Trade-offs in zebrafish (*Danio rerio*) associated with development in a low pH-environment

Liam Tigert

Thesis submitted to the University of Ottawa in partial fulfillment of the requirements for the MSc degree in Biology

Department of Biology
Faculty of Science
University of Ottawa

Abstract

Low water pH is an ionoregulatory challenge to freshwater teleosts. Larval zebrafish (*Danio rerio*) exposed to pH 4 water experience increased loss of Na^+ and respond with increases in ionocyte abundance and whole-body concentrations of cortisol. Because cortisol plays a role in regulating early development, particularly of the stress axis, the present study asked whether the increase in cortisol in embryos exposed to pH 4 water causes dysregulation of the stress axis in later life. Baseline whole-body cortisol levels measured at 4, 6, and 15 days post-fertilization (dpf) did not differ between pH 4-exposed and control fish. At 6 dpf, pH 4-exposed fish had higher concentrations of cortisol compared to control fish following a stressor, but no difference was detected at 15 dpf. In addition, transcript abundances for key genes of the stress axis did not differ between control and pH 4-exposed fish. Based on these results, exposure to pH 4 water in early life does not influence the stress axis or cortisol responses later in life.

Increases in ionocyte abundance in response to low pH have the potential to alter gill morphology, thereby impairing gas transfer, a trade-off known as the osmorespiratory compromise. The present study tested the hypothesis that zebrafish reared in pH 4 water have reduced gas transfer capacity in accordance with the osmorespiratory compromise. Indicators of gas transfer and ionoregulation were measured at 6, 15, 30 and 90 dpf. Across all ages examined, fish reared in pH 4 water had significantly higher whole-body concentrations of Na^+, higher ionocyte abundances and thicker gills than control fish. These differences were accompanied by higher ventilation frequencies and higher critical PO_2 (P_{crit}) values. Additionally, adult fish raised in low pH had a significantly higher rate of oxygen consumption compared to control fish. These results support the hypothesis that development in water of low pH impairs gas transfer, as predicted by the osmorespiratory compromise.

Résumé

L'ionorégulation dans l'eau à un pH bas est un défi pour les téléostéens d'eau douce. Les larves de poissons zèbres (*Danio rerio*) exposées à de l'eau ayant un pH de 4 subissent une perte accrue de Na^+ et vont répondre avec une augmentation de l'abondance d'ionocytes et de la concentration de cortisol corporelle. Sachant que le cortisol joue un rôle dans la régulation du développement précoce, particulièrement sur l'axe de stress, l'étude présente se demande si l'augmentation de cortisol dans les embryons exposés à de l'eau ayant un pH de 4 provoque une dérégulation sur l'axe de stress plus tard dans la vie. Le niveau de base de cortisol corporelle mesuré à 4, 6 et 15 jours post-fertilisation (jpf) ne diffère pas entre les poissons exposés à de l'eau ayant un pH de 4 et les poissons témoins. À 6 jpf, les poissons exposés à un pH de 4 démontre de plus grande concentration de cortisol comparé aux poissons témoins suivant un facteur de stress, par contre aucune différence n'a été observé à 15 jpf. De plus, l'abondance de transcription pour les gènes clés de l'axe de stress ne diffère pas entre les témoins et les poissons exposés au pH de 4. Basé sur ces résultats, l'exposition à de l'eau avec un pH de 4 au début de la vie n'influence pas l'axe de stress ou la réponse de cortisol plus tard dans la vie.

L'augmentation de l'abondance d'ionocytes en réponse à un pH bas a le potentiel de modifier la morphologie des branchies, altérant ainsi le transfert de gaz, un compromis connu sous le nom de compromis osmorespiratoire. L'étude présente a testé l'hypothèse selon laquelle le poisson zèbre élevé dans de l'eau à un pH de 4 a une capacité de transfert de gaz réduite conformément au compromis osmorespiratoire. Des indicateurs de transfert de gaz et d'ionorégulation ont été mesuré à 6, 15, 30 et 90 jpf. À tous les âges examinés, les poissons élevés dans de l'eau à un pH de 4 ont une concentration corporelle significativement plus élevée de Na^+, une abondance d'ionocytes plus élevée et des branchies plus épaisses que les poissons

témoins. Ces différences sont accompagnées par des fréquences de ventilation plus élevées et des valeurs de PO_2 critiques (P_{crit}) plus élevées. De plus, les poissons adultes élevés dans de l'eau à pH bas ont un taux de consommation d'oxygène significativement plus élevé que les poissons témoins. Ces résultats supportent l'hypothèse que le développement dans de l'eau à pH bas détériore le transfert de gaz, comme le prédit le compromis osmorespiratoire.

Acknowledgements

First, I would like to thank Dr. Katie Gilmour, for her support and guidance over the past two years. She made me feel welcomed instantly when I moved to Ottawa. Katie helped me hone my research skills in the lab to a higher standard and has helped me progress in my academic career. Katie goes above and beyond for her students in and out of the lab, and for that I will be forever grateful.

I would also like to thank Dr. Steve Perry, and Dr. Jan Mennigen, for being on my advisory committee, and helping me with experiments, and providing advice and critical feedback throughout my thesis.

I would also like to thank the students who helped me out with various experiments. I'd like to thank Katie, Brenna Wilson, and Carol Best for taking care of my experimental fish over Christmas break. I'd also like to thank Carol for teaching me molecular biology, and I'd like to thank Hong Meng Yew, for doing the qPCR analysis for this thesis. Additional thanks to Yihang Pan for helping troubleshoot many experiments, specifically the immunohistochemistry, metabolic rate and ventilation experiments. Another thanks to Alex Hare, for teaching me how to do the cortisol ELISA, and Alex Zimmer for teaching me how to do the Na^+ assay. I'd also like to thank Kaz Shuji for helping me with all the fish care, keeping up with the feedings and water changes, doing the larval length and mass measurements, and for putting up with all my demands as my research assistant. I'd also like to thank Pier-Anne Ménard for providing the French translation for the abstract section of this thesis.

I'd also like to thank my lab members in the Gilmour and Perry labs, for all the fun times in and out of the lab (pre-pandemic). Specifically, I'd like to thank Brittany Bard, Emma Kunert, and Brenna Wilson for tolerating sharing an office space with me, we may not have been the most productive in there, but we did have a lot of excellent banter and deep conversations. Thanks for listening to all my rants. I could not have asked for a better lab to go through a global pandemic with.

Table of contents

List of abbreviations

[ ] – concentration

11ßHSD2 – 11ß-hydroxysteroid dehydrogenase type 2

ACTH – adrenocorticotropic hormone

BSA – bovine serum albumin

cDNA – complimentary DNA

conA – concanavalin A

CRF – corticotropin releasing factor

dpf – days post-fertilization

ELISA - enzyme-linked immunosorbent assay

f_v – ventilation frequency

GR – glucocorticoid receptor

h – hour

hpf – hours post fertilization

HPI – hypothalamic-pituitary-interrenal

HR – V-type H^+-ATPase-rich

LOE – loss of equilibrium

min – minute

$\dot{M}O_2$ –rate of oxygen consumption

MR – mineralocorticoid receptor

NaR – Na^+-K^+-ATPase-rich

NCC – Na^+,Cl^- cotransporter

PBS – phosphate buffered saline

PBST – phosphate buffered saline with tween

P_{crit} – critical oxygen tension

PO_2 – partial pressure of oxygen

qPCR – quantitative polymerase chain reaction

StAR – steroidogenic acute regulatory protein

List of tables and figures

Chapter 1: General introduction

1.1 Introduction

Evolution through natural selection does not lead to "perfect" adaptation – that is, there is no combination of physiological and morphological traits that allows an organism to be perfectly suited to every set of environmental conditions (Stearns, 1989a; Wernicke von Siebenthal et al., 2018). Consequently, trade-offs occur, where the efficiency or function of one system is compromised to improve the efficiency or function of another system so as to maximize the animal's overall fitness (Stearns, 1989a; Wernicke von Siebenthal et al., 2018). These types of trade-offs may occur in an evolutionary time frame, reflecting the fact that animals are integrated suites of traits and selection acts at the level of the whole organism (Stearns, 1989a; Stearns, 1989b). Trade-offs may also occur within an individual animal's lifetime as a result of phenotypic plasticity. Plasticity allows for changes in physiological function and/or morphology to enable an animal to deal with a change in environmental conditions, and can involve reversible or irreversible changes (Stearns, 1989a). Acclimatization, in which phenotypic change occurs in an individual in response to natural environmental variation (or acclimation, for controlled environmental change in a laboratory setting), is an example of reversible plasticity (Botero et al., 2015; Edelaar et al., 2017; Hollander et al., 2015). Developmental plasticity, in which the environment in which development occurs shapes the subsequent phenotype of the mature individual may be reversible or irreversible (e.g. Burggren and Reyna, 2011; Edenbrow and Croft, 2011; Hare et al., 2020; Mendez-Sanchez and Burggren, 2017). Regardless, the phenotypic changes that occur in response to environmental change may have impacts on other aspects of the animal's physiology, resulting in trade-offs.

The present thesis explores the concept of trade-offs in developing zebrafish, *Danio rerio*, in response to rearing in a low pH (pH 4) environment. Chapter 2 examines potential

trade-offs between ionoregulatory function and the development and function of the hypothalamic-pituitary-interrenal (HPI) axis, or stress axis, that may occur as a result of the dual role played by the corticosteroid hormone cortisol in regulating ion balance and stress responses in fish. Chapter 3 examines the osmorespiratory compromise, trade-offs that occur at the gill as a result of the conflicting structural requirements for gas transfer versus ion/water balance. Low water pH serves as an ionoregulatory challenge with potential impacts on the morphology of the gill, which in turn may affect gas transfer across the gill. In this opening chapter, background information relevant to these two topics will be briefly reviewed, and the hypotheses to be tested in the thesis will be presented. More specific development of the hypotheses is presented in the introduction to each data chapter, to reduce duplication of information.

1.2 Ontogeny of the stress axis and stress response in fish

The stress axis is important in achieving a return to homeostasis following exposure to a stressor (Schreck and Tort, 2016; Wendelaar Bonga, 1997). The stressors that activate this response range from interactions with intra- or inter-specific individuals (e.g. Abreu et al., 2018; Ros et al., 2014; reviewed by Barton, 2002), to handling stress (e.g. Ramsay et al., 2009), to a wide range of environmental conditions(e.g. McBryan et al., 2013; Takei and Hwang, 2016; reviewed by Barton, 2002). When a stressor is encountered, corticotropin releasing factor (CRF) released from the hypothalamus stimulates the pituitary to release adrenocorticotropic hormone (ACTH), which in turn stimulates the steroidogenic interrenal cells located in the head kidney to produce the steroid cortisol, which is the main hormone responsible for mediating the stress response in teleost fishes (Alderman and Bernier, 2009; reviewed by Gorissen and Flik, 2016; Wendelaar Bonga, 1997). Cortisol activates physiological processes that favour short-term survival in the face of a stressor (Barton, 2002; Wendelaar Bonga, 1997). When the stressor is

acute, circulating cortisol levels rapidly return to baseline levels. However, chronic stress leading to extended periods of elevated cortisol can be detrimental owing to the tendency of cortisol to prioritize short-term survival, for example by mobilizing energy reserves, a response that does not favour growth (Barton, 2002; Gorissen and Flik, 2016; Wendelaar Bonga, 1997).

In zebrafish, as in other teleost fishes, the individual components of the HPI axis begin to develop early in embryogenesis, with the precursor to the hypothalamus beginning to form around 12 hours post fertilization (hpf) and hypothalamic tissue beginning to differentiate around 18 hpf (Nesan and Vijayan, 2013a). The precursors for the pituitary and interrenal cells begin to develop early as well, around 10 and 22 hpf, respectively (Nesan and Vijayan, 2013a). Similarly, transcripts associated with the HPI axis are detected early in development, with *crf* detected before 24 hpf, although these are also maternally derived (Alderman and Bernier, 2009; Nesan and Vijayan, 2013b). Despite the development of the HPI axis early in embryogenesis, it does not begin to function in cortisol production or stress activation until later in development (Alsop and Vijayan, 2008; Hare et al., 2020; Jeffrey and Gilmour, 2016). Endogenous cortisol production begins around 48 hpf, with the cortisol that is present before 48 hpf being derived from maternally deposited sources (Jeffrey and Gilmour, 2016; Nesan and Vijayan, 2016). Cortisol production in response to a stressor appears around 3 days post fertilization (dpf), but only in response to severe stressors, with robust responses to a variety of stressors developing by about 6 dpf (Alderman and Bernier, 2009; Alsop and Vijayan, 2008; Jeffrey and Gilmour, 2016). Importantly, experimental evidence suggests that elevated cortisol levels in embryogenesis and early life, whether from maternal deposition or endogenous production, lead to dysregulation and alterations of HPI axis function later in life (Best and Vijayan, 2017; Hare et al., 2020; Nesan and Vijayan, 2016; Vindas et al., 2016; reviewed by Sopinka et al., 2017). These changes in

function reflect the key role that cortisol plays in orchestrating the events of early development (Nesan and Vijayan, 2013b; see also below), and include elevated baseline levels of cortisol, and reduced levels of cortisol generated in response to a stressor.

1.3 Cortisol as a multi-functional hormone in fish

In addition to its role as a stress hormone, cortisol plays key roles in other physiological processes, with its roles in early development and ionic/osmotic regulation being most relevant to the current study. As the key hormone mediating stress responses, cortisol affects many physiological processes to maximize short-term survival during and immediately following exposure to a stressor (Gorissen and Flik, 2016; Wendelaar Bonga, 1997). One such effect is the mobilization of stored energy through glycolysis, to provide immediate energy to respond to the stressor. Cortisol also stimulates lipid metabolism by activating hormone-sensitive lipase through transcriptional regulation to release fatty acids from storage lipids, and these free fatty acids are available for energy metabolism (Kostyniuk et al., 2018; Kostyniuk et al., 2019). Carbohydrate metabolism is stimulated by cortisol through its effects on phosphoenolpyruvate carboxykinase, which carries out an important role in gluconeogenesis, providing glucose that is the main energy source for various organs including the brain and gills (Kostyniuk et al., 2018; Kostyniuk et al., 2019). Other effects include repressing the immune and digestive systems, to lower their energy demand so that energy can be diverted to dealing with the stressor (Gorissen and Flik, 2016; Wendelaar Bonga, 1997). However, long term elevation of cortisol can be detrimental. Diversion of energy from the immune system can leave the fish vulnerable to disease, and the changes in intermediary metabolism to favour mobilization of energy reserves rather than energy storage can reduce growth (Gorissen and Flik, 2016; Wendelaar Bonga, 1997).

Cortisol also plays an important role in developmental programming (Best et al., 2017; Nesan and Vijayan, 2013a; Nesan and Vijayan, 2016). Maternally-deposited cortisol orchestrates the earliest stages of embryonic development, with *de novo* cortisol production after 48 hpf also having programming effects. Systems in which development is directly impacted by cortisol include cardiogenesis, metabolic pathways, and the HPI axis itself (Nesan and Vijayan, 2013b; Nesan and Vijayan, 2016; Wilson et al., 2013). Modification of cortisol levels during early development can disrupt the development of these systems. For example, exogenous cortisol exposure in early development results in deformities of the heart (Nesan and Vijayan, 2013b). Similarly, exposure to dexamethasone, a synthetic glucocorticoid, results in increases in circulating glucose concentrations in adulthood (Wilson et al., 2013). These dexamethasone-treated fish also displayed alterations of the HPI axis, having significantly higher levels of cortisol post-stressor than untreated control fish (Wilson et al., 2013). Through both knockdown and knockout experiments, cortisol has been found to carry out these actions through both glucocorticoid receptors (GR) and mineralocorticoid receptors (MR) (Alsop and Vijayan, 2008). Notably, zebrafish express only a single GR, which differs from the situation in most other teleost fishes, where two GRs are present (Alsop and Vijayan, 2008).

A third important role of cortisol in teleost fish is its involvement in regulating ionic and osmotic balance (e.g. Breves et al., 2010; Kumai et al., 2012; Kwong and Perry, 2013; Kwong et al., 2016; Lin et al., 2016a; Takei, 2012; reviewed by Guh et al., 2015; Kwong et al., 2016; McCormick, 2001). Cortisol is well known for its important role in the fresh water to salt water transition experienced by fish with anadromous life histories (Takei, 2012; Vindas et al., 2016). However, cortisol also regulates ion balance in freshwater fishes. For example, cortisol has been demonstrated to increase the expression of proteins involved in Na^+ (Kumai et al., 2012; Kwong

and Perry, 2013; reviewed by Hwang, 2009) and Ca^{2+} uptake (Kumai et al., 2015; Lin et al., 2016b) through transcriptional regulation. Cortisol treatment also increases the abundance of ionocytes, cells that are responsible for ion uptake into the body (Cruz et al., 2013; Lin et al., 2016a). These processes are crucial for freshwater fishes, which are hyperionic to their environment and therefore constantly lose ions to the surrounding fresh water through passive diffusion (Evans, 2008; Takei and Hwang, 2016). Interestingly, the ionoregulatory functions of cortisol appear to be expressed through GR rather than MR (Nesan and Vijayan, 2013b; Wilson et al., 2013). For example, cortisol acts through GR to activate ionoregulatory responses to environmental challenges such as low water pH (Kumai et al., 2012). The effects of cortisol are diverse, and while it is not clear that there is cross-talk between these roles, some evidence has been found that they may interact (Hare et al., 2020). Additionally, it is important to note that cortisol acts through GR to mediate both ionoregulatory and stress-related effects, and zebrafish only have one GR receptor (Alsop and Vijayan, 2009), which may be the mechanism for interactions.

1.4 Low pH as an ionoregulatory challenge

Low pH serves as an ionoregulatory challenge because it exacerbates the passive ion loss experienced by freshwater fishes (reviewed by Kwong et al. 2014). Some species are adapted to life in acidic water, while other species, such as zebrafish, can tolerate and acclimate to low pH conditions (Hirata et al., 2003; Matey et al., 2011; Takei and Hwang, 2016; reviewed by Kwong et al., 2014). Low pH environments amplify ion loss by disrupting tight junctions in the gill, allowing higher rates of ion movement through paracellular pathways, which leads to decreases in plasma and/or whole-body ion content (Kumai et al., 2012; Kwong et al., 2014). An additional challenge is that the uptake of some ions, particularly Na^+, is linked to H^+ secretion, and this

transport mechanism is disrupted by the increased concentration of H^+ ions in a low pH environment (Guh et al., 2015; Kwong et al., 2014). For example, zebrafish appear to use two ionocyte types for Na^+ uptake. In the V-type H^+-ATPase-enriched (HR) cell, Na^+ uptake occurs via Na^+/H^+ exchangers (NHE3b) and/or acid-sensing ion channels (ASIC4b), driven by proton efflux via NHE3b and/or H^+-ATPase (reviewed by Guh et al., 2015). Uptake via this ionocyte is expected to be impaired by acidic conditions (Kumai et al., 2012; reviewed by Kwong et al., 2014) The second ionocyte type involved in Na^+ uptake is enriched in Na^+,Cl^--cotransporters (NCC) and would not be expected to be negatively impacted by low environmental pH (Guh et al., 2015; Kwong et al., 2014). To cope with the increased loss of ions in a low pH environment, zebrafish increase the expression of proteins involved in ion uptake pathways as well as the abundance of ionocytes, thereby increasing the rate of ion uptake to balance the higher rate of ion loss (Chang et al., 2009; Kumai and Perry, 2011; reviewed by Kwong et al., 2014). Cortisol appears to play a major role in these responses. For example, larval zebrafish exposed to low pH exhibit an increase in whole-body cortisol levels, and pharmacological inhibition or knockdown of the GR prevents the expression of low pH-induced responses (Kumai et al. 2012). Through this suite of responses, zebrafish are able to acclimate to acid conditions as low as pH 4.

1.5 Ontogeny of the gill – O_2 versus ion uptake

The ionocytes that are essential for the maintenance of ion balance in freshwater fishes are located in the gill of adult fish, but the skin of developing fish, necessitating a developmental transition of ionoregulatory function from skin to gill. Similarly, gas transfer occurs across the body surface during early development, transitioning to the gill as the gill and circulatory system develop (Brauner and Rombough, 2012; Rombough, 1999; Rombough, 2007). Thus, the gill is a multi-functional organ in fishes, with roles beyond gas exchange and ionic/osmotic regulation

including gas sensing, acid-base balance and ammonia excretion (Evans et al., 2005; Hughes, 1966; Hughes and Morgan, 1973). Reflecting the requirements for these roles, the morphology of the gill is highly specialized. The gill structure in mature fish consists of gill arches, each of which supports two rows of branching filaments, from which extend many small, plate-like lamellae, the site of gas transfer (Hughes, 1966; Hughes and Morgan, 1973). The branchial epithelium covering the filaments and lamellae is composed largely (>90% of cells) of squamous epithelial cells, or pavement cells, that allow for a short blood-to-water diffusion distance. Nestled into this epithelium is a variety of specialized cell types, including the ionocytes responsible for ion uptake, neuroepithelial cells that are involved in gas sensing, and mucus cells (Wilson and Laurent, 2002). Notably, these specialized cell types are often larger and more ovoid than the pavement cells (Wilson and Laurent, 2002), and increases in the abundance of ionocytes, in particular, have been associated with increases in the blood-to-water diffusion distance of the gill (reviewed by Perry, 1998; Wilson and Laurent, 2002).

In zebrafish, the four gill arches appear around 48 hpf (Kimmel et al., 1995), developing from the pharyngeal arches, with filament primordia beginning to develop at around 72 hpf, and lamella primordia appearing around 12 dpf (Jonz and Nurse, 2005; Shadrin and Ozerniuk, 2002). The gill is not fully developed until about 40 dpf (Shadrin and Ozerniuk, 2002). Despite this extended period of development, the gill contributes to gas transfer and ionic regulation from an early stage of development. In zebrafish, ionocytes appear on the gill as early as 5 to 7 dpf (Rombough, 2002). Skin ionocytes decline in abundance from about 5 dpf, and the gill becomes the primary site for ionoregulation as early as 7 dpf (Rombough, 2002; Rombough, 2007). By contrast, the gill and circulation are not required for oxygen uptake in zebrafish until about 14 dpf (Hughes et al., 2019; Jacob et al., 2002; Pelster and Burggren, 1996; Rombough and Drader,

2009). For example, in developing fish prevented experimentally from ventilating their gills, larvae of 14 dpf and older were not able to survive without supplemental oxygen, whereas larvae 7 dpf and younger were still able to meet their O_2 demand through cutaneous respiration (Rombough, 2002).

1.6 The osmorespiratory compromise

The architectural complexity of the gill results in a very high surface area as well as a low blood-to-water diffusion distance, both factors that maximize the rate of diffusion according to the Fick equation (Gonzalez, 2011; Hughes, 1966; Hughes and Morgan, 1973). Although this structure is beneficial in terms of maximizing the diffusion of respiratory gases, it is not favourable from the perspective of ionic/osmotic regulation. Because large diffusion gradients exist between freshwater teleosts and the surrounding water, the high surface area and low blood-to-water diffusion distance that provide for efficient gas transfer also provide conditions that favour ion loss (Gonzalez, 2011; Perry et al., 2012). These ideas have been formalized in the concept of the osmorespiratory compromise, which points out that the structure of the gill must reflect a compromise between the requirement for oxygen uptake that is adequate to support metabolism, and the need to reduce diffusive ion losses to a level that can be matched via ion uptake mechanisms (Gilmour and Perry, 2018; Gonzalez, 2011; Matey et al., 2011; Nilsson et al., 2012). In some fish species, gill structure appears to favour reductions in ion loss, with reductions in the overall surface area, although this strategy may place limits on oxygen uptake (Hirata et al., 2003; Matey et al., 2011). In other species, reversible remodelling of the gill is used to manage the osmorespiratory compromise (Mitrovic et al., 2009; Nilsson et al., 2012; Sollid et al., 2003). For example, in goldfish and Crucian carp, the lamellae are protected by

interlamellar cell masses that are shed when there is a need to increase gill surface area for oxygen uptake, such as during hypoxia or when oxygen demand is increased during exercise (Perry et al., 2012; reviewed by Gilmour and Perry, 2018). However, this increased area for exchange comes at a cost of higher rates of loss of certain ions, such as Cl^- (Perry et al., 2012). Ionocytes located on the gill epithelium counteract diffusive ion losses (Guh et al., 2015), but under conditions such as ion-poor water where ionocyte abundance increases, the resulting thickening of the blood-to-water diffusion distance can impair gas transfer (reviewed by Perry, 1998). These examples illustrate the dynamic nature of the osmorespiratory compromise as well as the plasticity that exists in gill morphology.

1.7 Hypothesis and predictions

Overall, the main objective of the present thesis was to examine trade-offs associated with the development of zebrafish in a low pH environment. In Chapter 2, we hypothesized that there is a trade-off between HPI axis development, and the requirement for elevated cortisol to allow developing zebrafish to respond to the ionoregulatory challenge of low pH exposure. We predicted that this necessary increase in endogenous cortisol levels would have detrimental effects on HPI axis development, similar to the effects of cortisol treatment or early life stress. In Chapter 3, we investigated developmental effects on the osmorespiratory compromise. We hypothesized that the osmorespiratory compromise would apply throughout development in a low pH environment, and that developmental plasticity will not be able to alleviate this compromise. This hypothesis leads to the prediction that the increases in ionocyte abundance that occur to compensate for increased ion losses under low pH conditions will impair gas transfer and reduce hypoxia tolerance. Alternatively, developmental plasticity may result in changes in gill morphology that compensate for the impact of higher ionocyte abundance.

Chapter 2: The dual roles of cortisol in stress and iono-/osmoregulation in zebrafish (*Danio rerio*) exposed to an ionoregulatory challenge during early development

2.1 Introduction

The glucocorticoid hormones, with cortisol being the primary glucocorticoid in teleost fishes, are well known as key mediators of the stress response (Gorissen and Flik, 2016; Schreck and Tort, 2016; Wendelaar Bonga, 1997). In teleost fishes that are exposed to a stressor, the HPI axis is activated to increase the production of cortisol. Corticotropin releasing factor (CRF) produced by neurons of the preoptic area is released onto corticotropes of the anterior pituitary, which respond by releasing adrenocorticotropic hormone (ACTH). The ACTH stimulates the steroidogenic interrenal cells of the head kidney to increase production of cortisol, through effects on proteins such as steroidogenic acute regulatory protein (StAR) and P450 side chain cleavage enzyme (P450scc) that mediate rate-limiting steps in cortisol production. The effects of cortisol on target tissues, which include mobilization of stored energy, enhancement of vascular reactivity, and increases in anti-inflammatory and immunosuppressive actions, all of which are beneficial in dealing with a stressor, are activated by its interaction with corticosteroid receptors, of which there are two types in fishes, glucocorticoid (GR) and mineralocorticoid (MR) (Faught et al., 2016; Wendelaar Bonga, 1997). These receptors, particularly the GR, also mediate a second key role of cortisol in teleost fishes, which is the regulation of ionic and osmotic balance (McCormick, 2001; Takei and Hwang, 2016).

Freshwater fish are hyperosmotic and hyperionic to their environment, and as a result, lose ions by passive diffusion. To counter ion loss, branchial ionocytes actively take up ions from the surrounding fresh water (Guh et al., 2015). Both passive ion loss and active ion uptake appear to be influenced by cortisol. For example, larval zebrafish exposed to waterborne cortisol experienced increases in Na^+ uptake and whole-body Na^+ concentrations, responses that were GR-mediated (Kumai et al., 2012). Exposure to exogenous cortisol also increased the abundance

of ionocytes responsible for Na^+ uptake in larval zebrafish, including cells that express Na^+,Cl^- cotransporters (NCC cells; (Lin et al., 2016a), and ionocytes enriched in V-type H^+-ATPase (HR cells; Cruz et al., 2013), which likely contributed to the increases in Na^+ uptake and content. Cortisol treatment also reduced paracellular permeability in larval zebrafish, which in turn reduced diffusive Na^+ loss in zebrafish exposed to acidic water (Kwong and Perry, 2013). Although these effects were elicited by treatments that likely resulted in supra-physiological elevation of circulating cortisol levels, a recent study using an air-exposure stressor to elicit an endogenous stress response also reported increases in ion uptake rates, whole-body ion content, and ionocyte abundance (Hare et al. 2020). Thus, it appears that activation of the stress axis can alter ionoregulatory function during early development in zebrafish. The goal of the present study was to determine whether the opposite also occurs, i.e. whether elevation of circulating cortisol levels in response to an ionoregulatory challenge can alter HPI axis function during early development.

In teleost fish, development of the HPI axis is affected by glucocorticoid exposure during early life (Alderman and Bernier, 2009; Alsop and Vijayan, 2008; Nesan and Vijayan, 2013b; Wilson et al., 2016). For example, larval zebrafish exposed to the synthetic glucocorticoid dexamethasone exhibited a higher increase in cortisol post-stressor than a control group (Wilson et al., 2016). However, other studies found that cortisol exposure attenuated the cortisol response to a stressor (Nesan and Vijayan, 2013b). Endogenous cortisol elevation in response to stressor exposure also affected baseline and stress-induced cortisol levels later in development, suggesting that exposure to a stressor can influence HPI axis development (Hare et al., 2020). However, the stressor used by Hare et al. (2020) was air exposure rather than an ionoregulatory challenge.

In the present study, exposure to low pH (pH 4) water was used as an ionoregulatory challenge because it was reported to increase endogenous cortisol levels in developing zebrafish (Kumai et al., 2012). Exposure to acidic water increases rates of ion loss that are countered by compensatory increases in ion uptake achieved through increases in both ionocyte abundance and the expression of ion transport proteins (reviewed by Kwong et al., 2014). These compensatory responses are linked to cortisol, because pH 4-exposed larvae treated with the GR antagonist RU-486 or experiencing functional knockdown of the GR failed to increase Na^+ uptake (Kumai et al., 2012). Thus, exposure to pH 4 water serves as an ionoregulatory challenge that activates a cortisol response with functional consequences for the fish.

Given this background, we hypothesized that exposure to pH 4 water in early life would influence development of the HPI axis and hence cortisol responses later in development. We predicted that the cortisol response to pH 4 water would lead to increases in baseline cortisol concentrations, an attenuated cortisol response to a standardized stressor, and decreases in the transcript abundances of genes of the stress axis. Zebrafish were examined at three stages. The earliest time-point was 4 dpf, to confirm the cortisol response to pH 4 reported previously (Kumai et al. 2012; Kumai et al. 2015). The second time-point was 6 dpf, the earliest age at which larval zebrafish mount a robust cortisol response to a stressor (Alsop and Vijayan, 2008; Hare et al., 2020). Finally, fish were examined at15 dpf, to identify longer-term effects of early life exposure to a stressor, as in Hare et al. (2020).

2.2 Materials and methods

2.2.1 Experimental animals

The breeding colony of adult zebrafish at the University of Ottawa aquatics facility was initially established using animals purchased from the pet trade (Big Al's, Ottawa, ON, Canada).

Fish used in the present experiments were collected by breeding wild-type zebrafish (*Danio rerio*) from this stock. Adult fish were held in 10 L tanks with semi-recirculating flow-through water, at a density of 4 fish per litre. Fish were held in dechloraminated city of Ottawa tap water ("system water"; in mmol L^{-1}, 0.8 Na^{+}, 0.4 Cl^{-}, 0.25 Ca^{2+}; pH 7.2) maintained at 28°C. A photoperiod of 14 h light:10 h dark was maintained, and fish were fed 5% body mass per day of No. 1 crumble-Zeigler commercial fish food (Aquatic Habitats, Apopka, FL, USA). Embryos were obtained from breeding two male and three female fish in an acrylic breeding trap with a perforated bottom insert that allowed for embryo collection.

Embryos were reared at 28.5°C in system water or system water acidified to pH 4 with sulphuric acid (see below) in 50 mL Petri dishes, with a maximum of 40 eggs per dish. Full water changes were carried out daily following the removal of dead embryos and larvae. At 5 dpf, larvae were transferred to 500 mL tanks, with an average density of 35 larvae per tank. Again, water changes were carried out every day after the removal of dead larvae. Larvae were fed a satiating meal of GEMMA 75 micro fish feed (Skretting USA, Westbrook, ME, USA) daily following the water change. Survival was quantified by counting the number of dead fish in each Petri dish or tank daily. Hatch rate was measured by counting the number of larvae that had hatched in each Petri dish hourly between 48- and 60-hpf. All holding and experimental protocols were approved by an institutional animal care committee (protocol BL-2118) and followed the guidelines of the Canadian Council on Animal Care (CCAC) for the use of animals in teaching and research.

2.2.2 Experimental design

The goal of these experiments was to evaluate the effects of an ionoregulatory stressor during early life on the development of the stress axis and the stress response. The

ionoregulatory stressor chosen was (system) water acidified to pH 4 with sulphuric acid, because it was shown to be an effective osmoregulatory challenge to larval zebrafish (Kumai et al., 2012). Additionally, water of high or low Na^+ concentration was used as a second osmoregulatory stressor (Lin et al., 2016a). To assess the stress response, whole-body cortisol concentrations were measured under resting conditions or following exposure to a stressor at 4, 6 or 15 dpf. These sampling times were chosen because they correlate with specific developmental milestones in zebrafish. At 4 dpf, larvae synthesize cortisol *de novo* and begin to mount a cortisol response to a stressor, although it is not a robust response at this time (Alsop and Vijayan 2008). Responses to osmotic stressors have been detected at 4 dpf (Alderman and Bernier, 2009). By 6 dpf, the cortisol response to a stressor is robust (Alsop and Vijayan, 2008; Hare et al., 2020). At 15 dpf, zebrafish are reliant on the gill for gas transfer as well as ionic regulation (Rombough, 2007). Transcript abundances of stress axis genes were measured at 4, 6 and 15 dpf as an index of development of the stress axis.

For experiments involving pH 4 water, embryos were randomly allocated to one of five treatment groups; control, early exposure, late exposure, four day exposure, or last day exposure. Exposure groups were held in pH 4 water from 0 to 2 dpf (early), from 2 to 4 dpf (late), from 0 to 4 dpf (four day), or from 3 to 4 dpf (last day). Changes between pH 4 and control water were carried out during the daily water change. At 5 dpf, all fish were transferred to control (system) water in the 500 mL tanks.

For experiments in which water Na^+ concentration was manipulated, embryos at 0 dpf were randomly allocated to one of three treatment groups; control (system water, $[Na^+] = 0.8$ mmol L^{-1}), low $[Na^+]$ (0.04 mmol L^{-1}), and high $[Na^+]$ (10 mmol L^{-1}). Low and high $[Na^+]$ treatment water were produced from artificial fresh water, with supplemental ion addition to

match the control water osmolality (800 µmol L^{-1} Cl^-; 250 µmol L^{-1} Ca^{2+}; 150 µmol L^{-1} Mg^{2+}, and 40 µmol L^{-1} K^+; Zimmer et al., 2018). As with the pH 4 treatments, larvae were exposed to altered [Na^+] conditions until 5 dpf, when they were transferred to system water in 500 mL tanks.

Baseline whole-body cortisol levels were measured at 4, 6 or 15 dpf. Groups of larvae (for N = 1, 25 larvae per sample were pooled at 4 and 6 dpf, and 15 larvae per sample at 15 dpf) were euthanized by immersion in Tris-buffered MS-222 (0.72 mg mL^{-1}, tricaine methanesulphonate; Syndel Laboratories, Nanaimo, BC, Canada), and samples were flash frozen in liquid N_2. Whole-body cortisol levels were also measured following exposure to an acute stressor in 6 and 15 dpf larvae. No post-stressor measurements were carried out at 4 dpf, because at this age larvae do not reliably mount a cortisol response to acute stress (Alsop and Vijayan, 2008; Hare et al., 2020). A repeated air-exposure stressor was selected (Hare et al., 2020; Ramsay et al., 2009). Groups of larvae were exposed to air for 3 min, returned to water for 3 min, and exposed to air for another 3 min. After the second air exposure, larvae were returned to water for 10 min, and then euthanized and flash frozen as described above. Samples were stored at -80°C until processing for measurement of cortisol concentrations or transcript abundance.

2.2.3 Measurement of whole-body cortisol concentrations

Cortisol extraction, and measurement by enzyme-linked immunosorbent assay (ELISA; Neogen, Lansing, MI, USA), were carried out as described by Jeffrey and Gilmour (2016). In brief, samples were homogenized in 200 µL of extraction buffer (Neogen) using a handheld homogenizer (Bio-vortexer homogenizer, Daigger Scientific, Hamilton, NJ, USA). Diethyl ether (1 mL) was added to the samples, which were vortexed thoroughly, incubated at room temperature for 30 min, centrifuged at 3,000 *g* for 5 min and flash frozen at -80°C for 30 min. After removal of the liquid phase, two additional rounds of diethyl ether extraction were carried

out, and the combined liquid phase of all three extractions was evaporated under forced air. The extract was reconstituted by adding 250 µL of extraction buffer (Neogen), vortexing, and incubating at 65°C for 2 x 5 min. Reconstituted samples were stored at -80°C until analysis. In our hands, this protocol yields an extraction efficiency of 88.9%. Cortisol concentrations were measured in duplicate according to the manufacturer's instructions. Inter-assay variability was 7.9% CV, and intra-assay variability was 12.8% CV.

2.2.4 Transcript abundance of stress axis genes

Based on the lack of significant differences in cortisol responses among the early, late or four day exposure treatment groups (see section 2.3.3), transcript abundances were compared between control and four day exposure groups. Samples were homogenized on ice using a pestle grinder (Kimble Chase Kontes, Rockwood, TN, USA) and total RNA was extracted in TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The quantity and quality of RNA were assessed (NanoDrop 2000; ThermoFisher Scientific, Ottawa, ON, Canada), and cDNA was synthesized from 1 µg of total RNA using the iScript cDNA synthesis kit (Bio-Rad, Mississauga, ON, Canada) according to the manufacturer's directions. Gene-specific primer sequences (Table 2.1) were identified from the literature for the target genes which included corticotropin releasing factor (*crf*), steroidogenic acute regulatory protein (*star*), 11ßhydroxysteroid dehydrogenase type 2 (*11ßhsd2*), glucocorticoid receptor (*gr*), and mineralocorticoid receptor (*mr*). Two housekeeping genes were used, *ß-actin* and elongation factor alpha-1 (*elfa-1*), with primer sequences from McCurley and Callard (2008). Transcript abundances were assessed by semi-quantitative real-time RT-PCR using the SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) and a CFX96 Touch Real-Time PCR System (Qiagen). Reactions were carried out according to the manufacturer's instructions, but volumes

were scaled to 10 µL. Samples were assayed in duplicate, and negative controls (a no-template sample in which cDNA was replaced with water, and a no-RT sample in which the cDNA synthesis reaction was carried out without reverse transcriptase) were included in each assay. Cycling conditions were 5 min at 95°C, 40 cycles of 10 s at 95°C, 10 s at 60°C and 10 s at 72°C, and were followed by melt curve analysis, as per the manufacturer's instructions. Serially diluted pooled samples were used to generate standard curves for each target and housekeeping gene. Relative transcript abundances were calculated according to Pfaffl (2001).

2.2.5 Statistical analysis

Statistical analysis was conducted using R (v3.5.0) and RStudio (v1.0.153). All data were checked before analysis for violations of the assumptions of parametric tests. Normality was checked using the Kolmogorov–Smirnov test, and equal variance using Levene's test. For all tests, α was set to 0.05. Comparisons among groups were carried out using analysis of variance (ANOVA). Data are generally presented as boxplots, with the lower limit of the box representing the 25^{th} percentile, and the upper limit representing the 75^{th} percentile. The whiskers represent the highest and lowest recorded values that were not statistical outliers. The mean is represented by a solid line within the box. Individual data points are presented as symbols.

Table 2.1. Primer pairs used for semi-quantitative real-time RT-PCR.

Gene	Primer Pair (5'-3')	Accession Number	Amplicon size (bp)	Efficiency	Reference
crf	F: CAC CGC CGT ATG AAT GTA GA R: GAA GTA CTC CTC CCC CAA GC	NM001007379.1	113	88.7%	Nesan and Vijayan, 2016
star	F: TCA AAT TGT GTG CTG GCA TT R: CCA AGT GCT AGC TCC TGC TC	NM131663.1	122	107.5%	Alsop and Vijayan, 2008
11ßhsd2	F: TGC TGC TGG CTG TAC TTC AC R: TGC ATC CAA CTT CTT TGC TG	NM212720.2	123	101.8%	Alsop and Vijayan, 2008
gr	F: ACA GCT TCT TCC AGC CTC AG R: CCG GTG TTC TCC TGT TTG AT	NM001020711.3	116	110.2%	Alsop and Vijayan, 2008
mr	F: CCC ATT GAG GAC CAA ATC AC R: AGT AGA GCA TTT GGG CGT TG	NM001100403.1	106	105.5%	Alsop and Vijayan, 2008
ß-actin	F:GTC CCT GTA TGC CTC TGG T R: AAG TCC AGA CGG AGG ATG	NM181601.5	120	97.9%	McCurley and Callard, 2008
elf-1a	F: CTT CTC AGG CTG ACT GTG C R: CCG CTA GCA TTA CCC TCC	NM131263.1	358	95.7%	McCurley and Callard, 2008

2.3 Results

2.3.1 Survival and hatch rate

Exposure to pH 4 water had no significant impact on survival. All treatment groups exhibited some mortality during the first 24 hpf, but survival to 1 dpf was not significantly influenced by treatment group (Fig. 2.1, ANOVA, $p = 0.51$). From 1 to 4 dpf, mortality was very low, and no significant differences in survival among treatment groups were detected at 4 dpf (Fig 2.1, ANOVA, $p = 0.51$). A significant effect of treatment on hatch rate was detected at 52 hpf, but at no other time (Fig 2.2, ANOVA, $p < 0.0001$). Specifically, treatment groups that were exposed to pH 4 conditions before 48 hpf (i.e. the early and four day exposure groups) hatched more rapidly than the control group, with a greater proportion of (hatched) larvae per dish at 52 hpf. The switch from control conditions to pH 4 water at 48 hpf (i.e. the late treatment group) did not seem to affect hatch rate; the hatch rate for the late exposure group was not significantly different from that of the control group.

2.3.2 Whole-body cortisol concentrations

No significant differences in baseline whole-body cortisol levels were detected among treatment groups at 4 dpf (Fig 2.3A, ANOVA, $p = 0.166$). To confirm this result, two additional trials were carried out. In one, baseline whole-body cortisol levels in 4 dpf larvae raised under control conditions were compared with those of larvae transferred to pH 4 water at 48 hpf (Fig. 2.3B). In the second, baseline whole-body cortisol levels were compared in 4 dpf larvae raised under control conditions versus those transferred to pH 4 water at 72 hpf (Fig. 2.3C). Again, no significant differences were detected between control fish and those exposed to pH 4 water at 48 hpf (ANOVA, $p = 0.3$), nor between the control group and the group exposed to pH 4 water at 72 hpf (ANOVA, $p = 0.27$).

At 6 dpf, no significant differences in baseline cortisol levels were detected across treatment groups (Fig 2.3D), and in all groups, cortisol levels post-stressor were significantly higher than baseline values (2-way ANOVA, $p_{treatment} = 0.045$, $p_{stressor} < 0.001$, $p_{treatmentxstressor} = 0.0014$). Additionally, larvae exposed to pH 4 (early, late, and four day treatment groups) had significantly higher cortisol levels post-stressor than the control group (Tukey HSD, $p = 0.032$, 0.045, and 0.001, respectively).

At 15 dpf, there were no systematic differences detected. Treatment group did not affect cortisol levels under baseline or post-stress conditions, but post-stressor cortisol levels were significantly higher than baseline levels (Fig. 2.3E, 2-way ANOVA, $p_{treatment} = 0.79$, $p_{stressor} < 0.001$, $p_{treatmentxstressor} = 0.09$).

Owing to the absence of significant differences in whole-body cortisol concentrations among the pH 4 exposure groups (early, late and four day), transcript abundances of genes of the stress axis were compared between control and four day exposure groups only. No significant differences in relative transcript abundances were detected (Table 2.2).

Using the alternative ionoregulatory challenge of varying water Na^+ concentrations, a significant effect on baseline whole-body cortisol levels was detected at 4 dpf, (Fig 2.4A, ANOVA, $p = 0.0032$), with larvae in the high Na^+ treatment group having significantly higher cortisol levels than those in the control group but not the low Na^+ treatment group. At 6 dpf, cortisol concentrations were not affected by treatment group under baseline or post-stressor conditions, but larvae that had been exposed to the stressor had significantly higher cortisol concentrations than those measured under baseline conditions (Fig. 2.4B, 2-way ANOVA, $p_{treatment} = 0.016$, $p_{stressor} < 0.001$, $p_{treatmentxstressor} = 0.68$).

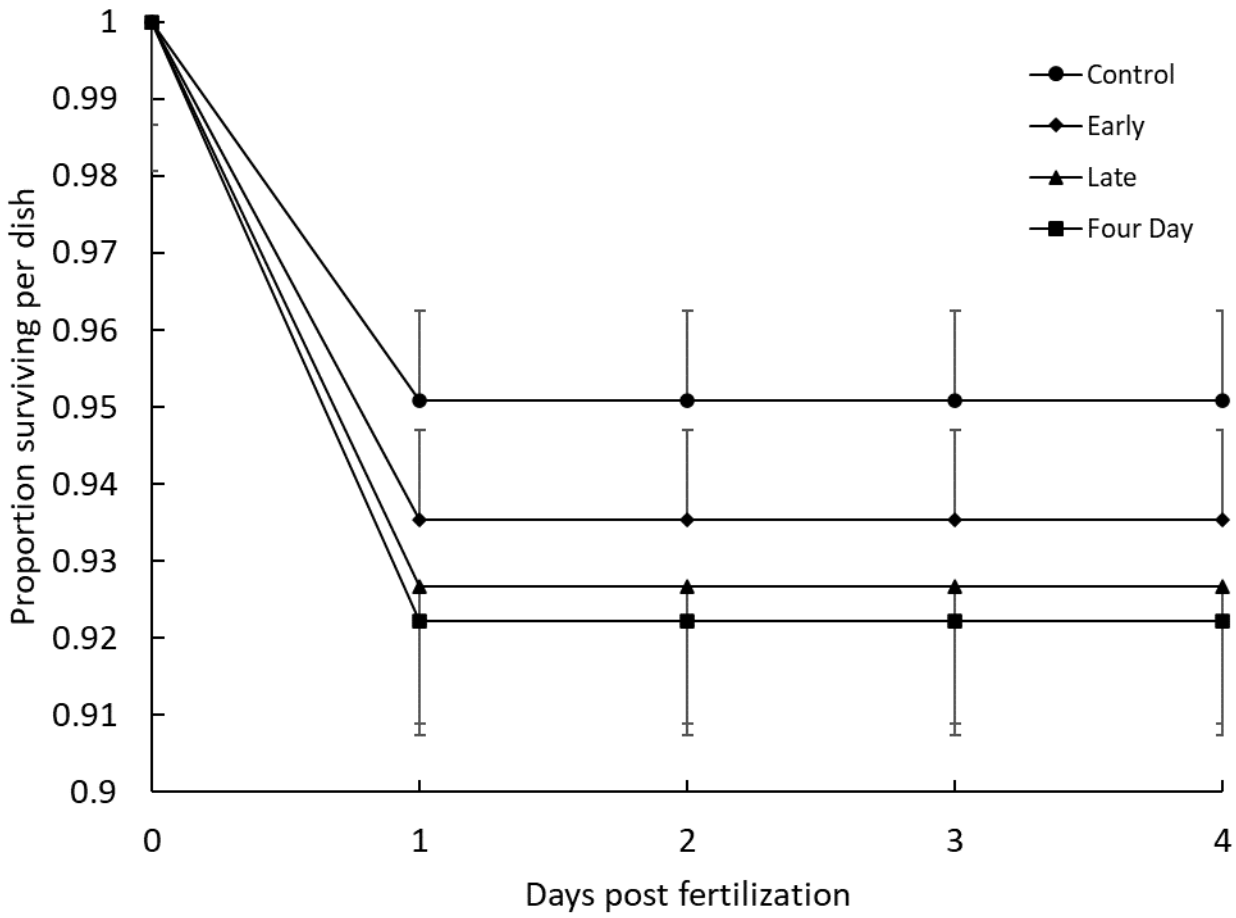

Figure 2.1. Cumulative survival (as a proportion of the initial number of embryos in a Petri dish) of zebrafish (*Danio rerio*) embryos and larvae exposed to water of pH 4 for different periods. Treatment groups included control (no pH 4 exposure), early (pH 4 exposure from 0 to 2 dpf), late (pH 4 exposure from 2 to 4 dpf), and four day exposure (pH 4 exposure from 0 to 4 dpf). Values are means ± SEM, N = 20-22 Petri dishes per treatment group. No significant differences in survival were detected at 1 or 4 dpf (ANOVA, N = 22, p = 0.51 for each time point).

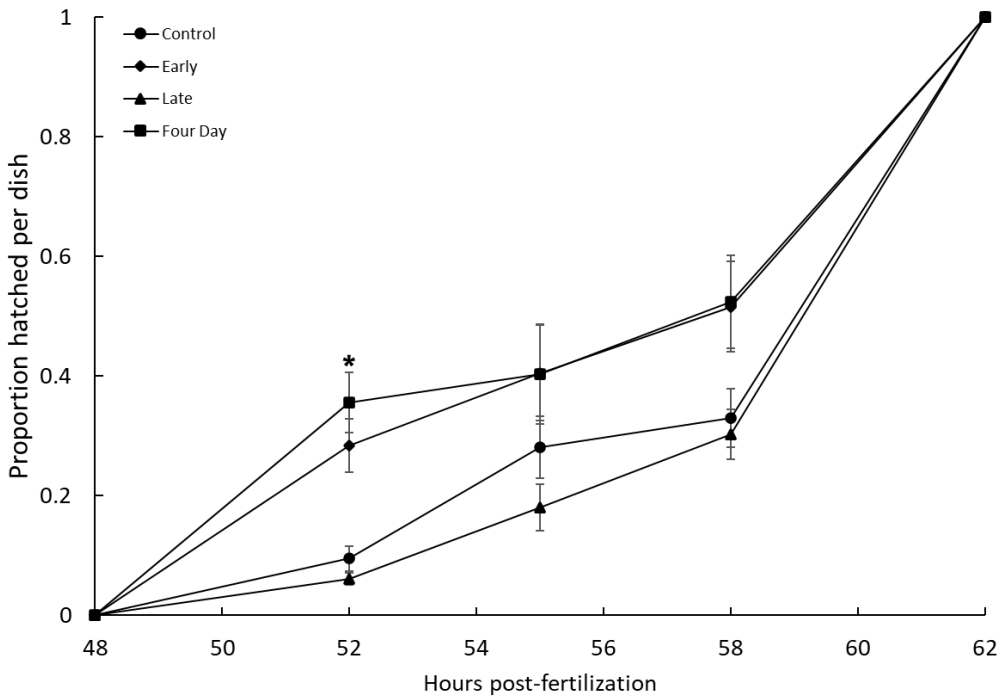

Figure 2.2. Proportion of individuals per Petri dish that hatched as a function of time for zebrafish (*Danio rerio*) embryos exposed to water of pH 4 for different periods. Treatment groups included control (no pH 4 exposure), early (pH 4 exposure from 0 to 2 dpf), late (pH 4 exposure from 2 to 4 dpf), and four day exposure (pH 4 exposure from 0 to 4 dpf). Values are means ± SEM, *N* = 9-10 Petri dishes per treatment group. An asterisk denotes a significant difference from the control group (ANOVA, $p < 0.001$, 0.061 and 0.061 for 52, 55 and 58 hpf, respectively). No larvae in any treatment group had hatched at 48 hpf, and all larvae in every treatment group had hatched by 62 hpf.

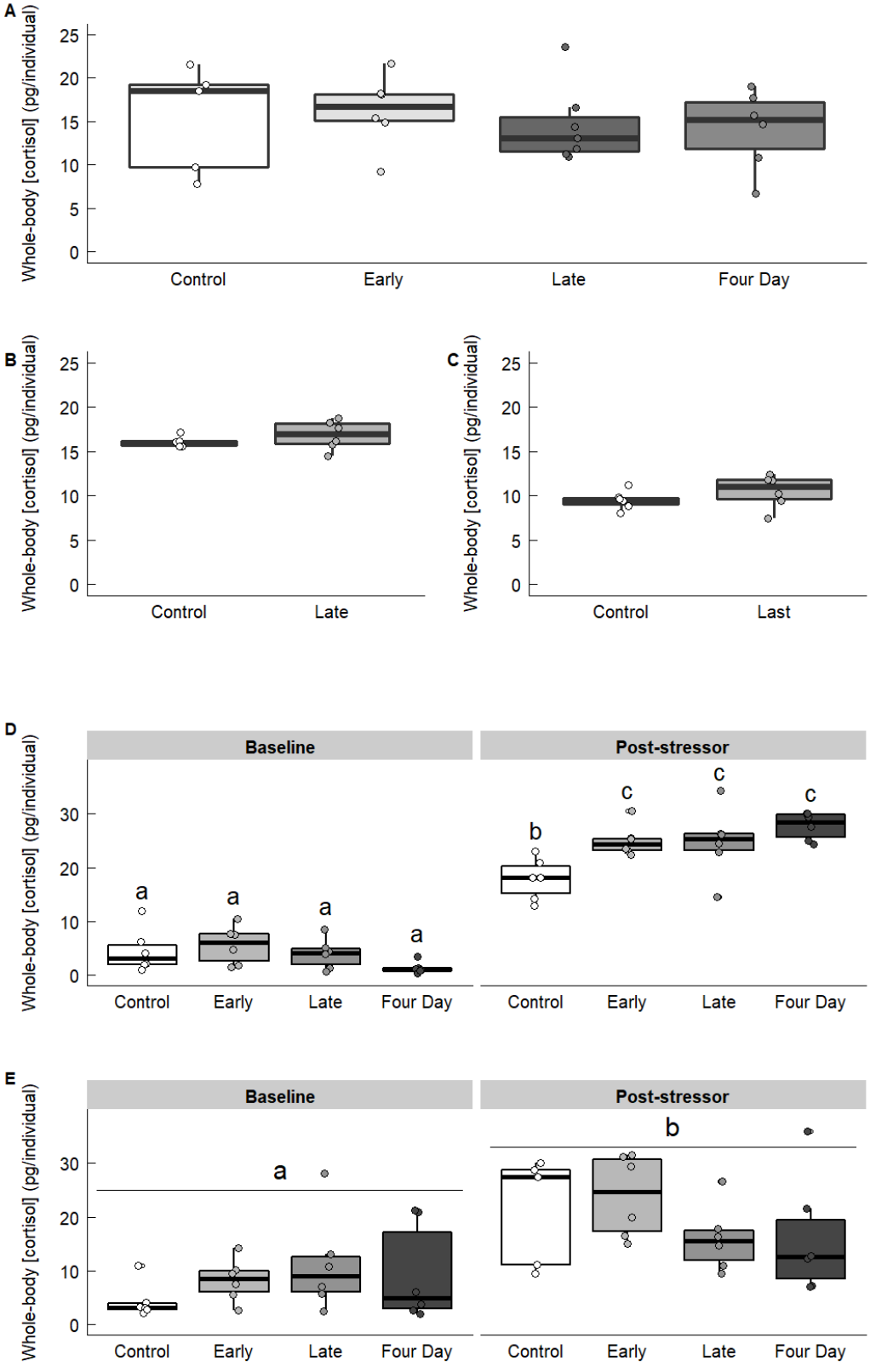
A
Whole-body [cortisol] (pg/individual)
25
20
15
10
5
0
Control
Early
Late
Four Day
B
Whole-body [cortisol] (pg/individual)
Control
Late
C
Whole-body [cortisol] (pg/individual)
Control
Last
D
Whole-body [cortisol] (pg/individual)
Baseline
Post-stressor
30
20
10
0
a
a
a
a
b
c
c
c
Control
Early
Late
Four Day
E
Whole-body [cortisol] (pg/individual)
Baseline
Post-stressor
a
b
Control
Early
Late
Four Day

Figure 2.3. Whole-body cortisol concentrations in 4 dpf (A-C), 6 dpf (D), and 15 dpf (E) zebrafish (*Danio rerio*) that were exposed to water of pH 4 for different durations during early development. Treatment groups included control (no pH 4 exposure), early (pH 4 exposure from 0 to 2 dpf), late (pH 4 exposure from 2 to 4 dpf), four day exposure (pH 4 exposure from 0 to 4 dpf), and late exposure (pH 4 exposure from 3 to 4 dpf). Cortisol levels were measured under baseline conditions or following an air-exposure stressor. Post-stressor cortisol levels were not measured at 4 dpf because zebrafish of this age do not mount a robust cortisol response to a stressor. Bars or groups that share a letter are not significantly different from one another (panel A, ANOVA, $p = 0.167$; panel B, Student's *t*-test, $p = 0.3$; panel C, Student's *t*-test, $p = 0.23$; panel D, 2-way ANOVA, $p_{treatment} = 0.045$, $p_{stressor} < 0.001$, $p_{treatmentxstressor} = 0.0014$; panel E, 2-way ANOVA, $p_{treatment} = 0.79$, $p_{stressor} < 0.001$, $p_{treatmentxstressor} = 0.09$). Data are presented as boxplots, with the upper and lower limits of the box representing the 75^{th} and 25^{th} quartiles, respectively. The bold line across the box indicates the mean value. Individual data points are indicated by the symbols; $N = 6$ per group except the 4 dpf data for the late exposure treatment group where $N = 7$.

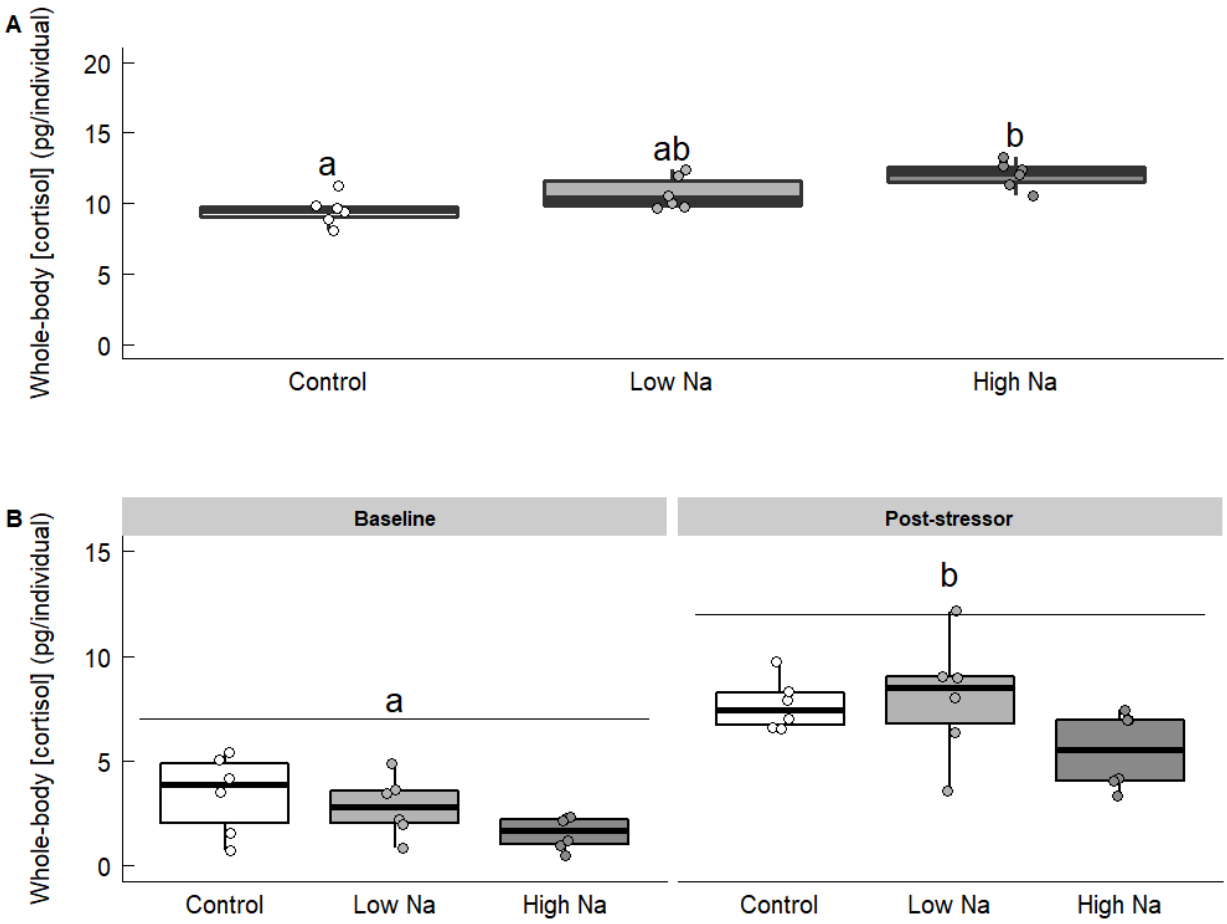

Figure 2.4. Whole-body cortisol concentrations in 4 dpf (A) and 6 dpf (B) zebrafish (*Danio rerio*) that were exposed to water of different Na^+ concentrations. Cortisol levels were measured under baseline conditions or following an air-exposure. Post-stressor cortisol levels were not measured at 4 dpf because zebrafish of this age do not mount a robust cortisol response to a stressor. Bars or groups that share a letter are not significantly different from one another (panel A, ANOVA, $p = 0.0032$; panel B, 2-way ANOVA, $p_{treatment} = 0.016$, $p_{stressor} < 0.001$, $p_{treatmentxstressor} = 0.68$). Data are presented as boxplots, with the upper and lower limits of the box representing the 75^{th} and 25^{th} quartiles, respectively. The bold line across the box indicates the mean value. Individual data points are indicated by the symbols; $N = 6$ in all groups.

Table 2.2. Relative transcript abundances for genes of the stress axis in 4, 6 and 15 dpf zebrafish (*Danio rerio*) raised under control conditions or in water of pH 4 from 0-4 dpf.

	Gene	Control	pH 4	p-value
4 dpf	*crf*	1.01 ± 0.06	1.26 ± 0.15	0.95
	star	1.18 ± 0.29	1.32 ± 0.28	0.86
	11bhsd2	1.05 ± 0.13	1.34 ± 0.36	0.82
	gr	1.05 ± 0.13	1.47 ± 0.21	0.98
	mr	1.06 ± 0.15	0.93 ± 0.22	0.37
6 dpf	*crf*	1.05 ± 0.13	0.97 ± 0.05	0.26
	star	1.12 ± 0.29	0.93 ± 0.01	0.23
	11bhsd2	1.25 ± 0.38	0.73 ± 0.06	0.11
	gr	1.18 ± 0.27	0.81 ± 0.11	0.14
	mr	1.28 ± 0.43	0.56 ± 0.064	0.08
15 dpf	*crf*	1.02 ± 0.1	1.48 ± 0.41	0.87
	star	1.05 ± 0.14	1.9 ± 0.47	0.96
	11bhsd2	1.25 ± 0.4	1.51 ± 0.57	0.81
	gr	1.03 ± 0.12	2.08 ± 0.77	0.91
	mr	1.03 ± 0.11	1.2 ± 0.52	0.64

Values are means ± SEM, $N = 6$ for all groups. The p values were determined using one sample Student's *t*-tests on the pH 4 values. Transcript abundances were calculated using *ß-actin* and *ef1α* as housekeeping genes and were expressed relative to the value for the corresponding control group.

2.4 Discussion

The goal of this study was to determine whether exposure to pH 4 water in early development has a lasting effect on the function of the HPI axis. Previous studies reported that larval zebrafish exposed to pH 4 water at 3-4 dpf exhibit elevated whole body cortisol concentrations compared to larvae raised in control water (Kumai et al., 2012; Kumai et al., 2015). For example, Kumai et al. (2012) found cortisol concentrations of 300 pg/individual in larval zebrafish exposed to pH 4 water for 24 h at 4 dpf, compared to 150 pg/individual for control fish. Similarly, cortisol levels in larval zebrafish exposed to pH 4 water for 48 h were 1.6-fold higher than those in control fish (Kumai et al., 2015), although the absolute values in this study were much lower than those reported by Kumai et al. (2012), likely owing to differences in the cortisol extraction protocols used in the different studies. Accompanying these differences in whole-body cortisol levels, Kwong et al., (2014) noted, anecdotally, that elevated GR transcript abundance occurred in pH 4-exposed larvae. To our surprise, however, we were unable to detect any differences in baseline cortisol concentrations with exposure to pH 4 water, regardless of the duration or window of exposure, or the age of the fish examined. This difference may be explained by the way in which samples were collected. In the current study, multiple batches of larvae were collected from genetically different fish, to ensure that a diverse pool of individuals was tested, and this approach likely caused higher variation in the reported values. It is possible that previous studies collected less diverse samples, making it easier to detect small changes in baseline cortisol values. Alternatively or additionally, individual zebrafish may differ in their tolerance of pH 4 water. For example, JavadiEsfahani and Kwong (2019) found that pH 4 exposure significantly reduced survival to 3 dpf (to ~75%), whereas the survival of pH 4-exposed fish in the present study exceeded 90%. In agreement with the lack of

impact on baseline cortisol concentrations in the present study, no differences in transcript abundances of stress axis or cortisol receptor genes were detected between control and pH 4-exposed fish.

To explore the effects of pH 4 exposure on the stress axis in more detail, whole-body cortisol responses to a standardized air-exposure stressor were assessed at 6 and 15 dpf, ages where a robust cortisol response is present (Alderman and Bernier, 2009; Alsop and Vijayan, 2008; Hare et al., 2020; Jeffrey and Gilmour, 2016). At 6 dpf, larvae that had been exposed to pH 4 water during early development had higher post-stressor cortisol concentrations than control fish. By 15 dpf, however, there was no longer a difference in post-stressor cortisol levels between pH 4-exposed fish and control fish. Several studies have reported that early life stress in zebrafish influences stress responses later in life. For example, Hare et al. (2020) found that larval zebrafish exposed to a repeated air-exposure stressor at 4 dpf had higher post-stressor cortisol levels compared to a control group at 15 but not 7 or 35 dpf. However, larvae that were exposed to the stressor at 7 dpf did not exhibit significant differences in cortisol levels post-stressor later in development (Hare et al., 2020). Similarly varied responses have been reported for zebrafish exposed to other stressors, including elevated water ammonia levels (Williams et al., 2017), anoxia (Ivy et al., 2017), visual contact with a predator (Abreu et al., 2018), or high water flow (Castillo-Ramírez et al., 2019; see Table 1 in Hare et al. 2020 for a summary). To date it appears that there is some capacity for the cortisol stress response to be shaped by events during larval and juvenile development, but it appears limited relative to the greater level of plasticity occurring in response to cortisol manipulation during embryonic development (Hare et al. 2020). The results of the present study are consistent with this trend.

To complement experiments with pH 4 water, exposure to altered water Na^+ levels also was examined, because Lin et al. (2016) found that 3 dpf zebrafish raised in water with a high concentration of Na^+ had significantly lower whole-body cortisol levels than those raised in water of low Na^+ concentration. Using the same Na^+ levels as Lin et al. (2016) with the addition of a control Na^+ concentration that matched that of the water in our facility, we found that 4 dpf, zebrafish raised in high Na^+ water had significantly higher levels of cortisol than the control group. By 6 dpf, however, no significant differences in baseline cortisol levels were detected, although it should be noted that fish were transferred to control water at 5 dpf, to match the experimental design of the pH 4 trials. Post-stressor cortisol levels at 6 dpf similarly were unaffected by Na^+ conditions during rearing. As with the pH 4 trials, the cause of these differences between studies was not clear.

The current study aimed to determine whether exposure to an ionoregulatory challenge during early development affects HPI axis development or activity. Previous studies found that increased cortisol levels alter ionoregulation, regardless of whether the elevated cortisol stems from exogenous sources or is produced endogenously through a stress response. For example, larval zebrafish treated with exogenous cortisol exhibited increases in Ca^{2+} and Na^+ uptake (Kumai et al., 2012; Kumai et al., 2015), and larval zebrafish exposed to a stressor in early life had higher whole-body concentrations of Na^+ and Ca^{2+} (Hare et al., 2020). The present study aimed to determine whether the reverse is also true, that is whether elevation of cortisol in response to an ionoregulatory disturbance alters development or activity of the stress axis. Overall, the findings of the present study did not provide strong evidence that exposure to an environment that presents an ionoregulatory challenge in early life affects the development and function of the HPI axis later in development. However, our ability to test this hypothesis was

limited by the apparently high tolerance of our zebrafish larvae to the ionoregulatory challenges that they faced. In contrast with previous studies, neither pH 4 water nor manipulation of water Na^+ concentrations caused significant elevation of cortisol levels. Thus, although the present data do not support a role for ionoregulatory challenges in altering stress axis development or function, it remains possible that more severe ionoregulatory disturbances may impact HPI axis function.

Chapter 3: The osmorespiratory compromise in a low pH environment, using zebrafish (*Danio rerio*) as a model.

3.1 Introduction

The fish gill is a multi-functional organ that plays several crucial roles for teleost fish, particularly in gas transfer and ionic regulation (Evans et al., 2005). As a gas transfer organ, the structure of gills should maximize the rate of passive O_2 diffusion by having a high surface area and permeability, low blood-to-water diffusion distance, and effective mechanisms for ventilation and perfusion to deliver O_2 to the gill and carry it away to the tissues, respectively (Evans et al., 2005; Huang and Lin, 2016; Hughes, 1966; Hughes and Morgan, 1973). These structural requirements are met in the fish gill through the presence of four gill arches, each with two rows of filaments. Each filament carries many small lamellae formed of two thin epithelial layers, and these lamellae serve as the site of gas transfer (Hughes and Morgan, 1973). However, the large surface area and thin epithelium of the gill also facilitate the undesirable passive diffusion of ions and water.

Freshwater teleost fish are hyperosmotic and hyperionic to their environment and experience passive ion loss and water gain (Evans, 2008; Laurent and Perry, 1991; Takei and Hwang, 2016). To counter passive ion loss, specialized cells known as ionocytes actively take up ions from the surrounding fresh water (Guh et al., 2015; Takei and Hwang, 2016). Ionocytes are found in the gill epithelium of adult fish and on the skin of developing fish (Guh et al., 2015; Takei and Hwang, 2016). In zebrafish (*Danio rerio*), at least five ionocyte types exist, including H^+-ATPase-rich (HR) cells that function in Na^+ uptake, Na^+-Cl^- cotransporter (NCC) cells that function in Na^+ and Cl^- uptake, Na^+-K^+-ATPase-rich (NaR) cells that function in Ca^{2+} uptake, solute carrier 26-expressing cells that function in acid-base balance, and K^+ secreting cells (Guh et al., 2015; Hwang, 2009; Takei and Hwang, 2016). In HR cells, proton and ammonia secretion are linked to the uptake of Na^+ (Guh et al., 2015), and environmental conditions such as low pH

can disrupt this mechanism (Kumai and Perry, 2011; Kumai et al., 2012; Kwong et al., 2014). Low pH conditions not only impair Na^+ uptake, but also increase passive Na^+ loss, leading to an overall decrease in whole body Na^+ content (Kumai et al., 2012; Kwong et al., 2014). In response, zebrafish exposed to low pH conditions experience increases in the expression of ion transporters and associated proteins involved in Na^+ uptake, as well as the proliferation of HR and NCC ionocytes (Guh et al., 2015; Kumai et al., 2015; Kwong et al., 2014; Lin et al., 2016a). The present study aimed to determine whether these responses aimed at maintaining ion balance in low pH water have a trade-off in terms of negatively impacting gas transfer.

The potential trade-offs between gas transfer and ion balance at the fish gill are formalized in the concept of the osmorespiratory compromise (Gilmour and Perry, 2018; Gonzalez, 2011; Nilsson et al., 2012; Wood and Eom, 2021). According to the osmorespiratory compromise, gill structure represents a compromise between the minimum rate of O_2 transfer that is sufficient for metabolism and the maximum rates of passive ion and water movements that can be tolerated. Thus, changes in gill morphology that favour O_2 uptake by increasing gill surface area or reducing blood-to-water diffusion distances would be expected to increase rates of passive ion and water movement, a prediction that has some empirical support (e.g. Sollid et al. 2003; but see Gilmour and Perry 2018). Similarly, proliferation of ionocytes, which tend to be larger cells than the flattened pavement cells that make up the bulk of the lamellar epithelium, might hinder gas exchange by increasing the blood-to-water diffusion distance. For example, proliferation of ionocytes in the gill of rainbow trout increased the blood-to-water diffusion distance and impaired hypoxia tolerance and exercise performance (Bindon et al., 1994a; Bindon et al., 1994b; Greco et al., 1995; Greco et al., 1996; Perry et al., 1996; reviewed by Perry, 1998). Although many experiments have examined the osmorespiratory compromise in response to

short-term exposures to environmental conditions expected to alter the balance of this trade-off, fewer studies have examined longer-term challenges, particularly in the context of development.

The present study addressed the question of whether gill morphology and function were altered in accordance with the osmorespiratory compromise in zebrafish raised in a low pH environment. Although the ionoregulatory responses of zebrafish to low pH water have been characterized in some detail (Guh et al., 2015; Kwong et al., 2014), these studies have focused essentially exclusively on fish up to 4 days post-fertilization (dpf). Whether these ionoregulatory responses are maintained as the fish develop and mature has not been examined. In keeping with the osmorespiratory compromise, we hypothesized that the ionoregulatory challenges of life in a low pH environment would cause changes in gill morphology that would impair gas transfer in zebrafish raised under these conditions. A first step in testing this hypothesis was to determine whether ionoregulatory responses to low pH were maintained as fish developed and matured. Whole-body cortisol concentrations and Na^+ content as well as abundances of the ionocytes responsible for Na^+ uptake, the HR and NCC cells, were measured in zebrafish at 6, 15, 30 and 90 dpf for this purpose. To invoke the osmorespiratory compromise, the ionoregulatory responses must alter gill structure, and therefore we assessed indices of gill surface area and epithelial thickness. The osmorespiratory compromise predicts that gas transfer is impaired as a consequence of the alterations in gill structure. Thus, zebrafish reared under low pH conditions would be predicted to exhibit reduced hypoxia tolerance, which in the present study was assessed by measuring ventilation, the critical oxygen tension (P_{crit}), and the time to loss of equilibrium (LOE) during hypoxic exposure.

3.2 Materials and methods

3.2.1 Experimental animals

The fish used for the present experiments were bred from wild-type zebrafish (*Danio rerio*) from the University of Ottawa stock. The adult breeding stock were held at a density of 4 fish L^{-1} in 10 L tanks supplied with flowing, dechloraminated, city of Ottawa tap water ("system water"; in mmol L^{-1}, 0.8 Na^+, 0.4 Cl^-, 0.25 Ca^{2+}; pH 7.2) at 28°C. Fish were fed 5% body mass per day of No. 1 crumble-Zeigler commercial fish food (Aquatic Habitats, Apopka, FL, USA) and held on a 14L:10D photoperiod. To obtain embryos, two male and three female fish were bred in an acrylic breeding trap with a perforated bottom insert that allowed for embryo collection.

Embryos were randomly allocated to either a control group that was maintained in system (pH 7.2) water or a pH 4 treatment group that was maintained in system water adjusted to pH 4 with H_2SO_4. Embryos were reared in an incubator at 28.5°C in Petri dishes with 20-35 embryos per 50 mL dish; water was changed daily and dead embryos were removed. At 5 dpf, larvae were transferred to 500 mL tanks at a density of ~20-35 larvae per tank. The volume of water in the tank was increased from 100 mL to 500 mL over four days, after which water was changed daily. Larvae were fed daily with a satiating meal of GEMMA 75 micro fish feed (Skretting USA, Westbrook, ME, USA) starting at 6 dpf. At 15 dpf, juveniles were transferred to glass aquaria (19 L volume) at a density of 40 larvae per tank. The volume of water in the aquaria was increased from 4 L to 19 L over four days and then changed (50% replacement) on a daily basis. From 15 to 30 dpf, juveniles were fed daily with a satiating meal of GEMMA 150 (Skretting), which was changed to GEMMA 300 (Skretting) for 30 to 60 dpf. After 60 dpf, fish were fed No. 1 crumble-Zeigler (Aquatic Habitats).

Measurements were carried out at 6, 15, 30 and 90 dpf, time-points that correspond to significant developmental milestones. At 6 dpf, larvae exhibit a robust cortisol response to a stressor (Alderman and Bernier, 2009; Alsop and Vijayan, 2008), and begin to use the gill for ion uptake and gas transfer (Rombough, 2002); by 15 dpf, they rely on the gill for these processes (Jonz and Nurse, 2005; Rombough, 2002). The 30 dpf time-point occurs just prior to sexual differentiation (which is ~35 dpf in domesticated zebrafish, Uchida et al., 2002), and zebrafish reach sexual maturity at ~90 dpf.

All holding and experimental protocols were approved by an institutional animal care committee (protocol BL-2118) and were in compliance with the guidelines of the Canadian Council on Animal Care (CCAC) for the use of animals in teaching and research.

3.2.2 Experimental design

The aim of the present experiment was to investigate whether development in pH 4 water, which serves as an ionoregulatory challenge for zebrafish (reviewed by Kwong et al. 2014), alters gill morphology and hence gas transfer in accordance with the osmorespiratory compromise. Thus, indicators of ion balance and respiratory function were measured together with indicators of gill morphology. Length and mass were measured to assess growth. Whole-body Na^+ and cortisol concentrations together with ionocyte abundance were measured as indicators of ion balance. Indicators of gas transfer included morphometric indices of gill blood-to-water diffusion distance, ventilation frequency (f_V), rates of oxygen consumption ($\dot{M}O_2$), P_{crit}, and time to LOE during acute exposure to hypoxia.

3.2.3 Growth

Fish younger than 90 dpf were individually photographed at 20x magnification using a digital camera attached to a dissecting microscope. The length of each fish was determined from

its photograph using ImageJ v1.53a (National Institute of Health, USA) and a 1 mm scale bar included in each image. Fish length was measured as the straight-line distance from the anterior end of the head to the base of the caudal fin. Following imaging, the mass of each 30 dpf individual was measured using a microbalance with a limit of detection of 0.0001 g (UMX2 Ultra-microbalance; Mettler Toledo, Mississauga, ON, Canada). Fish were euthanized in a solution of Tris-buffered MS-222 (0.72 mg mL^{-1} tricaine methanesulphonate; Syndel Laboratories, Nanaimo, BC, Canada). An individual fish was placed on a pre-weighed paper, and lightly dabbed with a Kimwipe to remove water droplets. Mass was recorded immediately because exposure to air caused rapid dehydration. A regression of mass on length (mass = 0.0006·length - 0.0027, $R^2 = 0.87$) was used to estimate mass from measurements of length where the mass of individual fish was required ($\dot{M}O_2$; see below). At 6 and 15 dpf, the masses of 20 individual larvae in each of the control and pH 4 treatment groups were measured using the same approach, and mean masses for the control and pH 4 groups were used in calculations of $\dot{M}O_2$.

A ruler and analytical balance (ED124S Extend Analytical Balance; Sartorius Canada, Oakville, ON, Canada) were used to measure the fork length and mass, respectively, of 90 dpf zebrafish. Fish were lightly dabbed with paper towel to remove excess water prior to weighing.

3.2.4 Whole-body cortisol concentrations

Baseline whole-body cortisol concentrations were measured for pooled 6 dpf larvae (25 larvae for $N = 1$) and 15 dpf juveniles (15 fish for $N = 1$), and on individual 30 and 90 dpf fish. Pools of 6 dpf larvae and 15 dpf juvenile fish were euthanized in a solution of Tris-buffered MS-222 (0.72 mg mL^{-1}; Syndel Laboratories). For 30 dpf fish, individuals were photographed as described above and allowed to recover from handling stress for 3 h prior to being euthanized in

buffered MS-222. The 90 dpf fish were quickly netted from their holding tank, euthanized within 2 min using buffered MS-222, as described above, and weighed. All samples were flash frozen in liquid N_2 and stored at -80°C for later extraction and measurement of whole-body cortisol concentrations by ELISA (Neogen, Lansing, MI, USA).

For larvae and juvenile fish, cortisol was extracted from homogenized samples using the protocol of Jeffrey and Gilmour (2016), as described in section 2.2.3. Fish of 90 dpf were homogenized in 10:1 vol/vol of extraction buffer (Neogen). Homogenates were extracted three times with 1 mL of diethyl ether each time. After each addition of diethyl ether, samples were vortexed, incubated for 30 min at room temperature, centrifuged at room temperature at 3,000 *g* for 5 min, and frozen at -80°C for 30 min. The liquid phase was then removed to a new microcentrifuge tube and evaporated under forced air. The combined extracts were reconstituted in 200 µL of extraction buffer (Neogen), vortexed, incubated at 65°C for 2 x 5 min, and stored at -80°C until analysis.

All extracts were measured in duplicate by ELISA according to the manufacturer's instructions (Neogen). Inter-assay variability was 3.0% CV, and intra-assay variability was 10.8% CV.

3.2.5 Whole-body Na^+ concentrations

As with cortisol, whole-body Na^+ concentrations were measured for pools of 6 dpf larvae (25 larvae for $N = 1$) and 15 dpf juveniles (15 fish for $N = 1$), but individual 30 dpf and 90 dpf fish. Fish were euthanized in MS-222 as described above. Samples were digested at 65°C overnight for 6, 15 and 30 dpf fish, and over two days for 90 dpf fish, in 100 µL (6 and 15 dpf samples) or 10 mL per g of body mass (30 and 90 dpf samples) of 2 N HNO_3. Body mass for each 30 dpf fish was estimated from its length using the previously mentioned regression of mass

on length; 90 dpf fish were weighed prior to digestion. After the overnight incubation, samples were thoroughly vortexed, and an aliquot was diluted 12-fold (6 and 15 dpf fish) or 20-fold (30 and 90 dpf fish) for measurement of Na^+ concentrations by atomic absorption spectrophotometry (Spectra AA 220FS; Varian, Palo Alto, CA). Measured Na^+ concentrations were determined from absorbances measured for NaCl standards containing the same concentration of HNO_3.

3.2.6 Ionocyte abundance

Ionocyte abundance was assessed using immunohistochemistry (IHC) and confocal microscopy to identify ionocytes in the skin (6 dpf larvae) or gill (15, 30 and 90 dpf fish). Three ionocyte types were assessed. Concanavalin A (conA; 6 dpf larvae) or V-type H^+-ATPase IHC (15, 30 and 90 dpf fish) was used to identify HR cells (Lin et al., 2006), Na^+,K^+-ATPase IHC was used to identify NaR cells (Liao et al., 2007), and Na^+,Cl^- co-transporter IHC was used to identify NCC cells (Kwong and Perry, 2016). Fish used for IHC were euthanized by immersion in a buffered solution of MS-222 and fixed overnight at 4°C in 4% paraformaldehyde (PFA; Santa Cruz Biotechnology, Santa Cruz, CA, USA). For 90 dpf fish, only the head was fixed, following removal of the right operculum.

Prior to being euthanized, 6 dpf larvae were first stained with conA conjugated to Alex Fluor 488 (Invitrogen, Waltham, MA, USA) by holding the fish for 30 min in system or pH 4 water, as appropriate, containing 0.05 mg mL^{-1} conA. Fixed larvae were washed 3 x 5 min in phosphate-buffered saline (PBS) at room temperature, and antigen retrieval was carried out using pH 9 buffered Tris for 15 min at room temperature. After washing [5 x 5 min in PBS containing 0.1% Tween 20 (PBST)], larvae were incubated for 2 h at room temperature in blocking solution (0.8% Triton X-100 and 3% bovine serum albumin (BSA) dissolved in PBST) and then with primary antibodies (Table 3.1), again for 1 h at room temperature. After rinsing 5 x 5 min with

PBST, larvae were incubated in the dark on a shaker for 1 h with the appropriate secondary antibodies (Table 3.2) in 0.8% Triton X-100 in PBST solution. Finally, samples were rinsed (5 × 5 min with PBST) and mounted with Vectashield mounting medium containing 4′,6-diamidino-2-phenylindole (DAPI; Vector Laboratories Inc., Burlingame, CA) for imaging.

After fixation, 15 dpf and 30 dpf fish and the heads of 90 dpf fish were washed (3 x 5 min in PBS) and incubated in 30% sucrose at 4°C for 48 h. Prior to this cryo-protection step, 90 dpf samples were decalcified by immersion in 10% HCl for 20 min at room temperature. Cryo-protected samples were embedded in optimal cutting temperature (OCT; Thermo Fisher Scientific, Ottawa, ON, Canada) compound for 45 min. Larvae (15 and 30 dpf) were cryosectioned (CM3050 S, Leica Biosystems, Concord, ON, Canada) at 30 µm intervals in the horizontal plane from the ventral surface. Samples from 90 dpf fish were sectioned at 30 µm intervals in the sagittal plane, starting from the right side where the operculum had been removed. Sections were collected onto Fisherbrand Superfrost Plus slides (ThermoFisher Scientific) and stored at -80°C until IHC was carried out. Prior to IHC, slides were brought to room temperature and washed 3 x 5 min with PBS to remove the cryomedium. Antigen retrieval was carried out using pH 9 buffered Tris as described above. Slides were incubated in blocking solution for 1 h at 4°C as described above, and then incubated with primary antibodies (Table 3.1) for 2 h at room temperature. Following 5 x 5 min washes with PBST, slides were incubated with shaking in the dark at room temperature for 1 h with the appropriate secondary antibodies (Table 3.1) in 0.8% Triton X-100 in PBST. Slides were washed again (5 x 5 min with PBST) and mounted with Vectashield mounting medium containing DAPI for imaging.

Imaging for 6 dpf larvae was carried out using a confocal laser scanning microscope (A1R+; Nikon Instruments, Melville, NY). Images were collected as a z-stack with an optical

slice thickness of 2 µm across the sample and were viewed as a maximum intensity image. Gill sections for 15, 30 and 90 dpf fish were imaged in the same fashion but using a different confocal microscope (Olympus FV1000 BX61, Olympus Canada, Richmond Hill, ON, Canada). To estimate ionocyte abundance, HR, NaR and NCC cells on the yolk sac (6 dpf fish) or in gill tissue sections (15, 30 and 90 dpf fish) were counted using the point-to-point function in ImageJ, and the corresponding yolk sac or gill area was measured using ImageJ to express ionocyte abundance as cells mm^{-2}. For 15 and 30 dpf fish, four sections usually included gill tissue and the middle two were used for quantification of ionocytes. For 90 dpf fish, eight sections usually included gill tissue and the middle three were used for quantification of ionocytes.

3.2.7 Indices of gill structure

Morphometric measurements were carried out on gill tissue sections stained with hematoxylin and eosin. Fish younger than 90 dpf were euthanized, fixed, processed, and sectioned as described above for IHC, with the procedures for 6 dpf larvae being the same as those for 15 and 30 dpf fish. One set of samples was sectioned in the sagittal plane and a separate set of samples was sectioned in the frontal plane, to provide two different views of the gills. Slides were washed 3 x 5 min with PBS to remove the cryomedium, and then stained using hematoxylin for 5 min. Following this incubation, slides were rinsed with deionized water for 1 min, dipped in Scott's tapwater (15 g L^{-1} $MgSO_4$, 2 g L^{-1} $NaHCO_3$) 6 times, as a blueing agent, and rinsed in deionized water a second time. Slides were then stained with eosin for 2 min, and rinsed 3 x 5 min with 100% ethanol. Following 10 min of drying, slides were mounted using a xylene-based mounting medium (Cytoseal 60, Thermo Scientific, ON, Canada).

Samples for 90 dpf fish were fixed as described above, washed 3 x 5 min with PBS, and decalcified by immersion in 10% HCl for 20 min at room temperature. Samples were then

dehydrated by immersion in a series of ethanol solutions (30%, 50%, 70%, 80%, and 99%, 30 min per solution at room temperature), followed by immersion in a 50% ethanol, 50% xylene solution for 30 min, and finally immersion in 100% xylene (30 min). The dehydrated samples were embedded in paraffin wax (Sigma-Aldrich, Oakville, ON), and sectioned at 10 µm thickness in the sagittal plane (Leica CM 3050 S, Leica Microsystems Inc. Concord, ON). Sections were collected onto Fisherbrand SuperFrost Plus slides (ThermoFisher Scientific) and incubated at 65°C for 30 min to remove the wax. The slides were then washed to remove any remaining wax, 3 x 5 min in 100% xylene, followed by 1 min each in 99%, 90% and 80% ethanol, and finally, 1 min in water. Slides were stained with hematoxylin for 6 min, washed (1 min in water), and dipped 6 times in Scott's tapwater. Following a 1 min rinse in water, slides were stained with eosin for 2 min, washed 2 x 1 min in 95% ethanol, and rinsed 3 x 5 min in xylene. Slides were dried and mounted as described above.

Imaging was carried out using a compound microscope (Zeiss Axiophot upright microscope; Carl Zeiss Canada Ltd., Toronto, ON). For 6, 15 and 30 dpf fish, four sections usually included gill tissue and the middle two were measured, with all 4 gill arches being measured (Figs. 3.1, 3.2). For 90 dpf, eight sections usually included gill tissue and the middle three were analysed. Using the second gill arch in each case, 20 randomly selected lamellae were measured (Fig. 3.3).

3.2.8 Ventilation frequency

Video microscopy was used to measure f_V in 6, 15 and 30 dpf fish under both normoxic and hypoxic conditions, essentially as described by Pan et al. (2019). An individual larva or juvenile fish was placed in a glass capillary tube (inner diameter 1 mm; 6 and 15 dpf) or pipette (inner diameter 5 mm; 30 dpf) and video recorded using an iPhone SE mounted on a dissecting

microscope (Stereo trinocular microscope, AmScope, Irvine, USA). Water of pH appropriate to the treatment group (pH 7.2 for the control group, pH 4 for the pH 4 group), containing 0.05 mg mL^{-1} Tris-buffered MS-222, and maintained at 28.5°C was gravity-fed through the glass tube at a rate of 1.6-1.8 mL min^{-1}. The low level of anaesthetic was needed to minimize movement of the fish and facilitate the detection of breathing movements (Jonz and Nurse, 2005). Fish were allowed to acclimate for 5 min to the chamber conditions with normoxic (water PO_2 = 153 Torr) water flow. A trial consisted of video recording the fish under normoxic conditions for 15 min followed by 15 min under hypoxic (water PO_2 = 30 Torr) conditions; this level of hypoxia was chosen to correspond to the P_{crit} of developing zebrafish (Mandic et al., 2020). The desired level of hypoxia was achieved by equilibrating the header tank with a mixture of N_2 and air delivered by a custom-built gas mixer (University of Ottawa) and confirmed by measuring water PO_2 (FireStingO2, PyroScience, Aachen, Germany). Average f_V was determined by counting all breaths for the full 15 min period using either buccal or opercular movements depending on the orientation of the fish in the chamber and the visibility of the mouth versus opercula. Ventilation amplitude was not measured owing to the absence of an established method to measure this variable in developing zebrafish.

Ventilation in 90 dpf fish was recorded using the method of Vulesevic et al. (2006). In brief, fish were placed in cylindrical plastic chambers fitted with two electrodes, one at each end of the chamber, separated from the fish by a piece of mesh, that detected opercular displacements. The analog output of the electrodes was amplified using a custom-built amplifier (University of Ottawa) and converted to digital data using a data acquisition system and software (AcqKnowledge, BioPac Systems, Galeta, CA, USA) set to a sampling frequency of 500 Hz. Each chamber was supplied by gravity with continuous water flow at 1.6 mL min^{-1} from a header

tank containing aerated water of appropriate pH for the treatment group (i.e. pH 7.2 for the control group, pH 4 for the pH 4 treatment group). Fish were placed in the recording chambers for a 2 h acclimation period under normoxic (water PO_2 = 153 Torr) conditions. After 35 min of recording ventilation under normoxic conditions, the water flow to the chamber was switched to a header tank that had been pre-equilibrated as described above to water PO_2 = 60 Torr for 45 min of hypoxic exposure. The level of hypoxia was chosen to elicit a robust but not maximal hyperventilatory response (Vulesevic et al. 2006), with the aim of allowing differences in ventilation between treatment groups to be detected. Average f_V was determined for the final 15 min each of the normoxic and hypoxic periods by counting the number of voltage peaks over a set time interval. Adult zebrafish increase f_V but not breathing amplitude during hypoxia (Vulesevic et al. 2006). Analysis of amplitude data for the recordings collected in the present study revealed a similar pattern and therefore ventilation amplitude data are not presented.

3.2.9 Oxygen consumption and P_{crit}

Oxygen consumption and P_{crit} were measured by closed-system respirometry. Larvae or juvenile fish were placed in individual wells of 80 µL (6 dpf) or 500 µL (15 and 30 dpf) volume in a 24-well glass microplate (Loligo Systems, Viborg, Denmark) in which each well was fitted with an O_2 sensor spot (PreSens, Regensburg, Germany) and contained water corresponding to the appropriate treatment condition (control or pH 4 water). The microplate was sealed with adhesive tape (AB0580, ThermoFisher Scientific) and placed into a water bath maintained at 29°C. The water bath containing the sealed microplate was attached to an O_2 sensor (SDR SensorDish Reader, PreSens) linked to a laptop computer running MicroResp software (Loligo Systems) and water PO_2 in each well was measured until the oxygen was depleted. Following the respirometry trial, 30 dpf fish were imaged and mass was determined from the mass-length

regression described above. The mass-specific rate of oxygen consumption (μmol g^{-1} h^{-1}) was calculated as: $\dot{M}O_2 = (\Delta PO_2 \cdot \alpha O_2 \cdot V)/m$, where ΔPO_2 is the slope of the fall in water PO_2 over time, using sequential 2 min intervals, αO_2 is the solubility coefficient of O_2 in freshwater at 28°C (Boutilier et al., 1984), V is the volume of water in the respirometer well, and m is the mass of the fish. The volume of the fish accounted for 5-10% of the respirometer volume and therefore was not taken into account in determining V. Normoxic $\dot{M}O_2$ was calculated as the average $\dot{M}O_2$ for water PO_2 values above 110 Torr. The 'broken-stick' regression approach of Yeager and Ultsch (1989) was used to determine P_{crit}. With this approach, $\dot{M}O_2$ is plotted against water PO_2 and regression lines are fitted to the oxy-regulatory and oxy-conforming phases of the plot using REGRESS software (www.wfu.edu/~mudayja/software/o2.exe). The intersection of the best fit linear regression lines is deemed to be the P_{crit}.

Closed system respirometry on 90 dpf, fish was carried out by placing fish in individual 15.6 mL glass chambers fitted with O_2 sensor spots (horizontal mini chamber system, Loligo Systems). Fish were allowed to recover overnight under conditions where the respirometers were flushed continuously with aerated, 28°C water from a 20 L header tank of water of the appropriate pH (control or pH 4 water). To initiate the respirometry trial, the flush pump providing water to the chamber was turned off while a recirculating pump remained on to provide mixing. Water PO_2 within the chamber was recorded continuously from the O_2 sensor spot using a fibre optic reader (Witrox, Loligo Systems) linked to a laptop computer running AutoResp software (Loligo Systems). Water PO_2 fell as the fish consumed the O_2 in the respirometer and the trial was terminated when water PO_2 plateaued. The calculation of $\dot{M}O_2$ and P_{crit} was carried out as described above, with the mass of the fish being measured on an

analytical balance before it was placed in the chamber. For 90 dpf fish, respirometer volume was corrected for the volume of water displaced by the fish.

3.2.10 Time to LOE during acute hypoxia

Time to LOE was measured only for 90 dpf fish. Zebrafish were placed individually in a container with a perforated end, which was in turn submerged in a 20 L aquarium containing 29°C water of the appropriate pH (control or pH 4 water). This system prevented fish from accessing the surface of the water to perform aquatic surface respiration during hypoxia. A fibre optic probe to measure water PO_2 (FireStingO2, PyroScience) was introduced into the chamber containing the fish, and the fish were given an overnight acclimation period. Prior to beginning the trial, the surface of the water was covered with bubble wrap to limit O_2 diffusion into the water from air. Hypoxia was achieved by gassing the aquarium with N_2 to reach a water PO_2 of 12 Torr within 15 min, which was then maintained. This level of hypoxia was chosen based on the results of Mandic et al. (2020), who reported that most (wildtype) zebrafish lost equilibrium within 5 h when exposed to water of PO_2 = 12 Torr. Fish were monitored continuously, and the time required to lose equilibrium was recorded for each individual. A fish was considered to have lost equilibrium when it failed to right itself for at least 5 s. The experiment was terminated after 5 h, and fish that had not lost equilibrium were given a time to LOE of 5 h.

3.2.11 Statistical analysis

Student's *t*-tests were used to compare values for control versus pH 4-reared fish for all variables except f_V. For f_V, a 2-way repeated measures analysis of variance (RM ANOVA) was used to assess the effects of treatment group (control vs pH 4), and oxygen level (normoxia vs hypoxia). Where significant differences were detected, Tukey's HSD test was used to test all pair-wise differences. Additionally, a Chi-squared test was used to compare the proportions of

control and pH 4 fish that lost equilibrium during the LOE trial. All statistical tests were carried out in Rstudio (v1.01.153). Data were checked for normality using the Shapiro-Wilk test, and tested for equal variances using the Levene's test. Data are generally presented as boxplots, with the lower limit of the box representing the 25^{th} percentile, and the upper limit representing the 75^{th} percentile. The whiskers represent the highest and lowest recorded values that are not statistical outliers. The mean is represented by a solid line within the box. Individual data points are presented as symbols. The variables that we tested either are known to change with age, such as whole-body cortisol concentration (Hare et al., 2020), or exhibit structural differences with age that necessitated different types of measurements, such as gill structure (Jonz and Nurse, 2005). Therefore, control and pH 4 treatments were compared within an age group and comparisons between ages were not carried out.

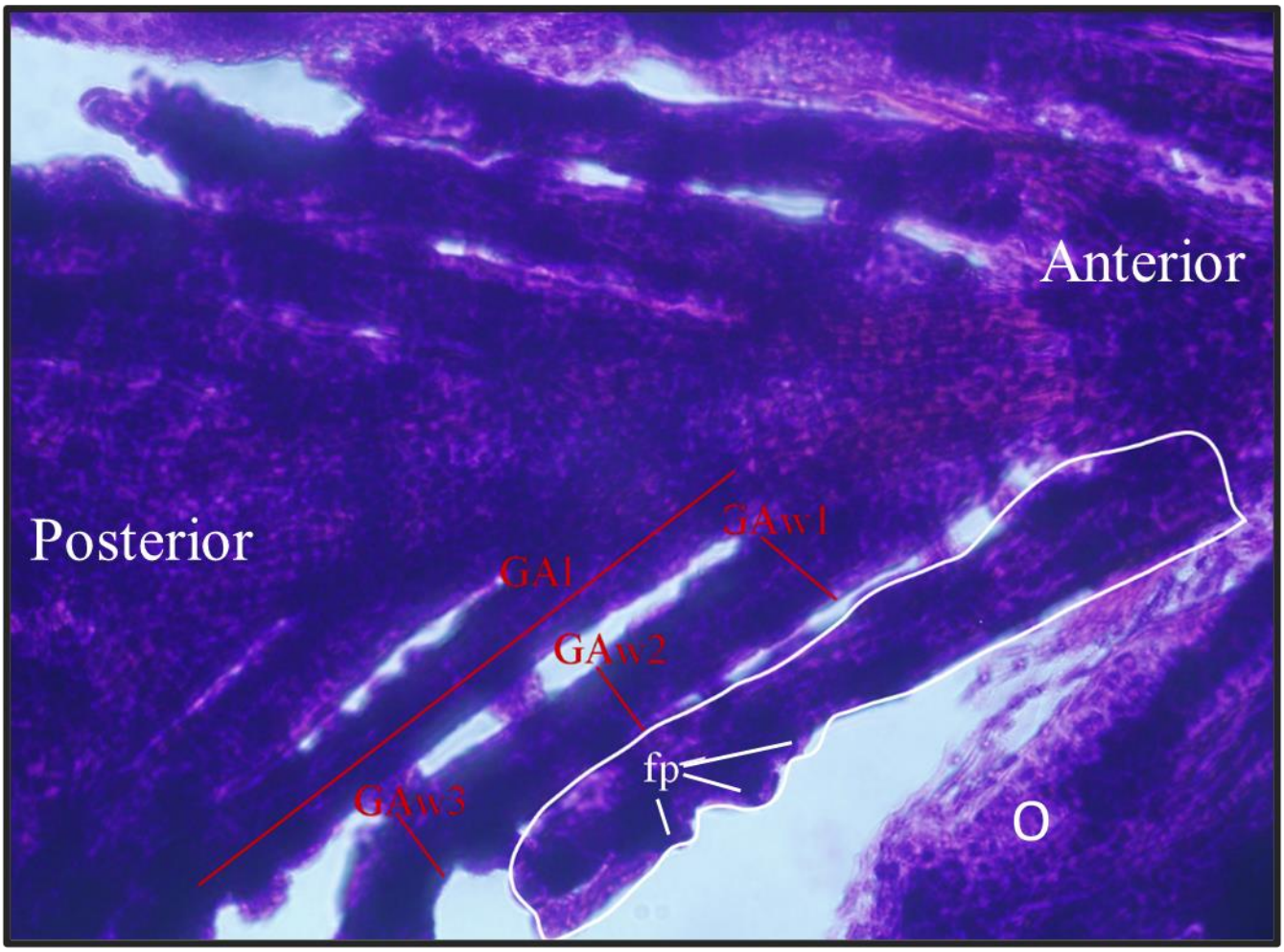

Figure 3.1. Horizontal plane section of a zebrafish (*Danio rerio*) larva, at 40 times magnification, serving as an example of how gill arch width and length were measured. The area outlined in white identifies the first gill arch, *fp* are filament primordia, and *O* is the operculum. *GAl* indicates the measurement of gill arch length, with the value for the animal being reported as the average value over all eight gill arches. *GAw1-3* represent the 3 measurements of gill arch width per gill arch that were averaged to provide the average width of each gill arch, with the value for the animal being reported as the average of all gill arches. All measurements were carried out in ImageJ.

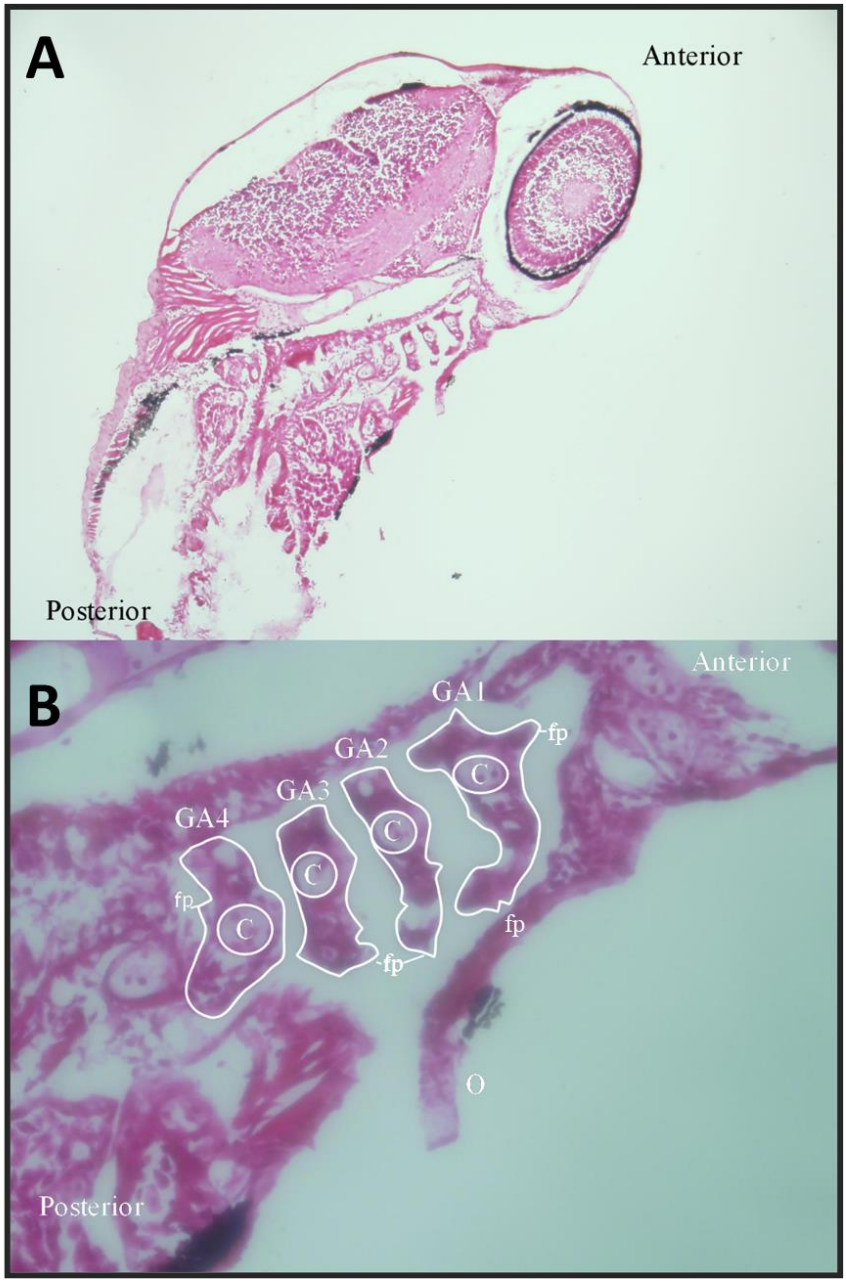

Figure 3.2. Sagittal sections at 10 times (A) and 40 times (B) magnification of a zebrafish (*Danio rerio*) larva, serving as an example of how gill arch cross-sectional area was measured. In (B), *GA1-4* are the four gill arches, *C* is the cartilage in the gill arches, *fp* are filament primordia, and *O* is the operculum. The perimeter of each gill arch, outlined in white, was measured, and the area was calculated in ImageJ. The average of the four arches was used as the value for the individual.

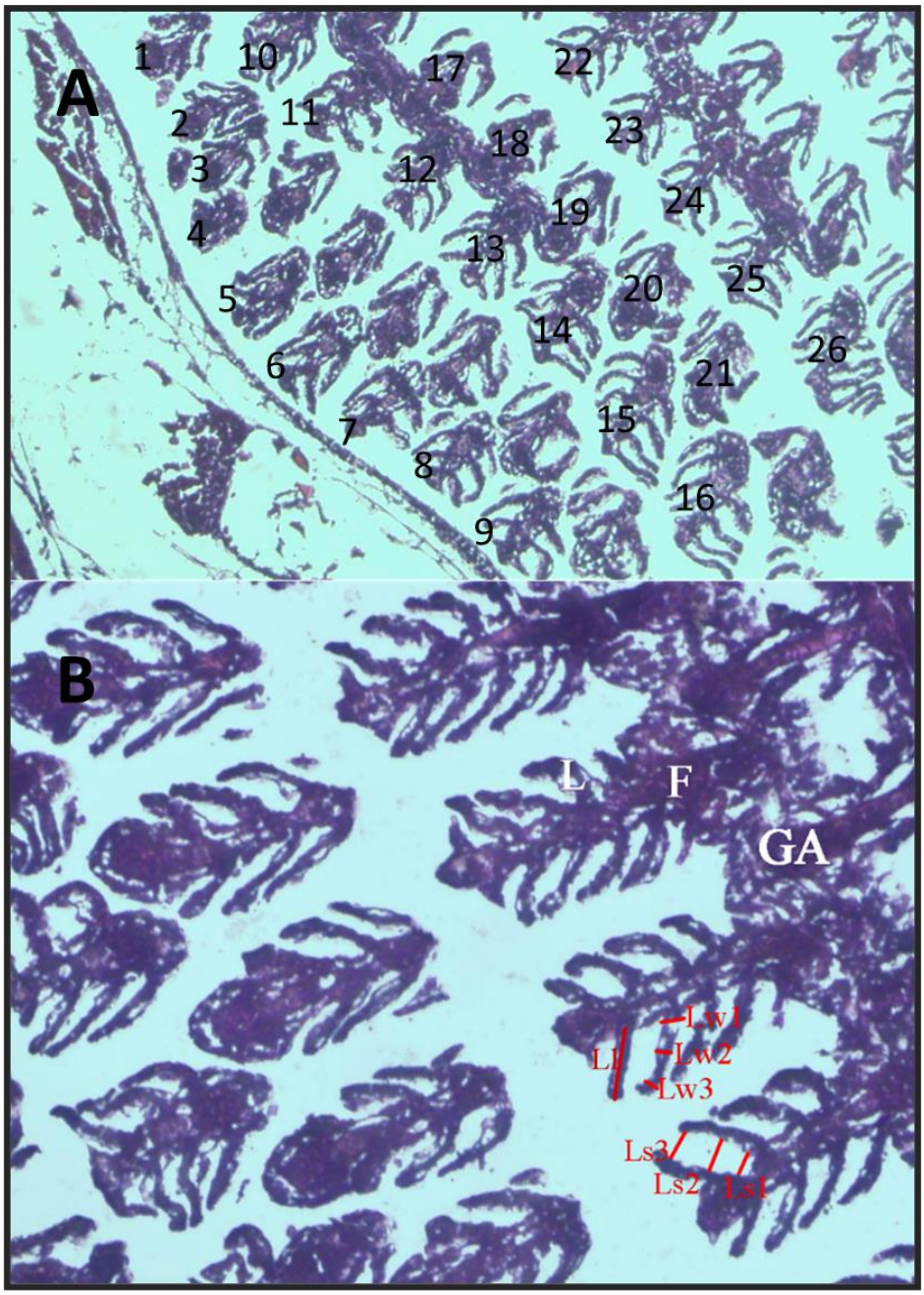

Figure 3.3. Sagittal gill sections of a 90 dpf zebrafish (*Danio rerio*) at 10 (A) and 20 (B) times magnification, serving as an example of how gill lamellae measurements were carried out. In (A), each filament section was assigned a number, and random number generator picked 20 numbers, representing the filaments that would be used for morphometric analysis. In (B), *GA* indicates the gill arch, *F* indicates a filament section, and *L*, a lamella. *L1* indicates the measurement of lamellar length. *Lw1-3* indicate the three measurements that were used to determine average lamella width. *Ls1-3* represent the three measurements of the space between lamellae used to determine average space between lamellae. All of these values collected across the 20 filaments were used to provide the average length, width, and space between lamellae for an individual fish.

Table 3.1 Antibodies used for immunohistochemistry.

Name of Antibody	Target	Dilution	Host	Source
Primary				
anti-NCC	NCC (SLC2A10.2)	1 to 500	rabbit, polyclonal	Genscript (Kwong and Perry 2016)
anti-H^+-ATPase	α-subunit of H^+-ATPase	1 to 500	rabbit, polyclonal	Dr. Uchuyama, University of Toyama
α5	α-subunit of Na^+-K^+-ATPase	1 to 500	mouse, monoclonal	Developmental Studies Hybridoma Bank, α5
Secondary				
alexa-594 conjugated anti-rabbit anti-NCC		1 to 500	donkey	Invitrogen, Burlington ON
alexa-488 conjugated anti-rabbit anti-H+-ATPase		1 to 500	donkey	Invitrogen, Burlington ON
alexa-637 conjugated anti-mouse α5		1 to 500	donkey	Invitrogen, Burlington ON

3.3 Results

3.3.1 The effects on growth of early development under pH 4 conditions

No significant differences in body length were detected between individuals raised in system water and those raised in water of pH 4 at 6, 15, 30 or 90 dpf (Table 3.2). Similarly, no significant differences in body mass were detected between control and pH 4 treatment groups at any age (Table 3.2).

3.3.2 The effects of development under pH 4 conditions on indicators of ion balance

Whole-body cortisol and Na^+ content as well as skin (6 dpf) or gill (all other ages) ionocyte abundances were evaluated as metrics of ion balance and ionoregulatory capacity in zebrafish raised under pH 4 versus control conditions. No significant differences between control and pH 4 treatment groups were detected in whole-body cortisol concentrations at 6 (Fig. 3.4, Student's *t*-test, $p = 0.17$), 15 (Student's *t*-test, $p = 0.10$), 30 (Student's *t*-test, $p = 0.67$), or 90 dpf (Student's *t*-test, $p = 0.19$). By contrast, whole-body Na^+ content of fish raised in pH 4 water was significantly higher than that of fish raised under control conditions at 6 (Fig. 3.5, Student's *t*-test, $p = 0.0056$), 15 (Student's *t*-test, $p = 0.0062$), and 90 dpf (Student's *t*-test, $p = 0.0052$). However, no significant difference was detected in 30 dpf fish raised under control versus pH 4 conditions (Student's *t*-test, $p = 0.67$).

Fish raised under pH 4 conditions had significantly higher HR cell abundances in the skin (6 dpf) and gill (15, 30 and 90 dpf) than control fish at all ages examined (Fig. 3.6, Fig. 3.7; Student's *t*-tests, 6 dpf, $p = 0.034$; 15 dpf, $p = 0.0059$; 30 dpf, $p = 0.0013$; 90 dpf, $p = 0.0059$). Similarly, significantly higher densities of NCC cells were detected in fish reared in pH 4 water relative to the control group (Fig. 3.8, Fig. 3.9; Student's *t*-tests, 6 dpf, $p = 0.049$; 15 dpf, $p = 0.0071$; 30 dpf, $p = 0.0062$; 90 dpf, $p = 0.028$). A significant difference in NaR cell density was

detected at 6 dpf (Fig. 3.10, Fig. 3.11; Student's *t*-test, p = 0.048); however, we were unable to measure NaR cell densities in 15, 30 or 90 dpf fish, owing to lack of success with immunohistochemical staining.

3.3.3 The effects of early development under pH 4 conditions on indicators of gas transfer

Gill morphology, as well as measurements of f_V, $\dot{M}O_2$, P_{crit}, and (in 90 dpf fish only) time to LOE during acute hypoxia were evaluated as metrics of gas transfer in zebrafish raised under pH 4 versus control conditions.

Owing to the state of development of the gill at the earlier ages (6, 15 and 30 dpf), measurements focused on gill arches, with thickness and cross-sectional area used as indicators of the blood-to-water diffusion distance. Length was also measured; an increase in length would be indicative of an increase in gill surface area. At all three of these ages, zebrafish reared in pH 4 water had significantly thicker gill arches (Fig. 3.12, Fig. 3.14 D-F; Student's *t*-tests, 6 dpf, p = 0.0054; 15 dpf, p = 0.0055; 30 dpf, p = 0.002). For gill arch cross-sectional area, no significant difference was detected at 6 dpf (Fig. 3.13, Fig. 3.14 G-I; Student's *t*-test, p = 0.35), but individuals raised in pH 4 water had gill arches with a larger cross-sectional area at both 15 and 30 dpf (Student's *t*-tests, p = 0.00082 and p = 0.0047, respectively). No significant difference in gill arch length was detected (Fig. 3.12, Fig. 3.14 A-C; Student's *t*-tests, 6 dpf, p = 0.09; 15 dpf, p = 0.93; 30 dpf, p = 0.55).

For adult fish, we measured lamellar length, lamellar width, and interlamellar space. No significant differences in lamellar length were detected (Fig. 3.15, Fig. 3.16; Student's *t*-test, p = 0.91). However, fish raised in pH 4 water had thicker lamellae (Student's *t*-test; p = 0.0024) and less space between lamellae (Student's *t*-test; p = 0.015) than control fish.

Both rearing environment and acute exposure to hypoxia had significant effects on f_V, although the effects of rearing environment were not consistent across age (Fig. 3.17). At 6 dpf, f_V was significantly higher in hypoxic conditions than in normoxic conditions for both control fish and fish raised in pH 4 water. Additionally, fish raised at pH 4 had significantly higher f_V than control fish under normoxic conditions, but not under hypoxic conditions (Fig. 3.17A; 2-way RM ANOVA, $p_{hypoxia} < 0.001$) $p_{treatment} = 0.021$, $p_{treatmentxhypoxia} = 0.12$). At 15 dpf, only the effect of hypoxic exposure was significant, with higher f_V during hypoxia than normoxia (Fig. 3.17B; 2-way RM ANOVA, $p_{treatment} = 0.04$, $p_{hypoxia} < 0.001$, $p_{treatmentxhypoxia} = 0.72$). In 30 dpf fish, the effects of both rearing environment and hypoxic exposure were significant, but there was no significant interaction between these factors, with fish raised in pH 4 water having higher f_V than control fish (Fig. 3.17C; 2-way RM ANOVA, $p_{treatment} = 0.003$, $p_{hypoxia} = 0.0004$, $p_{treatmentxhypoxia} = 0.77$). Finally, a significant effect of hypoxic exposure but not rearing environment was detected in 90 dpf fish (Fig. 3.17D; 2-way RM ANOVA on ranks, $p_{treatment} = 0.32$, $p_{hypoxia} = 0.00022$, $p_{treatmentxhypoxia} = 0.15$). However, there was an increase in f_V after hypoxia exposure, which tended to be significantly greater in control fish (Student's *t*-test, $p = 0.0724$;118.5 ± 26.8, $N = 7$) compared to fish raised under pH 4 conditions (58.0 ± 9.6, $N = 6$).

At 6, 15 and 30 dpf, no significant differences were detected between fish raised in water of pH 4 and those raised in control water for either $\dot{M}O_2$ (Fig. 3.19; Student's *t*-tests, 6 dpf, p = 0.34; 15 dpf, p = 0.85; 30 dpf, $p = 0.13$) or P_{crit} (Fig. 3.20; Student's *t*-tests, 6 dpf, p = 0.1; 15 dpf, p = 0.25; 30 dpf, p = 0.8). However, 90 dpf fish raised in pH 4 water exhibited $\dot{M}O_2$ values that were nearly twice those of fish raised under control conditions (Fig. 3.19 D; Student's *t*-test, $p = 0.00068$). This difference was accompanied by significantly higher P_{crit} in fish raised in pH 4 water than in control fish (Fig. 3.20 D; Student's *t*-test, $p = 0.037$), but time to LOE during

exposure to acute hypoxia (12 Torr PO_2) did not differ significantly between fish reared under control versus pH 4 conditions (Fig. 3.21; Student's *t*-test, $p = 0.25$).

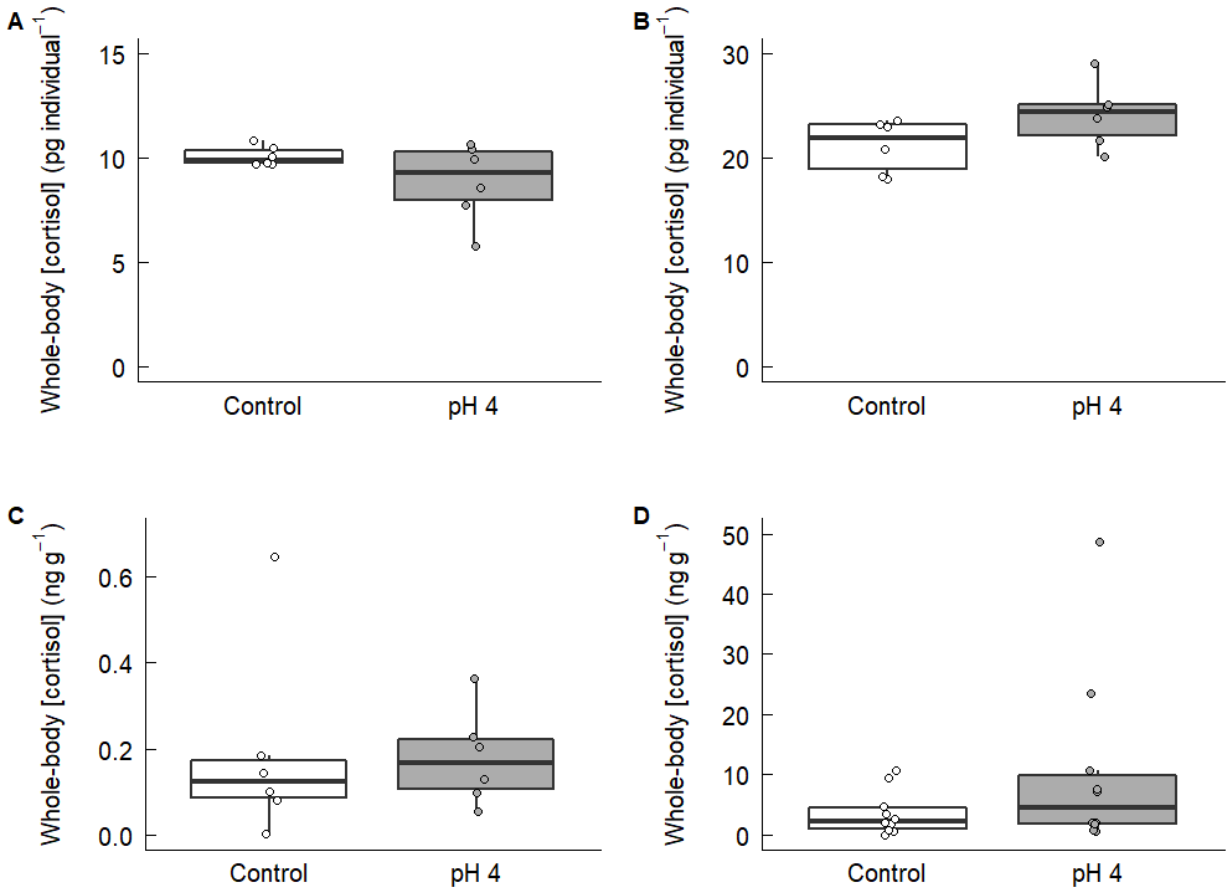

Figure 3.4. Whole-body cortisol levels in 6 dpf (A), 15 dpf (B), 30 dpf (C), and 90 dpf (D) zebrafish (*Danio rerio*) raised under control conditions or in water of pH 4. Data are shown as a boxplot, with upper and lower limits of the box representing the 75th and 25th quartiles, respectively. The bold line across the box represents the mean value. The white or grey symbols represent the individual data point for a sample. There were no significant differences in cortisol levels between treatment groups at any age (Student's *t*-tests; A, p = 0.17; B, p = 0.10; C, p = 0.67; D, p = 0.19).

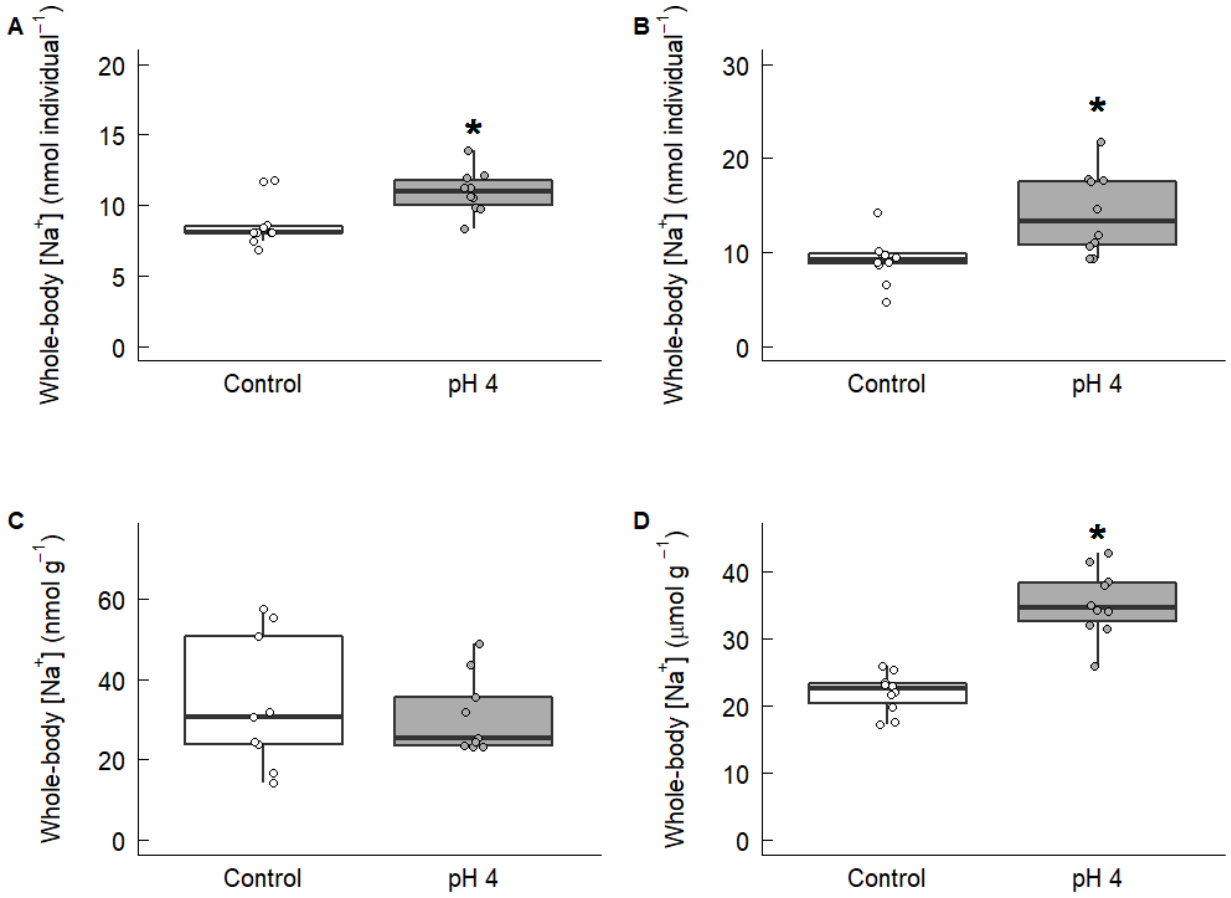

Figure 3.5. Whole-body Na^+ levels in 6 dpf (A), 15 dpf (B), 30 dpf (C), and 90 dpf (D) zebrafish (*Danio rerio*) raised under control conditions or in water of pH 4. Data are shown as a boxplot, with upper and lower limits of the box representing the 75th and 25th quartiles, respectively. The bold line across the box represents the mean value. The white or grey symbols represent the individual data point for a sample. An asterisk denotes a significant difference between groups (Student's *t*-tests, A, p = 0.0056; B, p = 0.0062; C, p = 0.67; D, p = 0.0052).

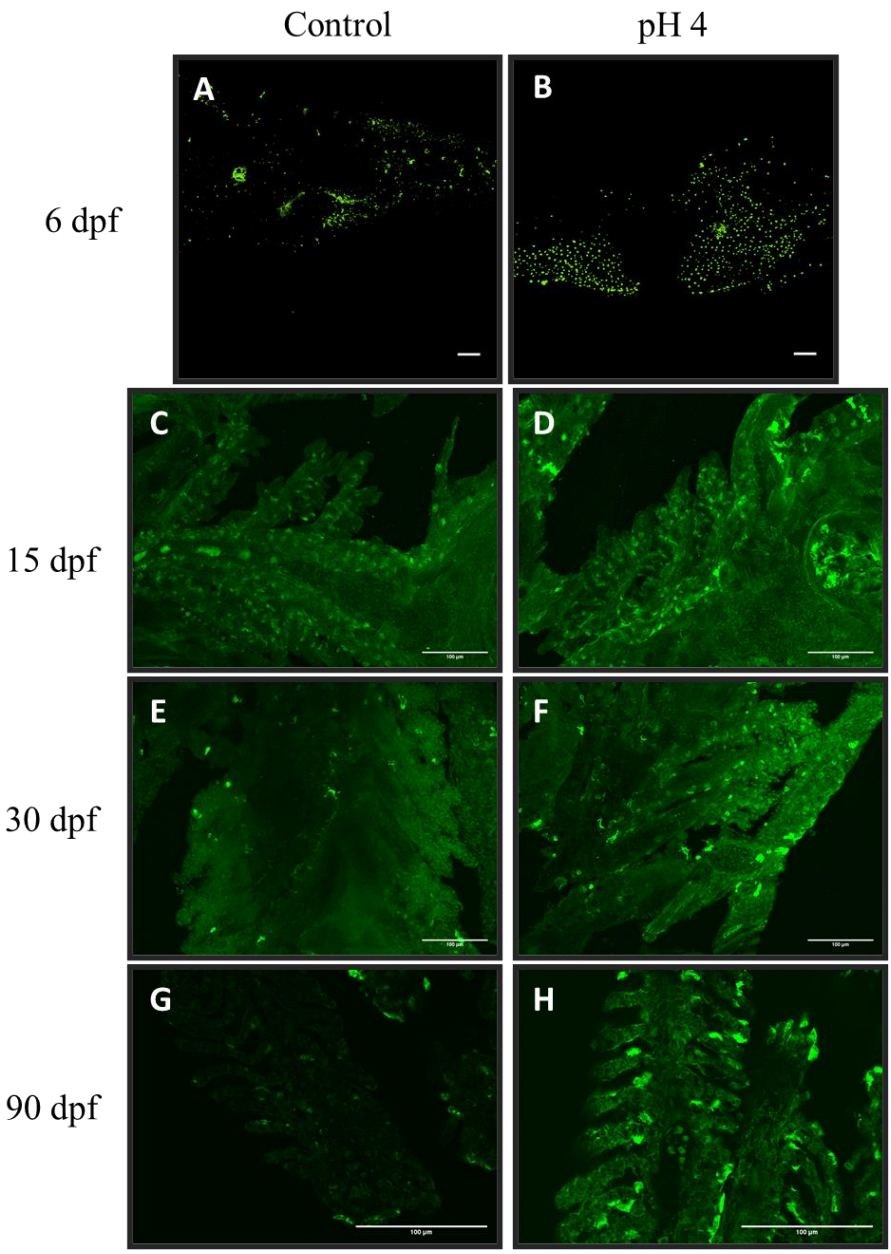

Figure 3.6. Representative images of 6 dpf larvae yolk sacs (A-B) and gills from 15 (C-D), 30 (E-F) and 90 dpf (G-H) zebrafish (*Danio rerio*). Samples were stained either using ConA (A-B), or antibodies against V-type H^+-ATPase (C-H), both of which identify HR ionocytes. Fish were raised in control (A, C, E and G) or pH 4 (B, D, F, H) water. Scale bar = 100 µm.

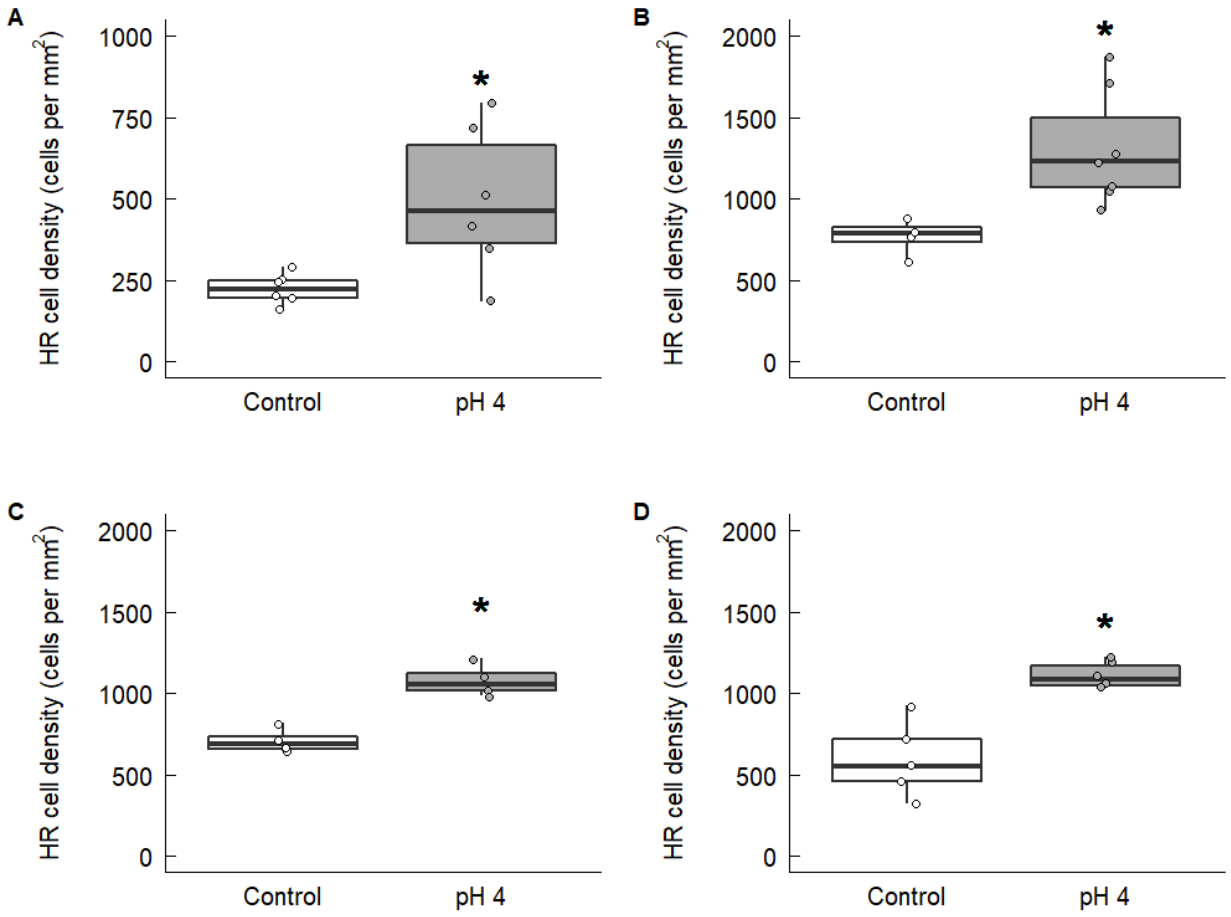

Figure 3.7. The abundance of HR ionocytes in 6 dpf (A), 15 dpf (B), 30 dpf (C), and 90 dpf (D) zebrafish (*Danio rerio*) raised under control conditions or in water of pH 4. Ionocytes were counted in the skin of 6 dpf zebrafish and in the gills of 15, 30 and 90 dpf individuals. Data are shown as a boxplot, with upper and lower limits of the box representing the 75th and 25th quartiles, respectively. The bold line across the box represents the mean value. The symbols represent individual data points. An asterisk denotes a significant difference between groups (Student's *t*-tests, A, p = 0.034; B, p = 0.0059; C, p= 0.0013; D, p = 0.0059).

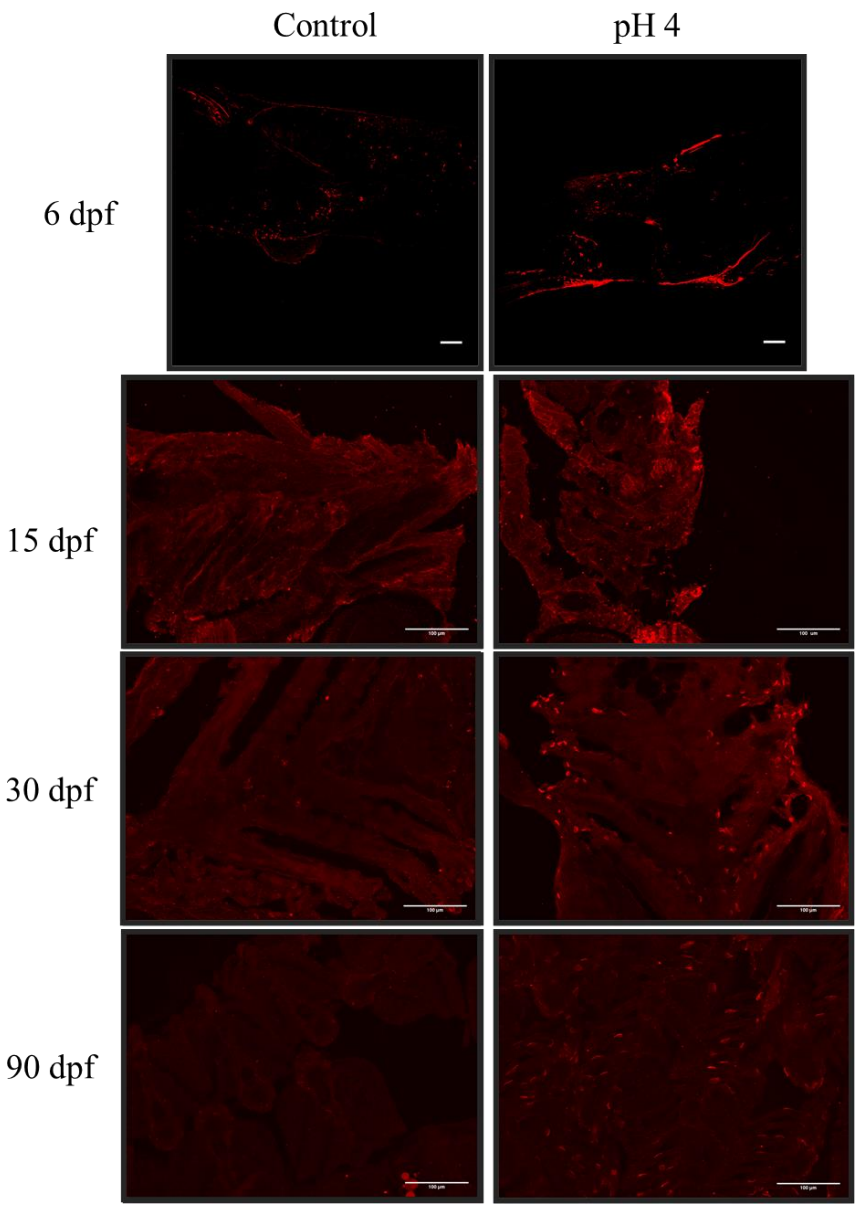

Figure 3.8. Representative images of 6 dpf larvae yolk sacs (A-B) and gills from 15 (C-D), 30 (E-F) and 90 dpf (G-H) zebrafish (*Danio rerio*). Samples were stained using an antibody against NCC, thereby identifying NCC cells. Fish were raised in control (A, C, E and G) or pH 4 (B, D, F, H) water. Scale bar = 100 µm.

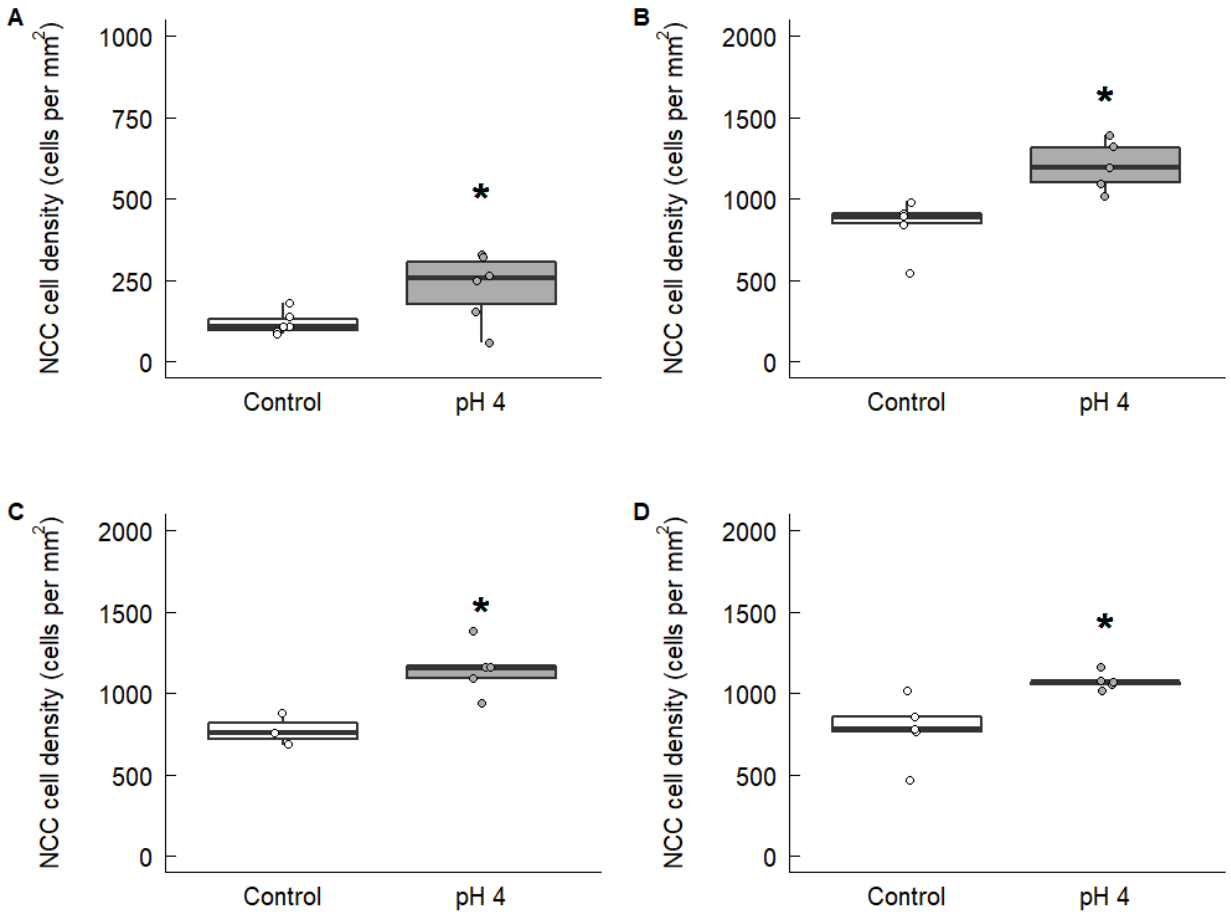

Figure 3.9. The abundance of NCC ionocytes in 6 dpf (A), 15 dpf (B), 30 dpf (C), and 90 dpf (D) zebrafish (*Danio rerio*) raised under control conditions or in water of pH 4. Ionocytes were counted in the skin of 6 dpf zebrafish and in the gills of 15, 30 and 90 dpf individuals. Data are shown as a boxplot, with upper and lower limits of the box representing the 75th and 25th quartiles, respectively. The bold line across the box represents the mean value. The symbols represent individual data points. An asterisk denotes a significant difference between groups (Student's *t*-tests, A, p = 0.049; B, p = 0.0071; C, p = 0.0062; D, p = 0.0028)

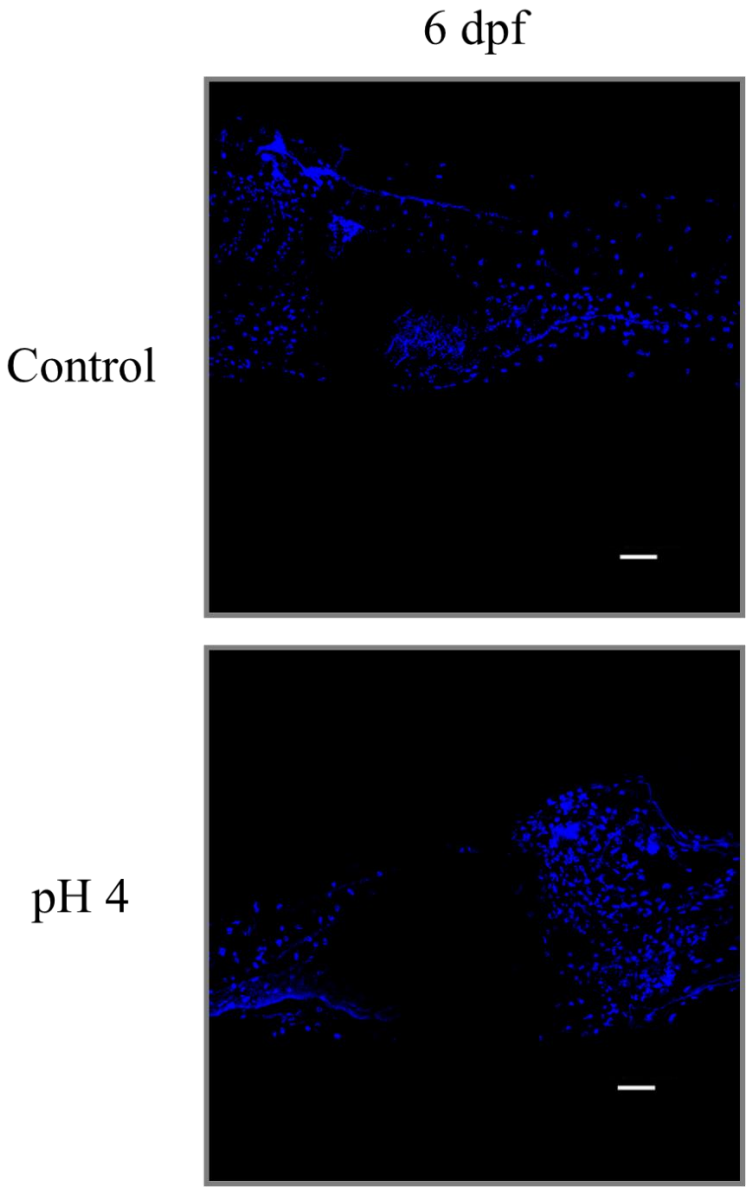

Figure 3.10. Representative images of the yolk sac of 6 dpf zebrafish (*Danio rerio*) larvae. Samples were stained using an antibody against Na^+,K^+-ATPase that identifies NaR cells. Fish were raised in control (A) or pH 4 (B) water. Scale bar = 100 µm.

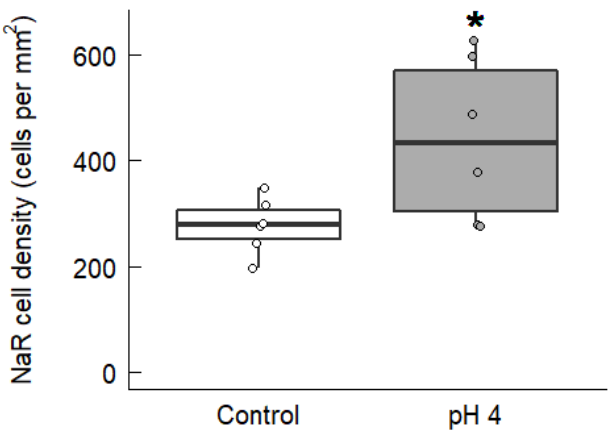

Figure 3.11. The abundance of NaR ionocytes in the skin of 6 dpf zebrafish (*Danio rerio*) raised under control conditions or in water of pH 4. Data are shown as a boxplot, with the upper and lower limits of the box representing the 75th and 25th quartiles, respectively. The bold line across the box represents the mean value. The symbols represent individual data points. An asterisk denotes a significant difference between groups (Student's *t*-test, p = 0.048)

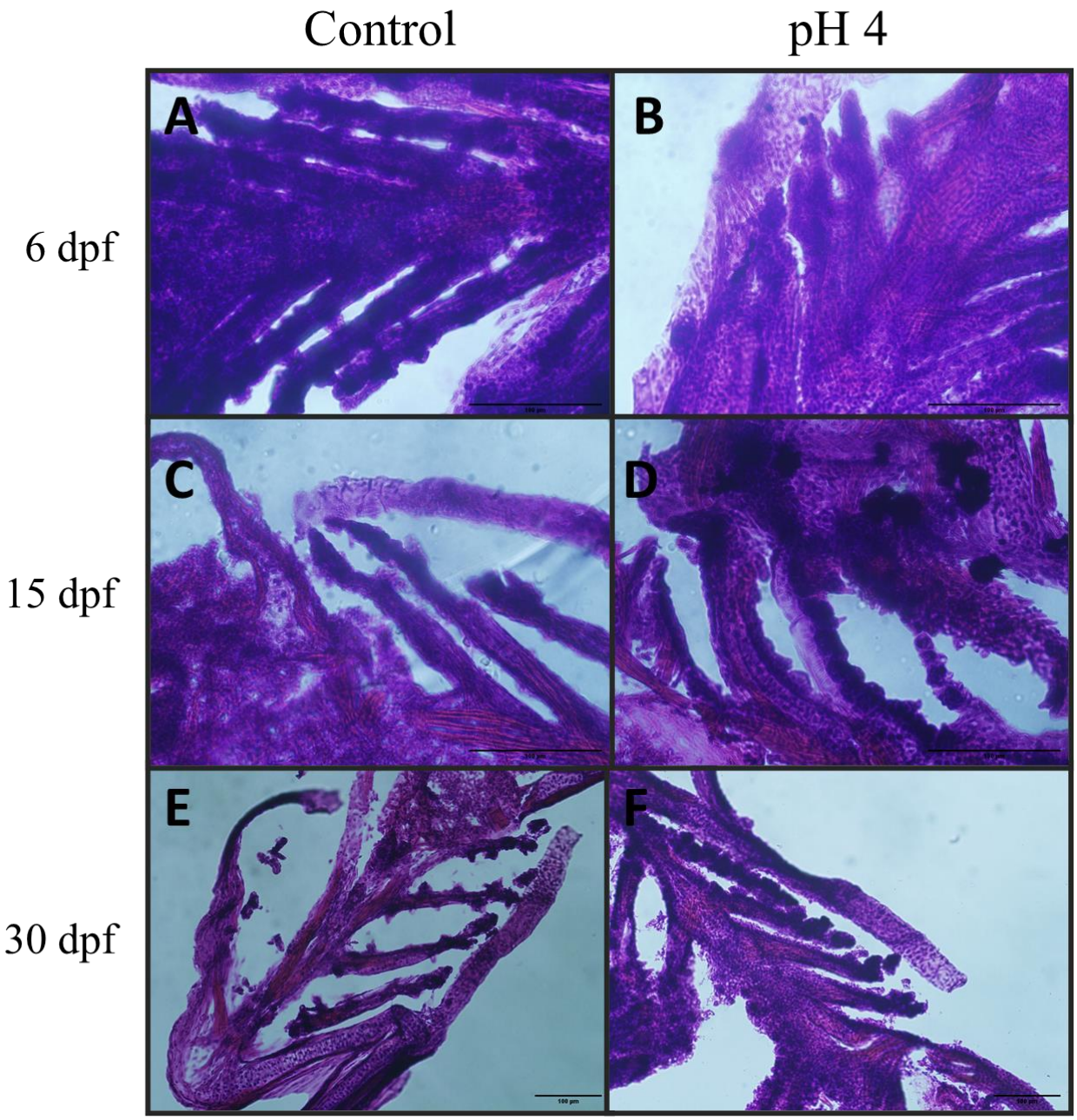

Figure 3.12. Histological sections of zebrafish (*Danio rerio*) gill arches, sectioned in the horizontal plane, ventrally at 6 (A-B), 15 (C-D), and 30 dpf (E-F). Fish were raised in control (A, C and D) or pH 4 (B, D and F) water. Scale bar = 100 µm.

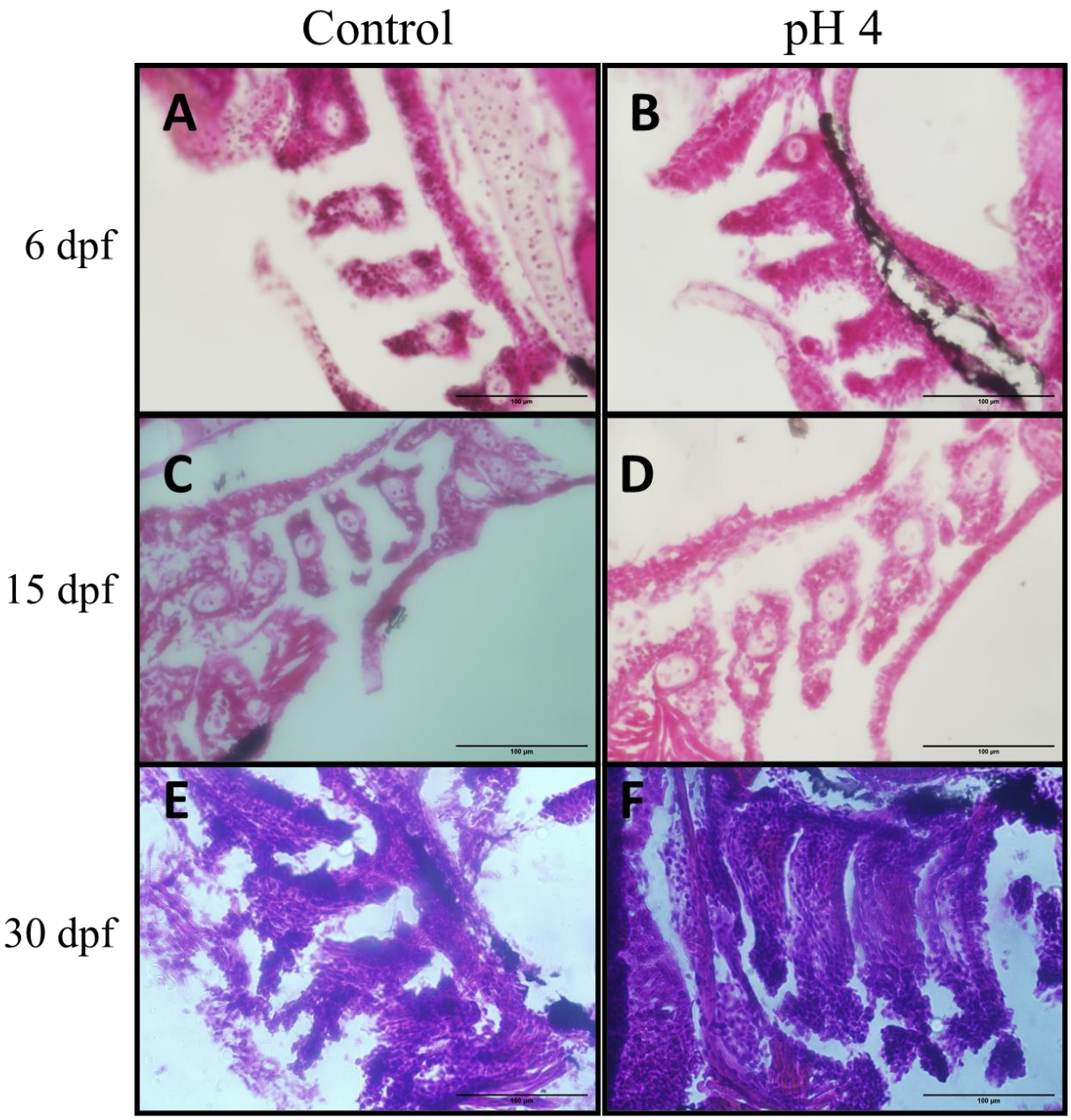

Figure 3.13. Histological sections of zebrafish (*Danio rerio*) gill arches, sectioned in the sagittal plane, at 6 (A-B), 15 (C-D), and 30 dpf (E-F). Fish were raised in control (A, C and D) or pH 4 (B, D and F) water. Scale bar = 100 µm.

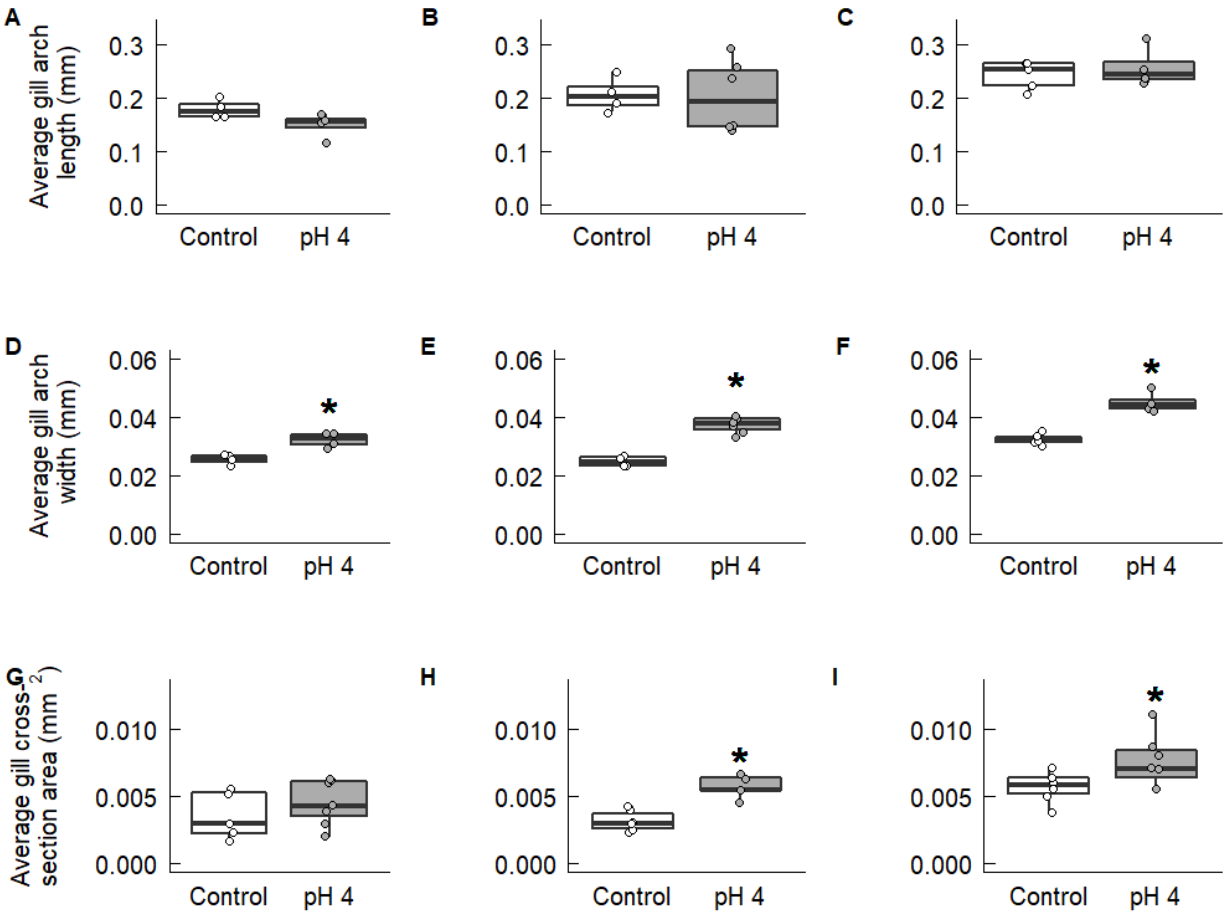

Figure 3.14. Average gill arch length (A-C), width (D-F), and cross-sectional area (G-I) in 6 dpf (A, D, G), 15 dpf (B, E, H), and 30 dpf (C, F, I) zebrafish (*Danio rerio*) raised under control conditions or in water of pH 4. Data are shown as a boxplot, with upper and lower limits of the box representing the 75th and 25th quartiles, respectively. The bold line across the box represents the mean value, and. The white or grey symbols represent individual data points. An asterisk denotes a significant difference between groups (Student's *t*-tests, A, $p = 0.09$; B, $p = 0.93$; C, $p = 0.55$; D, $p = 0.0055$; E, $p < 0.001$; F, $p = 0.002$; G, $p = 0.35$; H, $p = 0.00082$; I, $p = 0.047$).

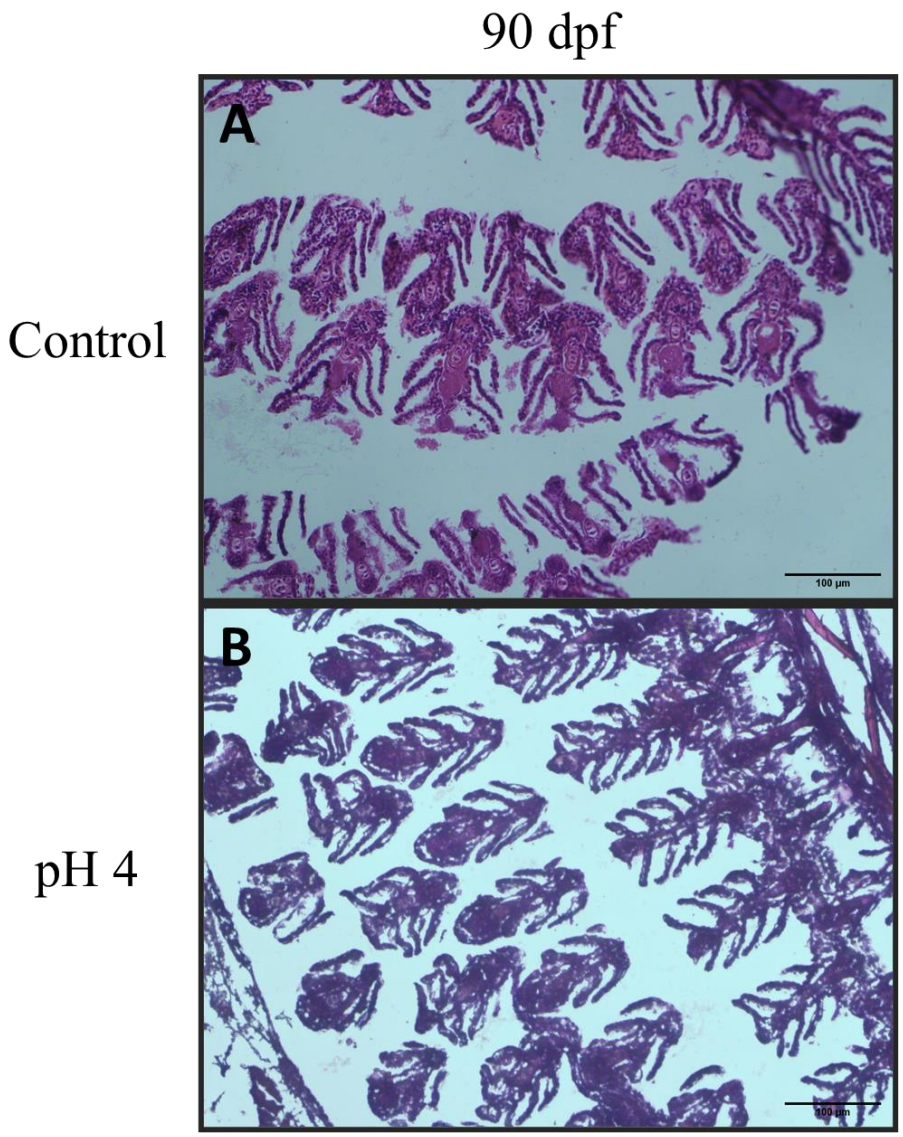

Figure 3.15. Histological images of gill lamellae of 90 dpf zebrafish (*Danio rerio*), raised in control (A) or pH 4 (B) water. Scale bar = 100 µm.

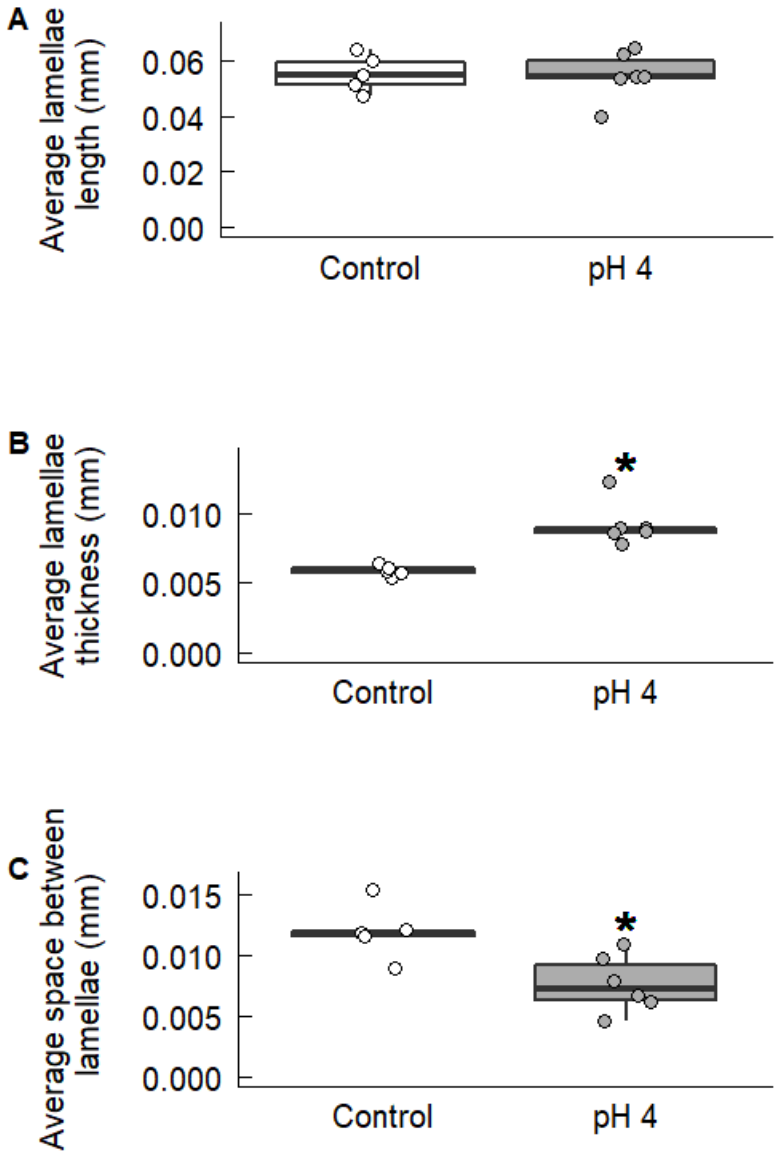

Figure 3.16. Average lamellar length (A), thickness (B), and inter-lamellar space (C) in the gills of 90 dpf zebrafish (*Danio rerio*) raised under control conditions or in water of pH 4. Data are shown as a boxplot, with upper and lower limits of the box representing the 75th and 25th quartiles, respectively. The bold line across the box represents the mean value. The white or grey symbols represent individual data points. An asterisk denotes a significant difference between groups (Student's *t*-tests, A, p = 0.91; B, p = 0.0024; C, p = 0.015).

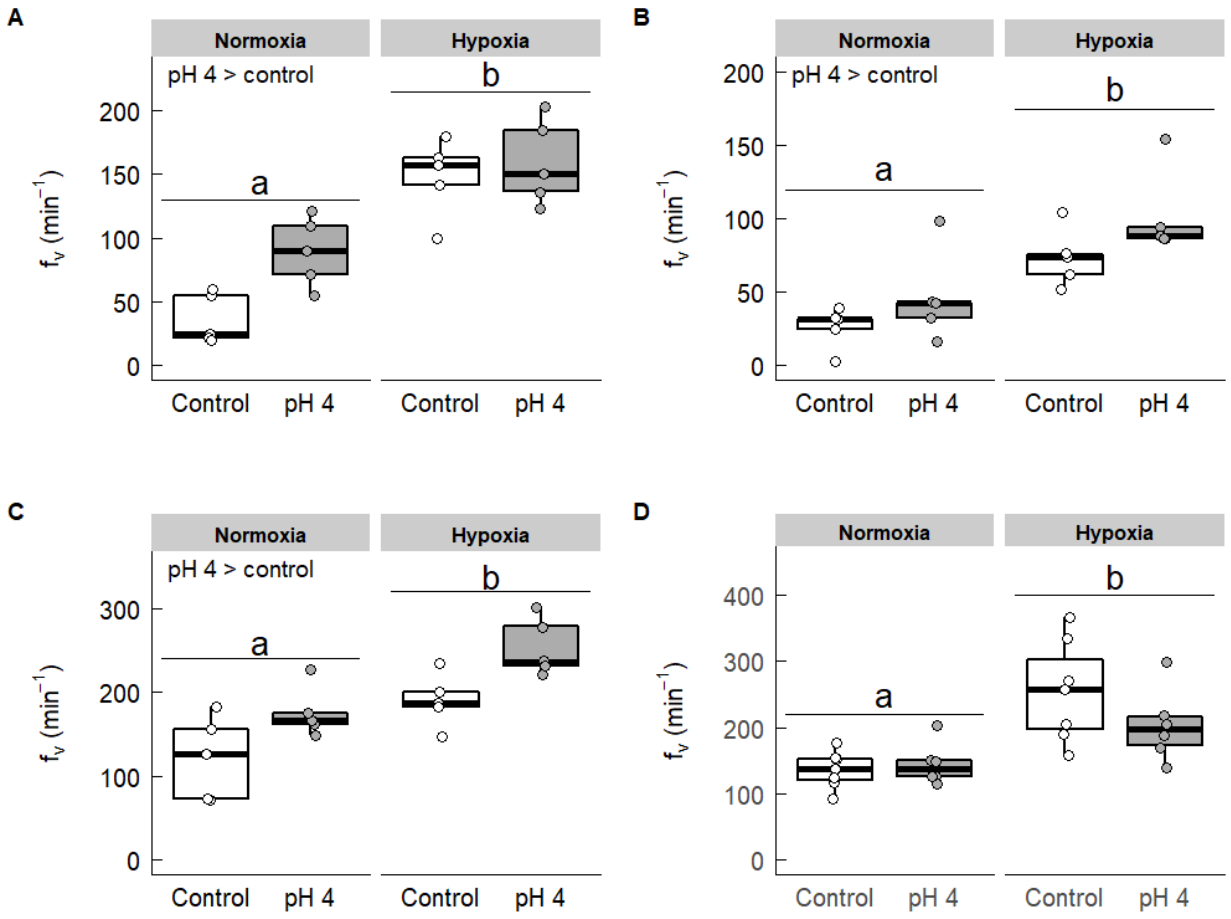

Figure 3.17. Ventilation frequency under normoxic (150 Torr) and hypoxic (30 Torr) conditions for 6 dpf (A), 15 dpf (B), 30 dpf (C), and 90 dpf (D) zebrafish (*Danio rerio*) that were raised under control conditions or in water of pH 4. Data are shown as a boxplot, with upper and lower limits of the box representing the 75th and 25th quartiles, respectively. The bold line across the box represents the mean value. The white or grey symbols represent individual data points. Groups that share a letters are not significantly different from one another (ANOVA, A, $p_{treatment} = 0.021$, $p_{hypoxia} < 0.001$, $p_{treatmentxhypoxia} = 0.12$; B, $p_{treatment} = 0.04$, $p_{hypoxia} = 0.00025$, $p_{treatmentxhypoxia} = 0.72$; C, $p_{treatment} = 0.0025$, $p_{hypoxia} = 0.00044$, $p_{treatmentxhypoxia} = 0.78$; D, $p_{treatment} = 0.32$, $p_{hypoxia} = 0.00022$, $p_{treatmentxhypoxia} = 0.15$).

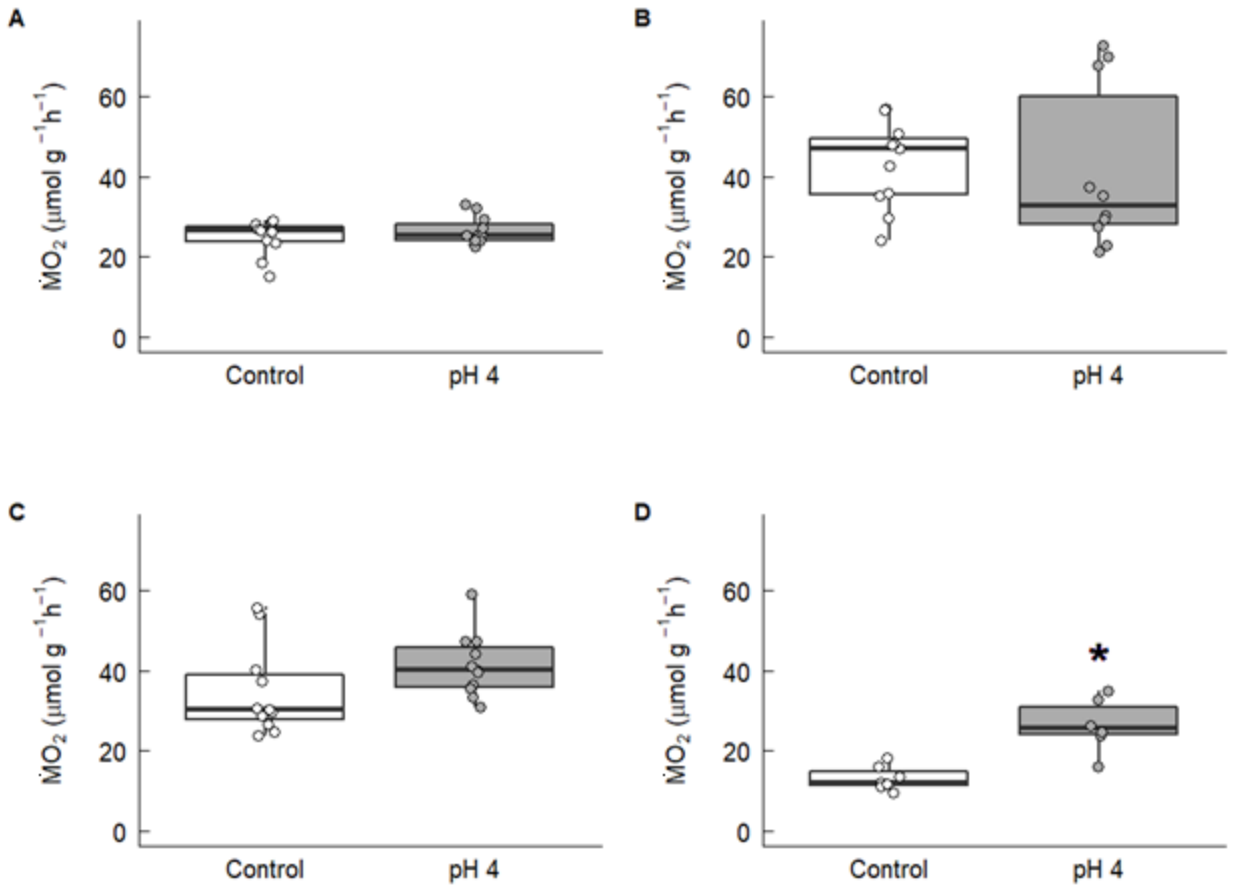

Figure 3.18. Rates of oxygen consumption for 6 dpf (A), 15 dpf, (B), 30 dpf (C), and 90 dpf (D) zebrafish (*Danio rerio*) raised under control conditions or in water of pH 4. Data are shown as a boxplot, with upper and lower limits of the box representing the 75th and 25th quartiles, respectively. The bold line across the box represents the mean value. The white or grey symbols represent individual data points. An asterisk denotes a significant difference between groups (Student's *t*-tests, A, p = 0.34; B, p = 0.85; C, p = 0.13; D, p = 0.00068).

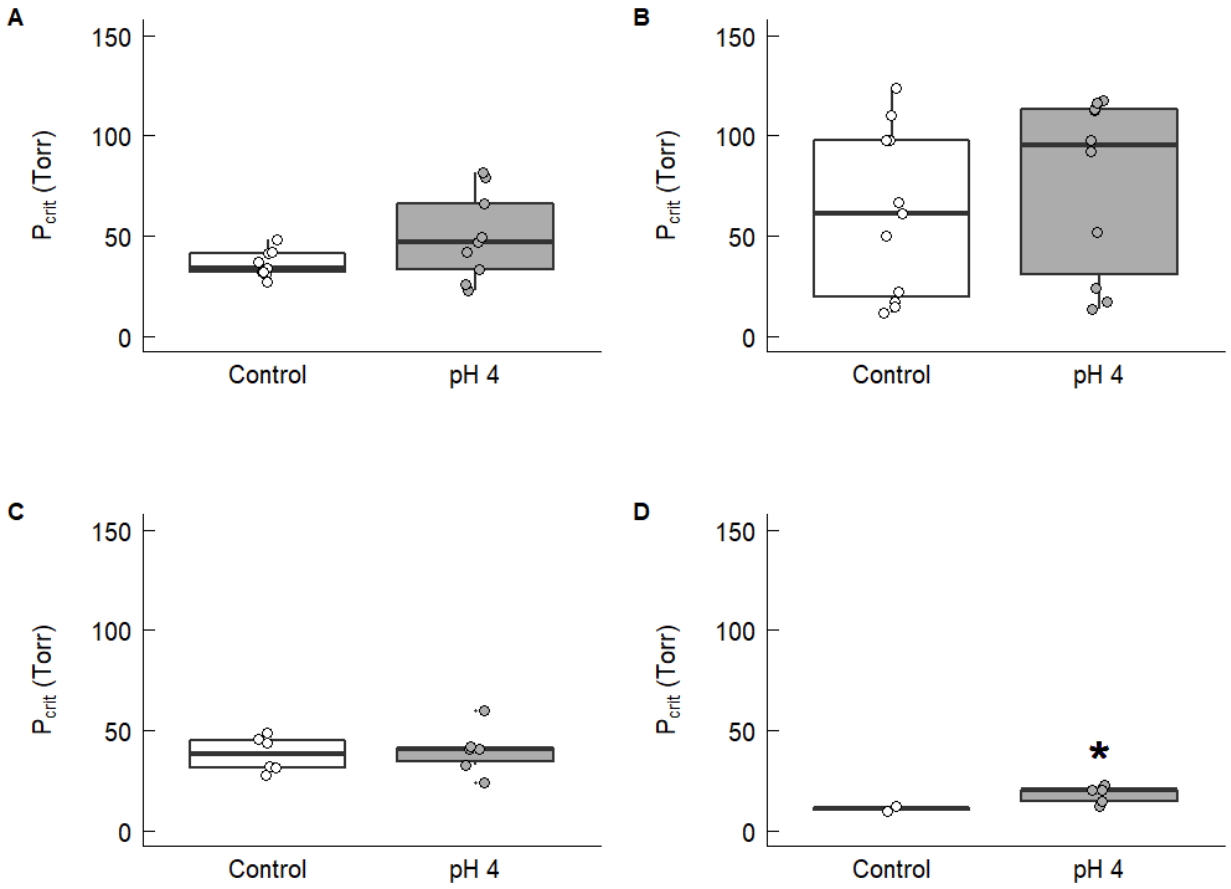

Figure 3.19. Critical oxygen tensions (P_{crit}) for 6 dpf (A), 15 dpf (B), 30 dpf (C), and 90 dpf (D) zebrafish (*Danio rerio*) raised under control conditions or in water of pH 4. Data are shown as a boxplot, with upper and lower limits of the box representing the 75th and 25th quartiles, respectively. The bold line across the box represents the mean value. The white or grey symbols represent individual data points. An asterisk denotes a significant difference between groups (Student's *t*-tests, A, p = 0.1; B, p = 0.8; C, p = 0.25; D, p = 0.037).

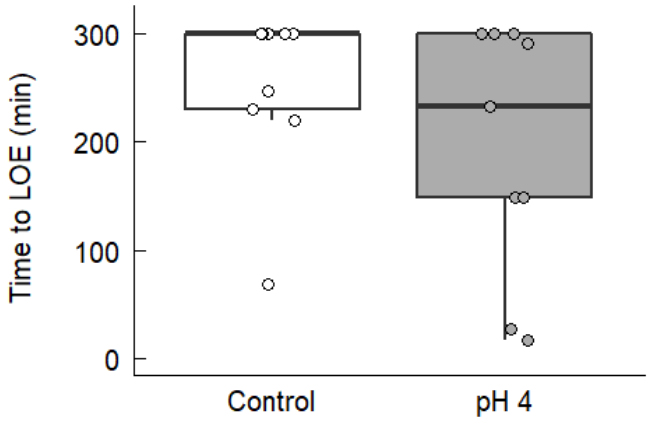

Figure 3.20. Time to loss of equilibrium (LOE) at a water PO_2 of 12 Torr for 90 dpf zebrafish (*Danio rerio*) raised under control conditions or in water of pH 4. Data are shown as a boxplot, with upper and lower limits of the box representing the 75th and 25th quartiles, respectively. The bold line across the box represents the mean value. The white or grey symbols represent individual data points. No significant difference was detected between fish raised under normal conditions and those raised in water of pH 4 (Student's *t*-test, p = 0.25).

Table 3.2. Length and mass of zebrafish (*Danio rerio*) raised under control conditions or in water of pH 4

Age	**Length (mm)**			**Mass (mg)**		
	control	**pH 4**	***p* value**	**Control**	**pH 4**	***p* value**
6 dpf	5.75 ± 0.03	5.65 ± 0.05	0.072	0.27 ± 0.01	0.28 ± 0.01	0.64
	(20)	(20)		(20)	(20)	
15 dpf	5.83 ± 0.07	5.84 ± 0.08	0.89	0.35 ± 0.01	0.34 ±0.01	0.47
	(20)	(20)		(20)	(20)	
30 dpf	5.92 ± 0.11	6.12 ± 0.08	0.19	0.85 ± 0.07	0.97 ± 0.05	0.19
	(17)	(17)		(17)	(17)	
90 dpf	348 ± 15	336 ± 18	0.68	57.5 ± 8.7	61.7 ± 7.2	0.71
	(28)	(24)		(38)	(41)	

Values are means ± SEM with the *N* number in parentheses. Mass was determined by weighing individuals of 6, 15 or 90 dpf, and from a length-mass regression curve (mass = 0.0006·length - 0.0027, $R^2 = 0.87$) for fish of 30 dpf (see text for details). Values for control and pH 4 treatment groups were compared using Student's *t*-tests.

3.4 Discussion

The present study tested the hypothesis that zebrafish raised in a low pH environment would experience changes in gill morphology to combat the ionoregulatory challenge of low pH, and these changes would negatively impact gas transfer in accordance with the predictions of the osmorespiratory compromise. Previous work reported that rainbow trout raised in ion-poor water experienced proliferation of branchial ionocytes that increased the blood-to-water diffusion distance and negatively impacted gas transfer as evidenced by impaired hypoxia tolerance (Greco et al., 1995; Greco et al., 1996; Perry et al., 1996; reviewed by Perry, 1998). A similar approach was adopted in the present study, but with a focus on early development, a period in which there is a high capacity for plasticity (Best and Vijayan, 2017; Burggren and Reyna, 2011; Burggren et al., 2016; Hare et al., 2020; Mendez-Sanchez and Burggren, 2017; Mendez-Sanchez and Burggren, 2019). Zebrafish were raised from embryonic development to sexual maturity in pH 4 water, allowing both long-term effects of low pH exposure and the potential for developmental plasticity to be examined. The findings of the present study indicate that key ionoregulatory responses to low pH, namely the proliferation of Na^+-transporting HR and NCC ionocytes, are maintained into adulthood when zebrafish are raised under low pH conditions. Further, these ionoregulatory responses alter gill morphology in a fashion that appears to impair gas transfer based on elevated ventilation and, in some cases, reduced hypoxia tolerance.

At 3-5 dpf, zebrafish raised in pH 4 water exhibit a suite of responses aimed at increasing Na^+ uptake to compensate for acid-induced increases in passive Na^+ loss (reviewed by Kwong et al. 2014). These responses include increases in transporters and proteins associated with transport pathways (summarized by Shir-Mohammadi and Perry, 2020)), as well as increases in HR cell density (Chang et al., 2009; Horng et al., 2009; Shir-Mohammadi and Perry, 2020), and

enable recovery of whole-body Na^+ content by one- to two weeks of age (Kumai and Perry, 2011). The elevation of cortisol levels appears to play a key role in activating these responses (Kumai et al., 2012). Therefore, to assess ionoregulatory responses to rearing in pH 4 water across development, we measured cortisol levels, whole-body Na^+ content, and ionocyte abundances. No significant difference in cortisol levels between control and pH 4-raised fish were detected at any age, suggesting that the initial elevation of cortisol in early development is sufficient to trigger the compensatory responses (Kumai et al., 2012; Kumai et al., 2015) and that prolonged exposure to pH 4 water does not result in chronic elevation of cortisol levels. Fish raised under pH 4 conditions had significantly higher whole-body Na^+ content than control fish at 6, 15 and 90 dpf, but not 30 dpf. Kumai et al. (2011) followed whole-body Na^+ content over the initial 72 h of development in pH 4 water and then at one and two weeks of exposure. Reduced Na^+ content was detected as early as 3 h following exposure to pH 4 water and persisted to 72 h, but had recovered to control values by one week of exposure (Kumai et al. 2011). Similarly, Horng et al. (2009) did not find differences in whole-body Na^+ content between control and pH 4-raised fish at 4 dpf. The findings of the present study are consistent with these patterns, and demonstrate that the recovery achieved during early development extends to sexual maturity. Further, the data suggest that the increased rates of Na^+ uptake triggered by exposure to low pH (Kumai et al., 2012; Kwong et al., 2014) can more than compensate for acid-induced increases in passive Na^+ loss, leading to elevated whole-body Na^+ content in pH 4-reared fish.

Across all time points, zebrafish of the present study that were raised in water of pH 4 had significantly higher abundances of HR and NCC cells than control fish. Additionally, the abundance of NaR cells was elevated in 6 dpf zebrafish; technical problems prevented measurements of this cell type in older fish. Increased HR and NaR cell densities in the skin of

larval zebrafish were reported previously for 4 dpf fish (Horng et al., 2009; Shir-Mohammadi and Perry, 2020) and the data of the present study show that this response is maintained to 6 dpf. Increased HR cell abundance is associated with a greater capacity for H^+ secretion (Horng et al., 2009) as well as Na^+ uptake (Kumai and Perry, 2011). However, NCC cells also contribute to Na^+ uptake in acid-exposed fish (Kwong et al., 2016), and the present study confirmed that NCC cell abundance is increased in the skin of pH 4-reared zebrafish. Ionoregulatory responses in developing zebrafish transition from skin ionocytes to gill ionocytes around 7 dpf (Rombough, 2002), and therefore ionocyte abundance was examined in the gills of 15 and 30 dpf fish. The elevated HR and NCC cell abundances observed in the gills of these juvenile fish, as well as in the gills of 90 dpf fish, indicate an ongoing requirement to maintain elevated rates of Na^+ uptake in the face of low environmental pH. Similarly, an increase in HR cell abundance was detected in the gill of adult zebrafish exposed to pH 4 water for 7 d (Chang et al., 2009).

Examination of gill structure revealed that increased ionocyte abundance and/or other factors associated with low pH had impacts on overall gill morphology from the earliest age investigated. At 6 dpf, larval zebrafish raised in pH 4 water had wider gill arches than control fish. However, this difference would not be expected to affect gas transfer, because at this early stage, O_2 uptake in larval zebrafish occurs primarily across the body surface (Rombough, 1988; Rombough, 1999; Rombough, 2007). At 15 and 30 dpf, when juvenile fish are dependent on the gill for gas exchange, the gill arches of fish raised in pH 4 water were wider and had a larger cross-sectional area than those of their control counterparts, suggesting an increased volume of the gill as a whole. Whether these morphological changes reflect increases in ionocyte abundances, or some other type of gill remodelling, perhaps to reduce passive ion loss, remains to be determined. It is important to note that the structure of the gill is not fully developed at the

earlier times examined, with filaments only beginning to bud at ~7 dpf, and lamellae beginning to bud around 14 dpf (Jonz and Nurse, 2005; Shadrin and Ozerniuk, 2002). The appearance of filaments and lamellae dramatically increases the effective surface area for gas exchange and ion diffusion. Evidence of pH 4-induced changes in gill morphology also was obtained for adult fish. At 90 dpf, pH 4-reared zebrafish had wider lamellae with decreased space between the lamellae. Similar changes in lamellar width and interlamellar space were reported in the gills of trout experiencing ionocyte proliferation, where they were correlated with increases in the blood-to-water diffusion distance (Greco et al., 1996; Sloman et al., 2001). If the observed changes in gill morphology in 15, 30 and 90 dpf zebrafish reared in pH 4 water reflect increases in the blood-to-water diffusion distance at the gill, then these changes would be predicted to impair gas transfer. Interestingly, neither gill arch length (in 6, 15 and 30 dpf fish) nor lamellar length (in 90 dpf fish) differed between control fish and fish raised in pH 4 water. Increases in length would be one potential mechanism for increasing effective gill surface area to compensate for the impact of increased blood-to-water diffusion distance on O_2 transfer. The absence of such differences suggests that these basic elements of gill structure are developmentally programmed and do not exhibit environmentally-induced plasticity.

Measurements of ventilation under normoxic and hypoxic conditions, P_{crit}, and time to LOE (in 90 dpf fish only) provided some evidence that the changes in gill morphology observed in pH 4-reared fish negatively impacted gas transfer. At 6 dpf, larvae that had been raised in pH 4 water had ventilation frequencies nearly double those of the control group. Zebrafish at this stage still rely on gas transfer across the body surface, with ventilatory movements and movements of the pectoral fins contributing to the convective water flow needed to maintain O_2 uptake (Parker et al., 2020; Zimmer et al., 2014). Despite higher f_V under normoxic conditions,

pH 4-reared fish increased f_V to the same extent as control fish under hypoxic conditions, and neither P_{crit} nor oxygen consumption differed between control and pH 4-reared fish at 6 dpf. These data suggest that hypoxia tolerance was not affected by rearing under pH 4 conditions. The lack of difference in normoxic, resting oxygen consumption between control and pH 4-reared fish was unexpected given that we anticipated higher energetic cost associated with elevated normoxic f_V in pH 4-reared fish based on previous studies (Kramer, 1983; Urbina et al., 2014). A possible explanation is that energy expended on other processes, such as growth, is reduced to compensate for the higher ventilatory expenditure in these animals. Consistent with this possibility, a trend for pH 4-reared fish to be shorter in length than control fish was detected at 6 dpf. Horng et al. (2009) reported a similar effect of pH 4-rearing on body length at 4 dpf.

At ~15 dpf, zebrafish become dependent on the gill and circulatory system for O_2 uptake and delivery (Jonz and Nurse, 2005; Rombough, 1999; Rombough, 2007). Despite the differences at this stage in gill morphology caused by rearing under pH 4 conditions, no significant differences in normoxic f_V, the f_V response to hypoxia, the rate of oxygen consumption under normoxic conditions or P_{crit} were observed. Either the morphological changes observed in the gills of pH 4-reared fish did not impact key variables such as the blood-to-water diffusion distance, or pH 4-reared fish were able to compensate for any decrement in O_2 uptake at the gill, perhaps with continuing transcutaneous O_2 uptake. Zebrafish at 30 dpf appeared similarly resilient to the effects of pH 4-rearing on gill morphology, with no difference in P_{crit}. However, the pH 4-reared fish had significantly higher f_V than control fish regardless of whether normoxic or hypoxic conditions were examined. This observation suggests some impact of the morphological differences in gill structure.

Arguably, the strongest evidence of impaired gas transfer in pH 4-reared fish was collected for 90 dpf fish. Although no significant difference in f_V was detected between pH 4-reared and control groups under normoxic or hypoxic conditions, a trend for the ventilatory response to hypoxia to be smaller in pH 4-reared fish relative to control fish was observed. The trend was driven largely by the failure of pH 4-reared fish to elevate f_V under hypoxic conditions, rather than by elevated normoxic f_V in these fish. Normoxic resting oxygen consumption was significantly higher in pH 4-reared fish, which may have contributed to their apparently reduced ability to elevate f_V under hypoxic conditions. Additionally, the P_{crit} of pH 4-reared fish was significantly higher than that of control fish, suggesting reduced hypoxia tolerance (Regan et al., 2017; Rogers et al., 2016; Ultsch and Regan, 2019). However, measurement of the time to LOE under severe hypoxia did not differ between control and pH 4-reared fish. Rainbow trout experiencing branchial ionocyte proliferation as a result of hormone treatment or exposure to ion-poor water displayed similar indications of impaired gas transfer. For example, ventilation was elevated under normoxic conditions in some (Greco et al., 1995; Perry et al., 1996) but not all (Bindon et al., 1994a) studies, and hypoxia tolerance was reduced in trout experiencing ionocyte proliferation based on higher release thresholds for catecholamine stress hormones and lower blood O_2 tensions (Bindon et al., 1994a; Greco et al., 1995; Perry et al., 1996). In rainbow trout, branchial ionocyte proliferation was shown to increase the blood-to-water diffusion distance (Bindon et al., 1994a; Greco et al., 1995), suggesting that the impairment of gas transfer in zebrafish raised under pH 4 conditions reflects the impact of branchial ionocyte proliferation on the blood-to-water diffusion distance.

Collectively, the findings of the present study suggest that zebrafish cannot escape the osmorespiratory compromise through developmental plasticity. Rearing in low pH posed an

ionoregulatory challenge that was addressed through remodelling of the gill epithelium to increase ionocyte abundance. Increases in ionocyte abundance and/or other responses to pH 4 water, such as an increase in mucus cells, altered gill morphology (Kwong et al., 2014). Although gas transfer in larval and juvenile fish appeared to be resilient to the effects of this structural remodelling, adult zebrafish raised under pH 4 conditions exhibited responses such as higher P_{crit} that indicated an apparent impairment of gas transfer, a situation that is consistent with the osmorespiratory compromise.

Chapter 4: General Discussion

The objective of the present study was to investigate developmental trade-offs in zebrafish raised in a low pH environment. Chapter 2 asked whether low pH exposure during early life and the resulting elevation of cortisol to stimulate ionoregulatory responses would alter development of the HPI axis and its function later in life. The data collected did not provide support for this hypothesis, but our ability to test the hypothesis was limited by the finding that baseline cortisol levels were not elevated in fish exposed to pH 4 conditions. This result contrasted with those of previous studies, which demonstrated that larval zebrafish exposed to low pH conditions experienced elevated cortisol levels in early life (Kumai et al., 2012; Kumai et al., 2015; Lin et al., 2016a). The reason for the different results was not apparent. We used the approaches reported in these previous studies, including zebrafish of the same stock as those used by Kumai et al (2012; 2015). Given recent studies indicating that early life stress and the resultant elevation of cortisol impact ionoregulatory function in zebrafish (Hare et al. 2020), it remains of interest to determine whether ionoregulatory challenges that elevate cortisol impact HPI axis development and function. However, testing this hypothesis will require identification of an ionoregulatory challenge that elicits a robust cortisol response.

Chapter 3 investigated a different trade-off related to low pH, based on the osmorespiratory compromise. We predicted that zebrafish reared in low pH conditions from fertilization (0 dpf) to sexual maturity (90 dpf) would experience increases in ionocyte abundance for ionoregulatory purposes that would impair respiratory function of the gills. An alternative possibility was that plasticity during gill development would be sufficient to overcome the constraints of the osmorespiratory compromise – either ion balance would be achieved without increases in ionocyte abundance, or gill surface area would be increased to compensate for increases in the blood-to-water diffusion distance. We found that at all four time

points examined, fish raised in pH 4 water had a higher abundance of ionocytes. This finding was in agreement with those of previous studies (Chang et al., 2009; Horng et al., 2009), but also novel in that previous studies had focused only on larval fish, or on short-term acclimation to pH 4 water in adults. Our results demonstrated that elevated ionocyte abundances persisted to maturity in fish raised in pH 4 water, suggesting that other mechanisms, such as increases in the expression of transport pathway proteins or reductions in paracellular ion loss (Kwong et al. 2014), are not sufficient to maintain ion balance under acidic conditions.

Evidence of significant gill remodelling was apparent in pH 4-reared fish, with larval and juvenile zebrafish having thicker gill arches, and adult fish having thicker lamellae with less space between them. These changes would be expected to increase the blood-to-water diffusion distance, and therefore have the potential to impair gas transfer (Perry, 1998). We found some evidence of impaired gas transfer in low pH-reared fish. Fish that had been raised in low pH to 6 dpf had elevated ventilation frequency under normoxic conditions. At 30 dpf, low pH-reared fish exhibited significantly higher ventilation frequencies than the control fish regardless of whether normoxic or hypoxic conditions were examined. At 90 dpf, zebrafish raised under low pH conditions had higher P_{crit} than control fish, indicating that their hypoxia tolerance was reduced. These results are consistent with those observed in rainbow trout, where increases in ionocyte abundance and the blood-to-water diffusion distance resulted in changes in ventilation and hypoxia tolerance (Bindon et al., 1994a; Greco et al., 1996; Perry et al., 1996; reviewed by Perry, 1998). Collectively, these data support the conclusion that gas transfer in pH 4-reared fish was impaired by the ionoregulatory responses to pH 4 conditions, in accordance with the osmorespiratory compromise (Gonzalez, 2011), and that gill morphology was not altered through developmental plasticity to compensate for the increased blood-to-water diffusion distance.

These conclusions would be strengthened by a more detailed morphometric analysis of gill structure, specifically to quantify the impact of increases in ionocyte abundance on blood-to-water diffusion distance, as in previous studies on rainbow trout gills (Bindon et al., 1994b; Greco et al., 1996). Whether the effects of elevated ionocyte abundances are compounded by other consequences of low pH exposure, such as changes in mucus cell abundance or mucus production, also warrants investigation (see Kwong et al. 2014). In addition, a more detailed analysis of gas transfer is needed in zebrafish raised under low pH conditions. Previous studies, in rainbow trout, assessed blood gases under normoxic and hypoxic conditions (Bindon et al., 1994a; Greco et al., 1995; Greco et al., 1996). Although these approaches are not practical in a species as small as the zebrafish, alternative indices of gas transfer effectiveness, such as exercise capacity, should be considered. Finally, future studies could investigate how other environmental conditions affect the osmorespiratory compromise to provide further insight into the complexity of gill form and function and the role of developmental plasticity in shaping the gill.

Overall, the hypothesis that rearing in a low pH environment prioritizes ionoregulatory function at the gill, leading to a trade-off in respiratory function, was supported in zebrafish as in rainbow trout (Bindon et al 1994b; Greco et al 1995; Perry et al 1996). However, the novelty of the present work lies in its focus on early development, where the potential for plasticity is substantial. The results suggest that even developmental plasticity is not sufficient to allow fish to escape the osmorespiratory compromise. Collectively, the research presented in this thesis improves our understanding of the physiology of teleost fish in a low pH environment and demonstrates the physiological trade-offs that can occur under these conditions.

References

Abreu, M. S., Oliveira, T. A., Koakoski, G., Barreto, R. E. and Barcellos, L. J. G. (2018). Modulation of Cortisol Responses to an Acute Stressor in Zebrafish Visually Exposed to Heterospecific Fish During Development. *Zebrafish* **15**, zeb.2017.1509.

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

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

Alsop, D. and Vijayan, M. (2009). The zebrafish stress axis: Molecular fallout from the teleost-specific genome duplication event. *Gen. Comp. Endocrinol.* **161**, 62–66.

Barton, B. A. (2002). Stress in Fishes: A Diversity of Responses with Particular Reference to Changes in Circulating Corticosteroids. *Integr. Comp. Biol.* **42**, 517–525.

Best, C. and Vijayan, M. M. (2017). Cortisol elevation post-hatch affects behavioural performance in zebrafish larvae. *Gen. Comp. Endocrinol.*

Best, C., Kurrasch, D. M. and Vijayan, M. M. (2017). Maternal cortisol stimulates neurogenesis and affects larval behaviour in zebrafish. *Sci. Rep.* **7**, 1–10.

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

Bindon, S. D., Gilmour, K. M., Fenwick, J. C. and Perry, S. F. (1994b). *THE EFFECTS OF BRANCHIAL CHLORIDE CELL PROLIFERATION ON RESPIRATORY FUNCTION IN THE RAINBOW TROUT ONCORHYNCHUS MYKISS*.

Botero, C. A., Weissing, F. J., Wright, J. and Rubenstein, D. R. (2015). Evolutionary tipping points in the capacity to adapt to environmental change. *Proc. Natl. Acad. Sci. U. S. A.* **112**, 184–189.

Boutilier, R. G., Heming, T. A. and Iwama, G. K. (1984). Appendix: Physicochemical Parameters for use in Fish Respiratory Physiology.pp. 403–430. Academic Press.

Brauner, C. J. and Rombough, P. J. (2012). Ontogeny and paleophysiology of the gill: New insights from larval and air-breathing fish. *Respir. Physiol. Neurobiol.* **184**, 293–300.

Breves, J. P., Hirano, T. and Grau, E. G. (2010). Ionoregulatory and endocrine responses to disturbed salt and water balance in Mozambique tilapia exposed to confinement and handling stress. *Comp. Biochem. Physiol. Part A Mol. Integr. Physiol.* **155**, 294–300.

Burggren, W. W. and Reyna, K. S. (2011). Developmental trajectories, critical windows and phenotypic alteration during cardio-respiratory development. *Respir. Physiol. Neurobiol.* **178**, 13–21.

Burggren, W. W., Bautista, G. M., Coop, S. C., Couturier, G. M., Delgadillo, S. P., García, R. M. and González, C. A. A. (2016). Developmental cardiorespiratory physiology of the air-breathing tropical gar, *Atractosteus tropicus*. *Am. J. Physiol. Integr. Comp. Physiol.* **311**,

R689–R701.

Castillo-Ramírez, L. A., Ryu, S. and De Marco, R. J. (2019). Active behaviour during early development shapes glucocorticoid reactivity. *Sci. Rep.* **9**, 1–9.

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*). *Am. J. Physiol. Integr. Comp. Physiol.* **296**, R1192–R1201.

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 Mol. Integr. Physiol.* **164**, 249–257.

Edelaar, P., Jovani, R. and Gomez-Mestre, I. (2017). Should I change or should I go? Phenotypic plasticity and matching habitat choice in the adaptation to environmental heterogeneity. *Am. Nat.* **190**, 506–520.

Edenbrow, M. and Croft, D. P. (2011). Behavioural types and life history strategies during ontogeny in the mangrove killifish, Kryptolebias marmoratus. *Anim. Behav.* **82**, 731–741.

Evans, D. H. (2008). Teleost fish osmoregulation: What have we learned since August Krogh, Homer Smith, and Ancel Keys. *Am. J. Physiol. - Regul. Integr. Comp. Physiol.* **295**, 704–713.

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.

Faught, E., Best, C. and Vijayan, M. M. (2016). Maternal stress-associated cortisol stimulation may protect embryos from cortisol excess in zebrafish. *R. Soc. Open Sci.* **3**,.

Gilmour, K. M. and Perry, S. F. (2018). Conflict and compromise: Using reversible remodeling to manage competing physiological demands at the fish gill. *Physiology* **33**, 412–422.

Gonzalez, R. J. (2011). Role of the gills | The Osmorespiratory Compromise. In *Encyclopedia of Fish Physiology*, pp. 1389–1394. Elsevier Inc.

Gorissen, M. and Flik, G. (2016). *The Endocrinology of the Stress Response in Fish: An Adaptation-Physiological View*. Elsevier Inc.

Greco, A. M., Fenwick, J. C. and Perry, S. F. (1995). The effects of soft-water acclimation on respiratory gas transfer in the rainbow trout Oncorhynchus mykiss. *Cell Tissue Res.* **285**, 75–82.

Greco, A. M., Fenwick, J. C. and Perry, S. F. (1996). The effects of soft-water acclimation on gill structure in the rainbow trout Oncorhynchus mykiss. *Cell Tissue Res.* **285**, 75–82.

Guh, Y.-J., Lin, C.-H. and Hwang, P.-P. (2015). Osmoregulation in zebrafish: ion transport mechanisms and functional regulation. *EXCLI J.* **14**, 627–659.

Hare, A. J., Zimmer, A. M., LePabic, R., Morgan, A. L. and Gilmour, K. M. (2020). Early-

life stress influences ion balance in developing zebrafish (Danio rerio). *J. Comp. Physiol. B Biochem. Syst. Environ. Physiol.*

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

Hollander, J., Snell-Rood, E. and Foster, S. (2015). New frontiers in phenotypic plasticity and evolution. *Heredity (Edinb).* **115**, 273–275.

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

Huang, C. Y. and Lin, H. C. (2016). Different oxygen stresses on the responses of branchial morphology and protein expression in the gills and labyrinth organ in the aquatic air-breathing fish, Trichogaster microlepis. *Zool. Stud.* **55**,.

Hughes, G. M. (1966). The dimensions of fish gills in relation to their function. *J. Exp. Biol.* **45**, 177–195.

Hughes, G. M. and Morgan, M. (1973). The structure of fish gills in relation to their respiratory function. *Biol. Rev. Camb. Philos. Soc.* **48**, 419–475.

Hughes, M. C., Zimmer, A. M. and Perry, S. F. (2019). Role of internal convection in respiratory gas transfer and aerobic metabolism in larval zebrafish (Danio rerio). *Am. J. Physiol. - Regul. Integr. Comp. Physiol.* **316**, R255–R264.

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

Ivy, C. M., Robertson, C. E. and Bernier, N. J. (2017). Acute embryonic anoxia exposure favours the development of a dominant and aggressive phenotype in adult zebrafish. *Proc. R. Soc. B Biol. Sci.* **284**,.

Jacob, E., Drexel, M., Schwerte, T. and Pelster, B. (2002). Influence of hypoxia and of hypoxemia on the development of cardiac activity in zebrafish larvae. *Am. J. Physiol. - Regul. Integr. Comp. Physiol.* **283**,.

JavadiEsfahani, R. and Kwong, R. W. M. (2019). The sensory-motor responses to environmental acidosis in larval zebrafish: Influences of neurotransmitter and water chemistry. *Chemosphere* **235**, 383–390.

Jeffrey, J. D. and Gilmour, K. M. (2016). Programming of the hypothalamic-pituitary-interrenal axis by maternal social status in zebrafish (Danio rerio). *J. Exp. Biol.* **219**, 1734–1743.

Jonz, M. G. and Nurse, C. A. (2005). Development of oxygen sensing in the gills of zebrafish. *J. Exp. Biol.* **208**, 1537–1549.

Kimmel, C. B., Ballard, W. W., Kimmel, S. R., Ullmann, B. and Schilling, T. F. (1995).

Stages of embryonic development of the zebrafish. *Dev. Dyn.* **203**, 253–310.

Kostyniuk, D. J., Culbert, B. M., Mennigen, J. A. and Gilmour, K. M. (2018). Social status affects lipid metabolism in rainbow trout, Oncorhynchus mykiss. *Am J Physiol Regul Integr Comp Physiol* **315**, 241–255.

Kostyniuk Id, D. J., Zhang, D., Martyniuk, C. J., Gilmour, K. M. and Mennigen Id, J. A. (2019). Social status regulates the hepatic miRNAome in rainbow trout: Implications for posttranscriptional regulation of metabolic pathways.

Kramer, D. L. (1983). The evolutionary ecology of respiratory mode in fishes: an analysis based on the costs of breathing. *Environ. Biol. Fishes* **9**, 145–158.

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

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

Kumai, Y., Kwong, R. W. and Perry, S. F. (2015). A role for transcription factor glial cell missing 2 in Ca2+ homeostasis in zebrafish, Danio rerio. *Eur. J. Physiol.* **467**, 753–765.

Kwong, R. W. M. and Perry, S. F. (2013). 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. (2016). A role for sodium-chloride cotransporters in the rapid regulation of ion uptake following acute environmental acidosis: New insights from the zebrafish model. *Am. J. Physiol. - Cell Physiol.* **311**, C931–C941.

Kwong, R. W. M., Kumai, Y. and Perry, S. F. (2014). The physiology of fish at low pH: The zebrafish as a model system. *J. Exp. Biol.* **217**, 651–662.

Kwong, R. W. M., Kumai, Y. and Perry, S. F. (2016). Neuroendocrine control of ionic balance in zebrafish. *Gen. Comp. Endocrinol.* **234**, 40–46.

Laurent, P. and Perry, S. F. (1991). Environmental Effects on Fish Gill Morphology. *Physiol. Zool.* **64**, 4–25.

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, L. Y., Horng, J. L., Kunkel, J. G. and Hwang, P. P. (2006). Proton pump-rich cell secretes acid in skin of zebrafish larvae. *Am. J. Physiol. - Cell Physiol.* **290**, 371–378.

Lin, C. H., Hu, H. J. and Hwang, P. P. (2016a). Cortisol regulates sodium homeostasis by stimulating the transcription of sodium-chloride transporter (NCC) in zebrafish (Danio rerio). *Mol. Cell. Endocrinol.* **422**, 93–102.

Lin, C.-H., Kuan, W.-C., Liao, B.-K., Deng, A.-N., Tseng, D.-Y. and Hwang, P.-P. (2016b). Environmental and cortisol-mediated control of Ca2+ uptake in tilapia (Oreochromis

mossambicus). *J. Comp. Physiol. B* **186**, 323–332.

Mandic, M., Best, C. and Perry, S. F. (2020). Loss of hypoxia-inducible factor 1α affects hypoxia tolerance in larval and adult zebrafish (*Danio rerio*). *Proc. R. Soc. B Biol. Sci.* **287**, 20200798.

Matey, V., Iftikar, F. I., de Boeck, G., Scott, G. R., Sloman, K. A., Almeida-Val, V. M. F., Val, A. L. and Wood, C. M. (2011). Gill morphology and acute hypoxia: Responses of mitochondria-rich, pavement, and mucous cells in the Amazonian oscar (Astronotus ocellatus) and the rainbow trout (Oncorhynchus mykiss), two species with very different approaches to the osmo-respiratory com. *Can. J. Zool.* **89**, 307–324.

McBryan, T. L., Anttila, K., Healy, T. M. and Schulte, P. M. (2013). Responses to Temperature and Hypoxia as Interacting Stressors in Fish: Implications for Adaptation to Environmental Change. *Integr. Comp. Biol.* **53**, 648–659.

Mccormick, S. D. (2001). *Endocrine Control of Osmoregulation in Teleost Fish 1*.

McCormick, S. D. (2001). Endocrine control of osmoregulation in teleost fish. *Am. Zool.* **41**, 781–794.

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 Mol. Biol.* **9**, 1–12.

Mendez-Sanchez, J. F. and Burggren, W. W. (2017). Cardiorespiratory physiological phenotypic plasticity in developing air-breathing anabantid fishes (*Betta splendens* and *Trichopodus trichopterus*). *Physiol. Rep.* **5**, e13359.

Mendez-Sanchez, J. F. and Burggren, W. W. (2019). Hypoxia-induced developmental plasticity of larval growth, gill and labyrinth organ morphometrics in two anabantoid fish: The facultative air-breather Siamese fighting fish (*Betta splendens*) and the obligate air-breather the blue gourami (*Trichopodus trichopterus*). *J. Morphol.* **280**, 193–204.

Mitrovic, D., Dymowska, A., Nilsson, G. E. and Perry, S. F. file:///C:/Users/Lia. T. full. pd. (2009). Physiological consequences of gill remodeling in goldfish (Carassius auratus) during exposure to long-term hypoxia. *Am. J. Physiol. - Regul. Integr. Comp. Physiol.* **297**, 224–234.

Nesan, D. and Vijayan, M. M. (2013a). The transcriptomics of glucocorticoid receptor signaling in developing zebrafish. *PLoS One* **8**, 1–15.

Nesan, D. and Vijayan, M. M. (2013b). Role of glucocorticoid in developmental programming: Evidence from zebrafish. *Gen. Comp. Endocrinol.* **181**, 35–44.

Nesan, D. and Vijayan, M. M. (2016). Maternal cortisol mediates hypothalamus-pituitary-interrenal axis development in Zebrafish. *Sci. Rep.* **6**, 1–11.

Nilsson, G. E., Dymowska, A. and Stecyk, J. A. W. (2012). New insights into the plasticity of gill structure. *Respir. Physiol. Neurobiol.* **184**, 214–222.

Pan, Y. K., Mandic, M., Zimmer, A. M. and Perry, S. F. (2019). Evaluating the physiological

significance of hypoxic hyperventilation in larval zebrafish (Danio rerio). *J. Exp. Biol.* **222**,.

Parker, J. J., Zimmer, A. M. and Perry, S. F. (2020). Respirometry and cutaneous oxygen flux measurements reveal a negligible aerobic cost of ion regulation in larval zebrafish (Danio rerio). *J. Exp. Biol.* **223**,.

Pelster, B. and Burggren, W. W. (1996). Disruption of hemoglobin oxygen transport does not impact oxygen- dependent physiological processes in developing embryos of zebra fish (Danio rerio). *Circ. Res.* **79**, 358–362.

Perry, S. F. (1998). Relationships between branchial chloride cells and gas transfer in freshwater fish. *Comp. Biochem. Physiol. - A Mol. Integr. Physiol.* **119**, 9–16.

Perry, S. F., Reid, S. G., Wankiewicz, E., Iyer, V. and Gilmour, K. M. (1996). Physiological responses of rainbow trout (Oncorhynchus mykiss) to prolonged exposure to soft water. *Physiol. Zool.* **69**, 1419–1441.

Perry, S. F., Fletcher, C., Bailey, S., Ting, J., Bradshaw, J., Tzaneva, V. and Gilmour, K. M. (2012). The interactive effects of exercise and gill remodeling in goldfish (Carassius auratus). *J. Comp. Physiol. B Biochem. Syst. Environ. Physiol.* **182**, 935–945.

Pfaffl, M. W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* **29**, 0.

Ramsay, J. M., Feist, G. W., Varga, Z. M., Westerfield, M., Kent, M. L. and Schreck, C. B. (2009). Whole-body cortisol response of zebrafish to acute net handling stress. *Aquaculture* **297**, 157–162.

Regan, M. D., Gill, I. S. and Richards, J. G. (2017). Metabolic depression and the evolution of hypoxia tolerance in threespine stickleback, Gasterosteus aculeatus. *Biol. Lett.* **13**,.

Rogers, N. J., Urbina, M. A., Reardon, E. E., McKenzie, D. J. and Wilson, R. W. (2016). A new analysis of hypoxia tolerance in fishes using a database of critical oxygen level (Pcrit). *Conserv. Physiol.* **4**,.

Rombough, P. J. (1988). Respiratory gas exchange, aerobic metabolism, and effects of hypoxia during early life. *Fish Physiol.* **11**, 59–161.

Rombough, P. J. (1999). The gill of fish larvae. Is it primarily a respiratory or an ionoregulatory structure? *J. Fish Biol.* **55**, 186–204.

Rombough, P. (2002). Gills are needed for ionoregulation before they are needed for O2 uptake in developing zebrafish, Danio rerio. *J. Exp. Biol.* **205**, 1787–1794.

Rombough, P. (2007). The functional ontogeny of the teleost gill: Which comes first, gas or ion exchange? *Comp. Biochem. Physiol. - A Mol. Integr. Physiol.* **148**, 732–742.

Rombough, P. and Drader, H. (2009). Hemoglobin enhances oxygen uptake in larval zebrafish (Danio rerio) but only under conditions of extreme hypoxia. *J. Exp. Biol.* **212**, 778–784.

Ros, A. F. H., Vullioud, P., Bruintjes, R., Vallat, A. and Bshary, R. (2014). Intra- and interspecific challenges modulate cortisol but not androgen levels in a year-round territorial damselfish. *J. Exp. Biol.* **217**, 1768–1774.

Schreck, C. B. and Tort, L. (2016). *The Concept of Stress in Fish*. First Edit. Elsevier Inc.

Shadrin, A. M. and Ozerniuk, N. D. (2002). Development of the gill system in early ontogenesis of Danio and nine spike stickleback. *Ontogenez* **33**, 118–123.

Shir-Mohammadi, K. and Perry, S. F. (2020). Expression of ion transport genes in ionocytes isolated from larval zebrafish (*Danio rerio*) exposed to acidic or Na^+ -deficient water. *Am. J. Physiol. Integr. Comp. Physiol.* **319**, R412–R427.

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.

Sollid, J., De Angelis, P., Gundersen, K. and Nilsson, G. E. (2003). Hypoxia induces adaptive and reversible gross morphological changes in crucian carp gills. *J. Exp. Biol.* **206**, 3667–3673.

Sopinka, N. M., Capelle, P. M., Semeniuk, C. A. D. and Love, O. P. (2017). Glucocorticoids in fish eggs: Variation, interactions with the environment, and the potential to shape offspring fitness. *Physiol. Biochem. Zool.* **90**, 15–33.

Stearns, S. C. (1989a). Trade-Offs in Life-History Evolution. *Funct. Ecol.* **3**, 259.

Stearns, S. C. (1989b). *The Evolutionary Significance of Phenotypic Plasticity*.

Takei, Y. (2012). Hormonal Control of Fish Euryhalinity. *Fish Physiol.* **32**, 69–123.

Takei, Y. and Hwang, P. P. (2016). *Homeostatic Responses to Osmotic Stress*. Elsevier Inc.

Uchida, D., Yamashita, M., Kitano, T. and Iguchi, T. (2002). Oocyte apoptosis during the transition from ovary-like tissue to testes during sex differentiation of juvenile zebrafish. *J. Exp. Biol.* **205**, 711–718.

Ultsch, G. R. and Regan, M. D. (2019). The utility and determination of Pcrit in fishes. *J. Exp. Biol.* **222**,.

Urbina, M. A., Meredith, A. S., Glover, C. N. and Forster, M. E. (2014). The importance of cutaneous gas exchange during aerial and aquatic respiration in galaxiids. *J. Fish Biol.* **84**, 759–773.

Vindas, M. A., Madaro, A., Fraser, T. W. K., Höglund, E., Olsen, R. E., Øverli, Ø. and Kristiansen, T. S. (2016). Coping with a changing environment: the effects of early life stress. *R. Soc. Open Sci.* **3**, 160382.

Vulesevic, B., McNeill, B. and Perry, S. F. (2006). Chemoreceptor plasticity and respiratory acclimation in the zebrafish Danio rerio. *J. Exp. Biol.* **209**, 1261–1273.

Wendelaar Bonga, E. (1997). The Stress Response in Fish. *Physiol. Rev.* **77**, 591–625.

Wernicke von Siebenthal, E., Rehberger, K., Bailey, C., Ros, A., Herzog, E. and Segner, H. (2018). Trade-Offs Underwater: Physiological Plasticity of Rainbow Trout (Oncorhynchus mykiss) Confronted by Multiple Stressors. *Fishes* **3**, 49.

Williams, T. A., Bonham, L. A. and Bernier, N. J. (2017). High environmental ammonia

exposure has developmental-stage specific and long-term consequences on the cortisol stress response in zebrafish. *Gen. Comp. Endocrinol.* **254**, 97–106.

Wilson, J. M. and Laurent, P. (2002). Fish gill morphology: Inside out. In *Journal of Experimental Zoology*, pp. 192–213. J Exp Zool.

Wilson, K. S., Matrone, G., Livingstone, D. E. W., Al-Dujaili, E. A. S., Mullins, J. J., Tucker, C. S., Hadoke, P. W. F., Kenyon, C. J. and Denvir, M. A. (2013). Physiological roles of glucocorticoids during early embryonic development of the zebrafish (Danio rerio). *J. Physiol.* **591**, 6209–6220.

Wilson, K. S., Tucker, C. S., Al-Dujaili, E. A. S., Holmes, M. C., Hadoke, P. W. F., Kenyon, C. J. and Denvir, M. A. (2016). Early-life glucocorticoids programme behaviour and metabolism in adulthood in zebrafish. *J. Endocrinol.* **230**, 125–42.

Wood, C. M. and Eom, J. (2021). The osmorespiratory compromise in the fish gill. *Comp. Biochem. Physiol. Part A* **254**, 110895.

Zimmer, A. M., Wright, P. A. and Wood, C. M. (2014). What is the primary function of the early teleost gill? Evidence for Na+/NH+4exchange in developing rainbow trout (Oncorhynchus mykiss). *Proc. R. Soc. B Biol. Sci.* **281**,.

Zimmer, A. M., Dymowska, A. K., Kumai, Y., Goss, G. G., Perry, S. F. and Kwong, R. W. M. (2018). Assessing the role of the acid-sensing ion channel ASIC4b in sodium uptake by larval zebrafish. *Comp. Biochem. Physiol. -Part A Mol. Integr. Physiol.* **226**, 1–10.