Developmental plasticity in zebrafish (*Danio rerio*): effects of early life exposure to a stressor

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Abstract

Experience of stress and/or cortisol, the end-product of activation of the hypothalamic-pituitary-interrenal (HPI) axis, may serve as a cue to trigger developmental plasticity. In fish, most research in this area has focused on effects of maternal stress or maternal cortisol levels on development, particularly with respect to the HPI axis and stress responses, and little attention has been paid to the effects of an endogenous stress response during early life. In the current study, zebrafish (*Danio rerio*) at four developmental stages (4, 7, 15 or 35 days post fertilization, dpf) were subjected to an air exposure stressor twice a day for two days. Individuals stressed early in life exhibited decreased survival and growth, increased whole-body Na^+ and Ca^{2+} concentrations, and altered HPI axis activity associated with changes in anxiety-related behaviour at 7 to 35 dpf, with most effects diminishing with increasing age. Stress at 7 dpf was particularly effective at eliciting phenotypic changes, suggesting this age represents a critical window for cortisol to influence development. Finally, stress at 35 dpf induced masculinization, suggesting that cortisol influences sexual differentiation in zebrafish. These findings demonstrate that early-life stress in zebrafish triggers developmental plasticity, with effects on physiology and behaviour mediated by the HPI axis in an age-dependent manner.

Résumé

L'exposition au stress et/ou au cortisol, le produit final de l'activation de l'axe hypothalamique-pituitaire-interrénal (HPI), peuvent agir comme signaux déclencheurs pour la plasticité développementale. Chez les poissons, la majorité de la recherche dans ce domaine s'est concentrée sur les effets du stress maternel et du taux de cortisol maternel sur le développement, en particulier sur l'action de l'axe HPI et des réponses au stress, mais peu d'attention a été donnée aux effets de la réponse endogène au stress pendant les stades de développement précoces. Dans cette étude, des poissons-zèbres (*Danio rerio*) à quatre stades de développement (4, 7 15 et 35 jours post fertilisation ou dpf) ont été stressés deux fois par jour pendant deux jours en étant exposés à l'air. Les individus stressés pendant leur vie précoce ont montré une survie et une croissance réduites, une augmentation de leur concentrations totales de Na^+ et Ca^{2+} , ainsi qu'une modulation d'activité de l'axe HPI associée à des changements de comportement lié à l'anxiété entre 7 et 35 dpf. La plupart de ces réponses se sont affaiblies avec l'âge. Le stress à 7 dpf était particulièrement susceptible de provoquer des changements phénotypiques, ce qui suggére que cet âge représente la fenêtre de temps critique pendant laquelle le cortisol influence le développement. Finalement, le stress à 35 dpf a induit une masculinisation, ce qui suggère que le cortisol influence la différenciation sexuelle chez le poisson-zèbre. Ces résultats démontrent que le stress précoce déclenche une plasticité développementale chez le poisson zèbre, avec des effets sur la physiologie et le comportement qui sont modulés par l'axe HPI et qui dépendent de l'âge.

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List of Abbreviations

Abbreviation	**Full Name**
11βHSD2	11-β-hydroxysteroid dehydrogenase type 2
ACTH	Adrenocorticotropic hormone
ANOVA	Analysis of variance
CRF	Corticotropin releasing factor
df	Degrees of freedom
dpf	Days post fertilization
EIA	Enzyme linked immunosorbent assay
GR	Glucocorticoid receptor
GSI	Gonadosomatic index
HIF	Hypoxia inducible factor
hpf	Hours post fertilization
HPI	Hypothalamic-pituitary-interrenal
HR	H^+-ATPase-rich
IGF	Insulin-like growth factor
K	Fulton's condition factor
LED	Light emitting diode
log	Logarithmic
MC2R	Melanocortin 2 receptor
MCO_2	Rate of CO_2 production
MO_2	Rate of O_2 consumption

MR	Mineralocorticoid receptor
mRNA	Messenger ribonucleic acid
N	Sample size
NCC	Na^+/Cl^- co-transporter
NHE	Na^+/H^+ exchanger
NKA	Na^+, K^+-ATPase
P450scc	P450 side chain cleavage enzyme
RER	Respiratory exchange ratio
SEM	Standard error of the mean
StAR	Steroidogenic acute regulatory protein

Chapter 1: Introduction

1.1 Overview

It has been well documented across many taxa that environmental conditions can have effects ranging from mild to substantial on the phenotype of individuals. This interplay between the environment and phenotype is known as phenotypic plasticity, and allows organisms to respond adaptively to a constantly changing world (reviewed by Smith-Gill, 1983; West-Eberhard, 1989). Long-term changes to phenotype are most pronounced when triggered during early developmental stages (Hayward and Wingfield, 2004; Oomen et al., 2010; Vindas et al., 2016; Wagner et al., 2011), where a range of pathways can influence the development of both physiology and behaviour. For example, the glucocorticoid hormone cortisol plays a key role in orchestrating early development in fish (Nesan and Vijayan, 2013; Nesan and Vijayan, 2016) but is also the end-product of the hypothalamic-pituitary-interrenal (HPI) axis, which is activated in response to stress (Wendelaar Bonga, 1997). Increased egg cortisol concentrations, reflecting maternal stress, influence both the physiology and behaviour of the offspring that develop from these eggs, yet the long-term adaptive significance of these changes is still poorly understood (reviewed by Sopinka et al., 2017). Similarly, exposure to a stressor early in life with concomitant elevation of endogenous glucocorticoids also might be expected to influence subsequent development, but this possibility has received relatively little research attention to date.

The objective of the present study was to determine the impacts of early-life stress on growth, metabolism and ion regulation as well as HPI axis function and anxiety-related behaviour in developing zebrafish (*Danio rerio*). To this end, the following sections review the concept of developmental plasticity, the stress response, and the effects of cortisol, identifying significant knowledge gaps in our understanding of how early-life stress influences the development of distinct phenotypes. Along with this overview, the rationale for the developmental points that were selected for examination is discussed. The aim of the current study was to provide a broad-ranging examination of how activation of an endogenously regulated stress response in early life can influence the physiology and behaviour of a developing zebrafish. The overarching hypothesis of the present study was that early life stress would cause changes in the physiology and behaviour of developing zebrafish by modifying HPI axis activity in an age-dependent manner.

1.2 Developmental plasticity

The ability of a single genotype to produce multiple morphological, physiological, and/or behavioural phenotypes in response to variation in the environment is known as phenotypic plasticity (West-Eberhard, 1989). Acting through a variety of biological pathways, and across a wide temporal range, phenotypic plasticity allows organisms to respond adaptively to variable environmental conditions, providing a significant advantage over a rigid, fixed phenotype (Groothuis and Taborsky, 2015; West-Eberhard, 1989). Developmental plasticity, in turn, is considered to be a form of phenotypic plasticity that occurs during maturation from juvenile life stages to adulthood (Marks et al., 2005; Nettle and Bateson, 2015). This phenomenon has been well documented in fish, where environmental stress has been shown to induce development of distinct polymorphisms (Donelson et al., 2011; Hall and Johnston, 2003). In one such study, hypoxic conditions during early development increased circulatory system vascularization (Pelster, 2002), while another study found increased thermal tolerance in zebrafish raised in a highly variable thermal environment (Schaefer and Ryan, 2006). Although polymorphisms related to developmental plasticity typically are considered to be non-reversible (Love and

Williams, 2008a; West-Eberhard, 1989), such as environmental sex determination in many reptile and fish species (Ferguson and Joanen, 1982; Fernandino et al., 2013), changes may be reversed later in life owing to the effects of phenotypic plasticity (Gabriel et al., 2005). Examples of this phenomenon include the oxygen carrying-capacity of mammalian blood cells in response to altitude (Tufts et al., 2013), the social behaviour and brain morphology seen in cooperatively breeding cichlids (Fischer et al., 2015), or the reversal of reproductive phenotype seen in Japanese flounders in response to elevated cortisol (Yamaguchi et al., 2010). Although later plasticity in phenotypes that arise through developmental plasticity may be costly, requiring large scale physiological changes (Groothuis and Taborsky, 2015), they are prevalent in a wide range of taxa and presumably function to increase fitness in response to a dynamic environment (Dufty et al., 2002).

Developmental plasticity may also be considered as a specific case of a carry-over effect (Bennett and Murray, 2012). Carry-over effects occur when the previous experience of an organism affects its current performance (O'Connor et al., 2014), and are most frequently used to describe seasonal differences in adult individuals. It has been argued that changes that occur between developmental periods should not be classified as carry-over effects, but rather be considered simply as developmental changes (Harrison et al., 2011). This is largely due to the difference in scale of phenotypic changes (Groothuis and Taborsky, 2015). In zebrafish, for example - while sexually mature individuals may go through relatively large-scale physiological changes, such as altered neuronal development (von Krogh et al., 2010), there are many more drastic changes that occur prior to reaching sexual maturity (Eaton and Farley, 1974; Hisaoka and Battle, 1958; Rombough, 2002). Although the more inclusive definition of carry-over effects does provide a common term for diverse fields of biology to use, the argument can be made that developmental plasticity is adequately described by its current definition (Marks et al., 2005; Nettle and Bateson, 2015), and that the term carry-over effect is better restricted to use in adult organisms (Harrison et al., 2011). For the purposes of the present study, the latter approach will be used.

Often, the phenotypic changes that constitute developmental plasticity are dependent upon exposure to an environmental factor during a critical developmental window (Robertson et al., 2014; Vindas et al., 2016). A critical developmental window is a limited period of time during early development, where environmental cues can lead to changes in gene expression (Alsop and Vijayan, 2009; Robertson et al., 2014). These in turn give rise to large scale changes in physiological processes. For example, zebrafish exposed to hypoxia at 24 to 36 hours post fertilization (hpf) exhibited increased hypoxia tolerance and male biased sex ratios, but earlier or later exposure to hypoxia did not elicit these effects (Robertson et al., 2014). The impacts of these critical developmental windows also can influence behavioural traits, as has been demonstrated in zebrafish, where larval experience with conspecifics determined shoaling preferences, and was non-reversible once reaching the juvenile life stage (Engeszer et al., 2007).

Exposure to an environmental cue during early development may be separated from the phenotypic consequences of this exposure by a relatively large scale of time (Marks et al., 2005). For example, exposure of zebrafish embryos to hypoxia for several hours prior to hatching had significant impacts on the sex of adult fish at sexual maturity (Robertson et al., 2014). This situation can result in a mismatch between the environment in which the phenotype would have been adaptive originally and the environment in which the organism eventually finds itself (Chevin and Hoffmann, 2017). The effects of a mismatch between phenotype and environment were demonstrated in wild populations of the great tit (*Parus major*), where heritable plasticity in laying dates caused by climate change have caused a mismatch between hatching dates and peak food availability (Nussey et al., 2005). Experimentally stressed female sockeye salmon provide a second example; the offspring of these stressed females displayed higher levels of burst swimming initiation in response to a chase stressor and decreased swimming endurance, indicating distinct metabolic costs, traits which would perhaps become maladaptive in an environment with low predation risk and high conspecific competition (Sopinka et al., 2014).

The mechanisms underlying developmental plasticity, or the mechanisms through which environmental cues are translated into polymorphisms, often remain poorly understood. With respect to environmental stressors, glucocorticoid hormones are of interest in this regard. Glucocorticoids play a key role in growth and survival during embryonic development in vertebrates (Eriksen et al., 2006; Gagliano and McCormick, 2009), and are essential for normal development (Moisiadis and Matthews, 2014; Nesan and Vijayan, 2013). Experimental elevation of embryonic cortisol levels in zebrafish caused increased incidence of cardiac malformation, decreased resting heart rate (Nesan and Vijayan, 2012), increased primary neurogenesis in both the pallium and preoptic region (Best et al., 2017), and increased HPI axis gene expression related to a diminished cortisol response (Wilson et al., 2013). Conversely, lowering of embryonic cortisol levels increased the cortisol response to a stressor (Nesan and Vijayan, 2016; Wilson et al., 2013) and caused mesoderm malformation (Nesan and Vijayan, 2016), demonstrating the essential role of cortisol in development. In response to a stressor, vertebrate species undergo a highly conserved physiological stress response (Barton, 2002), and a significant component of this stress response is the production of glucocorticoid stress hormones, such as cortisol in fish (Wendelaar Bonga, 1997). Given the essential role of cortisol in development, alteration of cortisol levels owing to maternal stress or early life experience of a stressor may be associated with developmental polymorphism.

1.2.1 The influence of maternal stress on offspring development

In fish, developing embryos are incapable of producing endogenous cortisol prior to hatching (Nesan and Vijayan, 2016). Hence, the cortisol required for normal development during this period is maternally derived, and is transferred to the ova during the period of yolk deposition, known as vitellogenesis (Alsop and Vijayan, 2008; McCormick and Nechaev, 2002). The extent to which maternal glucocorticoid deposition depends on maternal cortisol levels is unclear at present. Although heightened maternal glucocorticoid levels translated to increased embryonic glucocorticoids in some cases (Giesing et al., 2011; McCormick et al., 1998; Stratholt et al., 1997), there are as many studies that found no relationship between maternal stress and embryonic cortisol levels (Jeffrey and Gilmour, 2016; Redfern et al., 2017; Sopinka et al., 2014). This variability suggests that maternal transfer of cortisol is not a passive process but may be regulated through maternal and/or embryonic mechanisms (reviewed by Sopinka et al., 2017). Lending support to this hypothesis, cortisol deposition in the ovarian follicles has been found to be buffered by metabolism of cortisol to its inactive form cortisone, mitigating the impacts of elevated maternal cortisol levels on embryonic development (Faught et al., 2016; Li et al., 2014). However, even in situations where maternal stress or cortisol treatment failed to affect embryonic cortisol levels, presumably owing to this buffering capacity, cortisol treatment simulating maternal stress in largemouth bass (*Micropterus salmoides*) increased the mass of hatching larvae, diminished their cortisol response to a stressor, and increased thigmotaxis in developing individuals (Redfern et al., 2017). These findings were mirrored in zebrafish, where maternal stress influenced mRNA levels of key HPI axis genes after hatching, while also reducing the cortisol response to an acute stressor (Jeffrey and Gilmour, 2016). Similarly, maternal stress in sockeye salmon (*Oncorhynchus nerka*) resulted in elevated transcript abundance of glucocorticoid receptors in the pre-optic area of the brain, and of proteins involved in cortisol synthesis (steroidogenic acute regulatory protein, StAR, cytochrome P450 side chain cleavage enzyme, P450scc) in offspring, as well as a diminished cortisol response to a stressor (Sopinka et al., 2016). Although it has been argued that this modification of stress responsiveness could be adaptive, in that it may prime offspring for an environment similar to that experienced by their parents (Groothuis and Taborsky, 2015), there is not a clear consensus as to the adaptive significance of variation in maternal glucocorticoid transfer at present (Sopinka et al., 2017).

Experimental manipulation of egg or embryo cortisol concentrations has been used as a proxy for maternal stress or to examine the effects of cortisol itself on early developmental stages (Burton et al., 2011; Nesan and Vijayan, 2016; Wilson et al., 2013). Such studies have focused on a wide variety of developmental endpoints (Auperin and Geslin, 2008; Mccormick and Nechaev, 2002). Experimentally-increased egg or embryo glucocorticoid concentrations simulating the effects of maternal stress dampened the glucocorticoid response to a stressor in a range of taxa, including birds (Hayward and Wingfield, 2004; Love and Williams, 2008a) and fish (Best et al., 2017; Gagliano and McCormick, 2009). In addition, increased cortisol resulted in both increased egg mortality, and increased larval survival to a later age in the embryos that hatched, suggesting that maternal cortisol deposition has the ability to influence offspring competitive ability (Gagliano and McCormick, 2009).

Increased cortisol during early development also significantly influenced growth rates, with studies finding decreased growth, more irregular patterns of maturation (Burton et al., 2011; Mccormick and Nechaev, 2002), and increased metabolic rates (Sloman, 2010). Changes to the HPI axis and associated behaviours have been demonstrated, including diminished HPI axis activity (Wilson et al., 2013), and behaviour more typical of a proactive coping style, such as increased aggression towards conspecifics (Bell and Sih, 2007; Sloman, 2010), decreased thigmotaxis, and increased activity (Best et al., 2017). Conversely, experimental reduction in maternally deposited cortisol through a cortisol antibody injection caused heightened stress responsiveness in juvenile zebrafish (Nesan and Vijayan, 2016). Given these findings, it is evident that cortisol plays a key role during early development, but the connection between these effects and early-life stress is still poorly understood.

1.2.2 The influence of cortisol during early-life development

Relatively few studies have examined the impacts of an endogenous stress response during early life, yet those that have done so have found distinct effects on development. Exposure of zebrafish to hypoxia prior to hatching significantly increased aggression and dominance in social interactions in these fish as adults, relating to increased circulating testosterone levels during adulthood (Ivy et al., 2017). Early-life hypoxia also increased hypoxia tolerance and induced masculinization in adult zebrafish (Robertson et al., 2014). In both of these studies, effects of hypoxia exposure were attributed primarily to the effects of hypoxia-inducible-factor (HIF) (Ivy et al., 2017; Robertson et al., 2014), providing evidence that early-life non-maternal factors can shape development of phenotype in zebrafish.

In the relatively few studies that have examined the impacts of early-life stress following hatching, a variety of long-term impacts on phenotype have been demonstrated in juvenile fish. In European sea bass (*Dicentrarchus labrax*) exposed to an unpredictable, chronic, low-intensity stressor at the stages of flexion and fin formation, increased baseline plasma cortisol concentrations and diminished impacts of an acute stressor on neural cell proliferation were seen in juvenile individuals measured 5 months following the stress protocol (Fokos et al., 2017). Similarly, rainbow trout (*Oncorhynchus mykiss*) exposed to an acute stressor after hatch, during yolk absorption, exhibited an attenuated cortisol response to stress when measured as 5 month old fingerlings (Auperin and Geslin, 2008). Also, Atlantic salmon parr (*Salmo salar*) exposed to an unpredictable chronic stressor protocol over several months exhibited decreased hypothalamic catecholaminergic and serotonergic responses to acute stress, along with increased growth rates (Vindas et al., 2016). Building on what has been demonstrated in juvenile fish, exposure to chronic stress influenced the development of sex in zebrafish, with both increased conspecific density and dietary cortisol during juvenile development inducing masculinization in adult fish (Ribas et al., 2017).

1.2.3 Cortisol and sexual differentiation

There is mounting evidence in the literature that cortisol may play a significant role in sexual differentiation in zebrafish, as mediated through a variety of environmental stressors. For example, crowding density during early development and the period of sexual differentiation produced male-biased sex ratios in zebrafish, while dietary cortisol administration resulted in complete masculinization of experimental individuals (Ribas et al., 2017). Through domestication, most laboratory strains of zebrafish have lost the chromosomal sex determination system associated with the telomeric region of chromosome 4 that is typical of wild strains of the species (Wilson et al., 2014). In lieu of this ZW/ZZ system (Wilson et al., 2014), most domestic strains exhibit polygenic sex determination based on interactions between the environment and genotype (Liew et al., 2012). Recent work linked this polygenic system to loci on chromosomes 3, 4, 5, and 16 (Anderson et al., 2012; Bradley et al., 2011; Howe et al., 2013), and pointed towards the HPI axis and cortisol as playing a critical role (Ribas et al., 2017). However, the mechanism responsible for stress-induced masculinization in zebrafish remains unclear. One possibility is that increased expression of *hsd11b2*, the enzyme that converts cortisol into the inactive form cortisone, in response to chronically increased cortisol converts 11ß-hydroxyandrogens into 11-ketotestosterone, an androgen with stronger masculinizing effects than testosterone (Fernandino et al., 2013). This possibility was examined in pejerry (*Odontesthes bonariensis*), where increased environmental temperature from the time post hatch until adulthood resulted in male biased sex ratios, related to increased concentrations of *hsd11b2* via a negative feedback mechanism involving heightened cortisol concentrations (Fernandino et al., 2012). Further studies with both medaka (*Oryzias latipes*) (Hayashi et al., 2010) and Japanese flounder (*Paralichthys olivaceus*) (Yamaguchi et al., 2010) reported that increased cortisol induced through high environmental temperature caused masculinization of genetically female individuals. This effect was suggested to be mediated by suppression of female germ cell proliferation and expression of follicle-stimulating hormone receptor (*fshr*) mRNA in medaka (Hayashi et al., 2010), and through suppression of ovary-type aromatase (*cyp19a1*) mRNA expression in the Japanese flounder (Yamaguchi et al., 2010), demonstrating the key role that cortisol plays in influencing signalling in gonadal endocrine pathways.

Despite the evidence suggesting a role for the HPI axis and cortisol as contributing factors behind sexual differentiation in domestic zebrafish and at least some other fish species (Hayashi et al., 2010; Ribas et al., 2017; Yamaguchi et al., 2010), there is still no consensus on the magnitude of its effect or the mechanism through which cortisol influences sex determination. It appears possible that critical developmental windows may play a role in cortisol's masculinizing actions, as demonstrated by the masculinizing effects of dietary cortisol administered to zebrafish during the period of sexual differentiation (Ribas et al., 2017), yet this relationship is still poorly understood, because treatments were applied over broad periods of time (Hayashi et al., 2010; Ribas et al., 2017; Yamaguchi et al., 2010). For the relationship between cortisol and sexual differentiation to be better understood, further research into the interplay between critical developmental windows for sexual differentiation and stress is required.

In summary, most studies that have examined developmental plasticity in regards to the stress axis and critical developmental windows have focused on the effects of maternal stress (reviewed by Sopinka et al., 2017) or hypoxic stress experienced during embryonic development (Ivy et al., 2017; Nesan and Vijayan, 2012; Robertson et al., 2014), with less regard paid to later effects or development over a longer period of time (Love and Williams, 2008b). Because the HPI axis is active throughout life, and plays critical roles in a wide variety of physiological functions (Mommsen et al., 1999; Wendelaar Bonga, 1997), it is surprising that more research has not focused on the relationship between stress and phenotypic plasticity.

1.3 The physiological roles of cortisol in teleost fish

Cortisol is the end-product of activation of the HPI axis (Mommsen et al., 1999; Wendelaar Bonga, 1997). In response to stress, neurons originating in the preoptic area of the hypothalamus release corticotropin releasing factor (CRF) onto the corticotropes of the pituitary, which in turn triggers secretion of adrenocorticotropic hormone (ACTH). Adrenocorticotropic hormone travels through the blood stream to the steroid-producing interrenal cells of the head kidney, where it binds to melanocortin 2 receptors (MC2R), thereby stimulating the synthesis of cortisol from cholesterol (Alsop and Vijayan, 2009; Sopinka et al., 2016; Wendelaar Bonga, 1997). Activation of the HPI axis is arguably most studied with respect to responses to a stressor, where cortisol is involved in metabolic adjustments such as enhancement of hepatic gluconeogenesis (Mommsen et al., 1999). However, cortisol has mineralocorticoid functions in the regulation of osmotic and ionic balance (McCormick, 2001; Mommsen et al., 1999), and despite the presence of mineralocorticoid receptors (MR) in fish, this mineralocorticoid function appears to be largely controlled through cortisol's interaction with glucocorticoid receptors (GR) (McCormick et al., 2008; Prunet et al., 2006). Even with the large volume of research on the dual functions of cortisol, the relationship between the metabolic/stress response and ionic/osmotic regulatory functions remains uncertain and under-explored (Mommsen et al., 1999; Takei and McCormick, 2012; Takahashi, 1977).

1.3.1 Glucocorticoid functions of cortisol

Stress may be defined as a perturbation of homeostasis caused by intrinsic or extrinsic factors acting on an organism and its environment (Wendelaar Bonga, 1997). All organisms experience stress, and respond in a fashion that attempts to minimize costs while maximizing fitness. In teleost fish, the primary response to a perceived stressor can be divided into the two axes of the stress response (Barton, 2002). The first is the release of the catecholamine hormones, epinephrine and norepinephrine, from chromaffin cells in the head kidney which are innervated by the sympathetic nervous system (Reid et al., 1998). This response was first described as the “fight/flight” response (Cannon, 1929), occurring only in response to acute severe stress, and results in a significant increase in circulating catecholamine concentrations within seconds, allowing an individual to adjust oxygen delivery and access fuel reserves (Perry and Bernier, 1999).

The second axis triggered in response to a stressor is the HPI axis, activation of which culminates in elevation of circulating levels of the glucocorticoid hormone cortisol and occurs over a longer time-period of minutes (Wendelaar Bonga 1997, Mommsen et al. 1999). The primary role of cortisol as a glucocorticoid is to mobilize energy reserves, allowing an organism to respond to a stressor and restore homeostasis (Barton and Iwama, 1991; Wendelaar Bonga, 1997). These metabolic actions of cortisol are achieved through GR, with the GR-cortisol complex serving to modify the transcription of key genes that influence metabolism (Mommsen et al., 1999). Modulation of hepatic gluconeogenesis is a key metabolic action of cortisol in response to stress (Mommsen et al., 1999; Vijayan et al., 1994), and reflects transcriptional regulation of enzymes such as glucose 6-phosphatase, fructose 1,6-bisphosphatase and phosphoenolpyruvate carboxykinase (Vijayan et al., 1991; Vijayan et al., 2003). However, cortisol also modulates protein and amino acid metabolism (Mommsen et al., 1999). The effects of cortisol on metabolism have been well documented in salmonid fish, examining both the proximate and long term impacts. For example, increased circulating cortisol levels owing to chronic social stress increased standard metabolic rate (Sloman et al., 2000), while experimental elevation of cortisol levels using cortisol implants caused increased plasma glucose concentrations, CO_2 production, and respiratory quotient (De Boeck et al., 2001).

The long term impact of the metabolic changes associated with increased cortisol appear to primarily be reduced growth rates, as demonstrated in brown trout (*Salmo trutta*) (Burton et al., 2011). However, decreased growth rates in response to cortisol may also occur through both reduced intake of nutrients and decreased capacity to absorb nutrients from the food that is consumed, resulting in less energy being made available for growth (Bernier, 2006; Bernier and Peter, 2001a; Peters, 1982). In addition, cortisol elevation in response to a 24-hour confinement stressor decreased circulating levels of growth hormone, which could contribute to decreased growth in stressed individuals (Pickering et al., 1991). Although these processes are beneficial in the short-term in allowing individuals to respond more efficiently to an acute stressor, over prolonged periods of time, these effects of elevated cortisol concentrations prove detrimental to growth (Mommsen et al., 1999).

The cortisol response to a standardized stressor may vary among individuals, as do behavioural responses to a stressor, resulting in the description of distinct stress-coping styles (Koolhaas et al., 1999; Sørensen et al., 2013) that fall within a reactive-proactive continuum (Wilson et al., 1994; Wong et al., 2012). Reactive individuals generally exhibit greater anxiety-related behaviour such as immobility in response to a threat, a stronger thigmotaxic (i.e. wall-hugging) response to a novel environment, and a tendency to become subordinate in social interactions, whereas proactive individuals tend to be more active when faced with a threat, demonstrate lower levels of thigmotaxis in a novel environment, and tend to be more dominant in social interactions (Champagne et al., 2010; Dahlbom et al., 2011; Tudorache et al., 2015; Wilson et al., 1994). These behavioural coping styles are closely related to distinct HPI axis responses, with proactive individuals exhibiting a lower cortisol response to a stressor than reactive individuals (Koolhaas et al., 1999; Øverli et al., 2007; Tudorache et al., 2013).

Behavioural coping styles have traditionally been viewed as rigid and consistent throughout an organism's lifespan, but recent research suggested that they are in fact flexible and can respond to environmental changes and stress (Dingemanse et al., 2010; Stamps and Groothuis, 2010). For example, adult zebrafish exposed to an unpredictable chronic stressor developed greater anxiety-related behaviour compared to control animals (Piato et al., 2011). These behaviours included tighter shoal cohesion, increased immobility, and lower position in the water column (similar to a thigmotaxic response), and occurred in conjunction with increased basal whole-body cortisol levels (Piato et al., 2011). These findings demonstrate plasticity in behavioural/physiological responses to the environment. Changes in coping style elicited by the experience of stress and the consequent elevation of cortisol levels in fish require large-scale physiological changes to the associated neural network and/or biochemical signalling pathways (Sørensen et al., 2013). This prediction was met in zebrafish, where social stress caused increases in mRNA expression of key HPI axis genes (Pavlidis et al., 2011), as well as in the cichlid species *Neolamprologus pulcher*, where social interaction and subsequent isolation affected the size of several brain structures, including the optic tectum and hypothalamus (Fischer et al., 2015). These findings all point towards the widespread glucocorticoid actions of cortisol and the associated HPI axis on influencing the phenotype in response to stress.

1.3.2 Mineralocorticoid functions of cortisol

Although the roles of cortisol as both a glucocorticoid and a mineralocorticoid hormone are well studied independently, there is a lack of research into how the stress axis functions of this hormone cross over into the control of salt and water balance (McCormick, 2001). In the majority of terrestrial tetrapod species, aldosterone is the primary mineralocorticoid hormone that functions to control water and ion balance (McCormick et al., 2008). However in fish, concentrations of aldosterone are relatively low, and cortisol plays a significant mineralocorticoid role (Prunet et al., 2006; Takahashi and Sakamoto, 2013; Wendelaar Bonga, 1997). Although MR is present, there is little evidence for its involvement in the mineralocorticoid actions of cortisol, and indeed cortisol's mineralocorticoid actions appear primarily to be accomplished through binding to GR (Ivanis et al., 2008; Kelly and Chasiotis, 2011; Lin and Randall, 1993; Takahashi and Sakamoto, 2013).

In freshwater fish, maintaining ionic and osmotic balance is a constant challenge owing to high diffusion gradients between the internal and external environments that favour water gain and ion loss (McCormick, 2001). Cortisol appears to play a role in salt and water balance by regulating the uptake of ions such as Na^+ and Ca^{2+} from the environment (Hwang and Chou, 2013; McCormick, 2001). These effects have been well examined in salmonid fish, particularly during later developmental stages owing to the relative ease with which tissues of interest can be isolated for measurement, and the interest in the osmotic challenges faced by these often anadromous species (McCormick, 2001). In these studies, increased cortisol concentrations increased activity of Na^+, K^+-ATPase (NKA), a key driver of ion transport in the gill, gut and kidney, as well as increased mRNA levels of NKA isoforms α1a and α1b (Kiilerich et al., 2007; McCormick et al., 2008), leading to increased ion uptake from the environment.

In recent years, larval fish have become a much-used model to examine ion transport mechanisms, with many of these studies focusing on larval zebrafish in particular (Hwang and Chou, 2013; Hwang et al., 2011). In response to waterborne cortisol treatment, larval zebrafish significantly increased their uptake of environmental Na^+ by upregulation of Na^+/H^+ exchanger (NHE) isoform 3b (Kumai et al., 2012). Similarly, cortisol treatment and GR activation was linked to increased transcription of a Na^+/Cl^- co-transporter (NCC), which functions in concert with NHE to manage Na^+ balance (Lin et al., 2016a). Environmental Ca^{2+} uptake in larval zebrafish also increased following cortisol treatment owing to an increased number of calcium channels in a pathway mediated by GR (Lin et al., 2011). Along with changes in the expression of ion transport proteins, changes in the expression of ion-transporting cells or ionocytes in larval zebrafish appear to be mediated by cortisol signalling. For example, in response to an acidic environment, the number of H^+-ATPase-rich (HR) cells in the skin of zebrafish larvae was increased (Horng et al., 2009), primarily through differentiation of ionocyte progenitors mediated by cortisol-GR signalling. Although progress has been made in understanding the effects of cortisol on ion transport, little of this work was done in the context of development, neglecting the large scale physiological changes that occur as fish mature. Moreover, despite the clear impact that cortisol has on both salt/water balance and the HPI axis, there is a surprising lack of cross-talk between the two lines of research. Most studies simply regard either the glucocorticoid or mineralocorticoid functions of cortisol and fail to examine the possibility of interplay between these two functions.

1.4 Objectives and predictions

The objective of the present study was to determine the impacts in zebrafish of early-life stress on a wide variety of physiological and behavioural endpoints over several key developmental stages. These endpoints included survival, growth, metabolic rate, ion balance, the cortisol response to an acute stressor, anxiety-related behaviour, and sexual differentiation. The present study provided a broad overview of how physiological and behavioural variables are dynamically influenced by an early-life, endogenously-regulated stress response. The primary hypothesis of the present study was that early life stress induces changes in both the physiology and behaviour of developing zebrafish via modification of HPI axis responsiveness in an age-dependent manner. Zebrafish provide an excellent study species in which to examine the effects of exposure to early-life stress for a number of reasons, including the large volume of literature describing their physiology and development (Eaton and Farley, 1974; Hisaoka and Battle, 1958; Uchida et al., 2002), a fast generation time with high fecundity in a laboratory setting (Buske and Gerlai, 2011; Lin et al., 2009), and their relative ease to work with and care for as compared to rodent models (Maximino et al., 2010; Ramsay et al., 2006). Along with these practical advantages to the use of zebrafish, the development of their HPI axis and other key organ systems has been documented (Alderman and Bernier, 2009; Alsop and Vijayan, 2009), providing several key developmental stages to examine.

Based on the overarching hypothesis of the present thesis and the effects of cortisol discussed above, individuals exposed to early-life stress would be predicted to develop a more proactive stress coping style, typified by a lower cortisol response to an acute stressor (Best et al., 2017; Nesan and Vijayan, 2016) and a reduction in anxiety-related behaviour such as thigmotaxis (Best et al., 2017). Individuals that experienced early-life stress also would be predicted to exhibit increased metabolic rates (Sloman, 2010) and decreased growth rates (Eriksen et al., 2006). Based on the effects of cortisol treatment in zebrafish embryos and larvae, exposure to early-life stress would be predicted to increase whole-body concentrations of both Na^+ and Ca^{2+} (Kumai et al., 2012; Lin et al., 2011). Finally, exposure to early-life stress would be predicted to result in male-biased sex ratios in experimental populations of zebrafish (Ribas et al., 2017). In line with the idea of critical developmental windows (Robertson et al., 2014; Vindas et al., 2016), it is likely that the effects of early life stress depend on the developmental stage at which the stress is experienced. In the present study, developing zebrafish were subjected to an acute air exposure stressor at 4-5, 7-8, 15-16 and 35-36 days-post-fertilization (dpf). The air exposure stressor was selected based on the results of a pilot study examining the relative efficacy of a single one-minute air exposure versus a repeated three-minute air exposure with an intermediate three-minute recovery period that had previously been used to evoke a stress response in adult zebrafish (Ramsay et al., 2009).

Selection of important developmental stages at which to examine the effects of stress was facilitated for zebrafish by the large volume of research describing their development (Eaton and Farley, 1974; Hisaoka and Battle, 1958; Rombough, 2002; Uchida et al., 2002). Immediately after hatching, at ~3 dpf, the HPI axis is fully developed and *de novo* cortisol synthesis occurs, but the cortisol response to a stressor is not yet robust, with some but not all stressors eliciting a cortisol response (Alderman and Bernier, 2009; Alsop and Vijayan, 2009; Jeffrey and Gilmour, 2016; Wilson et al., 2013). Along with *de novo* production of cortisol, 3 dpf is the time at which zebrafish gill primordia start to function in ion regulation, with their importance in that function relative to that of the skin continuing to increase until the gills are more completely developed at ~14 dpf (Rombough, 2002). At approximately 6 dpf, all major organ systems are fully developed and larvae transition to exogenous feeding from feeding on yolk (Chu and Sadler, 2009). This point is particularly relevant from the perspective of stress responsiveness because it is at this point that the cortisol response to a stressor becomes robust (Alderman and Bernier, 2009; Alsop and Vijayan, 2009). Another developmental milestone occurs when the gills become necessary for gas transfer, at around 14 dpf (Jonz and Nurse, 2005). Although the gill filament primordia are present as early as 3 dpf, they are not essential as gas transfer surfaces until 14 dpf (Rombough, 2002). Finally, the point of sexual differentiation at approximately 35 dpf (Uchida et al., 2002) is of interest because pronounced neuroendocrine differences have been noted between male and female zebrafish (Filby et al., 2010), stemming from this period of sexual differentiation, yet little research has examined the role of cortisol on development during this period of large-scale reorganization (Spence et al., 2008; Takahashi, 1977; Uchida et al., 2002).

Chapter 2: Materials and methods

2.1 Experimental animals

All experimental fish were collected by in-house breeding of adult wild-type zebrafish (*Danio rerio*) using in-tank breeding traps. Adult fish were held under a 14L:10D photoperiod with semi-recirculating flow-through water in 10 L tanks at a density of ~3 fish per litre, and were fed ~5% body mass per day of No.1 crumble-Zeigler (Aquatic Habitats, Apopka, FL, USA) commercial fish food. Holding tanks were supplied with dechloraminated city of Ottawa tap water at 28.5°C (0.25 mmol L^{-1} Ca^{2+}, 0.78 mmol L^{-1} Na^{+}, 0.02 mmol L^{-1} K^{+}, 0.15 mmol L^{-1} Mg^{2+}; pH 7.6). Breeding traps were composed of a 1 L catch-tank and a filter that was filled with marbles and artificial aquatic plants to simulate suitable egg deposition substrate. The filter allowed eggs to fall into the catch-tank through slots that were large enough for eggs to fall through but prevented adult access to the fertilized eggs. Traps were placed in holding tanks the night prior to collection, and were removed after 10:30 am the following day, allowing time for adults to spawn after being triggered by the room lights turning on at 9 am.

Prior to 7 dpf, embryos and larvae were raised in 50 mL Petri dishes filled with embryo medium (0.33 mM $CaCl_2 \cdot 2H_2O$, 0.17 mM KCl, 0.33 mM $MgSO_4 \cdot 6H_2O$, 5 mM NaCl, 0.0001% methylene blue) at a set density of 40 embryos per petri dish. Fish used for measurements of whole-body ion concentrations and for assessment of sex ratio at sexual maturity were raised in system water. Petri dishes were held in an incubator set to 28.5˚C and embryo medium/system water was changed once daily with two-thirds replacement. Dead embryos/larvae were removed at each water change.

Fish between the ages of 7 and 11 dpf were held in 1 L standing tanks with 400 mL of system water at 28.5°C on a 14L:10D photoperiod, and were fed to satiation with a light sprinkle of GEMMA 75 once daily. Upon reaching 11 dpf, the volume of water in each tank was increased to 800 mL. Water in these tanks was changed and dead fish were removed at 11, 15, 20, 25, and 30 dpf. Instead of being transferred to 1 L standing tanks, fish used for the assessment of sex ratio at sexual maturity were transferred to 3 L tanks at a volume of 400 mL at a density of less than 10 fish per 100 mL, and were fed a mixed diet of GEMMA 75 and rotifer culture until 15 dpf, when the rotifer culture was removed with a water change. GEMMA 75 was replaced with the larger GEMMA 150 food between 20 and 25 dpf depending on the size of the individuals in the tank, and then replaced again at ~60 dpf with No. 1 crumble-Zeigler commercial fish food. At 11 dpf, tanks were converted from standing to flow-through by turning system water on to a slow drip, which was then increased to a steady flow at ~20 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 research and teaching.

2.2 Experimental design

Experiments involved the comparison of fish stressed at one of several early developmental stages to fish that were raised identically without exposure to the stress protocol. This approach allowed the relative impact of stress at key developmental points to be assessed. Individuals from randomly mated adult zebrafish were separated into Petri dishes (40 embryos per dish) and at one of three developmental time points, 4-5, 7-8, or 15-16 dpf (identified as 4, 7 and 15 dpf stress treatments), were subjected to a stressor consisting of repeated air-exposure. Air exposure involved netting fish and removing them from the water for 3 min, allowing them to recover in water for 3 min, and repeating the 3 min air exposure (Ramsay et al., 2009). The repeated air-exposure was selected for its ability to consistently evoke a cortisol response at both early developmental ages (see below) and later in life (Ramsay et al., 2009). This stressor was repeated twice daily over two days, with repeated bouts separated by a minimum of 5 h. Individuals from these 'stressed' treatment groups were then sampled at 7, 15, and 35 dpf along with control individuals that were housed under identical conditions, resulting in a total of 9 different treatment groups. These treatment groups were repeated across five separate experiments to collect data on survival, growth, metabolic rate, whole-body ion concentrations, whole-body cortisol concentrations, and anxiety-related behaviour. An additional experiment was carried out to assess the effects of early life adversity on sex ratio and other morphological parameters at sexual maturity, where control fish as well as fish stressed as described above at 4, 7, 15 or 35 dpf were raised to sexual maturity (120-130 dpf).

2.2.1 Pilot studies – selection and validation of the stress protocol

An air-exposure stressor was chosen owing to its ability to consistently induce a cortisol response at a wide range of developmental ages, from larvae to adult (Fuzzen et al., 2010; Ramsay et al., 2009; Redfern et al., 2017). Several pilot studies were conducted to determine the most appropriate air-exposure protocol. To determine the time course of the cortisol response to an air-exposure stressor, 4 and 7 dpf zebrafish larvae were exposed to air (in a net) for one minute (Fuzzen et al., 2010; Redfern et al., 2017). Separate groups (N = 5-12 at each time, where each N was a group of 20 larvae) of 4 and 7 dpf zebrafish larvae were euthanized as unstressed controls, or stressed using the one-minute air-exposure stressor and sampled at 5, 10, or 60 min post-stressor. This trial was repeated using sampling times of 10, 30, 60, 480 and 1140 min post-stressor and the air-exposure stressor of Ramsay et al. (2009); i.e. 3 min in air, 3 min in water, 3 min in air. Larvae were euthanized in a solution of Tris-buffered MS-222 (0.72 mg mL^{-1} 3-

aminobenzoic acid ethyl ester, 21 mM Tris, pH 7; Sigma-Aldrich, St. Louis, MO, USA) and whole-body cortisol concentrations were measured by enzyme-linked immunosorbent assay (EIA), as described below (*section 2.2.5*). These pilot studies indicated that 10 min post-stressor was an appropriate time at which to measure peak whole-body cortisol concentrations.

Using the 10 min post-stressor sampling time, the magnitude of the cortisol response was compared between the two air-exposure protocols. Baseline and peak whole-body cortisol concentrations were measured for samples of 20 larvae ($N = 1$) at 4 and 7 dpf. This pilot experiment revealed that the repeated air-exposure protocol (Ramsay et al., 2009) was more reliable, and therefore it was selected for all subsequent experiments.

To assess the consistency of the cortisol response to the air-exposure stressor over the four repeated air exposures used in the stress protocol, whole-body cortisol concentrations were measured in 4 dpf zebrafish subjected to 1, 2 or 4 episodes of the air-exposure stressor. Baseline whole-body cortisol concentrations were measured in separate groups of control fish. Whole-body cortisol concentrations were measured for groups of 20 individual fish pooled for each sample ($N = 1$). The results of this trial indicated that repeated bouts of air-exposure significantly increased whole-body cortisol concentrations in a consistent manner at the earliest developmental point used in the remaining experiments. On this basis, the stress protocol used in all subsequent experiments was chosen to be two applications of the air-exposure stressor per day for two days.

2.2.2 Survival and growth

The goal of this experiment was to assess the effects of early life exposure to a stressor on fish survival and growth during early life, using changes in length and mass as indices of growth.

Measurements of length and survival were carried out concurrently with measurements of whole-body cortisol concentrations, with a subset of ~5 individuals from each group of fish used for cortisol measurements being set aside for the measurement of length and the percent of the initial forty individuals allocated per tank surviving to sampling being used for survival. Measurements of mass were carried out concurrently with measurements of metabolic rate.

Fish at 7 and 15 dpf were photographed individually using a digital camera attached to a dissecting microscope. Maximum length for each fish was determined from its photograph through comparison of the maximum of each fish in the image at a set height to a fixed scale photographed from the same height using ImageJ v1.49 (National Institute of Health, USA). Maximum length was measured as the straight-line distance from the most anterior part of each individual's head to the end of the caudal fin. Measurements of 35 dpf fish were conducted in a similar fashion for fish photographed against a ruler, with fork length being recorded rather than maximum length. Measurements for each individual in a sample were averaged to determine the mean length for that sample ($N = 1$).

Wet mass of fish at 7, 15, and 35 dpf was measured using modified 1.5 mL microcentrifuge tubes in which the bottom of the tube was replaced with fine-grade mesh. The sample of fish used for the measurement of metabolic rate in micro-respirometry chambers (see below) was placed in the modified tube and lightly centrifuged to remove excess water. The combined mass of fish and tube was then recorded to the nearest mg using an analytical balance (ED 1245, Sartorius, GER) and the previously measured mass of the tube alone was subtracted to yield the mass of the fish. The average mass of an individual was obtained by dividing the measured mass by the number of individuals in the sample ($N = 1$).

2.2.3 Metabolic rate

This experiment served to determine whether exposure to stress early in development affects metabolic rate, measured as the rates of oxygen consumption ($\dot{M}O_2$) and CO_2 production ($\dot{M}CO_2$), allowing calculation of the respiratory exchange ratio (RER = $\dot{M}CO_2/\dot{M}O_2$). The basic method of Talbot et al. (2015) was followed, with the number of individuals in the 2 mL micro-respirometry chamber being adjusted by age. For 7 dpf fish, a sample ($N = 1$) consisted of 15 individuals, whereas 10 individuals were used per sample at 15 dpf, and at 35 dpf, each sample consisted of a single individual.

Fish were placed in the micro-respirometry chamber and given a 30 min acclimation period with two-thirds water replacement every 10 min. All trials were carried out at 28.5˚C in a custom-built water bath with a continuous flow of water from an SL-381 submersible pump (Panegy, CHN) that functioned to minimize temperature gradients within the water bath. Micro-respirometry chambers were held in the water bath above small magnetic stir pads (Thermo Electron LED GmbH, GER) which rotated magnetic stir bars located in the bottom of each microrespirometry chamber, separated from the fish in the chamber by a mesh screen (Loligo Systems, Tjele. DNK).

The partial pressure of O_2 (PO_2) in the water in the micro-respirometry chamber was measured continuously over the course of each 30 min trial using fibre-optic O_2 electrodes (FOXY AL-300, Ocean Optics, FL, USA) linked to a laptop running Ocean Optics software. Immediately prior to and after the 30-minute respirometry period, 0.5 mL water samples were removed from the chamber for the measurement of initial and final water total CO_2 concentrations. These samples were analyzed in triplicate 50 µL aliquots using a custom-built total CO_2 analyzer (Talbot et al., 2015). The difference between the initial and final water CO_2

concentrations for a trial was used to calculate $\dot{M}CO_2$, taking into account time, fish mass, and the volume of water in the chamber. Oxygen consumption was calculated from the rate of change of water PO_2, taking into account the volume of water in the chamber, fish mass, and the solubility coefficient of O_2 in water at 28.5°C (Boutilier et al., 1984). Empty chamber runs were used to assess background rates of O_2 production and CO_2 production, and fish mass was determined as described above (*section 2.2.2*).

2.2.4 Whole-body ion concentrations

The aim of this experiment was to examine the effects of early life stress on whole-body Na^+ and Ca^{2+} concentrations. The number of individual fish used per replicate ($N = 1$) depended on the age of sampling, with samples of 7 dpf fish consisting of 20 individuals, samples of 15 dpf fish consisting of 15 individuals, and samples of 35 dpf fish being comprised of a single individual per replicate. The basic method used was that of Kwong and Perry (2013). Fish were euthanized in a solution of Tris-buffered MS-222 (see above) and then rinsed three times in separate baths of deionised water. Samples were transferred to microcentrifuge tubes, excess water was removed and 5 N HNO_3 was added to each tube (0.2, 0.3, and 0.8 mL, respectively, for 7, 15 and 35 dpf fish). Samples were incubated at 65˚C for 48 h to digest all organic matter into solution and then diluted with 0.8 mL (7 and 35 dpf) or 0.7 mL (15 dpf) deionised water for measurement of ion concentrations by flame emission spectrometry (AA240 Atomic Absorption Spectrometer; Varian, USA). Average whole-body ion concentrations per individual were then calculated by taking into account the number of fish in each sample and the dilution used in sample preparation.

2.2.5 Whole-body cortisol concentrations

The goal of this experiment was to assess the effects of early life stress on baseline cortisol concentrations as well as the cortisol response to a stressor. To measure baseline cortisol concentrations, fish were sampled directly from the holding tank at the designated age. To assess the cortisol response to a stressor, fish were stressed at the designated sampling age using the air-exposure stressor described above (Ramsay et al., 2009) and were sampled after 10 min recovery in their holding tank. The 10 min recovery period allowed circulating cortisol levels to rise to their peak value (*section 2.2.1*).

Fish were euthanized by immersion in a solution of Tris buffered MS-222 (see above) and flash frozen in liquid nitrogen in 1.5 mL microcentrifuge tubes. The number of fish used for each sample ($N = 1$) varied with fish age; at 7 dpf, 20 individuals made up a sample, at 15 dpf, samples ranged from 12 to 20 individuals per sample, and 5 individuals per sample were used for 35 dpf fish. Samples were stored at -80˚C for later extraction and measurement of whole-body cortisol concentrations.

The extraction and measurement of whole-body cortisol concentrations followed the basic method of Jeffrey and Gilmour (2016). In brief, samples were homogenized in 200 µL of 5X diluted cortisol extraction buffer (ELISA, Neogen, MI, USA) using a battery-powered, handheld pestle grinder (Kimble Chase, NJ, USA). Cortisol was extracted from the homogenized samples using 3 x 1 mL of diethyl ether (Fisher Scientific, ON, CAN), with 30 min (first incubation) or 15 min incubation on ice each time. After incubation, samples were centrifuged at 3,000 *g* for 5 min and then frozen at -80˚C for 30 min. The liquid diethyl ether containing dissolved cortisol was separated from the frozen sample, and evaporated under forced air at room temperature in 1.5 mL microcentrifuge tubes. The extracted cortisol (combined from the three extractions) was then dissolved in 10 μL of extraction buffer per larvae of the original sample for 7 and 15 dpf fish, or 40 μL of extraction buffer per individual for 35 dpf fish. Samples were vortexed and heated to 65˚C for 5 min three times to aid in reconstituting the sample. Reconstituted samples were frozen and stored at -80˚C until analysis for cortisol concentrations using a commercial EIA (Neogen, MI, USA). Extraction efficiency was 88.9%, while inter-assay variability was 11.1%, and intra-assay variability was 0.6%.

2.2.6 Anxiety-related behaviours

In this experiment, the effects of early life exposure to a stressor on activity and thigmotaxis, both of which are considered to be anxiety-related behaviours, were investigated using an open-field test (Best et al., 2017; Champagne et al., 2010; Simon et al., 1994). Groups of fish to be tested were held for a minimum of 1 h of acclimation in an incubator that was in close proximity to the test chamber. Fish were tested in sets of 6 individuals from a given group, with a maximum of 3 sets of 6 individuals per group and at least 1 h between sets within a group. For these trials, a sample ($N = 1$) was an individual fish regardless of age (see *section 2.3*). Open-field trials were carried out in a custom-built arena over which was mounted a digital camcorder (Vixia HF R300, Canon Inc., JPN). The arena was composed of a 30 L Plexiglas tank, partially filled with water held at 28.5˚C with two aquarium heaters, and a 6-well microplate (Cellvis, CA, USA) that was partially submerged to maintain arena temperature. Each well in the 6-well plate was 3.5 cm in diameter and had opaque black walls. Trials were carried out in a dark room with the arena illuminated from below using LED light strips (Striberg, IKEA, SWE) diffused through several layers of laminated wax paper.

Each trial consisted of a 3 min acclimation period after the camera had been set to record and the observer had left the room, followed by a 5 min recording period. Video recordings were edited to the 5 min test period using Aiseesoft Video Converter V. 8.0.22, and contrast and brightness were enhanced. The 5 min video recordings were then analysed using the Zebralab software package (Viewpoint Behavior Technology, FRA). Activity was quantified as distance travelled, using a movement threshold defined according to Best et al. (2017) for the same software and modified at each sampling age based on the mean length of the fish at that age. To estimate thigmotaxis, each circular well was divided into inner and outer areas, with the inner area comprising half of the diameter of the well and positioned centrally. Thigmotaxic tendency was expressed as the proportion of time that an individual spent in the outer area over the course of each 5 min trial.

2.2.7 Sex ratio

The goal of this experiment was to investigate the effects of early-life stress on the determination of sex. Fish were euthanized at 120-130 dpf in ice-cold system water and the total number of fish per tank was recorded as a metric of survival. Two-thirds of the fish in each tank were measured against a ruler to determine fork length. Weight was measured for these individuals by drying each fish with a Kimwipe before measuring mass to the nearest mg on a 1702 analytical balance (Sartorius, GER). Measurements of fork length and mass were then used to calculate Fulton's condition factor, $K = 100 \times (\text{wet weight} / \text{forklength}^3)$ (Froese, 2006). Upon dissection, the sex of each individual was recorded by inspection of the gonads, which were subsequently weighed to the nearest µg using an MX5 microbalance (Mettler Toledo, OH, USA). Gonad mass was compared to the previously recorded mass of each individual to calculate gonadosomatic index, GSI = 100 x (gonad mass / body mass), a rough measure of reproductive condition (Devlin and Nagahama, 2002).

2.3 Statistical analyses

Data are presented as means ± one standard error of the mean (SEM). Prior to statistical analysis, data were transformed if the assumptions of normality and equal variance were not met. In order to select the appropriate data transformation, eight separate transformations were performed in a specific order (log, square root, natural log, squared, cubed, cube root, sine, and arcsine), with the first transformation which met the assumptions being chosen. If transformation was not sufficient to meet the assumptions of parametric statistics, a non-parametric alternative was used. Although the variables examined in the present study would be expected to change with developmental age, the goal of the present study was to evaluate effects of early-life exposure to stress within a developmental age. Therefore, statistical comparisons were not carried out across developmental ages, but rather the significance of exposure to early life stress was examined statistically within each developmental age. The significance of stress protocol treatment effects on survival, growth, metabolic rate, and whole-body ion concentrations was assessed in 7 dpf fish using Student's *t*-tests and in 15 and 35 dpf fish using one-way analysis of variance (ANOVA). Where significant differences were detected between treatments via ANOVA, Tukey-HSD post-hoc multiple comparisons tests were conducted. Data on tank density were collected for fish used for the measurement of metabolic rate, and therefore effects of density were first examined at each age using ANCOVAs with density as a covariate. All whole-body cortisol data were assessed statistically utilizing two-way ANOVAs at each age, with stress treatment and sampling time (baseline vs stress-induced) as the two factors. Behavioural data were analysed using linear mixed-effects models, with treatment as a fixed effect and test well as a random effect to control for the position of each fish on the tray, allowing for each individual fish run to be considered a replicate. Morphological measurements of adult fish were analysed separately for female and male fish using ANCOVAs with density as a covariate. Sex ratio was compared among treatments using a generalized linear model with sex as a binary response variable and tank density as a covariate. All tests were conducted at a level of significance of $\alpha = 0.05$ using R (Version 3.2.2 for Mac OSX).

Chapter 3: Results

3.1 Selection and validation of the stress protocol

A series of validation experiments were carried out to develop an appropriate stress protocol. In the first of these trials, the effect of a single one-minute air exposure on whole-body cortisol concentrations was examined in 4 and 7 dpf zebrafish (Fig. 3.1). Although one minute of air exposure failed to induce a significant increase in whole-body cortisol levels at 4 dpf (Fig. 3.1a; one-way ANOVA, $F_{3,25} = 1.8427$, $P = 0.165$), at 7 dpf, whole-body cortisol concentrations were significantly increased 5 and 10 min post-stressor, with a return to baseline levels by 60 min post-stressor (Fig 3.1b; one-way ANOVA, $F_{3,26} = 12.424$, $P < 0.001$). Similar results were obtained using the 3 min in air, 3 min in water, 3 min in air stress protocol of Ramsay et al. (2009). No significant changes in whole-body cortisol concentrations were detected in 4 dpf fish (Fig. 3.2a; Kruskall-Wallis rank sum test, $H = 3.4271$, degrees of freedom, $df = 5$, $P = 0.633$), despite a tendency for cortisol levels to be elevated 10 min post-stressor. At 7 dpf, whole-body cortisol concentrations were significantly elevated 10 min post-stressor, returning to baseline by 60 min post-stressor, and remaining within baseline levels to 24 h post-stressor (Fig.3.2b; one-way ANOVA on log transformed data, $F_{5,26} = 9.0746$, $P < 0.001$).

The efficacy of the repeated air-exposure stressor of Ramsay et al. (2009) relative to a single air-exposure stressor in developing zebrafish was tested in 4 and 7 dpf zebrafish larvae (Fig. 3.3). Although no significant effect of either air-exposure protocol was apparent at 4 dpf (Fig 3.3a; two-way ANOVA on log transformed data, treatment, $F_{1,26} = 2.1555$, $P = 0.154$, sampling point, $F_{1,26} = 2.2787$, $P = 0.143$, treatment x sampling point, $F_{1,26} = 0.0111$, $P = 0.917$), whole-body cortisol concentrations were significantly elevated by both air-exposure protocols in 7 dpf zebrafish, with the repeated air-exposure protocol resulting in significantly higher whole-body cortisol concentrations (Fig. 3.3b; two-way ANOVA on log transformed data, treatment, $F_{1,26}$

$=12.5112$, $P = 0.002$, sampling point, $F_{1,26} = 25.5086$, $P < 0.001$, treatment x sampling point, $F_{1,26} = 0.7796$, $P = 0.3854$). On this basis, the repeated air-exposure of Ramsay et al., (2009) was selected for subsequent experiments.

To test the consistency of the cortisol response to multiple applications of the air-exposure stressor, the whole-body cortisol response was measured in 4 dpf zebrafish larvae over one, two or four repetitions of the air-exposure stressor (Fig. 3.4). A significant cortisol response was detected at each repetition of the air-exposure stressor, with no significant differences among repetitions (two-way ANOVA on log transformed data, number of air-exposure stressors, $F_{2,30} =0.8445$, $P = 0.440$, sample time, $F_{1,30} = 47.2796$, $P <0.001$; number of air-exposure stressors x sampling time, $F_{2,30} = 2.6552$, $P = 0.087$).

The results of these pilot trials demonstrated that repeated bouts of the air-exposure stressor (where the air-exposure stressor consisted of 3 min in air, 3 min in water, 3 min in air as per Ramsay et al. (2009)) significantly increased whole-body cortisol concentrations in larval zebrafish in a consistent manner. For these reasons, the stress treatment or stress protocol used in subsequent experiments consisted of two bouts per day, for two days, of the air-exposure stressor.

3.2 Survival and growth

Exposure to a stressor during early development had significant effects on both survival and growth of larval and juvenile zebrafish. At 7 dpf, fish that had been exposed to stress at 4 dpf exhibited a significant reduction in survival (Fig. 3.5a; Student's *t*-test, $t = -2.4795$, $df = 27$, $P = 0.020$). Although there was no significant effect of the stressor on maximum length (Fig. 3.6a;

Student's *t*-test, t = 0.1986, df = 17, P = 0.845), fish stressed at 4 dpf exhibited lower mass at 7 dpf than control fish (Fig. 3.7a; Student's *t*-test, t = -3.1489, df = 9, P = 0.012).

In fish sampled at 15 dpf, exposure to a stressor at either 4 dpf or 7 dpf significantly decreased survival compared to the control treatment (Fig. 3.5b; one-way ANOVA, $F_{2,23}$ = 20.604, P < 0.001). Exposure to stress at 4 dpf significantly decreased maximum length (Fig. 3.6b; one-way ANOVA, $F_{2,21}$ = 3.9934, P = 0.034) but not mass (Fig. 3.7b; one-way ANOVA, $F_{2,19}$ = 5.711, P = 0.011), whereas the opposite was true for fish exposed to the stressor at 7 dpf (Figs. 3.6b, 3.7b).

At 35 dpf, survival was significantly decreased only by exposure to the stressor at 7 dpf (Fig. 3.5c; one-way ANOVA on log transformed data, $F_{3,47}$ = 13.259, P < 0.001). Fish stressed at 7 dpf also exhibited significantly lower fork length, as did fish stressed at 15 dpf (Fig. 3.5c; one-way ANOVA on log transformed data, $F_{3,47}$ = 5.0143, P=0.004). The mass of 35 dpf fish was unaffected by earlier exposure to a stressor (Fig. 3.7c; one-way ANOVA, $F_{3,23}$ = 0.13628, P = 0.937).

3.3 Metabolic rate

The effects of early-life exposure to an acute stressor on metabolic rate were examined by measuring $\dot{M}O_2$ and $\dot{M}CO_2$, and calculating the respiratory exchange ratio (RER). No significant effects of exposure to a stressor were detected at 7 and 35 dpf, whereas $\dot{M}O_2$ at 15 dpf was significantly reduced in response to early-life stress at both 4 dpf and 7 dpf (Table 3.1). In addition to this significant treatment effect, density also played a significant role on $\dot{M}O_2$ at 15 dpf, as well as on $\dot{M}CO_2$ (ANCOVA, treatment, $F_{1,7}$ = 0.3468, P = 0.574, density, $F_{1,7}$ = 6.8791, P = 0.034) and RER (ANCOVA, treatment, $F_{1,5}$ = 1.5697, P = 0.266, density, $F_{1,5}$ = 10.632, P = 0.022) at 7 dpf.

3.4 Whole-body ion concentrations

Although whole-body Na^+ concentrations at 7 dpf were unaffected by exposure to a stressor (Fig. 3.8a; Student's *t*-test, t = 0.55124, df = 12, P = 0.592), significant effects of early-life stress on whole-body Na^+ concentrations were detected at 15 and 35 dpf. At 15 dpf, whole-body Na^+ concentrations were significantly elevated by exposure to a stressor at 7 dpf but not 4 dpf (Fig.3.8b; one-way ANOVA, $F_{2,18}$ = 109.784, $P < 0.001$). Exposure to a stressor at either 4 or 7 dpf resulted in significantly higher whole-body Na^+ concentrations than in control fish at 35 dpf (Fig. 3.8c; one-way ANOVA on log transformed data, $F_{3,44}$ = 7.5855, $P < 0.001$).

Whole-body Ca^{2+} concentrations were significantly increased by early life exposure to a stressor for all but one treatment group (Fig. 3.9). In response to stressor exposure at 4 dpf, whole-body Ca^{2+} concentrations were elevated at 7 dpf (Fig. 3.9a; Student's *t*-test on log transformed data, t = 17.573, df = 12, $P < 0.001$). Similarly, exposure to a stressor at 4 dpf elevated whole-body Ca^{2+} levels in 15 dpf fish, as did stress exposure at 7 dpf (Fig. 3.9b; one-way ANOVA, $F_{2,17}$ = 138.91, $P < 0.001$). No significant difference in whole-body Ca^{2+} concentrations at 35 dpf was detected for fish stressed at 4 dpf, yet exposure to stress at either 7 or 15 dpf resulted in significantly higher whole-body Ca^{2+} concentrations (Fig. 3.9c; Kruskall-Wallis rank sum test, H = 34.936, df = 3, $P < 0.001$).

3.5 Baseline and stressor-induced whole-body cortisol concentrations

Although 7 dpf control fish and fish stressed at 4 dpf both responded to the stressor with a significant elevation of whole-body cortisol concentrations, there were no significant differences between treatment groups for either baseline or stress-induced cortisol levels (Fig. 3.10a; two-way ANOVA on log transformed data, treatment, $F_{1,31} = 0.747$, $P = 0.394$, sampling time, $F_{1,31} = 15.944$, $P < 0.001$, treatment x sampling time, $F_{1,31} = 0.242$, $P = 0.626$).

At 15 dpf, fish in all treatment groups mounted a significant cortisol response to the air-exposure stressor, but early exposure to a stressor was associated with significant differences in baseline and stress-induced cortisol concentrations (Fig. 3.10b). Baseline whole-body cortisol concentrations in fish stressed at 4 or 7 dpf were significantly lower than those of control individuals, and fish stressed at 4 dpf also exhibited significantly higher stress-induced whole-body cortisol concentrations than control fish (Fig. 3.10b; two-way ANOVA on log transformed data, treatment, $F_{2,29} = 22.52$, $P < 0.001$, sampling time, $F_{1,29} = 169.412$, $P < 0.001$, treatment x sampling time, $F_{2,29} = 18.222$, $P < 0.001$).

Measurement of whole-body cortisol concentrations in 35 dpf fish revealed loss of the cortisol response to the air-exposure stressor in fish stressed at 15 dpf (Fig. 3.10c). No significant differences among control, stressed at 4 dpf and stressed at 7 dpf treatment groups were detected for either baseline or stressor-induced whole-body cortisol concentrations, but fish stressed at 15 dpf exhibited cortisol concentrations that were intermediate between baseline and stress-induced values for both sampling points (Fig. 3.10c; two-way ANOVA on square root transformed data, treatment, $F_{3,39} = 3.5057$, $P = 0.024$, sampling time, $F_{1,39} = 2.3909$, $P = 0.130$, treatment x sampling time, $F_{3,39} = 4.8397$, $P = 0.006$).

3.6 Anxiety-related behaviours

Activity in an open-field arena was measured when the arena was illuminated by under-tank lights. Under these conditions, larval and juvenile zebrafish are expected to exhibit light-induced inhibition of movement (MacPhail et al., 2009), and therefore decreased activity relative to a control is indicative of increased anxiety. In 7 dpf larvae, fish stressed at 4 dpf travelled a significantly shorter distance over the course of the 5 min trial compared to control individuals (Fig. 3.11a; linear mixed-effects model on log transformed data, $F_{1,92} = 17.87$, $P < 0.001$). No significant differences in distance travelled were detected among treatment groups in 15 dpf fish (Fig.3.11b; linear mixed-effects model on log transformed data, $F_{2,79} = 2.0865$, $P = 0.131$). At 35 dpf, the distance travelled by fish that were stressed at 7 dpf was significantly greater than that of control fish and fish stressed at 4 or 15 dpf (Fig. 3.11c; linear mixed-effects model, $F_{3,132} = 9.6043$, $P < 0.001$).

Thigmotaxis, or wall-hugging behaviour, also was examined for the open-field trials, with increased proportion of time spent in the outer area of the arena, nearer the edges, being indicative of greater anxiety. No significant effect of early-life exposure to a stressor was detected in the proportion of time 7 dpf fish spent in the outer portion of the arena (Fig. 3.12a; linear mixed-effects model on arcsine transformed data, $F_{1,75} = 0.00238$, $P = 0.961$). At 15 dpf, fish that were stressed at 7 dpf spent a significantly greater proportion of time in the outer area of the arena in comparison to control fish and fish stressed at 4 dpf (Fig. 3.12b; linear mixed-effects model on arcsine transformed data, $F_{2,74} = 16.8$, $P < 0.001$). In 35 dpf fish, early-life exposure to a stressor, regardless of whether fish were stressed at 4, 7, or 15 dpf, significantly increased the proportion of time spent in the outer area of the arena compared to control individuals (Fig. 3.12c; linear mixed-effects model on log transformed data, $F_{3,168} = 4.6103$, $P = 0.004$).

3.7 Sex ratio

To examine the effects of early-life exposure to a stressor on sexual differentiation, fish in all treatment groups plus an additional treatment group that was subjected to the stress protocol at 35 dpf were raised to sexual maturity (4 months post fertilization). No significant treatment effects were detected in morphological variables, i.e. mass, fork length, K, or GSI, for female or male fish (Table 3.2). Although stress exposure did not significantly influence body metrics measured during adulthood, exposure to a stressor at 35 dpf resulted in a significant increase in the proportion of fish per tank that developed into males when compared to the control treatment, with fish stressed at 4, 7 or 15 dpf exhibiting values intermediate between those of control fish and fish stressed at 35 dpf (Fig. 3.13; generalized linear model, $\chi = 35.131$, df = 4, $P = 0.013$).

Figure 3.1: Mean whole-body cortisol concentrations of 4 dpf (a) and 7 dpf (b) zebrafish (*Danio rerio*) larvae before (time = 0) or after being subjected to one minute of air exposure. Values are means + SEM with *N* = 5-12 replicates of 20 fish per sample at each time. Times that share a letter are not significantly different from one another (one-way ANOVA, see text for details).

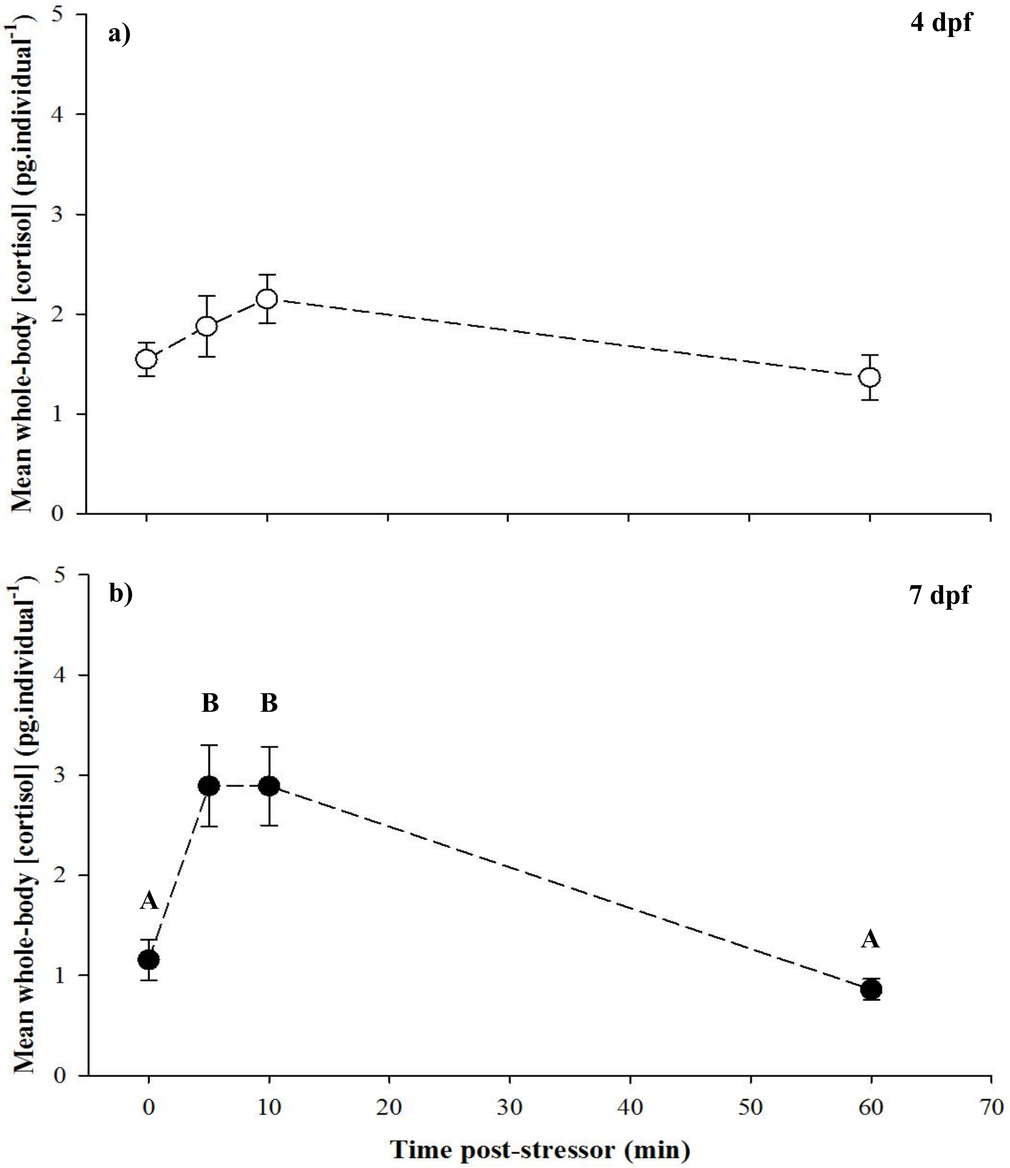

a)
4 dpf
b)
7 dpf
Mean whole-body [cortisol] (pg.individual^{-1})
0
1
2
3
4
5
A
B
B
A
0
10
20
30
40
50
60
70
Time post-stressor (min)

Figure 3.2: Mean whole-body cortisol concentrations of 4 dpf (a) and 7 dpf (b) zebrafish (*Danio rerio*) larvae before (time = 0) or after being subjected to an air-exposure stressor consisting of 3 min in air, 3 min in water, 3 min in air. Values are means ± SEM with *N* = 4-6 replicates of 20 fish per sample at each time. Values that share a letter are not significantly different from one another (one-way ANOVA on log transformed data, see text for details).

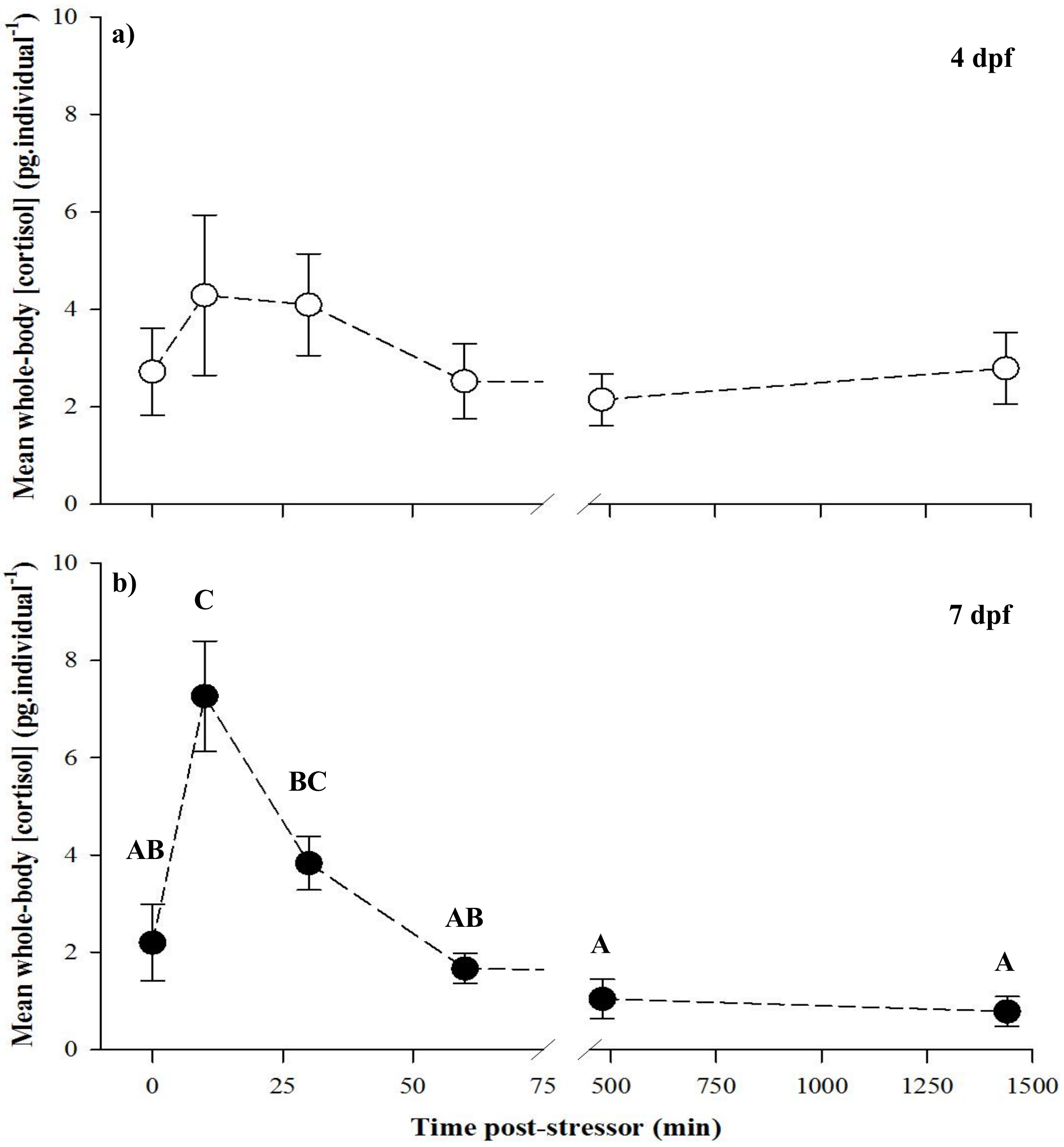

a)
4 dpf
b)
7 dpf
C
BC
AB
AB
A
A
Mean whole-body [cortisol] (pg.individual^{-1})
0
2
4
6
8
10
0
25
50
75
500
750
1000
1250
1500
Time post-stressor (min)

Figure 3.3: Mean whole-body cortisol concentrations of 4 dpf (a) and 7 dpf (b) zebrafish (*Danio rerio*) larvae before (baseline) or 10 min after (post-stressor) being subjected to either a single one-minute air-exposure stressor or repeated three-minute air exposures separated by 3 min of recovery in water. Values are means + SEM with *N* = 6-12 replicates of 20 fish per sample at each time. An asterisk indicates a significant difference between baseline and stress-induced cortisol concentrations, while groups that share a letter are not significantly different from one another (two-way ANOVA on log transformed data, see text for details).

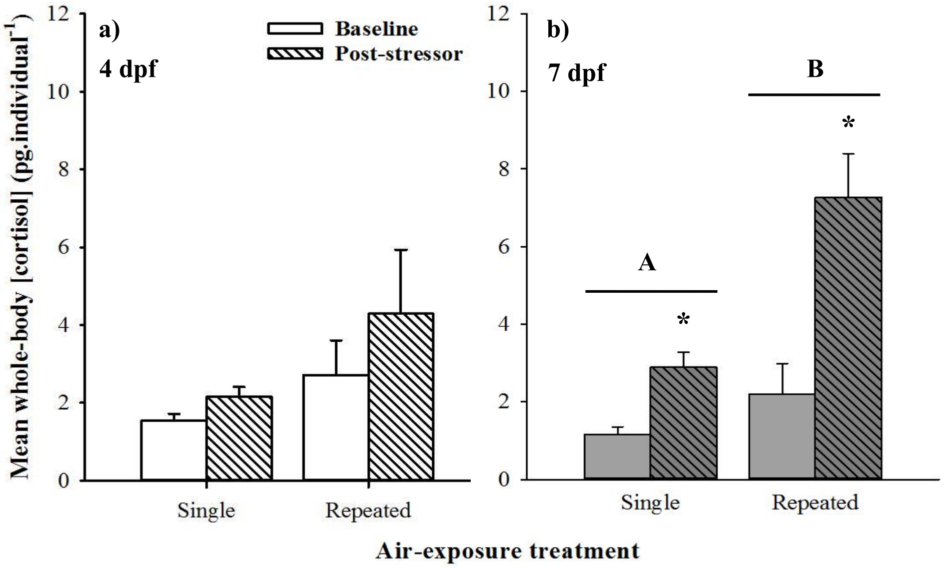
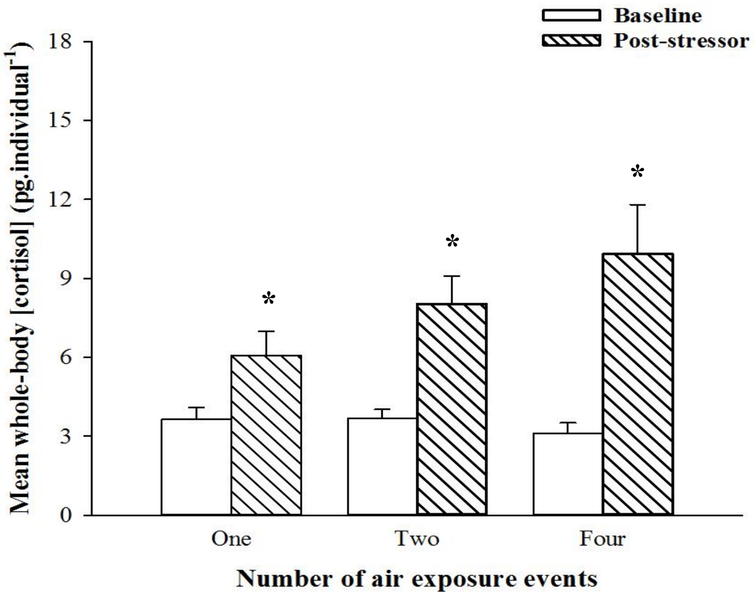

a)
4 dpf
Baseline
Post-stressor
b)
7 dpf
A
B
*
*
Mean whole-body [cortisol] (pg.individual^{-1})
0
2
4
6
8
10
12
Single
Repeated
Air-exposure treatment

Figure 3.4: Mean whole-body cortisol concentrations of 4 dpf zebrafish (*Danio rerio*) before (baseline) or 10 min after (post-stressor) being subjected to one, two, or four air-exposure stressors. Values are means + SEM with *N* = 6 replicates of 20 fish per sample at each time. An asterisk denotes a significant difference between baseline and post-stressor groups (two-way ANOVA on log transformed data, see text for details).



Baseline
Post-stressor
Mean whole-body [cortisol] (pg.individual^{-1})
18
15
12
9
6
3
0
*
*
*
One
Two
Four
Number of air exposure events

Figure 3.5: Mean survival as the percentage of zebrafish (*Danio rerio*) in a holding tank surviving to sampling at 7 dpf (a), 15 dpf (b), or 35 dpf (c). "Stress" treatment groups were subjected to four air-exposure stressors over two days, beginning at the indicated time, whereas "control" treatment groups were held untreated. Values are means + SEM with $N = 11\text{-}18$ for panel (a), $N = 12$ for panel (b), and $N = 11\text{-}16$ for panel (c). Each N represents a single tank. Groups that share a letter are not significantly different from one another (panel (a), Student's *t*-test; panel (b), one-way ANOVA; panel (c), one-way ANOVA on log transformed data; see text for details).

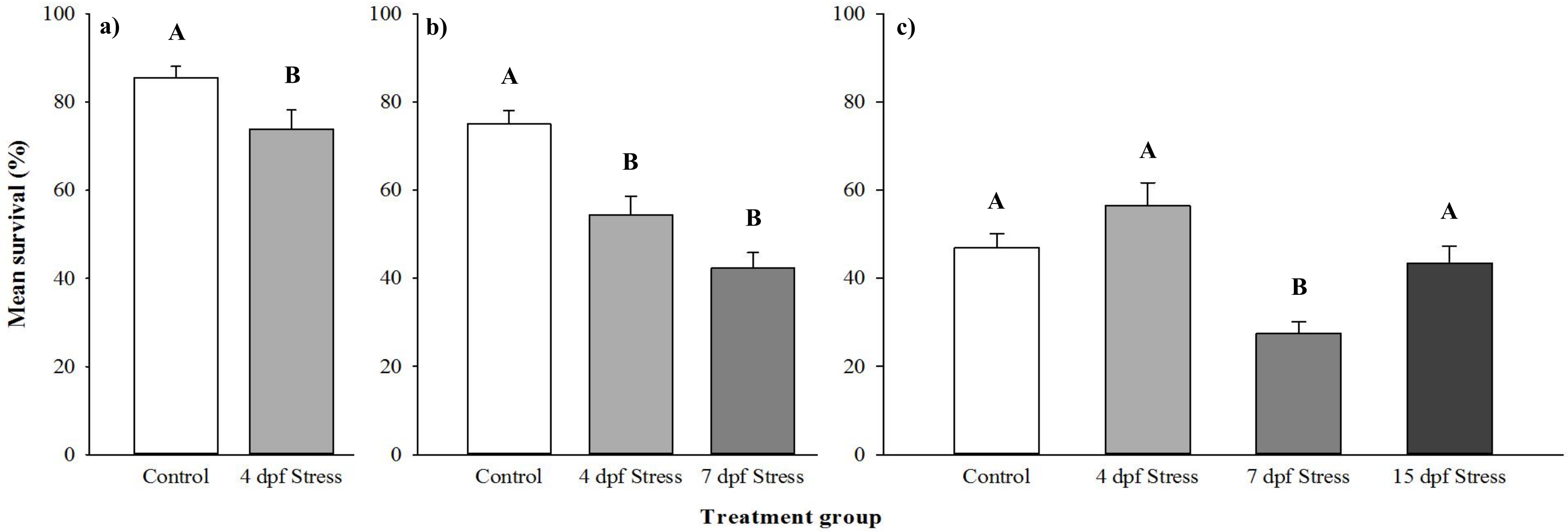

a)
b)
c)
Mean survival (%)
0
20
40
60
80
100
A
B
A
B
B
A
A
B
A
Control
4 dpf Stress
7 dpf Stress
15 dpf Stress
Treatment group

Figure 3.6: Mean maximum length of 7 dpf (a) and 15 dpf (b) fish, and mean fork length of 35 dpf (c) zebrafish (*Danio rerio*) subjected to four air-exposure stressors over two days, beginning at the indicated time ("stress" treatment groups) or held untreated ("control" treatment groups). Values are means + SEM with *N* = 6-13 (a), *N* = 6-11 (b), and *N* = 11-16 (c). Each *N* represents the mean value of at least 5 fish sampled from a single tank. Groups that share a letter are not significantly different from one another (panel (a), Student's *t*-test; panels (b) and (c), one-way ANOVA; see text for details).

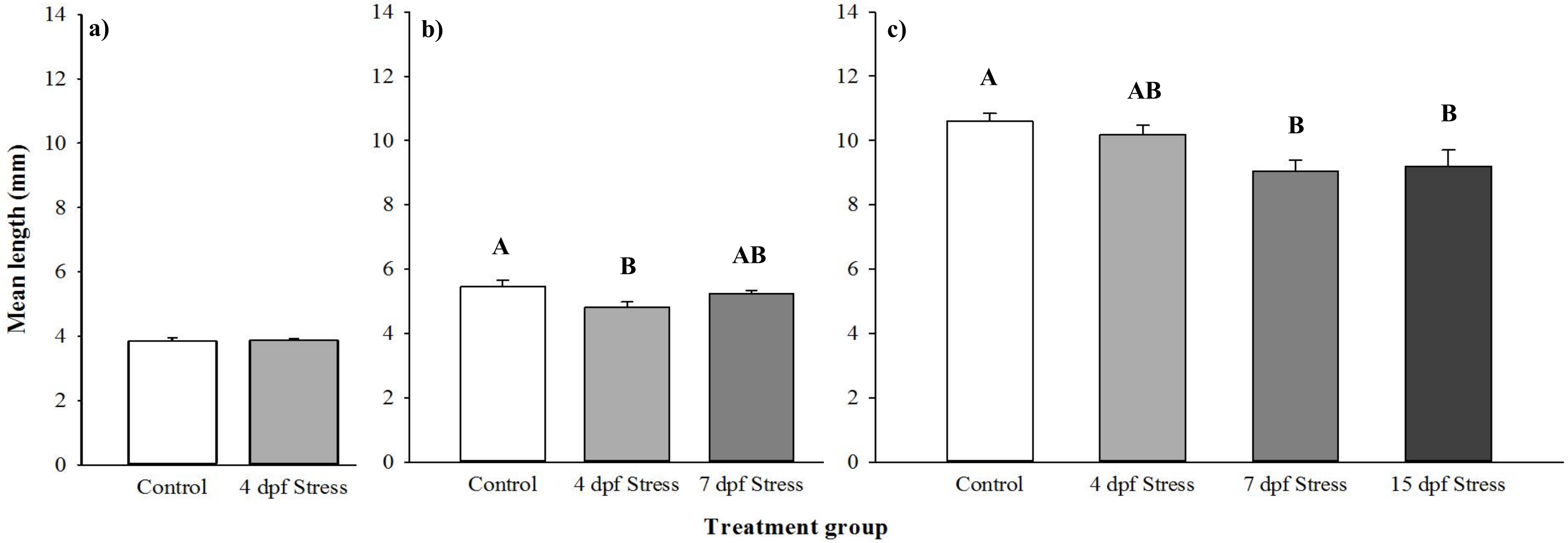

a)
b)
c)
Mean length (mm)
0
2
4
6
8
10
12
14
Control
4 dpf Stress
7 dpf Stress
15 dpf Stress
A
B
AB
A
AB
B
B
Treatment group

Figure 3.7: Mean mass of 7 dpf (a), 15 dpf (b) and of 35 dpf (c) zebrafish (*Danio rerio*) subjected to four air-exposure stressors over two days, beginning at the indicated time ("stress" treatment groups) or held untreated ("control" treatment groups). Values are means + SEM with $N = 6$ (a), $N = 6\text{-}9$ (b), and $N = 5\text{-}8$ (c). Each N represents fish sampled from a single tank. Groups that share a letter are not significantly different from one another (panel (a), Student's *t*-test; panels (b) and (c), one-way ANOVA; see text for details).

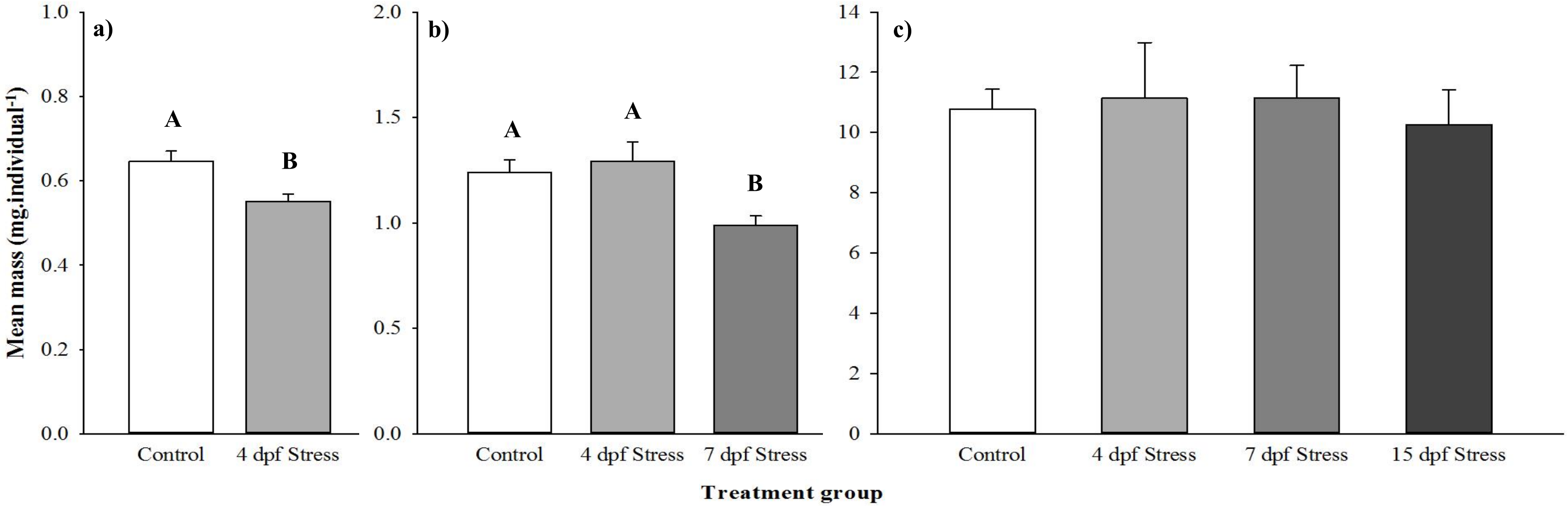

Mean mass (mg.individual^{-1})
a)
1.0
0.8
0.6
0.4
0.2
0.0
A
B
Control
4 dpf Stress
b)
2.0
1.5
1.0
0.5
0.0
A
A
B
Control
4 dpf Stress
7 dpf Stress
c)
14
12
10
8
6
4
2
0
Control
4 dpf Stress
7 dpf Stress
15 dpf Stress
Treatment group

Table 3.1: Mean rates of oxygen consumption ($\dot{M}O_2$) and CO_2 excretion ($\dot{M}CO_2$), as well as the respiratory exchange ratio (RER = $\dot{M}O_2/\dot{M}CO_2$), of 7, 15 and 35 dpf zebrafish (*Danio rerio*) subjected to four air-exposure stressors over two days, beginning at the indicated time ("stress" treatment groups) or held untreated ("control" treatment groups).

Age	Control	4 dpf stress	7 dpf stress	15 dpf stress	Statistical analysis
$\dot{M}O_2$ ($\mu mol\ g^{-1}\ h^{-1}$)					
7 dpf	36.4 ± 1.2 (5)	38.0 ± 2.9 (6)			$F_{1,7} = 0.3468, P = 0.574$
15 dpf	48.5 ± 1.8 (9)^{a}	39.5 ± 2.9 (6)^{b}	39.9 ± 2.5 (7)^{b}		**$F_{2,16} = 6.8383, P = 0.007$**
35 dpf	50.1 ± 4.0 (8)	43.2 ± 3.9 (5)	46.3 ± 7.2 (6)	41.3 ± 2.7 (8)	$F_{3,19} = 0.7712, P = 0.524$
$\dot{M}CO_2$ ($\mu mol\ g^{-1}\ h^{-1}$)					
7 dpf	36.4 ± 7.1 (4)	45.4 ± 5.9 (5)			$F_{1,5} = 1.5624, P = 0.267$
15 dpf	32.4 ± 3.3 (9)	33.1 ± 3.7 (6)	27.4 ± 3.5 (7)		$F_{2,16} = 0.836, P = 0.452$
35 dpf	39.6 ± 3.9 (8)	37.3 ± 5.4 (5)	32.7 ± 5.3 (6)	37.2 ± 5.3 (8)	$F_{3,19} = 0.2947, P = 0.829$
RER					
7 dpf	0.98 ± 0.19 (4)	1.2 ± 0.18 (5)			$F_{1,5} = 1.5697, P = 0.266$
15 dpf	0.67 ± 0.07 (9)	0.83 ± 0.05 (6)	0.67 ± 0.06 (7)		$F_{2,16} = 1.8138, P = 0.195$
35 dpf	0.82 ± 0.10 (8)	0.87 ± 0.09 (5)	0.75 ± 0.15 (6)	0.88 ± 0.09 (8)	$F_{3,19} = 0.2896, P = 0.832$

Data were analyzed by ANCOVA with density as covariate. Statistical analyses for effects of stress-exposure treatment are presented, with significant effects indicated by bold font; where a significant effect of treatment was detected, groups that share a letter are not significantly different from one another. Values are means ± SEM (*N*), and each *N* represents 15 fish at 7 dpf, 10 fish at 15 dpf, and 1 fish at 35 dpf. *dpf*, days post fertilization.

Figure 3.8: Mean whole-body Na^+ concentration of 7 dpf (a), 15 dpf (b) and of 35 dpf (c) zebrafish (*Danio rerio*) subjected to four air-exposure stressors over two days, beginning at the indicated time ("stress" treatment groups) or held untreated ("control" treatment groups). Values are means + SEM with $N = 6$-8 (panels a and b), and $N = 12$ (c). Each N represents 20 fish at 7 dpf, 15 fish at 15 dpf, and 1 fish at 35 dpf. Groups that share a letter are not significantly different from one another (panel (a), Student's *t*-test; panel (b), one-way ANOVA; panel (c), one-way ANOVA on log transformed data; see text for details).

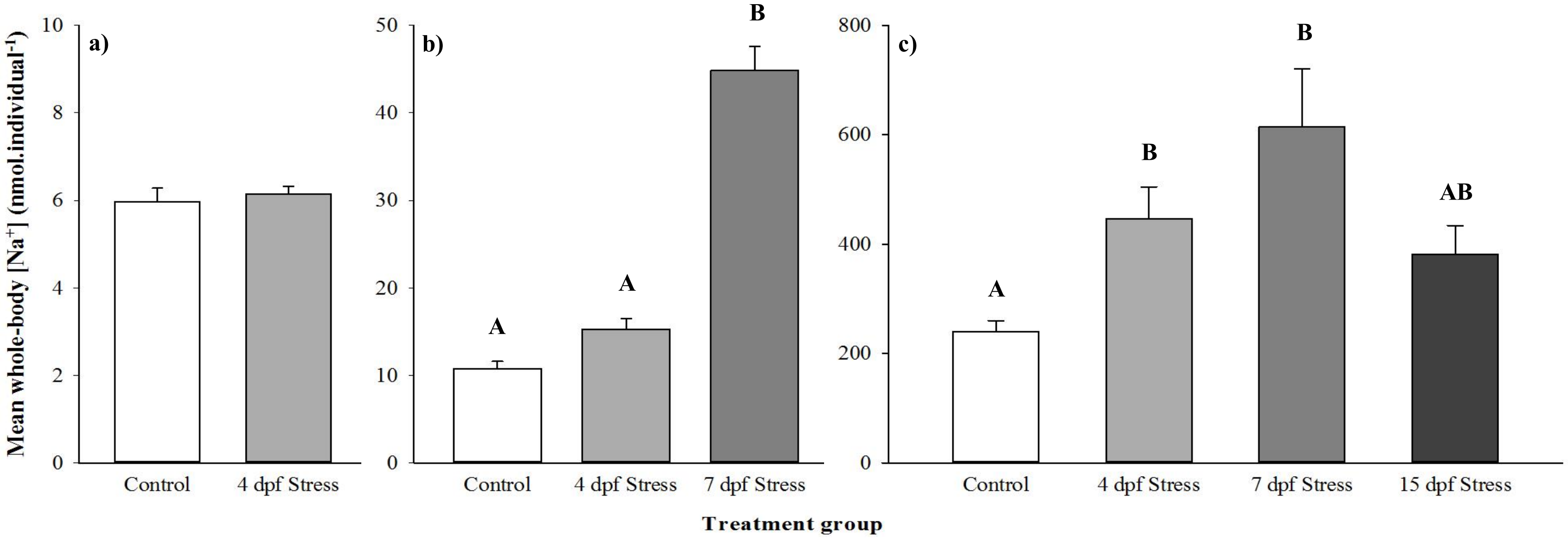

Mean whole-body [Na+] (nmol.individual-1)
a)
10
8
6
4
2
0
Control
4 dpf Stress
b)
50
40
30
20
10
0
A
A
B
Control
4 dpf Stress
7 dpf Stress
c)
800
600
400
200
0
A
B
B
AB
Control
4 dpf Stress
7 dpf Stress
15 dpf Stress
Treatment group

Figure 3.9: Mean whole-body Ca^{2+} concentration of 7 dpf (a), 15 dpf (b) and of 35 dpf (c) zebrafish (*Danio rerio*) subjected to four air-exposure stressors over two days, beginning at the indicated time ("stress" treatment groups) or held untreated ("control" treatment groups). Values are means + SEM with $N = 6\text{-}8$ (a), $N = 6\text{-}7$ (b), and $N = 12\text{-}16$ (c). Each N represents 20 fish at 7 dpf, 15 fish at 15 dpf, and 1 fish at 35 dpf. Groups that share a letter are not significantly different from one another (panel (a), Student's *t*-test on log transformed data; panel (b), one-way ANOVA; panel (c), Kruskall-Wallis rank sum test; see text for details).

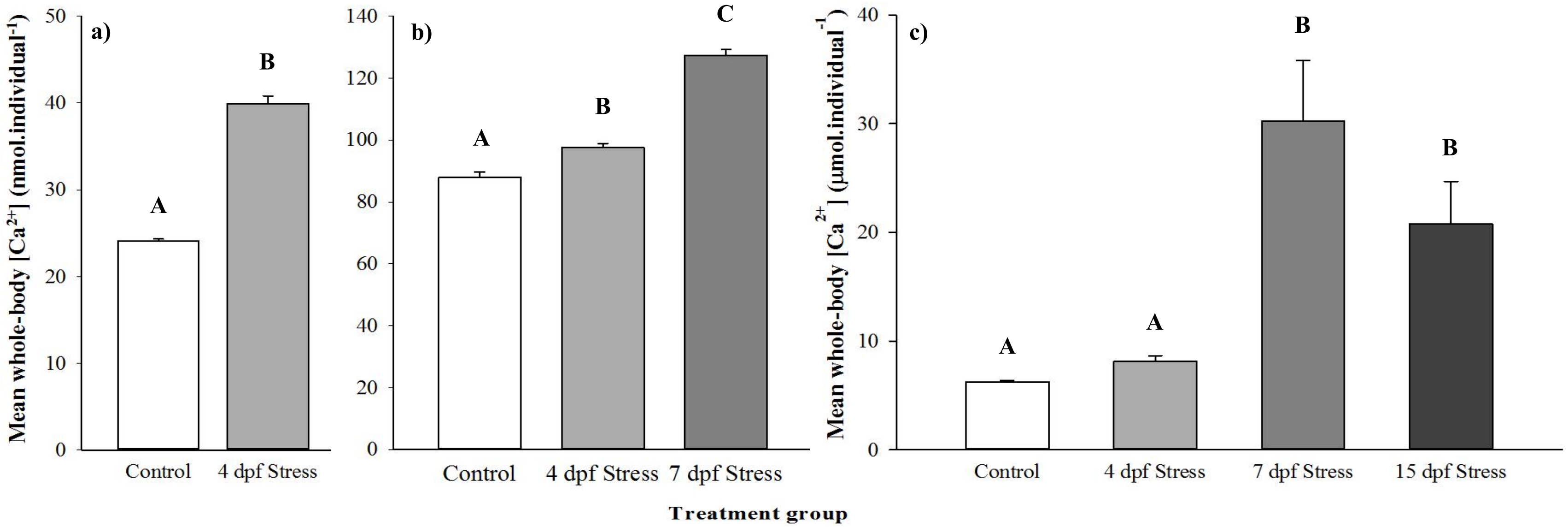

a)
b)
c)
Mean whole-body [Ca^{2+}] (nmol.individual^{-1})
Mean whole-body [Ca^{2+}] (µmol.individual^{-1})
0
10
20
30
40
50
0
20
40
60
80
100
120
140
A
B
A
B
C
A
A
B
B
Control
4 dpf Stress
7 dpf Stress
15 dpf Stress
Treatment group

Figure 3.10: Mean baseline and stress-induced (10 min post-stressor) whole-body cortisol concentrations of 7 dpf (a), 15 dpf (b) and 35 dpf (c) zebrafish (*Danio rerio*) that were subjected to four air-exposure stressors over two days, beginning at the indicated time (“stress” treatment groups) or held untreated (“control” treatment groups). Values are means + SEM with *N* = 5-12 (a), *N* = 5-7 (b), and *N* = 5-7 (c). Each *N* represents a group of 20 fish at 7 dpf, 12-20 fish at 15 dpf, and 5 fish at 35 dpf. An asterisk denotes a significant difference between baseline and stress-induced values in panel (a), while in panels (b) and (c), groups that share a letter are not significantly different from one another (panels (a) and (b), two-way ANOVA on log transformed data; panel (c), two-way ANOVA on square root transformed data; see text for details).

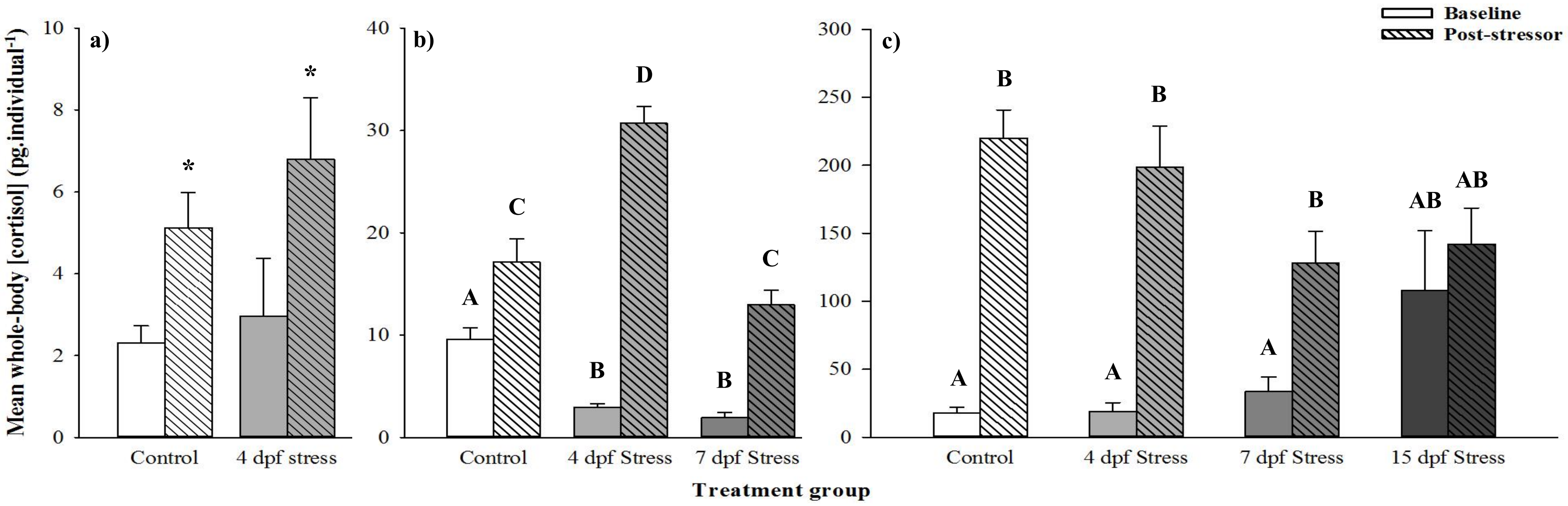
Baseline
Post-stressor
Mean whole-body [cortisol] (pg.individual^{-1})
a)
0
2
4
6
8
10
*
*
Control
4 dpf stress
b)
0
10
20
30
40
A
C
B
D
B
C
Control
4 dpf Stress
7 dpf Stress
c)
0
50
100
150
200
250
300
A
B
A
B
A
B
AB
AB
Control
4 dpf Stress
7 dpf Stress
15 dpf Stress
Treatment group

Figure 3.11: Mean distance travelled of 7 dpf (a), 15 dpf (b) and of 35 dpf (c) zebrafish (*Danio rerio*) in a 5-min open-field test. Fish were subjected to four air-exposure stressors over two days, beginning at the indicated time ("stress" treatment groups) or held untreated ("control" treatment groups). Values are means + SEM with N = 46-48 (a), N = 17-33 (b), and N = 34 (c), where each N represents an individual fish. Groups that share a letter are not significantly different from one another (panels (a) and (b), linear mixed-effects model on log transformed data; panel (c), linear mixed-effects model; see text for details).

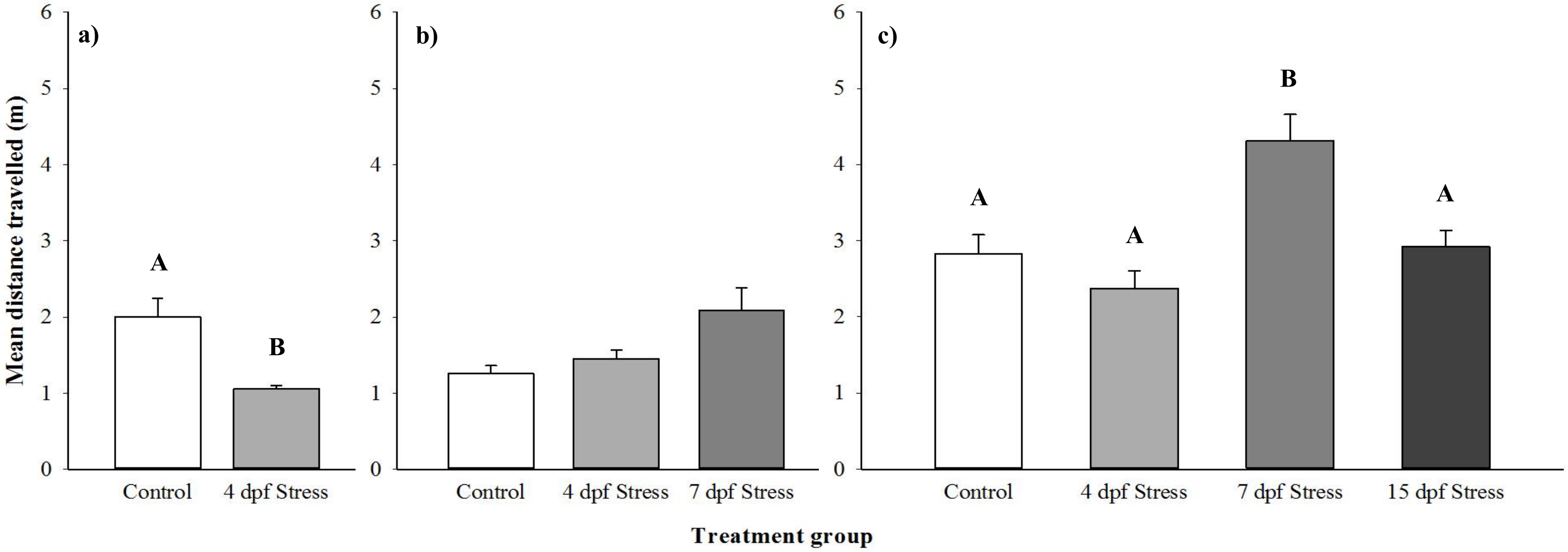

a)
b)
c)
Mean distance travelled (m)
0
1
2
3
4
5
6
A
B
A
A
B
A
Control
4 dpf Stress
7 dpf Stress
15 dpf Stress
Treatment group

Figure 3.12: Mean proportion of time spent in the outer area of an open-field arena for 7 dpf (a), 15 dpf (b) and of 35 dpf (c) zebrafish (*Danio rerio*) in a 5-min open field test. Fish were subjected to four air-exposure stressors over two days, beginning at the indicated time ("stress" treatment groups) or held untreated ("control" treatment groups). Values are means + SEM with $N = 35\text{-}47$ (a), $N = 20\text{-}31$ (b), and $N = 34\text{-}53$ (c), where each N represents an individual fish. Groups that share a letter are not significantly different from one another (panels (a) and (b), linear mixed-effects model on arcsine transformed data; panel (c), linear mixed-effects model on log transformed data; see text for details).

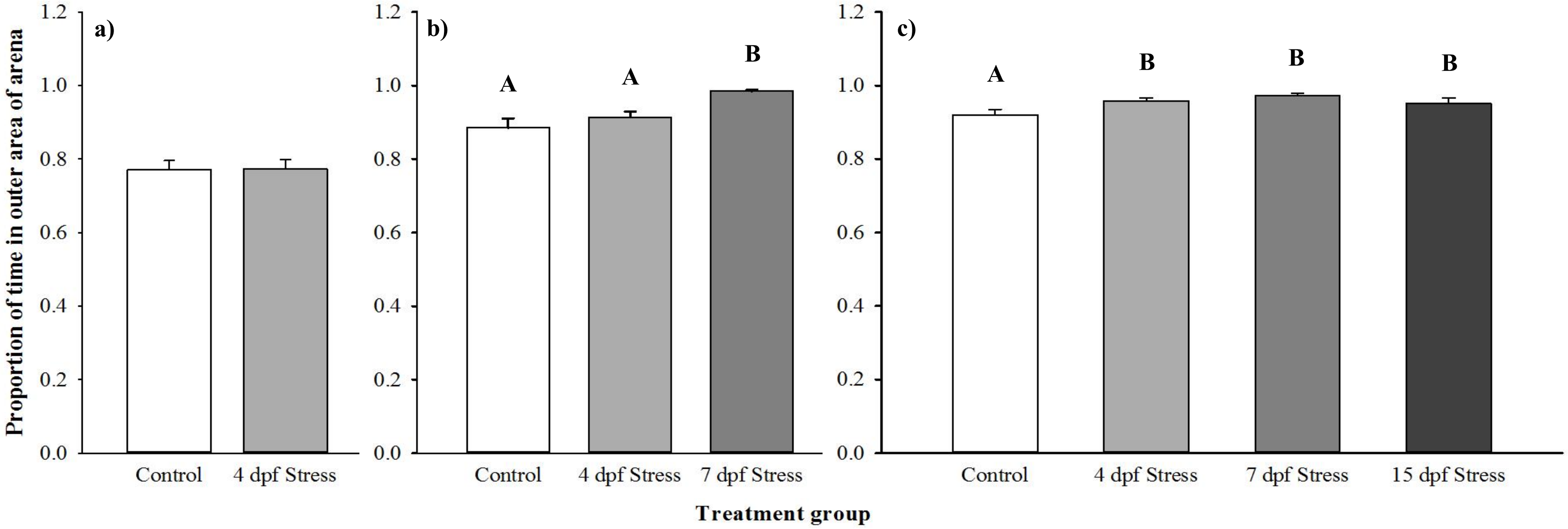
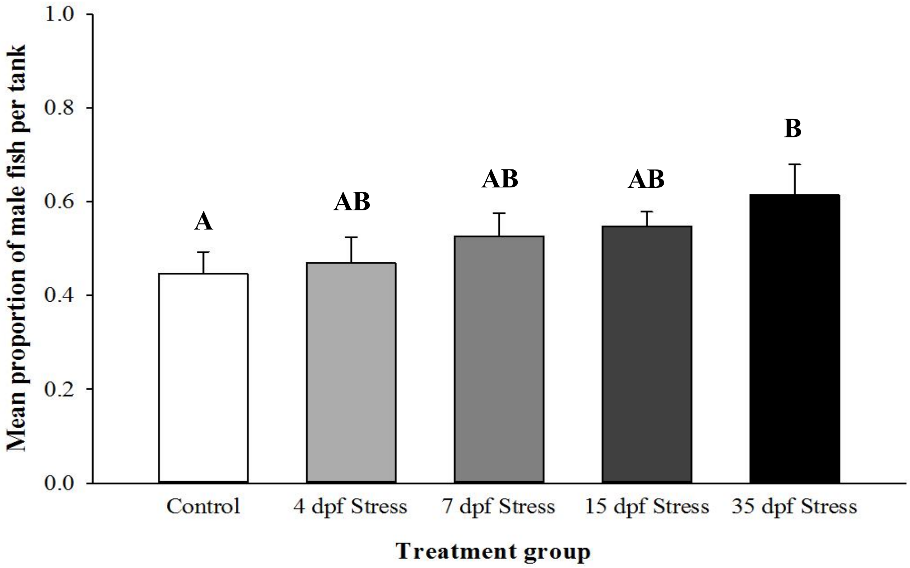

Proportion of time in outer area of arena
1.2
1.0
0.8
0.6
0.4
0.2
0.0
a)
Control
4 dpf Stress
b)
A
A
B
Control
4 dpf Stress
7 dpf Stress
c)
A
B
B
B
Control
4 dpf Stress
7 dpf Stress
15 dpf Stress
Treatment group

Table 3.2: Mean percent survival, mass, length, Fulton's condition factor (K), and gonadosomatic index (GSI), of male and female adult zebrafish (*Danio rerio*) subjected to four air-exposure stressors over two days, beginning at the indicated time ("stress" treatment groups) or held untreated ("control" treatment groups). Values are means ± SEM with *N* = 6-8, where *N* represents the mean value for a single tank and is presented in parentheses in the column headings.

Variable Measured	**Control (6)**	**4 dpf stress (7)**	**7 dpf stress (7)**	**15 dpf stress (8)**	**35 dpf stress (8)**	**Statistical analysis**
Percent survival	62.5 ± 7.0	62.9 ± 7.5	65.0 ± 5.6	65.3 ± 6.6	62.2 ± 10.3	$F_{4,31} = 0.0371$, P = 0.9972
Female						
Mass (mg)	399 ± 34	441 ± 47	396 ± 38	442 ± 62	489 ± 70	$F_{4,26} = 1.5292$, P = 0.223
Fork length (cm)	3.2 ± 0.1	3.3 ± 0.1	3.2 ± 0.1	3.2 ± 0.1	3.3 ± 0.1	$F_{4,26} = 1.9703$, P = 0.129
K	1.21 ± 0.06	1.18 ± 0.03	1.17 ± 0.04	1.26 ± 0.10	1.29 ± 0.10	$F_{4,26} = 0.7674$, P = 0.556
GSI (% body mass)	15.1 ± 2.8	12.5 ± 1.1	10.6 ± 1.2	12.5 ± 1.0	12.9 ± 1.5	$F_{4,26} = 1.9268$, P = 0.136
Male						
Mass (mg)	345 ± 29	358 ± 47	335 ± 18	352 ± 36	406 ± 55	$F_{4,26} = 2.0379$, P = 0.118
Fork length (cm)	3.2 ± 0.1	3.2 ± 0.1	3.2 ± 0.1	3.2 ± 0.1	3.3 ± 0.1	$F_{4,26} = 1.4743$, P = 0.239
K	1.01 ± 0.04	1.00 ± 0.03	0.97 ± 0.02	1.06 ± 0.05	1.02 ± 0.03	$F_{4,26} = 0.7431$, P = 0.571
GSI (% body mass)	1.10 ± 0.06	2.67 ± 1.14	1.09 ± 0.09	1.54 ± 0.45	1.75 ± 0.54	$F_{4,26} = 0.7933$, P = 0.540

Survival data were analysed by one-way ANOVA. Female data were analyzed by ANCOVA with density as covariate following log transformation, while male data were analyzed by ANCOVA with density as a covariate. Values are means ± SEM. *dpf*, days post fertilization.

Figure 3.13: Mean proportion of male fish per tank for adult zebrafish (*Danio rerio*), subjected to four air-exposure stressors over two days, beginning at the indicated time ("stress" treatment groups) or held untreated ("control" treatment groups). Values are means + SEM with *N* = 6-8, and each *N* representing an individual tank of fish. Groups that share a letter are not significantly different from one another (linear mixed-effects model, see text for details).


1.0
0.8
0.6
0.4
0.2
0.0
Mean proportion of male fish per tank
A
AB
AB
AB
B
Control
4 dpf Stress
7 dpf Stress
15 dpf Stress
35 dpf Stress
Treatment group

Chapter 4: Discussion

4.1 Overview

The present study examined the effects of an early-life, endogenously-produced stress response on cortisol levels and associated physiological and behavioural variables over a relatively long developmental period. Most studies have examined either the effects of maternal stress (e.g. Giesing et al., 2011; Jeffrey and Gilmour, 2016; Sopinka et al., 2016; reviewed by Sopinka et al., 2017) or the effects of cortisol alone using exogenous sources (Burton et al., 2011; Kumai et al., 2012; Lin et al., 2011; Wilson et al., 2013), without taking into account the importance of an endogenous stress response early in life. Moreover, many of these studies focused on the stress axis and stress responses at early developmental stages. Few studies have examined longer-term effects of early-life exposure to stress, and the role that the glucocorticoid stress hormone cortisol may play in other physiological processes, such as salt/water balance in teleost fish. Recent research highlighted the importance of critical developmental windows in shaping the phenotype of fish (Ivy et al., 2017; Robertson et al., 2014), and identified a role for cortisol in sexual differentiation in zebrafish and other teleost species with polygenic sex determination (Fernandino et al., 2012; Ribas et al., 2017). Thus, the current study aimed to address existing knowledge gaps through examination of the relationship between an endogenously produced stress response during critical developmental windows and the range of phenotypes that might arise as a result of this stress response through developmental plasticity.

To examine this relationship, the effects of a repeated acute stressor at one of four developmental stages were measured in zebrafish. Early life stress had marked impacts on survival and growth during early development, with only minimal effects on metabolic rate, and these effects diminished over longer periods of development with no treatment-related differences being detected by adulthood. Stress axis function, assessed through whole-body cortisol concentrations and anxiety-related behaviour, was also altered in response to early-life stress. Developing zebrafish exposed to a repeated acute stressor exhibited increased whole-body ion concentrations, likely owing to elevated cortisol concentrations. Finally, acute early-life stress during critical developmental windows was sufficient to induce masculinization of experimental populations. These results indicate that activation of the endogenous stress response during early development can play a significant but variable role in a range of physiological parameters, with the magnitude of the effect depending on the developmental window in which the stressor was experienced. Seemingly negative effects detected during early development often diminished over time, suggesting few impacts associated with early-life stress, apart from an effect on sex ratio that may be related to exposure to stress during a critical developmental window.

4.2 Decreased survival and growth without changes in metabolic rate

Exposure to early-life stress significantly reduced survival of zebrafish at 7 and 15 dpf, with effects diminishing by 35 dpf such that only stress experienced at 7 dpf caused a significant reduction in survival. The magnitude of the effect was most pronounced in response to the 7 dpf stress treatment, which saw a nearly 50% decrease in survivorship at both 15 and 35 dpf. Chronically increased cortisol has been demonstrated to have immunosuppressive effects, leading to increased mortality through disease (Gilmour et al., 2005; Pickering and Pottinger, 1989). For example, in adult brown trout, dietary or intraperitoneal cortisol implantation both led to increased fungal and bacterial infection rates, resulting in increased mortality (Pickering and Duston, 1983). Similarly, exposure of Atlantic salmon embryos to increased maternal cortisol levels decreased survival at the beginning of exogenous feeding (Eriksen et al., 2006). By adulthood, however, the zebrafish of the present study exhibited no significant effect of early-life exposure to stress on survival. This observation suggests that the higher mortality at early life stages in fish exposed to a stressor may be balanced out later in development by previously stressed fish becoming more resistant to stress, resulting in a net neutral impact on mortality in adulthood. These results also indicate that 7 dpf may be a developmental stage where fry are particularly susceptible to the effects of stress, possibly owing to the occurrence at this time of the transition to exogenous feeding from reliance on the yolk sac (Chu and Sadler, 2009), and therefore may act as a critical developmental window.

The trends detected in survival were mirrored to some extent in growth, with effects present in both length and mass at different stages and/or treatments. Effects of exposure to the stressor at 4 dpf generally were lost by 35 dpf, and although effects of exposure to the stressor at 7 or 15 dpf persisted at 35 dpf, these were lost by the time of sexual maturity. Maternal cortisol treatment increased offspring mass at hatching in largemouth bass with effects disappearing thereafter (Redfern et al., 2017), and also decreased growth rates in juvenile Atlantic salmon (Eriksen et al., 2006). Similarly, cortisol treatment reduced growth rates in channel catfish (*Ictalurus punctatus*) (Davis et al., 1985) and chronic stress decreased growth rates in rainbow trout (Pickering et al., 1991). However, few studies have examined the effects of early life stress on growth in adulthood. The findings of the present study indicate that individuals can recover from the detrimental impacts of stress on growth if given sufficient time, a trend that also was detected in Atlantic salmon in response to an unpredictable chronic stressor (Vindas et al., 2016), and runs parallel to what is observed with compensatory growth, the period of accelerated growth that occurs in fish when food is re-supplied following a period of deprivation (Ali et al., 2003). Compensatory growth reflects both higher-than-normal food intake and endocrine regulation, particularly involving growth hormone and insulin-like growth factor (IGF) (Won and Borski, 2013). Interestingly, both food intake and endocrine pathways that regulate growth are influenced by the HPI axis, and are modified in response to stress (Madison et al., 2015; Pickering et al., 1991).

Despite well-established relationships for the effects of cortisol on metabolic rate, at least in juvenile fish (De Boeck et al., 2001; Eriksen et al., 2006; Morgan and Iwama, 1996), changes in survival and growth in zebrafish fry exposed to a stressor early in life occurred almost completely in the absence of changes in metabolic rate or respiratory exchange ratio, an indicator of fuel use. A significant decrease in $\dot{M}O_2$ relative to control fish at 15 dpf was detected in response to stress at both 4 and 7 dpf, yet this trend was not present in $\dot{M}CO_2$, providing little evidence for a biologically-relevant change in metabolism. This result contrasts with data for brown trout, where increased embryonic cortisol, mimicking maternal stress, increased $\dot{M}O_2$ in juvenile fish (Sloman, 2010). Clearly, more investigation of the impact of cortisol treatment on metabolic rate during early development is warranted.

Alternatively, changes in growth may have been related to stress-induced reductions in energy intake, either from the yolk or via food consumption (Bernier, 2006; Bernier and Peter, 2001a). In adult goldfish (*Carassius auratus*), elevated mRNA levels of CRF reduced food intake (Bernier and Peter, 2001b), and juvenile rainbow trout exposed to chronically increased cortisol levels via micro-osmotic pumps exhibited reduced growth related to decreased food intake (Madison et al., 2015). The proposed mechanism behind this effect involved increased mRNA levels of liver leptin (*lep-a1*) and preoptic area CRF (*crf*), resulting from chronically increased hepatic insulin-like growth factor binding protein caused by the increased circulating cortisol levels, which together reduced growth and increased energy mobilization while supressing appetite (Madison et al., 2015). Whether similar effects occur during early-life development in fish exposed to elevated cortisol levels requires investigation. Individual food consumption was neither controlled nor monitored in the present study.

4.3 Increased whole-body Na^+ and Ca^{2+} concentrations

The impacts of early-life stress on whole-body ion concentrations were somewhat variable across developmental periods sampled, yet when effects were present there was a consistent increase in ion concentrations in response to stress treatment. These results are in agreement with previous research that demonstrated both increased Na^+ (Kumai et al., 2012; Lin et al., 2016a) and Ca^{2+} (Kumai et al., 2015; Lin et al., 2011; Lin et al., 2016b) uptake from the environment in response to cortisol treatment of zebrafish embryos. For whole-body concentrations of both Na^+ and Ca^{2+}, exposure to a stressor at 7 dpf had particularly pronounced effects across the developmental stages that were examined, with the greatest response seen in whole-body Ca^{2+} concentrations measured at 35 dpf, which were nearly 5-fold higher than those of control individuals. The disproportionate impact of exposure to a stressor at 7 dpf may reflect 7 dpf being an intermediate age between ionoregulatory functions being carried out via the integument, and the switch to reliance on gills for ion uptake that occurs at ~14-15 dpf (Rombough, 2002). The stronger effects on Ca^{2+} relative to Na^+ concentrations at later ages may be related to the relative importance of Ca^{2+} in mineralization of the zebrafish skeletal system during the second week of life (Du et al., 2001; Kumai et al., 2015).

These data are significant in demonstrating a role for stress-induced elevation of cortisol in influencing salt/water balance in developing zebrafish, even when separated from an ionic or osmotic stressor. Studies of cortisol's role in ionic regulation in developing zebrafish typically have employed waterborne exposure to relatively high cortisol levels (e.g. Kumai et al., 2012; Lin et al., 2011; Lin et al., 2016a; Lin et al., 2016b), although cortisol responses to ionoregulatory stressors such as acidic water (Kumai et al., 2012), low water Na^+ concentrations (Lin et al., 2016a), and seawater exposure (Alderman and Bernier, 2009) have been detected. The results of the present study indicate that endogenous cortisol elevation, even in the absence of an ionoregulatory stressor, is capable of influencing whole-body ion concentrations. It is likely that the effects of repeated acute stress on whole-body ion concentrations reflect the impact of cortisol in increasing ion uptake from the environment, via regulation of ion transport proteins (Kumai et al., 2012; Lin et al., 2016a) and/or ionocyte numbers (Cruz et al., 2013a; Cruz et al., 2013b). Interestingly, neither an acute air emersion stressor nor chronic social stress affected ionocyte numbers in the gills of juvenile rainbow trout (Sloman and Gilmour, 2000; Sloman et al., 2001), suggesting the possibility of a developmental window for the effect on ion balance of stressor-induced cortisol elevation.

4.4 Altered whole-body cortisol concentrations

Research, primarily focused on the effects of maternal stress, increasingly has found that chronically increased cortisol levels during embryonic development significantly impact HPI axis responses at later developmental stages in fish (Auperin and Geslin, 2008; Jeffrey and Gilmour, 2016; Redfern et al., 2017). A common finding is that maternal stress or cortisol exposure during embryonic development (to mimic maternal stress) results in an attenuated cortisol response (Auperin and Geslin, 2008; Faught et al., 2016; Fokos et al., 2017; Sopinka et al., 2016), a trait that is commonly associated with a proactive phenotype (Koolhaas et al., 1999). Although the mechanisms responsible for this relationship remain to be fully elucidated, manipulation of embryo cortisol levels by microinjection of cortisol or antibodies against cortisol (to lower embryo cortisol levels) revealed changes in transcript abundance of key genes in the HPI axis, including CRF and two enzymes involved in cortisol biosynthesis, 11ß hydroxylase and StAR (Nesan and Vijayan, 2016). Recent work on European sea bass reported that early life stress caused decreased neural cell proliferation, an effect that was unrelated to GR and MR mRNA expression (Fokos et al., 2017). The present study adds to this body of research by reporting that exposure to a stressor and the associated elevation of endogenously produced cortisol during larval development influences HPI axis activity of zebrafish fry.

In particular, exposure to a stressor at 4 or 7 dpf lowered baseline cortisol levels at 15 dpf, whereas exposure to a stressor at 15 dpf stress raised baseline cortisol levels at 35 dpf, effectively dampening the cortisol response. The mechanisms responsible for these effects require investigation. If lower growth rates in fish exposed to a stressor reflected reduced food intake (see above), this factor might also have contributed to the lowered baseline cortisol levels in 15 dpf fish because unfed zebrafish larvae exhibited significantly lower baseline cortisol concentrations than fed larvae (Alderman and Bernier 2009). Diminished HPI axis responsiveness at 35 dpf may serve to buffer against the potential masculinizing effects of elevated cortisol (see below; Ribas et al., 2017) at a time when developing zebrafish are entering the period of sexual differentiation (Uchida et al., 2002). Under these conditions, however, baseline cortisol concentrations might also have been expected to be reduced.

Although the underlying mechanisms remain to be determined, the findings of the present study suggest that development of the HPI axis exhibits plasticity in response to early life exposure to a stressor, suggesting in turn that there are selective advantages to modification of the cortisol response under certain conditions. However, there is evidence that these changes may become maladaptive in response to chronic stress (Barton and Iwama, 1991; Wendelaar Bonga, 1997), or in situations where there is a mismatch between the conditions that initially trigger them and the environment the organism eventually finds itself in (Chevin and Hoffmann, 2017; Sopinka et al., 2014). It is unclear what the adaptive significance of these short-term changes in cortisol production might be, but they are likely context dependent and strongly influenced by developmental stage, potentially imparting a selective advantage to the individual later in life based on previous environmental conditions.

4.5 Altered anxiety-related behaviours

Anxiety-related behaviours are often indicative of the coping style or behavioural reaction norms that individuals utilize in response to an acute stressor, and as such are closely related to HPI axis activity and the cortisol response (Dingemanse et al., 2010; Koolhaas et al., 1999). Behavioural reaction norms are the suite of behavioural responses that arise through interplay between consistent individual differences in behaviour that often are referred to as 'personalities' or behavioural syndromes (Bell, 2007; Biro and Stamps, 2008; Sih et al., 2004), and the effects of phenotypic plasticity over time (Dingemanse et al., 2010). Recent research demonstrated that cortisol treatment of embryos to mimic increased maternal cortisol deposition into oocytes led to a more proactive coping style as indicated by increased activity under lighted conditions (when zebrafish larvae normally exhibit low activity) and reduced thigmotaxic tendencies (Best et al., 2017). These differences in behaviour following cortisol treatment were accompanied by increased neurogenesis in specific brain regions (Best et al., 2017), suggesting a mechanism through which elevated cortisol may influence behaviour. By contrast, thigmotaxic behaviour was unaltered in 3 dpf zebrafish larvae exposed to waterborne cortisol for 24 h to mimic early life exposure to a stressor, and these fish exhibited elevated activity under both light and dark conditions (Best and Vijayan, 2017). In 5 dpf zebrafish larvae that had been exposed from 1 hour-post-fertilization to waterborne dexamethasone (a synthetic glucocorticoid), elevated thigmotaxis was detected with no change in activity (Wilson et al., 2013). Exposure to the cortisol synthesis inhibitor metyrapone or the GR antagonist RU486 to block cortisol signalling, however, was without effect on thigmotaxis and reduced activity (Wilson et al., 2013). These studies demonstrate that the effects of cortisol on behaviour in developing zebrafish depend on the developmental stage at which cortisol levels are altered, and likely also on the cortisol levels that are achieved, emphasizing the importance of studying the effects of endogenous elevation of cortisol in response to a stressor.

When measured at 7 dpf, zebrafish stressed at 4 dpf had significantly decreased activity relative to control fish, indicating increased anxiety-related behaviour and a more reactive coping style (Koolhaas et al., 1999). This finding is in contrast to previous results for larval zebrafish exposed to waterborne cortisol, which resulted in increased activity (Best and Vijayan, 2017), and occurred without any change in thigmotaxis, which might be expected to increase following a typical reactive coping style (Koolhaas et al., 1999). When measured at later ages, stress treatment increased thigmotaxis, with the effects being most prominent when measured at 35 dpf, where all stress treatments significantly increased thigmotaxis relative to control fish. These effects suggest that early-life stress results in a more reactive coping style in zebrafish fry, yet the opposite trend was seen in the activity of 35 dpf fish. Fish stressed at 7 dpf had increased activity relative to both control and the other stress treatments when measured at 35 dpf, a response more typical of a proactive coping style. These trends in activity and thigmotaxis are in accordance with previous findings for larval zebrafish (Best and Vijayan 2017; Best et al., 2017;

Wilson et al., 2013), yet at this age represent a distinct contrast in what are typically considered closely related behavioural traits encompassing coping styles (Dingemanse et al., 2010; Koolhaas et al., 1999).

Together the data of the present study indicate that anxiety-related behaviour in zebrafish larvae and fry is influenced by early-life exposure to a stressor, with the responses to early-life stress being dependent on the developmental age at which the stressor was experienced. The adaptive significance of these trends remains to be determined. Similar to the findings for the HPI axis, it is likely that the development of these behavioural phenotypes provides adaptive benefits when matched with the same environmental conditions that initially triggered their development (Love and Williams, 2008b). Evidence for this possibility has come from studies examining maternal effects on offspring behaviour, where predation pressure on mothers resulted in offspring behaviour that reduces predation risk, such as increased shoal cohesion (Giesing et al., 2011) and enhanced burst swimming capability (Sopinka et al., 2014). Where effects may become maladaptive is when there is a mismatch between the initial conditions that triggered the development of these phenotypes and the conditions the organism is currently experiencing (Chevin and Hoffmann, 2017). For example, the enhanced burst swimming capability of sockeye salmon offspring in response to maternal stress may prove adaptive in habitats with high predation risk, but may also incur metabolic costs and reduce competitive ability in an environment with low predation risk (Sopinka et al., 2014). In combination with the effects observed for whole-body cortisol concentrations, it is evident that there is a significant degree of developmental plasticity in HPI axis function, allowing both stress responsiveness and anxiety-related behaviour to be modulated in response to relevant environmental stimuli in an age-dependent manner.

4.6 Sexual differentiation

In the current study, experimental tanks of zebrafish fry exposed to a repeated acute air-exposure stressor at 35 dpf exhibited a significantly higher proportion of males per tank than control tanks. The 35 dpf stress treatment coincided with a period of intense structural and hormonal reorganization that is associated with sexual differentiation in most strains of domestic zebrafish (Spence et al., 2008; Takahashi, 1977; Uchida et al., 2002). Based on the results obtained in the present experiment, this time-period appears to be a critical developmental window, where the effects of stress are strongly influential in determining adult sex. Notably, exposure to the same stressor earlier in development (at 4, 7 or 15 dpf) did not have a significant impact on the proportion of males in experimental tanks. Previous studies also found that early-life stress may play a role in sexual differentiation. For example, exposure to high stocking densities as a chronic stressor, or use of dietary cortisol supplementation from 15-45 dpf to chronically increase cortisol levels, both induced masculinization in zebrafish (Ribas et al., 2017). These results directly implicated cortisol in the masculinizing effects of chronic stress, possibly through up-regulation of 11ßHSD2, which has been shown to increase androgen levels, and hence induce masculinization (Fernandino et al., 2012; Fernandino et al., 2013). Whether a similar mechanism operates to elicit masculinization with activation of the endogenous stress response, as in the present study, remains to be determined.

Exposure to hypoxic conditions as early as 24-36 hours post fertilization also induced masculinization (Robertson et al., 2014). At this point, prior to hatching, all cortisol present is maternally derived (Alsop and Vijayan, 2008), and therefore the masculinizing effect of hypoxic exposure is unlikely to reflect cortisol signalling. Rather, the effects of hypoxic exposure on sexual differentiation may be related to hypoxia inducible factor (HIF-1; Robertson et al., 2014). Embryonic decreases in gonadal aromatase levels are elicited by hypoxia, likely through a HIF-mediated mechanism, and result in decreased testosterone concentrations (Ivy et al., 2017). Despite the strong effects of stress at 35 dpf on sexual differentiation in the present study, no impact was noted on GSI in female or male fish. The only significant influence on these measures of reproductive success was the density at which fish were housed, implicating resource limitation as the likely cause behind decreased reproductive condition, as all tanks were fed the same amount daily.

Given the results of the current study, developmental plasticity appears to play a key role in sexual differentiation in domesticated zebrafish. It is possible that stress during the period of sexual differentiation primes individuals to adopt a more adaptive phenotype given environmental constraints (Ivy et al., 2017). However, it is difficult to infer any real adaptive significance behind these systems, as polygenic sex determination is restricted to domesticated zebrafish and was incurred through domestication (Wilson et al., 2014). These potential links among the HPI axis, behavioural reaction norms, and polygenic sex determination in zebrafish require further research, but may provide insights into the adaptive significance of developmental plasticity in sex determination for other polygenic species (Fernandino et al., 2012; Hayashi et al., 2010; Yamaguchi et al., 2010). Aside from the implications for sexual differentiation in other species, the current study builds on the findings of Ribas et al. (2017) by demonstrating that an acute stressor, rather than a long-lasting chronic stressor, can induce masculinization when experienced during the period of sexual differentiation.

4.7 Future directions

While identifying key areas to target future research, the scope of the present study did not allow in-depth examination of the mechanisms underlying the effects of early life exposure to stress on physiological variables. Additional work is essential to link the events of an acute endogenous stress response directly to development. In response to early-life stress, changes in growth were largely independent of metabolic changes. The relationship between early-life stress, food intake (Bernier and Peter, 2001a; Madison et al., 2015), and circulating levels of growth hormone (McCormick et al., 1998; Pickering et al., 1991) should be examined further to determine the cause of altered growth rates. In examining potential alterations to the HPI axis, levels of StAR, P450scc, and 11βHSD2 should be measured to characterize the HPI axis response to an acute stressor, as well as the mechanisms behind long-term modification and dampening of the cortisol response in juvenile fish (Alsop and Vijayan, 2009). Along these lines, the interaction between the HPI axis and production of sex hormones should be more closely examined. In particular, the role 11βHSD2 plays in synthesis of androgens (Fernandino et al., 2013), and the negative impacts of increased cortisol on estrogen synthesis (Devlin and Nagahama, 2002; Lethimonier et al., 2000) should be further examined. To determine the mechanisms responsible for increased ion uptake in fish exposed to early life stress, attention should be directed to the transport proteins responsible for Na^+ and Ca^{2+} uptake, including NHE, NCC, and NKA, as well as ionocyte abundance (Kumai et al., 2012; Lin et al., 2011; Lin et al., 2016a). The relative importance of GR versus MR in crosstalk between glucocorticoid and mineralocorticoid functions of cortisol also remains unclear. Previous work has identified GR as playing a more significant role than MR in response to an osmotic stressor (Ivanis et al., 2008; Kelly and Chasiotis, 2011; Lin and Randall, 1993; Takahashi and Sakamoto, 2013), yet how these two receptors function in concert with cortisol to modify ion uptake in response to a non-osmotic stressor is unknown. Finally, variables measured in the current study should be followed to adulthood, which would be a necessary next step in understanding the long-term impacts of early-life stress.

4.8 General conclusions

The current study examined a range of physiological and behavioural variables at several key developmental stages to better understand the effects of early-life stress on the development of zebrafish. The findings of this study address several knowledge gaps in the literature. These include the impacts of an early-life, endogenously produced stress response rather than the effects of maternal cortisol, the long-term effects that stress during larval stages has on juvenile and sexually mature fish, the important dual nature of cortisol as both a glucocorticoid and mineralocorticoid, and interactions between environmental cues and critical developmental windows.

Across the range of variables measured, the effects of repeated activation of an endogenous stress response in early life were most prominent when measured shortly after treatment. Owing to this effect, findings for 7 dpf fish frequently fell into line with those of previous studies, which for the most part have focused on the larval developmental stage in zebrafish (Kumai et al., 2012; Nesan and Vijayan, 2016; Wilson et al., 2013). However, the results of the current study suggest that with greater latency between stress treatment and sampling, physiological impacts are diminished, indicating that individuals are able to compensate and recover from many of the early-life effects of stress, which often have been considered maladaptive (Groothuis and Taborsky, 2015; Wendelaar Bonga, 1997). This phenomenon is perhaps best exemplified by the effects of early-life stress on growth in the current study, which resulted in prominent negative effects when measured at earlier developmental stages, yet when measured at adulthood, had no significant effect on any of the morphological endpoints measured. Similarly, Vindas et al. (2016) found that stress in juvenile Atlantic salmon resulted in compensatory growth such that adult fish from control and treatment groups showed no significant differences in body weight and condition when measured following smoltification. The data of the current study suggest that zebrafish are for the most part able to recover from early-life stress, performing no differently from unstressed conspecifics if given adequate time.

Although the passage of time diminished the effects of stress in many of the variables and time points examined, stress treatment at 7 dpf had a disproportionately large impact on anxiety-related behaviour and whole-body ion concentrations measured at later ages. This time period coincides with the transition from larvae to fry, encompassing the beginning of exogenous feeding (Chu and Sadler, 2009), greater mobility (Müller and van Leeuwen, 2004), increased skeletal mineralization (Du et al., 2001), and the development of a robust response to external stressors (Alderman and Bernier, 2009; Alsop and Vijayan, 2009). The long-lasting effects on anxiety-related behaviour and ion regulation together with the large number of developmental changes that occur suggest that 7 dpf represents a critical developmental window for a variety of physiological functions. The few studies that have examined the long-term impacts of early-life stress on zebrafish development employed chronic stress (Ribas et al., 2017) or embryonic exposure to a stressor (Ivy et al., 2017; Robertson et al., 2014), leading to a diminished ability to detect critical developmental windows past hatching. The results of the present study are novel in demonstrating the likely existence of a critical developmental window at 7 dpf, and in providing evidence that a cortisol response to a non-osmotic stressor can influence mineralocorticoid functions of cortisol during early developmental stages in zebrafish, providing direct evidence of cross-talk between cortisol's two primary roles in teleost fish.

Collectively, the findings of the present study suggest that early-life exposure to a stressor can serve as an environmental cue to activate polymorphisms in zebrafish, with effects being mediated through the HPI axis in an age dependent manner. Many of the phenotypic responses to early life stress appear to dissipate given sufficient time, emphasizing the concepts of contextual adaptiveness and critical developmental windows in producing adaptive phenotypes. The present study also demonstrated crosstalk between the glucocorticoid (stress) and mineralocorticoid functions of cortisol, an area of research that has been relatively under-studied.

Zebrafish have become a widely-used model organism for studying development and human diseases (Howe et al., 2013), and an increased understanding of how early-life stress influences their physiology therefore is essential. Given the rapid rate of environmental change that is occurring owing to human-induced climate change (Walther et al., 2005), the findings of the present study on zebrafish may be useful in increasing our understanding of how other freshwater fish species may respond to the challenges of climate change. These may include changes in ion uptake in response to hypoxic and acidic water conditions caused by rising temperatures (Whitehead et al., 2009), as well as changes in coping style and associated HPI axis responsiveness caused by early-life stress related to range expansions of competing and predatory species (Rahel and Olden, 2008). By providing a broad view of the physiological impacts of early-life stress over the development of a commonly-used model species, the present study lays critical groundwork for further exploration of the mechanisms underlying developmental plasticity.

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