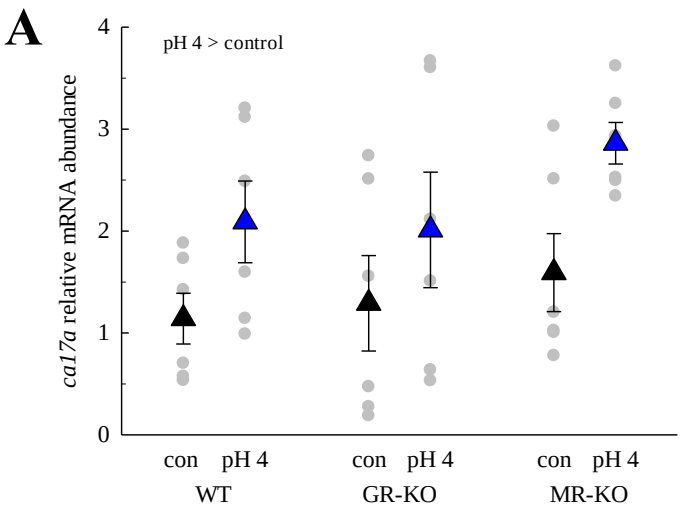
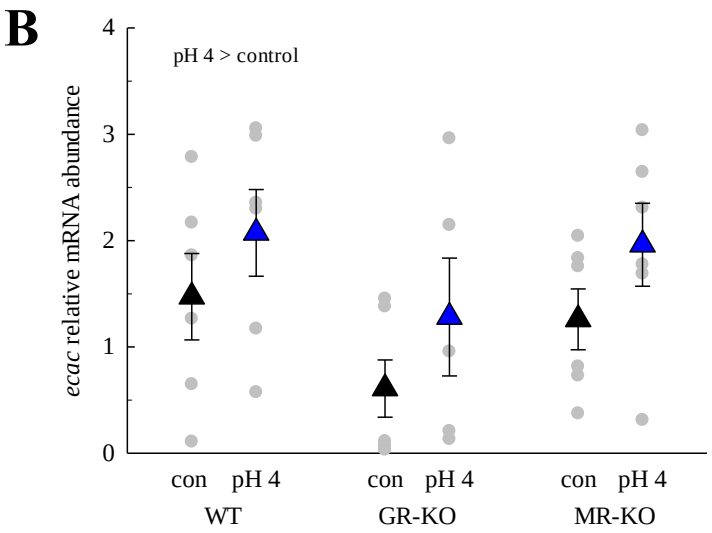
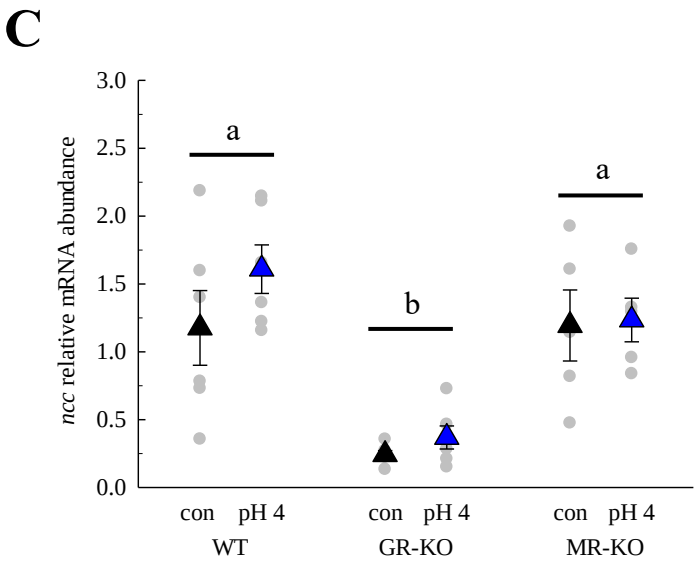
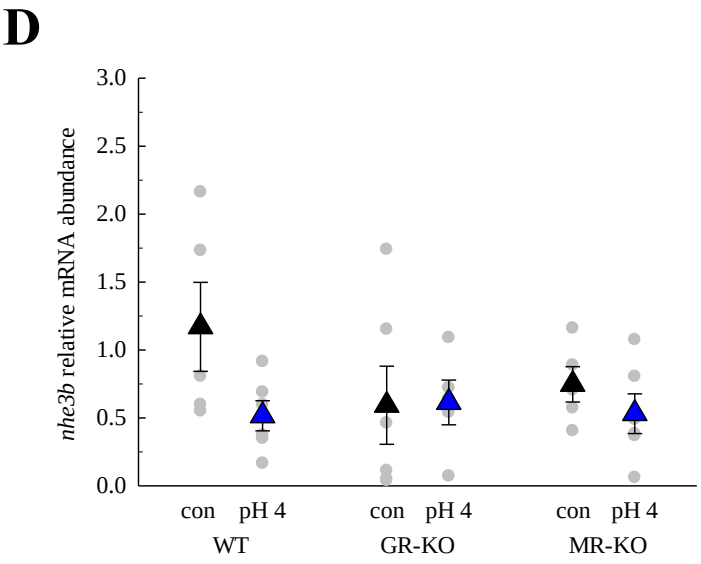

A
B
C
D
E
F

Figure 3.7. Representative images of three ionocyte types, (A-B) H^+-ATPase rich (HR) cells, (C-D) Na^+,K^+-ATPase rich (NaR) cells, and (E-F) Na^+,Cl^--cotransporter (NCC) cells in adult gill tissue (A, C, E) and the yolk sac of 4 dpf (B,D, F) zebrafish (*Danio rerio*). Scale bar = 100 µm


A
pH 4 > control
ca17a relative mRNA abundance
0
1
2
3
4
con pH 4
WT
con pH 4
GR-KO
con pH 4
MR-KO


B
pH 4 > control
ecac relative mRNA abundance
0
1
2
3
4
con pH 4
WT
con pH 4
GR-KO
con pH 4
MR-KO


C
ncc relative mRNA abundance
0.0
0.5
1.0
1.5
2.0
2.5
3.0
a
b
a
con pH 4
WT
con pH 4
GR-KO
con pH 4
MR-KO


D
nhe3b relative mRNA abundance
0.0
0.5
1.0
1.5
2.0
2.5
3.0
con pH 4
WT
con pH 4
GR-KO
con pH 4
MR-KO

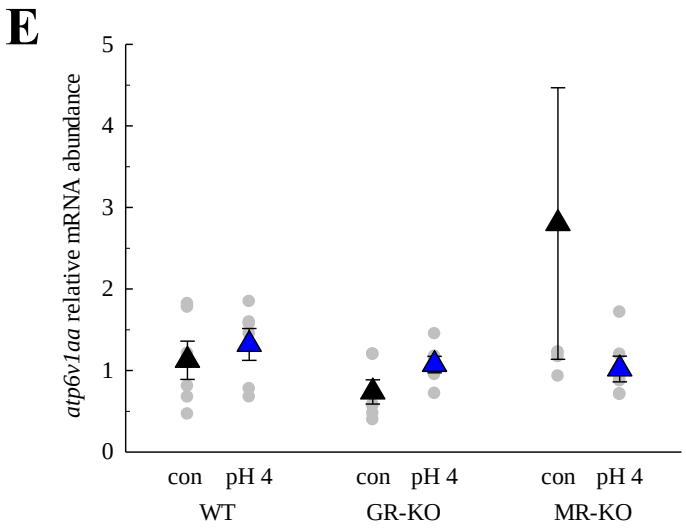

E
atp6v1aa relative mRNA abundance
0
1
2
3
4
5
con pH 4
WT
con pH 4
GR-KO
con pH 4
MR-KO

Figure 3.8. Transcript abundances of (A) *ca17a,* (B) *ecac*, (C) *ncc*, (D) *nhe3b*, (E) *atpv6v1aa* in gill tissue of adult wildtype (WT), GR-KO and MR-KO zebrafish (*Danio rerio*) exposed to control or pH 4 water for 7 d. Triangles represent mean values (± SEM, N = 5-6) and grey circles represent values for individual fish. Relative transcript abundances were normalized to the reference genes, *rpl13a* (NM_212784.1) and *eefl1a* (NM_131263.1) and expressed relative to the value for WT fish exposed to control conditions. Mean values that do not share a letter are significantly different from each other (two-way ANOVA; see text for details). Significant effects of water pH are indicated on the relevant panel figures in text (i.e. pH 4 < control; see text for details).

Chapter 4: Discussion

4.1 Overview

The present study aimed to expand our understanding of the role of cortisol in ionic regulation in a model freshwater fish, the zebrafish, with a specific focus on adult fish. Using CRISPR/Cas9 gene editing technology, we generated mutant zebrafish lines in which one of either *nr3c1* (GR) or *nr3c2* (MR) was deleted. We then assessed the ability of these knockout fish to tolerate exposure to pH 4 water, as low pH water challenges ion balance by increasing diffusive ion loss and inhibiting ion uptake (Kwong et al., 2014). Previous work on zebrafish larvae exposed to pH 4 water revealed that loss or impairment of GR function by knockdown or pharmacological inhibition significantly attenuated compensatory responses. By contrast, larvae experiencing MR knockout or pharmacological inhibition had no difficulty in re-establishing Na^+ and Ca^{2+} homeostasis (Kumai et al., 2012; Lin et al., 2016, 2015, 2011). These studies established that cortisol, acting via the GR, plays an important role in activating compensatory responses to low pH. The impaired ability of larvae experiencing GR knockdown to deal with low pH was reflected in decreased whole-body ion content, lower ionocyte abundances and decreased expression of genes related to ion transport when compared to control larvae (Cruz et al., 2013a, 2013b; Kumai et al., 2015, 2012; Lin et al., 2016, 2015, 2011). Thus, in the present study, we asked whether GR-KO fish would also have trouble compensating for the effects of pH 4 water. The use of a knockout line allowed us to probe the responses of both larvae and adult fish, addressing a knowledge gap around the role of cortisol in activating compensatory responses in adult fish. Somewhat unexpectedly, GR-KO larvae and adult fish were able to tolerate acidic conditions despite the absence of functional glucocorticoid receptors, maintaining normal or near normal whole-body Na^+ and Ca^{2+} contents.

4.2 Generation of GR-KO and MR-KO mutant lines

Although GR-KO and MR-KO mutant zebrafish lines have been generated previously using frameshift mutations that lead to premature stop codons and truncated proteins (Faught and Vijayan, 2018), our approach was to remove the entire gene. Work on GR-KO fish with truncated proteins has suggested that at least some non-canonical GR activities mediated by protein-protein interactions may occur even in the absence of GR-mediated transcriptional regulation (Facchinello et al., 2017); the gene deletion approach is expected to eliminate all GR-mediated actions. Whereas MR-KO fish exhibited normal baseline plasma cortisol levels, as in other MR-KO zebrafish lines (Facchinello et al., 2017; Faught and Vijayan, 2018), baseline cortisol levels were elevated in GR-KO fish. Elevated baseline cortisol levels were observed in other lines of GR-KO zebrafish, and are thought to reflect the loss of negative feedback regulation of the HPI axis, which is GR-dependent (Facchinello et al., 2017; Faught and Vijayan, 2020, 2019a, 2018). Adult GR-KO fish showed significantly larger body masses than WT or MR-KO fish. Higher body mass was also reported in adult GR-KO fish by Faught and Vijayan (2019a), and has been linked with greater accumulation of adipose tissue, probably via MR-mediated activation of genes involved in lipid synthesis (Facchinello et al., 2017; Faught and Vijayan, 2019b). During generation of the GR-KO line, we observed that males were more numerous than females, a trend that was not apparent in the WT or MR-KO fish and that does not appear to have been reported to date for other GR-KO lines. Although this observation requires more rigorous confirmation than we have carried out to date, it suggests that further study of sex differentiation in the absence of GR-mediated cortisol signalling is warranted. Domesticated zebrafish lines exhibit polygenic mechanisms of sex determination, but the factor or factors that trigger sex determination remain poorly understood (reviewed by Aharon and Marlow, 2021). These

observations suggest that stress and/or cortisol may contribute to male-biased sex ratios, an association that requires further study in light of the apparent male-biased sex ratio in our GR-KO zebrafish line. We also experienced difficulty in breeding GR-KO fish, an observation that has also been reported with another GR-KO line (Maradonna et al., 2020). GR-KO females have been reported to experience accelerated ovarian ageing (Faught et al., 2020; Maradonna et al., 2020) and this factor, in conjunction with disruption of sex steroids (Faught et al., 2020) may lead to challenges in breeding these fish.

4.3 Regulation of Na^+ under control and pH 4 conditions

Somewhat unexpectedly, GR-KO and MR-KO 4 dpf larvae exhibited higher whole-body Na^+ content than WT fish. In GR-KO larvae, HR cell abundance was lower but NCC cell abundance was higher than in WT or MR-KO larvae; the higher NCC cell abundance may have been more than sufficient to compensate for lower HR cell abundance, accounting for the elevated whole-body Na^+ levels in these fish. The WT and MR-KO larvae did not differ in the density of these Na^+ transporting ionocytes, raising questions about how MR-KO larvae achieved higher whole-body Na^+ content. The higher NCC abundance in GR-KO fish was unexpected because GR knockdown was associated with reductions in the abundance of both HR and NCC cells, whereas MR knockdown was without effect on ionocyte abundances (Cruz et al., 2013a, 2013b). In adult fish, whole-body Na^+ content did not differ among genotypes, suggesting that GR-KO and MR-KO fish did not experience impairment of Na^+ homeostasis. The density of HR cells in GR-KO fish gill tissue was significantly higher than that in WT or MR-KO fish, which was unexpected in light of the role GR is thought to play in regulating ionoycte abundance and the lowered HR cell density in GR-KO larvae. Although GR knockdown studies focused by necessity on larval fish, treatment of adult gill tissue with cortisol increased HR cell abundance (Cruz et al., 2013a),

suggesting a continuing role for cortisol in regulating ionocyte abundance in adult fish. However, it remains unclear to what extent the branchial HR and NCC ionocytes of adult fish resemble the cutaneous HR and NCC cells of larvae (Shih et al., 2022). The high density of branchial HR cells in GR-KO adults might have been expected to cause elevated whole-body Na^+ levels, but these fish also exhibited lower *ncc* transcript abundance, which may have offset the impact of higher HR cell density.

Water acidification impacts Na^+ balance by increasing passive Na^+ loss and zebrafish compensate for this effect by increasing Na^+ uptake (Kwong et al., 2014). In zebrafish larvae, increased Na^+ uptake was associated with increased whole-body cortisol levels, and Na^+ uptake was diminished in larvae experiencing GR knockdown, suggesting a key role for cortisol acting through GR in activating compensatory responses (Kumai et al., 2012; Lin et al., 2016). Transcript abundance of key genes implicated in Na^+ uptake, including *atp6v1aa*, *ca15a*, *ca17a* and *nhe3b* increased in zebrafish larvae exposed to pH 4 water (Lin et al., 2015; Shir-Mohammadi and Perry, 2020) and acid-exposed GR morphants failed to increase or showed a reduced rise in transcript abundances of these genes (Lin et al., 2015). Despite this evidence supporting the importance of cortisol in activating compensatory responses to acid exposure via GR, GR-KO larvae exposed to pH 4 water did not exhibit lowered whole-body Na^+ content, nor was there evidence that GR knockout prevented acid-induced increases in HR or NCC cell densityHowever, adult GR-KO fish exhibited slightly but significantly lower carcass Na^+ content after exposure to pH 4 water for 7 d and these fish also appeared to be unable to mount an increase in HR cell abundance as a response to pH water. By contrast, WT adults exhibited normal carcass Na^+ content after 7 d in pH 4 water and exhibited a significant increase in branchial HR cell abundance, as expected based on previous work (Chang et al., 2009; Kumai et

al., 2011; Shih et al., 2022). In addition, the increased transcript and protein abundances of *atp6v1a* observed in the gill of acid-exposed fish (Chang et al., 2009; Yan et al., 2007) were not apparent in the present study. Exposure of adult fish to pH 4 water resulted in higher transcript abundance of *ca17a*, an effect that was independent of genotype. This response is in agreement with current models of the role played by cytosolic carbonic anhydrase in acid-base regulation (Gilmour and Perry, 2009). Exposure to pH 4 water is expected to cause a metabolic acidosis (Wood et al., 1999) and increased cytosolic carbonic anhydrase activity in the gill is thought to support increased acid excretion (Gilmour and Perry, 2009).

4.4 Regulation of Ca^{2+} under control and pH conditions

As with whole-body Na^+ levels, GR-KO larvae had higher whole-body Ca^{2+} content than WT or MR-KO larvae, which did not differ. Moreover, the higher whole-body Ca^{2+} content of GR-KO larvae occurred despite lower density of the Ca^{2+}-transporting NaR cells in these fish, a response that is consistent with previous work implicating GR in the regulation of NaR cell abundance (Cruz et al., 2013b). Whole-body Ca^{2+} content in adult GR-KO fish did not differ from that of WT fish, although as in larvae, NaR cell density was significantly lower in GR-KO adult fish than in WT fish. Transcript abundance of *ecac*, which is responsible for the rate-limiting step in Ca^{2+} uptake (Hoenderop et al., 2005), did not differ with genotype in adult fish, which may account for the ability of GR-KO adult fish to maintain whole-body Ca^{2+} content.

Whole-body Ca^{2+} content was lowered in larvae raised in pH 4 water, an effect that was independent of genotype and consistent with previous reports (Horng et al., 2009a; Kumai et al., 2015). However, in contrast, to previous reports (Horng et al., 2009a; Kumai et al., 2015), pH 4-exposed larvae had lower density of NaR cells than those exposed to control conditions. Exposure to pH 4 for 7 d had no impact on whole-body Ca^{2+} levels in adult fish. There was no

evidence of elevated branchial NaR cell density in response to low pH in adult fish, but *ecac* transcript abundance was significantly higher in pH 4-exposed than control fish, and this may account for the apparently greater resilience of Ca^{2+} homeostasis in adult fish relative to larvae during exposure to low pH. Evidence suggests that cortisol plays a role in stimulating Ca^{2+} uptake in larvae, by increasing *ecac* transcript abundance and the density of NaR cells (Cruz et al., 2013b; Kumai et al., 2015; Lin et al., 2011). Given that cortisol increases in larvae exposed to pH 4 water (Kumai et al., 2015, 2012), it is possible that the compensatory increase in Ca^{2+} uptake triggered by low pH is mediated at least in part by cortisol. However, evidence as to whether NaR cells express GR is contradictory, with a lack of GR protein expression (Kumai et al., 2012; Kwong et al., 2016), despite the presence of GR mRNA (Cruz et al., 2013b). The general absence of genotype-specific effects in the Ca^{2+} data of the present study suggests that neither GR nor MR plays a key role in mediating Ca^{2+}-related compensatory responses to low pH. Other hormones are involved in regulating Ca^{2+} levels in zebrafish and may play a role in activating responses to low pH. For example, parathyroid hormone. Knockdown of parathyroid hormone has been shown to reduce whole-body Ca^{2+} levels and *ecac* expression in larval zebrafish (Kwong et al., 2016; Kwong and Perry, 2015; Lin et al., 2014). Additional work is needed to better understand the regulation of Ca^{2+} levels in zebrafish under low pH conditions.

4.5 Conclusions and perspectives

Regulatory responses may act over acute (short-term) or chronic (long-term) periods. During acute ionic challenges, pre-existing ionocytes are modified or enhanced via signalling cascades to respond to the challenge (Horng et al., 2009b; Hwang et al., 2011a; Hwang and Perry, 2010). In the longer-term, there is evidence changes in ionocyte abundance and/or the abundance of transporters may increase the functional capacity for ionic regulation (Chang et al., 2009; Craig

et al., 2007; Horng et al., 2009b; Hwang et al., 2011b; Pan et al., 2005; Wang et al., 2009). The present study utilized a 7 d exposure (in adult fish), with fish being sampled only at the end of the exposure period. It would be helpful to assess changes over time in whole-body ion content, ionocyte abundances, and transcript abundances to gain a better understanding of when compensatory responses are initiated following the increased ion loss elicited by acidic exposure. Recently experiments on adult zebrafish exposed acutely to low pH suggested that compensatory responses can be very rapid; the fish of this study re-established Na^+ balance in <12 h (Clifford et al., 2022). Pharmacological inhibition of NHE3b via EIPA or amiloride caused the most significant reduction in Na^+ uptake (Clifford et al., 2022), in contrast with early work that suggested a week or more was needed (Kumai et al., 2011). Using knockout lines to explore whether there is a role for cortisol in such acute responses would expand our understanding of the short-term hormonal regulation of ion balance.

Over the last decade, the zebrafish has been a popular and useful model to investigate the molecular and cellular mechanisms of ion regulation in fishes. Most studies have focused on larval fish, both because of the ease of accessing ionocytes in the skin relative to the gill, and because techniques such as protein knockdown are most easily used in larval fish. The present study demonstrated the importance of also examining ionoregulatory mechanisms in adult zebrafish, as responses may differ from those in larval fish. The use of GR and MR knockout lines allowed us to examine cortisol-mediated responses in adult fish, but this approach is not without challenges. For example, the apparent resilience of GR-KO fish to low pH raises questions about compensatory responses that may be activated in the absence of a key signalling pathway. The nature of knockout approaches provides the opportunity for the organism to adapt and compensate for the gene deletion, which can impact target pathways and signalling (Zimmer

et al., 2019). Also, although there may be no indication at the transcriptional level of a compensatory response to low pH, transcript levels are not always indicative of protein levels and these inconsistencies may be due to the translational delays for protein synthesis (Greenbaum et al., 2003). Further, there may be off-target effects of the gene editing technology; Cas9 nucleases have been reported to produce off-target mutations although refinements of CRISPR/Cas9 technology have improved target specificity (Tsai and Keith Joung, 2016; Zimmer et al., 2019).

Collectively, the research presented in the present thesis improves our understanding of the hormonal regulation of ion balance in teleost fish in low pH environments. It also highlights that there can be significant differences in compensatory responses between early development sexually mature fish, emphasizing the importance of examining ion regulation at different life stages.

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