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Developmental Effects of Cortisol on the Stress Response and Behaviour in Zebrafish

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Developmental Effects of Cortisol on the Stress Response and Behaviour in Zebrafish

by

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Abstract

The objective of the study was to determine whether maternal stress affects early development in zebrafish (*Danio rerio*). The hypothesis was that maternal stress will lead to excess cortisol deposition in the embryos, and this will compromise the larval stress response and locomotor activity. Maternal stress and the attendant rise in cortisol levels led to excess cortisol deposition, but only transiently in the embryos. This was kept in check by cortisol-induced stimulation of *11βHSD2*, a cortisol-degrading enzyme, in the ovarian follicles. Excess embryo cortisol content disrupted the larval stress response, locomotor activity, and was anxiolytic. These phenotypes corresponded with increased region-specific neurogenesis. Post-hatch elevation in cortisol levels by waterborne exposure, mimicking a stress response, increased locomotor activity but did not impact anxiety behaviour. Overall, maternal stress alters larval stress and behavioural phenotypes, underpinning a distinct developmental effect associated with elevated zygotic cortisol content in zebrafish.

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List of Symbols, Abbreviations and Nomenclature

Symbol	Definition
11βHSD2	11β hydroxysteroid dehydrogenase type 2
17,20P	17α, 20β-dihydroxy-4-pregnen-3-one
ACTH	adrenocorticotropic hormone
bHLH	basic helix-loop-helix
BMP	bone morphogenetic protein
CRF	corticotropin-releasing factor
cyp11a	cytochrome p450 11a (P450scc)
DAPI	4',6-diamidino-2-phenylindole (nuclear stain)
DOC	11-deoxycorticosterone
dpf	days post fertilization
DT	dorsal thalamus
EdU	ethynyl deoxyuridine
F	cortisol
GH	growth hormone
GR	glucorticoid receptor
GRE	glucocorticoid response element
GSI	gonadosomatic index
GVBD	germinal vesicle breakdown
H	hypothalamus
HB	hindbrain
hpf	hours post fertilization
HPI	hypothalamus-pituitary gland-interrenal tissue
hsd3b	3β-hydroxysteroid dehydrogenase
IGF	insulin-like growth factor
isl1	islet 1
K	condition factor
MB	midbrain
MC2R	melanocortin 2 receptor
MNE	mean normalized expression
MR	mineralocorticoid receptor
neurod4	neurogenic differentiation 4
otpb	orthopedia b
P	pallium
p450scc	cytochrome p450 11a (side chain cleavage)
POA/Po	preoptic area
POMC	proopiomelanocortin
PT	posterior tuberculum
Ret	retina
RU-486	Roussel-Uclaf 486, mifepristone (GR antagonist)
StAR	steroidogenic acute regulatory protein
Tel	telencephalon

Chapter One: General Introduction

The stress response is the coordinated set of physiological and behavioural responses that allow the animal to cope with a stressor and regain homeostasis. Both the catecholamine and glucocorticoid stress hormones mediate these responses and help the animal overcome the threat. Activation of the sympathetic nervous system and catecholamine release occurs within seconds of stressor perception (the flight-or-fight response), while glucocorticoid synthesis and release occurs more slowly, mobilizing and replenishing energy stores to cope with the stressor (Vijayan et al., 2010). Glucocorticoid hormones have been shown to affect the developmental programming events in vertebrates (reviewed by Harris and Seckl 2011, Nesan and Vijayan 2013b, Schurrmans and Kurrasch 2013, and Moisiadis and Matthews 2014), but the mechanisms are far from clear.

Recent studies examined the ontogeny of the stress axis in zebrafish (*Danio rerio*; Alsop and Vijayan 2008, 2009a), and demonstrated that glucocorticoid receptor (GR) signalling is essential for the regulation of early developmental events in this species (Nesan et al. 2012, Nesan and Vijayan 2012, 2013a, 2013b). However, it is unclear whether maternal stress will lead to the transfer of excess cortisol, the primary stress corticosteroid in teleosts, to the embryos, and if that would in turn affect developmental programming in fish. This thesis examined the transfer of cortisol from stressed mother to the embryo in an asynchronously breeding fish, the zebrafish, and also investigated how this excess zygotic cortisol deposition affects the stress response, behaviour and neurogenesis in the larval offspring.

1.1 Stress Physiology

The physiological response to stress is important for survival and, therefore, highly conserved among vertebrates. A stressor is defined as any stimulus that pushes an organism outside of its normal homeostatic limits (Wendelaar Bonga 1997). In response to stressor exposure, a coordinated set of physiological and behavioural responses are evoked that allows the organism to regain homeostasis (Wendelaar Bonga 1997). The glucocorticoid response to stress in teleosts is mediated by the hypothalamic-pituitary-interrenal (HPI) axis. The interrenal tissue in fish contains steroidogenic cells and is analogous to the adrenal gland in higher vertebrates (Vijayan et al. 2010). The response to stress begins after stressor perception by activation of CRF-secreting (corticotropin releasing factor) neurons in the preoptic area (POA) innervating the adenohypophysis (Bernier et al. 2004). In mammals, CRH (corticotropin releasing hormone) is one of six of the parvocellular neuroendocrine cells (also thyrotropin-releasing hormone, growth hormone-releasing hormone, somatostatin, gonadotropin-releasing hormone, and dopamine) that secrete to the anterior pituitary via the hypophyseal portal system (Löhr and Hammerschmidt 2011). In teleosts, most of these neuroendocrine secretory cells are localized to the preoptic region and directly innervate the anterior pituitary, as fish lack the hypophyseal portal system (Löhr and Hammerschmidt 2011, Pogoda and Hammerschmidt 2009). Transcripts of *crf* are expressed in the preoptic and tuberal hypothalamic nuclei, as well as in the olfactory bulbs and dorsal telecephalon (Alderman and Bernier 2007). In addition to its role as the major secretagogue for ACTH (adrenocorticotropic hormone), CRF is anorexigenic (De Pedro et al. 1993, Inui 1999, Lin et al. 2000, Matsuda 2009, Matsuda 2013) and has been shown to increase locomotor

and anxiety-like behaviour in fish (studies by Clements et al. 2002, Carpenter et al. 2007, Backström et al. 2011, reviewed by Backström and Winberg 2013).

CRF signaling results in the secretion of adrenocorticotropic hormone (ACTH) from the anterior pituitary into the blood (Wendelaar Bonga 1997). Once in circulation, ACTH binds the melanocortin 2 receptor (MC2R) on the steroidogenic cells of the teleost head kidney (Wendelaar Bonga 1997). The subsequent signalling cascade from this G-protein coupled receptor results in the shuttling of cholesterol from the outer to the inner mitochondrial membrane by the steroidogenic acute regulatory (StAR) protein, the rate limiting step in steroidogenesis (Stocco 2000). This is followed by the conversion of cholesterol into cortisol via cytochrome P450 enzymes and steroid dehydrogenases, ultimately leading to cortisol secretion into the blood for delivery to target tissues. Studies to date have not conclusively found a corticosteroid-binding globulin (CBG) in fish, which binds most of the circulating corticosteroids in mammals, controlling its bioavailability (Mommsen et al., 1999; Vijayan et al. 2010). Although plasma proteins, including albumins may bind (low affinity) cortisol (Mommsen et al., 1999), clearly further studies are warranted to understand how cortisol in transported in fish blood given the lack of a high affinity binding protein for this steroid (Mommsen et al. 1999).

Cortisol exerts the majority of its effects through the cytosolic glucocorticoid receptor (GR), a ligand-activated transcription factor (Mommsen et al. 1999). Cortisol diffuses through the cell membrane, binds to GR, which then translocates to the nucleus and binds to glucocorticoid response elements (GREs) in the promoter regions of target genes. Most teleost fish have two copies of *gr*, *gr1* and *gr2*, as the result of the teleost-specific genome duplication that occurred 320-350 MYA (Alsop et al. 2009b). However,

zebrafish are unique among teleosts in that they have only one *gr* gene with two splice variants, zgrα and zgrβ (Alsop and Vijayan 2008, 2009b, Schaaf et al. 2008). This is very similar to the glucocorticoid receptor system seen in humans, with the β-isoform lacking a portion of the transactivation domain resulting in a loss of transactivational activity, as well as a dominant negative inhibition of transactivation of the alpha isoform (Schaaf et al. 2008). Teleosts also have a mineralocorticoid receptor (MR) but lack aldosterone and its biosynthetic pathway (Sturm et al. 2005). While cortisol binds MR with high affinity and can have mineralocorticoid functions, including ion regulation and seawater adaptation, 11-deoxycorticosterone (DOC) is considered a good candidate for an MR-specific ligand in fish due to its high affinity for MR and not GR (Sturm et al. 2005, Pippal et al. 2011). Both receptors are expressed in many major tissues, but the role of MR is still unclear in fish (Sturm et al. 2005; Nesan and Vijayan, 2013).

In teleosts, cortisol affects all aspects of physiology, including metabolism, hydromineral balance, reproduction, growth, and immune function (Vijayan et al., 2010). Cortisol elevations increase glycolysis, gluconeogenesis (mainly from amino acid precursors), and lipolysis (Mommsen et al. 1999) and these effects are adaptive by providing substrates to cope with the increased energy demand during stress. However, persistent elevations in cortisol levels can be maladaptive due to energy repartitioning away from important life processes such as growth and immune function (Iwama 1998). Cortisol and stress have mainly inhibitory effects on reproductive performance and growth, as the metabolic demands of coping with the stressor divert energy away from these anabolic processes (reviewed by Schreck et al. 2001, Milla et al. 2009). Cortisol is metabolized in the body via processes that render this lipophilic molecule more soluble

for excretion, including hydroxylation by cytochrome P450 enzymes and glucuronidation (Vijayan et al. 2010). Cortisol may also be converted to inactive cortisone by 11β-hydroxysteroid dehydrogenase type 2 (11βHSD2), helping to protect glucocorticoid sensitive tissues from this steroid excess, and mediate MR-specific signaling (Alderman and Vijayan 2012).

1.1.1 Ontogeny of the Stress Response in Zebrafish

The zebrafish is a popular model in developmental biology because the embryo is transparent, their development is rapid, their maintenance is simple, and they reproduce abundantly (Link and Megason 2008). Immediately following fertilization, cells begin to divide and by 10 hours post fertilization (hpf), the zygote has gone through the cleavage, blastula and gastrula stages, and the primary germ layers have formed (Kimmel et al. 1995). The segmentation period occurs from 10-24 hpf, marked by the organization of the body axis into somites and the brain primordia into neuromeres, and the start of organogenesis (Kimmel et al. 1995). The pharyngula period occurs during the second day of development; organogenesis continues, pigmentation increases and fins and sensorimotor abilities develop in preparation for hatching (Kimmel et al. 1995). Hatching occurs over the third day of development and by 72 hpf, most morphogenesis is complete and after 5 dpf larvae begin to feed as the yolk depletes during the first two weeks (Figure 1).

Transcripts of *crf* are detectable in the whole body from fertilization indicating maternal deposition, while *de novo* synthesis of maternal transcripts commences from 6 hpf following the midblastula transition at 2.75 hpf (Kimmel et al. 1995, Alderman and Bernier 2009). The *crf* transcripts are found in the hypothalamus and telencephalon by 24

hpf (Chandrasekar et al. 2007). The pituitary begins formation at the start of the segmentation period and is a morphologically distinct structure by 24 hpf and is in its final position by 60 hpf (Pogoda and Hammerschmidt 2009). Initial detection of *pomc* (proopiomelanocortin) occurs at 18-20 hpf, which then forms a distinct cell cluster in the early pituitary by 24 hpf (Liu et al. 2003).

The adrenal analogue in teleosts is the head kidney, and this contains steroidogenic cells, as well as chromaffin cells (secrete catecholamines) and hematopoietic cells (Chai et al. 2003). The steroidogenic cells originate from the mesoderm while the chromaffin cells originate from the neuroectoderm (Chai et al. 2003). The earliest marker of interrenal development is *ff1b* (*ftz-f1*) at 22 hpf followed by transcripts of *star* at 24 hpf (Chai et al. 2003, To et al. 2007). Expression of *cyp11a2* (*p450 side chain cleavage,* cholesterol to pregnenolone) begins at 32 hpf (Parajes et al. 2013). Active enzymatic activity of *hsd3b1* (3β-hydroxysteroid dehydrogenase that converts pregnenolone to progesterone) appears at 28 hpf, followed by *mc2r* transcripts (Chai et al. 2003, To et al. 2007).

Although the stress axis develops only after hatch (48 hpf), most of the components of the HPI axis are in place by 32 hpf (Alsop and Vijayan 2008; Nesan and Vijayan 2013b). However, the earliest detected cortisol elevation in response to a stressor occurs at 72 hpf (Alderman and Bernier 2009, Nesan 2013). The lack of a cortisol response to stress during early development is known as the stress hyporesponsive period, and is observed in several fish species (Nesan and Vijayan 2013b), rodent models (Gunnar and Quevedo 2007), and possibly humans (Lupien et al. 2009), and is thought to have a protective role against the deleterious effects of glucocorticoids.

1.2 Maternal Effects and Programming

Progeny receive not only genetic information from their parents, but also information about any stressors due to environmental adversity that may influence their development (Khulan and Drake 2012). Developmental processes are highly conserved and regulated, but poor environmental conditions or stressors (e.g. undernutrition), can alter these developmental trajectories in a process known as developmental programming (Harris and Seckl 2011). Maternal stress and the attendant elevation in glucocorticoid hormone levels may exert such programming effects (Nesan and Vijayan 2013b).

1.2.1 Maternal Transfer

In teleosts, maternal steroids are thought to be incorporated into the potential offspring during oogenesis. Oogenesis in the zebrafish has been previously characterized, and the time required for primary oocytes to develop into mature follicles is 10 days (Wang and Ge 2004). Cortical alveoli appear during day 3 of oogenesis but no yolk, but substantial yolk formation is visible at the mid-vitellogenic stage (day 6) until the fully grown immature follicle (day 10). Mature oocytes (that have undergone germinal vesicle breakdown, GVBD) are then released from their follicle and expelled from the mother during the next spawning event for fertilization (Clelland and Peng 2009). Importantly, zebrafish breed asynchronously and their ovaries contain follicles undergoing several different stages of oogenesis that allows them to breed continuously, as opposed to synchronous development seen in seasonal breeders, including the rainbow trout (*Oncorhynchus mykiss*; Rocha and Rocha 2006).

The fish yolk is composed of lipoproteins and polyunsaturated fatty acids, including DHA (docosahexaenoic acid), which are selectively delivered to the oocyte

(Weigand 1996). Vitellogenin, the yolk protein precursor produced in the liver, as well as various hormones including cortisol are also delivered to the oocytes via the blood (Wiegand 1996, Mommsen and Walsh 1988). Maternal cortisol is incorporated into the yolk during the vitellogenic stage. While this can occur in oviparous animals, including fish species as well as birds (Henricksen et al. 2011), mammalian steroid transfer occurs via the placenta. The presence of 11βHSD2 in the placenta is protective, converting cortisol into inactive cortisone (Chapman et al. 2013), but a similar mechanism in non-placental species has not been confirmed. While low levels of *11βhsd2* transcripts have been detected in the developing zebrafish (Alsop and Vijayan 2008), its physiological role during development has not been characterized to date. However, ovarian follicular conversion of radiolabelled cortisol to cortisone in tilapia (*Oreochromis mossambicus*, Tagawa et al. 2000), as well as sulphation of cortisol in rainbow trout (Li et al. 2012) has been observed, but the regulation and the significance of this steroid breakdown on reproduction has not been well characterized in fish. Reflection of the mother's stress state in the offspring as cortisol elevation has been observed in fish species, including coho salmon (*Oncorhynchus kisutch*, Stratholt et al. 1997), threespined stickleback (*Gasterosteus aculeatus*, Giesing et al. 2011), and rainbow trout (Åberg Andersson et al. 2011).

1.2.2 Maternal Effects of Stress on Developmental Programming

The effects of glucocorticoids on development have been studied since the 1950s. Early experiments injecting cortisone into chick embryos and neonatal rats resulted in growth defects (Karnofsky et al. 1951, Field 1954). In several species, the effects of maternal stress or maternal glucocorticoid elevation on offspring include alterations in

HPI axis function as well as transcript abundances of key genes involved in the stress response (reviewed by Moisiadis and Matthews 2014) and an increase in basal cortisol level is often observed (rats, Green et al. 2011, Levitt et al. 1996; primates, Mustoe et al. 2014, Pryce et al. 2011). Disruption of the HPI response in offspring has also been shown in several species but the effects on stress-induced glucocorticoid levels are not consistent (no effect, Hauser et al. 2007, 2009; increase, Barbazanges et al. 1996, Hayward and Wingfield 2004, Mustoe et al. 2014; decrease, Hayward et al. 2006, Love and Williams 2008). Effects of prenatal stress on the brain of several vertebrate species are reviewed by Charil et al. (2010) and regions typically affected by prenatal stress include the hippocampus, amygdala and hypothalamus with changes observed in brain morphology, cell density and type, and neurogenesis (rats, Meyer 1983, Kawamura et al. 2006, Van den Hove et al. 2006, Llorente et al. 2009, Lucassen et al. 2009, mice, Noguchi et al. 2008; sheep, Dunlop et al. 1997; primates, Uno et al. 1990).

Developmental effects of maternal stress in model mammalian species have been well studied as indicated above, particularly in rodents. However, there is a dearth of knowledge in this subject area for teleosts. Nesan and Vijayan (2013b) have provided evidence for glucocorticoid-mediated programming of development in the zebrafish. When glucocorticoid signalling during early development is disrupted in this species, effects on several systems are impacted, including but not limited to defects in the mesoderm and disrupted myogenesis upon GR knockdown by morpholino (Nesan et al. 2012), cardiac development and performance with elevated cortisol deposition (Nesan and Vijayan 2012), reduced sodium uptake upon transient GR knockdown (Kumai et al. 2012), reduction of the stress response (Nesan 2013), and potentially several other

systems as determined by transcriptomic analysis, including neurogenesis (Nesan and Vijayan 2013a). Although these studies have shown developmental programming effects in the zebrafish, little is known about the effects on their developing brain and their behavioural outcomes.

1.3 Zebrafish Behaviour

The use of larval zebrafish as a behavioural model is desirable as their nervous system is well conserved and relatively simple at this stage, facilitating the ability to link observed behaviours with brain development (Fero et al. 2011). In addition, they can be used for high-throughput and automated assessment of behaviour, which allows for more statistically powerful behavioural analyses. This, in combination with the fully sequenced genome and suite of molecular tools available, makes zebrafish an excellent model to examine the genetic and physiological underpinnings of simple behaviours (Link and Megason 2008, Steenbergen et al. 2011).

The first movements made by the embryo occur at 17 hpf, with spontaneous coiling movements, which increase in contraction speed until hatching (Saint-Amant and Drapeau 1998). Swimming movements in response to touch appear as early at 26 hpf (Saint-Amant and Drapeau 1998). After hatch, the swim bladder inflates and the fish transitions from lying on its side to gaining positive buoyancy and being able to swim freely (Fero et al. 2011). By 96 hpf, larvae are capable swimmers, and able to change direction towards targets (Levin and Cerutti 2009). The optokinetic response (OKR), the ability of the larvae to track moving objects with their eyes, also develops at this stage (Colwill and Creton 2011). The onset of feeding occurs at 5 dpf, resulting in the start of

size differences between animals (Fero et al. 2011). Subsequent to feeding onset, social behaviours develop and activities become more complex (Fero et al. 2011) (see Fig. 1).

Locomotor responses in zebrafish have been studied in great detail, with movements in response to "startles" or sudden tactile, acoustic or visual stimuli being well characterized (Fero et al. 2011). Visual stimuli are very important to the early larva, and by 4 dpf they display locomotor circadian rhythmicity, with lower activity at night and higher activity during the day, although this gradually declines over the course of a day (Fero et al. 2011, MacPhail et al. 2009, Cahill et al. 1998). In mammals, the effects of light on physiology and behaviour are well-known in terms of circadian activity; however, less is known about the effects of masking, the direct and immediate effects of light on an organism, and most studies to date have been performed using rodents (reviewed by Redlin 2001). This masking effect appears to be conserved among fish, and during the circadian daytime, zebrafish respond to abrupt light increase by freezing (decreased locomotor activity), and abrupt darkness by increase in locomotor activity (Burgess and Granato 2007). The purpose of this conserved behavioural response in response to light and dark is not clear. It has been suggested that the hyperactivity observed in response to sudden darkness is not a predator escape response due to its longer latency in comparison to the acoustic startle response (Burgess and Granato 2007). Instead, increased activity in the dark may be an attempt to seek the light required to carry out their normal activities (Burgess and Granato 2007). The freezing response to increased light intensity has been interpreted as this stimulus being aversive to the fish (MacPhail et al. 2009).

While cortisol is known to play an important role in circadian activity of zebrafish, specifically by regulating circadian rhythms of S-phase in the cell cycle (Dickmeis et al. 2007), little is known about the effects of cortisol on their locomotor response to changes in light intensity. The locomotor reponse to light and dark is well conserved in zebrafish and provides information about sensorimotor function, making it a suitable model to assess behaviour in larval zebrafish (Irons et al. 2010, 2013, Fernandes et al. 2012, Emran et al. 2008, MacPhail et al. 2009, Prober et al. 2006, Easter and Nicola 1996).

Thigmotaxis is a behavioural model that has been used as a surrogate for the measurement of anxiety in various species and is also known as the open field test in rodents (Simon et al. 1994, Treit and Fundytus 1988). Positive thigmotaxis towards the walls of a testing arena is an indicator of anxiety that has been validated with both anxiolytic and anxiogenic drugs in rodents (Treit and Fundytus 1988) and recently in larval zebrafish (Schnörr et al. 2012). Given the strong links between stress physiology and anxiety, this model has a lot of potential in the integration of behaviour with not only neurobiology, but also stress physiology in fish (Steenbergen et al. 2011, Egan et al. 2009).

1.4 Development of the Nervous System in Zebrafish

As the stress response can occur as early at 72 hpf, and the earliest movements in the zebrafish occur prior to hatch at 17 hpf, it is clear that the development of the nervous system in zebrafish must be very rapid to allow the larvae to independently cope with stressors within 3 days of development. The nervous system develops from the dorsal ectodermal germ layer during gastrulation, and this patterning is dependent on families of

signaling factors, including BMPs (bone morphogenetic proteins), Wnt (wingless-integrated genes), Fgf (fibroblast growth factors), and Sox (SRY-box genes) (Schmidt et al. 2013). Primary neurogenesis begins with neurulation at the start of the segmentation period (10 hpf), and by 18 hpf, 10 neuromeres are apparent: the telencephalon, diencephalon, midbrain, and 7 rhombomeres (Kimmel 1995). During the segmentation period, primary sensory and motor neurons develop and extend their axons (Kimmel et al. 1995). This coincides with the earliest observed movements and tactile responses in the fish, which are in place even prior to hatching. In the brain primordia, many proneural genes are expressed in tightly regulated regional and temporal patterns to control the maintenance of neuronal precursors and differentiation of mature neurons. By 24 hpf (the pharyngula period), peak neurogenesis is occurring and the ventral diencephalon expands to form the hypothalamic primordium and the first transcripts of *crf* are detected in this region (Kimmel et al. 1995, Chandrasekar et al. 2007). Development of the retina begins at 28 hpf, and the optic axons reach the early optic tectum at 48 hpf (opsins appear at 50 hpf) (Raymond et al. 1995, Easter and Nicola 1996). This 48 hpf time point, when hatching occurs, marks the approximate beginning of postembryonic or secondary neurogenesis (Mueller and Wullimann 2003). In this stage, proliferation begins to be restricted to ventricular regions and continues to do so until just the neurogenic regions maintained during adulthood remain (Chapouton and Godinho 2010, Kizil et al. 2011).

Transcriptomic analysis identified neurogenesis as a major pathway affected following morpholino knockdown of *gr* (Nesan and Vijayan 2013a). These include transcripts of neurogenic differentiation genes, including *orthopedia b* (*optb*) which is involved in patterning in the preoptic region, and *islet 1* (*isl1*) which is essential for

motoneuron development. In addition, *neurogenic differentiation* (*neurod*) genes, which are bHLH (basic helix-loop-helix) transcription factors, promote differentiation of neurons in specific regions and patterns (Mueller and Wullimann 2002). *Neurod4*, homologous to *atonal* in drosophila, is involved in both early neurogenesis as well as late neurogenesis in the midbrain, hindbrain and retina (Park et al. 2003, Wang et al. 2003). *Islet 1* is an early marker of primary motoneurons and is required for the formation of these neurons in mice (Eisen 1999). *Orthopedia* is a homeodomain transcription factor that is essential in the patterning of the hypothalamus and preoptic region, particularly in the development of specific populations of dopaminergic and neuroendocrine cells, including *crf/crh* (Ryu et al. 2007, Fernandes et al. 2013). The disruption of brain development by maternal stress and glucocorticoids is well established in mammals (Charil et al. 2010), but little is known about its effect in lower vertebrates, including teleosts.

1.5 Objectives and Hypothesis

The physiological stress response in fish results in elevated levels of circulating glucocorticoids. However, little is known regarding the extent to which maternal glucocorticoids are incorporated into the oocyte in zebrafish or any other asynchronously breeding teleost. Additionally, the role this excess cortisol deposition in embryos may play in the regulation of early developmental events is not clear in fish (Nesan and Vijayan 2013b). The aim of this study was to determine the effect of maternal stress on larval development in zebrafish (Figure 2). The hypothesis was that maternal stress in zebrafish will lead to excess cortisol deposition in the embryos, and this will compromise the larval stress response and their locomotor activity. The specific objectives were to [1]

determine if maternal stress and the attendant rise in cortisol levels are transferred to the developing embryo and how this maternal transfer of steroid is regulated (chapter 2), [2] to determine if this elevated zygotic cortisol deposition alters the larval stress response and behaviour in the offspring, and if this involves changes in neurogenesis (chapter 3), and [3] determine how stress-mediated glucocorticoid signaling post-hatch alters larval behaviour (chapter 4).

1.6 Figures

Figure 1.1. Ontogeny of the HPI axis, behaviour, and neurogenesis in zebrafish. Timeline over the first 5 days of development is indicated for each system. The event, stage, onset of behaviour, or initial transcript detection are indicated with the associated time in hours post-fertilization (hpf) in parentheses. Maternally deposited cortisol (represented by the yellow line, top) decreases until hatch, then rises again. Acronyms: *crf:* corticotropin releasing factor, *pomc:* proopiomelanocortin, *star*: steroidogenic acute regulatory protein, *ff1b:* Ftz-F1b, *hsd3b1*: 3β-hydroxysteroid dehydrogenase, *mc2r*: melanocortin 2 receptor, *cyp11a2*: cytochrome P450 side chain cleavage, OKR: optokinetic response, n*eurod4*: neurogenic differentiation 4, *isl1*: islet 1, *otpb*: orthopedia b.

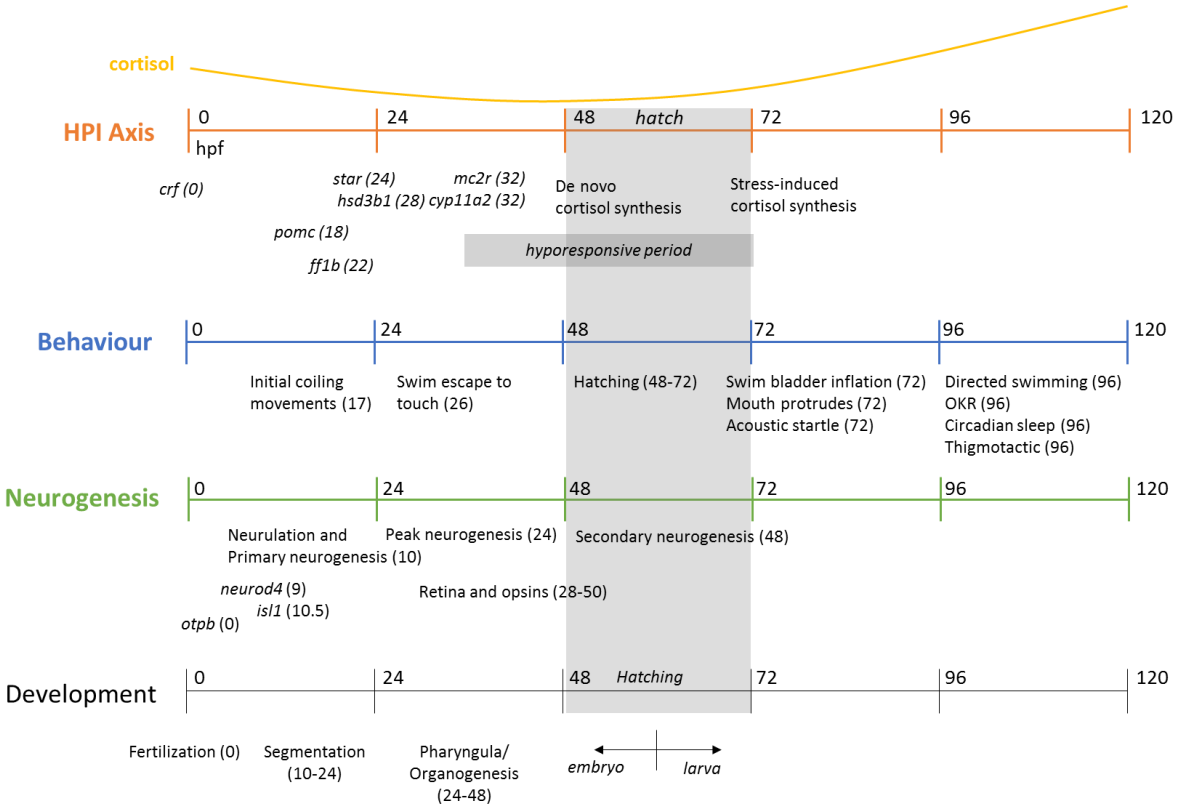
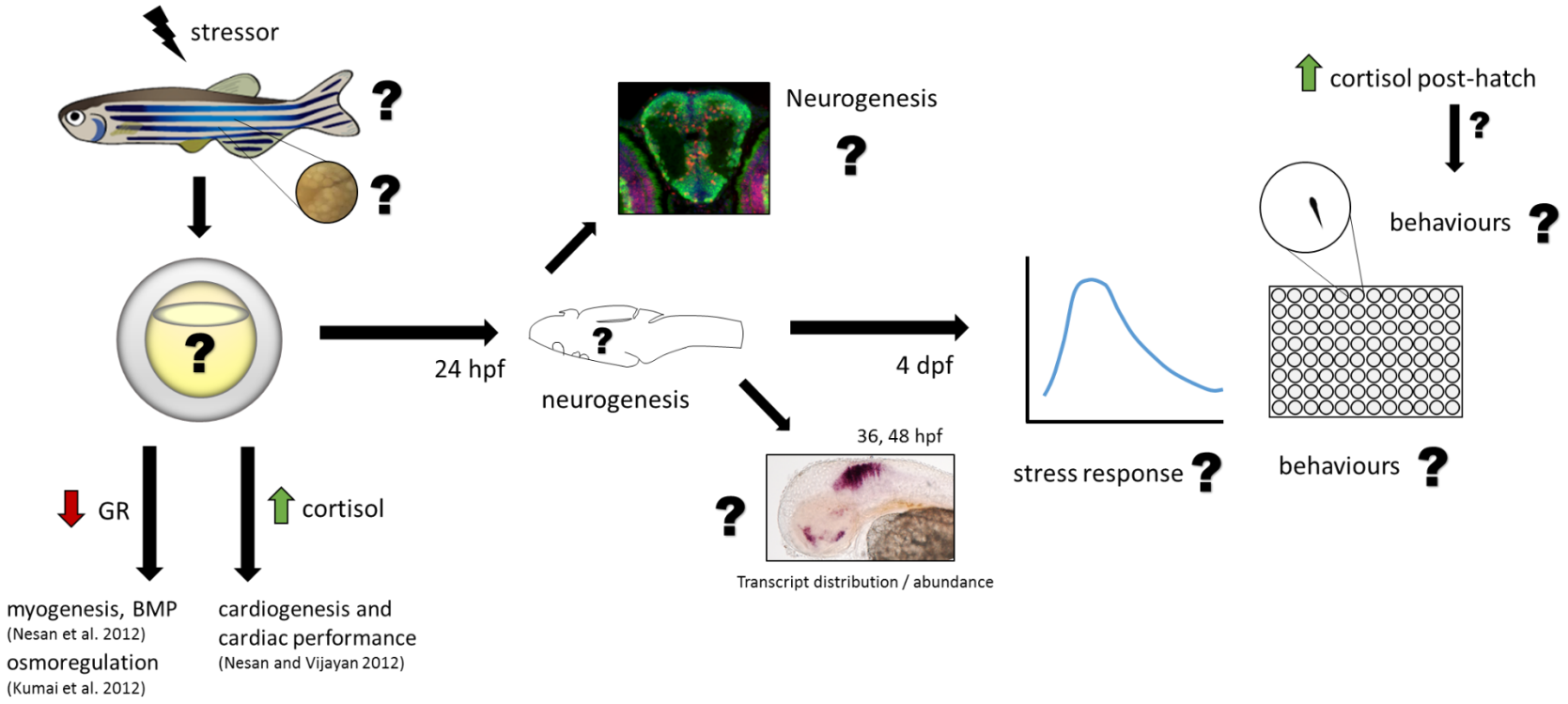

cortisol
HPI Axis
0
24
48
hatch
72
96
120
hpf
crf (0)
star (24)
hsd3b1 (28)
mc2r (32)
cyp11a2 (32)
De novo cortisol synthesis
Stress-induced cortisol synthesis
pomc (18)
ff1b (22)
hyporesponsive period
Behaviour
0
24
48
72
96
120
Initial coiling movements (17)
Swim escape to touch (26)
Hatching (48-72)
Swim bladder inflation (72)
Mouth protrudes (72)
Acoustic startle (72)
Directed swimming (96)
OKR (96)
Circadian sleep (96)
Thigmotactic (96)
Neurogenesis
0
24
48
72
96
120
Neurulation and Primary neurogenesis (10)
Peak neurogenesis (24)
Secondary neurogenesis (48)
neurod4 (9)
isl1 (10.5)
otpb (0)
Retina and opsins (28-50)
Development
0
24
48
Hatching
72
96
120
Fertilization (0)
Segmentation (10-24)
Pharyngula/ Organogenesis (24-48)
embryo
larva

Figure 1.2. Schematic overview of thesis objectives.

Topics addressed in this thesis are indicated by question marks. The thesis objectives are: 1) dynamics of maternal transfer of cortisol to ovary and embryo (chapter 2), 2) effects of cortisol on neurogenesis, stress and behaviour (chapter 3), and 3) role of stress-mediated glucocorticoid signaling on behaviour (chapter 4). Previous relevant research is indicated by citations.



Chapter Two: Maternal regulation of cortisol deposition in zebrafish embryos

2.1 Introduction

Plasma cortisol levels increase following exposure to a wide variety of stressors in fishes (Vijayan et al. 2010). The stress-induced increase in cortisol is the culmination of the hypothalamus-pituitary-interrenal (HPI) axis activation. The perception of a stressor stimulates the release of corticotropin releasing factor (CRF) from the hypothalamus, which stimulates the anterior pituitary to secrete adrenocorticotropic hormone (ACTH, Wendelaar Bonga 1997, Mommsen et al. 1999). ACTH acts on the interrenal tissue to release corticosteroids into the circulation. At target tissues, the role of cortisol action is mediated by intracellular receptors, including the glucocorticoid receptor (GR) and the mineralocorticoid receptor (MR) (Nesan and Vijayan 2013b). Nongenomic actions, independent of these receptors, have also been proposed (Nesan and Vijayan 2013b, Mommsen et al. 1999, Dindia et al. 2013). Further regulation of cortisol at the tissue level includes the conversion of this hormone to its biologically inactive equivalent, cortisone, by the enzyme 11β-hydroxysteroid dehydrogenase type 2 (11βHSD2) to reduce cortisol-mediated effects (Mommsen et al. 1999). Cortisol is not only playing an important role in the metabolic adaptation to stress, but is also involved in growth, energy metabolism, osmoregulation, and immune function (Wendelaar Bonga 1997, Mommsen et al. 1999).

In teleosts, HPI axis activation occurs only after hatch, and the stress hyporesponsive period during embryogenesis is thought to be important for normal developmental programming events (Nesan and Vijayan 2013b). However, egg-laying vertebrates, including fish, deposit maternal hormones into the egg during vitellogenesis,

including appreciable amounts of gonadal, thyroid, and interrenal steroid hormones (Mommsen and Walsh 1988, Tagawa et al. 2000). This maternally deposited cortisol plays an important role in early embryo development prior to *de novo* cortisol synthesis (Nesan and Vijayan 2013b). Studies suggest that maternal stress and the attendant elevation in plasma cortisol levels may modify embryo cortisol content in fishes. For instance, egg cortisol content corresponded with elevated maternal cortisol levels in the stickleback (*Gasterosteus aculeatus*; Giesing et al. 2011), rainbow trout (*Oncorhynchus mykiss*; Åberg Andersson et al. 2011) and Coho salmon (*Oncorhynchus kisutch*; Stratholt et al. 1997). Also, exposure of the mother to elevated exogenous cortisol levels elevated egg and embryo cortisol content in the common coral reef damselfish (*Pomacentrus amboinensis*; McCormick 1999), Atlantic salmon (*Salmo salar*; Eriksen et al. 2006) and Atlantic cod (*Gadus morhua*; Kleppe et al. 2013). Together, these results suggest that elevated cortisol levels associated with maternal stress may be reflected in the steroid content of the egg.

Studies have shown that abnormal increase in cortisol content in the embryos leads to developmental defects in zebrafish (Nesan and Vijayan 2013b). For instance, embryos exposed to exogenous glucocorticoids displayed developmental defects, including abnormal craniofacial phenotype (Hillegass et al. 2008) and cardiac defects (Nesan and Vijayan 2012). The above studies utilized exogenous cortisol manipulation of the embryos to mimic a maternal stress scenario. However, no study to date has examined maternal stress and the associated embryo cortisol deposition in an asynchronous breeder, including zebrafish (*Danio rerio*). As this species is a widely used model for developmental and genetic studies, it is essential to understand whether maternal stress

due to husbandry practices may lead to elevated embryo cortisol content, especially given the possibility that this steroid level in the embryo influences developmental trajectories and phenotype in this species (Nesan and Vijayan 2013b). Therefore, in the present study we tested the hypothesis that maternal stress and the resultant elevation in cortisol content will be transferred to the embryos in zebrafish. We tested this by subjecting zebrafish to a fasting stressor for 5 days, after which the fish were bred over a 10 d period to measure temporal changes in embryo cortisol content. This breeding regimen was followed because zebrafish are asynchronous breeders, and the timing of oogenesis to ovulation is around 10 days (Clelland and Peng 2009). To confirm if the observed changes were indeed due to elevated cortisol content, unstressed mature female zebrafish were fed control or cortisol-spiked food for 5 days to examine temporal changes in embryo cortisol content over a 10 d breeding regimen. We also carried out an *in vitro* study with isolated ovarian follicles to test whether elevated cortisol levels would limit its own deposition in the oocytes by upregulating the gene encoding the key steroid breakdown enzyme 11βHSD2 in zebrafish.

2.2 Materials and Methods

2.2.1 Animals

Adult zebrafish (TL strain) were maintained in 10 L tanks on a recirculating system with a 14:10 light: dark cycle (Pentair Aquatic Habitats, Apopka, FL). Water was maintained at 28 ºC, pH 7.6 and 750 µS conductivity. Animals were fed twice daily with Ziegler Adult Zebrafish Diet (Pentair Aquatic Habitats, Apopka, FL) in the morning and live *Artemia* in the evening. All protocols involved in the maintenance and experimental

use of zebrafish were approved by the University of Calgary Animal Care Committee, and abide by the guidelines set out by the Canadian Council on Animal Care.

2.2.2 Maternal Stress

Adult female zebrafish (n=48) were sorted into two groups (n=24), each housed in four 3 L tanks. One was fed as above, while the other was fasted for 5 days, as food deprivation increases cortisol response in this species (Ramsay et al. 2006). Fish were maintained in a recirculating system at a density of 3.5 g/ L, as recommended by Lawrence (2007). After 5 days, 12 fish (n=6 fish/treatment) from each group were sampled (see section 2.4), and the remaining fish (n=24; n=12/treatment) were transferred to breeding tanks and both groups were fed daily (prior to breeding) the following day onwards for 10 days (see section 2.5)

2.2.3 Exogenous Cortisol Treatment

Adult female zebrafish (n=48) were assigned one of two groups (n=24), and housed in two 10 L tanks, there were not replicate tanks. Fish in one tank were fed control zebrafish diet, while fish in the other tank were fed diet laced with 25 µg cortisol/g fish weight/day. Each diet was prepared by soaking food pellets in 100% ethanol either alone (control), or with 25 µg/g body weight hydrocortisone (Sigma-Aldrich, St. Louis, MO, USA) and allowing the ethanol to evaporate as previously described (Alderman et al. 2012). Fish were maintained in a recirculating system at a density of 3.5 g/ L and were fed 2.5% of their body weight twice per day. They were held under these conditions for 5 days, after which 12 were sampled (n=6/treatment; see section 2.4), and the remaining 36 fish (n=18/treatment) were transferred to breeding

tanks. Both groups were fed (unlaced feed) prior to breeding the following day onwards for 10 days (see section 2.5).

2.2.4 Fish Sampling

On day 5, a set of fish were euthanized with an overdose of 2-phenoxyethanol (1:1000 dilution; Sigma, Oakville, ON). They were blotted dry and the weight and fork length recorded to calculate condition factor ($K=W*100)/L^3$), and ovaries were weighed. Whole bodies and ovaries were snap frozen on dry ice and stored frozen at -80ºC for cortisol analysis.

2.2.5 Breeding

Female fish from each treatment (n=12 or 18/treatment; 12, maternal stress experiment; 18, exogenous cortisol experiment), were divided into 6 tanks (n=3/tank). Two unexposed males were added to each tank. Tanks were then divided into set 1 (2 or 3 tanks/treatment/day) and set 2 (2 or 3 tanks/treatment/day). Each set of fish were bred every other day for a total 5 spawnings/set over a 10 d period. This approach was taken to avoid fatigue, and to maximize the daily embryo yield. All fish resumed regular feeding as per section 2.1. For daily breeding setup (at 6 pm), fish were quickly netted and transferred into breeding traps in the same tank, and eggs were collected within one hour of light (at 8 am) the following morning. Embryos were counted and pools of 20 were snap frozen on dry ice and stored frozen at -80ºC for later cortisol analysis.

2.2.6 Cortisol Analysis

Frozen whole body was ground into a powder with a chilled mortar and pestle on dry ice. A known amount of tissue (whole body or ovary) was then homogenized and sonicated in a 5x volume of Tris buffer [50 mM Tris pH 7.5 with protease inhibitors

(Roche Diagnostics, Laval, QC)]. Extraction was performed as described previously (Alderman and Vijayan 2012). Briefly, the homogenate was double extracted with diethyl ether (1:5), the solvent fraction removed after freezing the aqueous phase on dry ice, evaporated and reconstituted with either kit extraction buffer (cortisol ELISA kit; Neogen, Lexington, KY) or PBS + 0.1% (w/v) BSA (in house ELISA, see below). The Neogen cortisol ELISA kit has been previously validated for zebrafish (Alderman and Vijayan 2012). Embryos were not extracted, as preliminary studies revealed no difference in the cortisol content between the extracted and unextracted embryos.

Additionally, an in-house competitive ELISA for zebrafish was used to quantify the cortisol levels in embryos and mothers of the feeding study, and embryos of the endogenous cortisol study, according to Yeh et al. (2013), with some modifications. Briefly, high binding 96 well plates (Immulon HB, VWR) were coated with 100 µl of cortisol monoclonal antibody (1.6 µg/ml; East Coast Bio, ME, USA) in phosphate buffered saline (1 x PBS; 10x stock:1.37M NaCl; 27 M KCl, 18 mM KH_2PO_4; Na_2HPO_4) for 16 h at 4°C. The plate was then washed with PBS with 0.05% Tween 20 (TPBS; 300 ul/well) and blocked with 0.1% bovine serum albumin (300 µl/well; BSA; Sigma-Aldrich, St. Louis, MO, USA) for 1 h at room temperature. Standards comprised of hydrocortisone (Sigma-Aldrich, St. Louis, MO, USA) serially diluted (0 – 25 ng/ml) in PBS and 50 µl of either standards or samples were added to the wells in duplicate. Cortisol conjugated to horseradish peroxidase (1:160 dilution; East Coast Bio, ME, USA) diluted in PBS was added to each well. Plates were incubated for 2 h, shaking, at room temperature. The plate was washed as described above and the detection reagent was added (41 mM TMB and 8 mM TBABH in 200 mM potassium citrate, pH 4). After 25

minutes the reaction was stopped with 1 M sulfuric acid. Wells were read at 450 nm using a microplate reader (VersaMax, Molecular Devices, CA, USA). The intra-assay and inter-assay coefficient of variation was 8.2% and 13.4% respectively, and the minimum detection limit was 0.5 ng/ml.

2.2.7 Cortisol Regulation by Ovarian Follicles

Female fish (n=4), were euthanized with an overdose of 2-phenoxyethanol (1:1000 dilution; Sigma, Oakville, ON, CAN). Intact ovaries were removed, and placed in a Petri dish of Leibovitz's (L15) medium (Gibco, Grand Island, NY). Ovarian follicles were mechanically dispersed using a Pasteur pipette and evenly divided into 6 wells of a 24-well plate. Follicles were immediately treated with cycloheximide (10 µg/ml; Sigma-Aldrich, St. Louis, MO, USA) or actinomycin-D-Mannitol (10 µg/ml; Sigma-Aldrich). These concentrations have been previously shown to block translation and transcription, respectively, in zebrafish ovarian follicles (Li et al. 2011). Cortisol (100 ng/ml hydrocortisone; Sigma-Aldrich) or vehicle control (0.01% ethanol) was added 30 minutes later. Treated ovarian follicles were left rocking at 28.5 ˚C. At 4 h post treatment, the follicles were collected, centrifuged at 13,000 rpm for 2 minutes, and media removed. Samples were stored at -80 ˚C for later analysis.

2.2.8 Transcript Analysis by Quantitative Real-Time PCR

Total RNA was extracted from ovarian follicles (n=4 per treatment) using Ribozol reagent (VWR, Mississauga, ON) according to the manufacturer's instructions, and quantified using the SpectraDrop Micro-Volume microplate (VersaMax, Molecular Devices, CA, USA). One µg of RNA was treated with DNase I (Thermo Scientific, Waltham, MA, USA) to remove genomic contamination prior to cDNA synthesis using

the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocols.

Transcript levels of *11βhsd2* and *β-actin* were measured by quantitative real time PCR (qPCR) in triplicates using gene specific primers (Table 1) as described previously (Sandhu and Vijayan 2011). A standard curve was used to determine relative transcript abundances according to established protocols (Sathiyaa and Vijayan 2003). All data was normalized to β-actin, as this transcript remained unchanged between treatments. Data is expressed as percent of the control.

2.2.9 Statistics

All data was analyzed using SigmaPlot 13 software (Systat Software Inc, San Jose, CA, USA). All data is expressed as mean ± standard error (SEM) and log transformed where necessary to mean the assumptions of normality and equal variance. Untransformed data is shown in all figures. For cortisol levels, data was analyzed by Student's t-test. If assumptions of normality could not be met, the nonparametric Mann-Whitney Rank Sum Test was used. For temporal embryo cortisol levels, a Kruskal-Wallis one-way ANOVA on ranks was performed (Dunn's post-hoc) as the data did not meet assumptions of normality or equal variances. A repeated measures one-way ANOVA was performed for the ovarian follicle study (Holm-Sidak post hoc). A significance level (α) of 0.05 was used in all cases.

2.3 Results

2.3.1 Maternal Stress

2.3.1.1 Whole Body and Ovary Cortisol Content

Whole body cortisol in fasted females was approximately four-fold higher than the fed controls (Fig. 2.1A; $p < 0.001$). There were no significant differences in ovary cortisol content normalized to tissue weight (Fig. 2.1B).

2.3.1.2 Embryo Yield and Cortisol Content

There was a clear difference in embryo yields between the fed and fasted mothers (Figure 2.2). The fed females had spawned fewer embryos compared to the fasted embryos. Fed females spawned on day 1 (73 embryos), day 5 (2 embryos), day 6 (23 embryos), and day 9 (3 embryos), whereas the fasted females bred on day 1 (142 embryos), day 2 (80 embryos), day 3 (53 embryos), day 7 (55 embryos), day 8 (102 embryos) and day 9 (179 embryos), and day 10 (124 embryos). There were not enough breeding events in the fed control group to show embryo cortisol levels for each day, therefore, only the embryos from fasted females were shown for daily embryo cortisol content, while the cortisol levels of fed control embryos over the entire breeding period were pooled (Figure 2.3B). The embryos from fasted females had significantly higher cortisol on day 9 (65 ng/ml; Figure 2.3B) compared to all other days within this treatment group (one way ANOVA, $p<0.05$). There was a statistically significant difference between the cortisol content of the fed embryos and the 9 d embryos from the fasted group (Fig. 2.3).

2.3.2 Cortisol Feeding

2.3.2.1 Maternal Cortisol

There was no change in whole body cortisol levels between control and cortisol-fed zebrafish (Figure 2.4A). There was a 2-fold increase in ovarian cortisol levels of the cortisol-treated fish compared to the controls (Figure 2.4B).

2.3.2.2 Embryo Cortisol and Yield

Both treatment groups yielded sufficient embryos daily to plot temporal embryo cortisol content (Fig 2.5). There was significantly higher embryo yield from cortisol-treated mothers compared to the control mothers (Figure 2.5 inset). Control (vehicle fed) females spawned on day 1 (314 embryos), day 2 (301 embryos), day 3 (214 embryos), day 4 (255 embryos), day 5 (14 embryos), day 6 (108 embryos), day 7 (275 embryos), day 8 (768 embryos), day 9 (365 embryos) and day 10 (388). Cortisol fed females bred on day 1 (521 embryos), day 2 (582 embryos), day 3 (695 embryos), day 4 (417 embryos), day 5 (244 embryos), day 6 (683 embryos), day 7 (391 embryos), day 8 (661 embryos) and day 9 (696 embryos), and day 10 (388 embryos).

Cortisol content of embryos from cortisol-fed mothers was significantly higher on days 3 and 4 compared to the embryos of control mothers. On day 9, cortisol levels in embryos of cortisol-treated mothers were significantly lower when compared to controls (Fig 2.6).

2.3.3 Ovarian Follicle 11βhsd2 Transcript Abundance

Transcript abundance of *11βhsd2* was 7-fold higher in cortisol treated oocytes (100 ng/ml) compared to vehicle treated (0.01% ethanol) control oocytes ($p<0.001$; Figure 2.7). Cycloheximide treatment (10 µg/ml) increased basal transcript abundance of

11βhsd2 4-fold. Cycloheximide/cortisol treated oocytes also had significantly higher transcript levels of this gene compared to both the cycloheximide control treated oocytes (4-fold), and the vehicle control oocytes (16-fold; $p<0.001$; Figure 2.7). Actinomycin-D (10 µg/ml), did not significantly alter transcript abundance of *11βhsd2*. However, actinomycin-D/cortisol treated oocytes had approximately 6x the transcript abundance of *11βhsd2* compared to transcript levels of actinomycin-D treated oocytes, as well as transcript levels of vehicle treated controls (3-fold; $p<0.001$; Figure 2.7). Cycloheximide/cortisol treated oocytes had significantly higher levels of *11βhsd2* transcripts then either just cortisol treated oocytes ($p=0.017$) or actinomycin-D/cortisol oocytes ($p<0.001$; Figure 2.7). Cortisol treated oocytes had significantly higher *11βhsd2* transcript levels (2-fold; $p=0.04$) compared to actinomycin/cortisol oocytes.

2.4 Figures

Figure 2.1. Maternal cortisol levels after fasting stressor.

A) Maternal cortisol levels. Whole-body cortisol content of the adult female zebrafish following 5 days of food deprivation. **B) Ovary cortisol content.** Cortisol levels in the ovary of adult zebrafish following 5 days of food deprivation. Values represent means ± SEM. Asterisks indicate significant effects (Student's t-test).

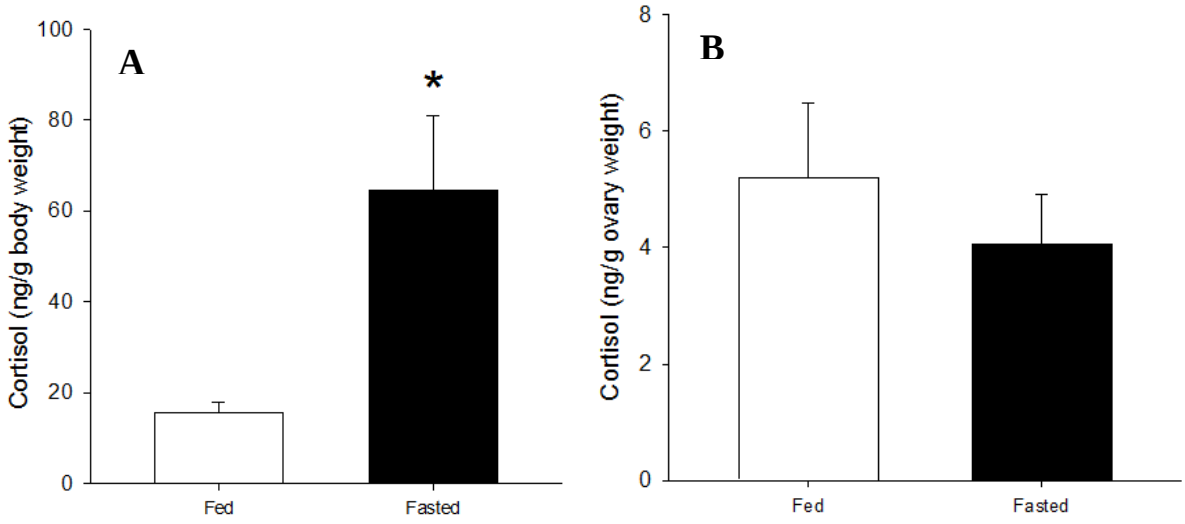


Figure 2.2. Daily embryo yield after fasting stressor.

Embryos were collected at 1 h post-fertilization of offspring yielded from fed or 5 day fasted females.

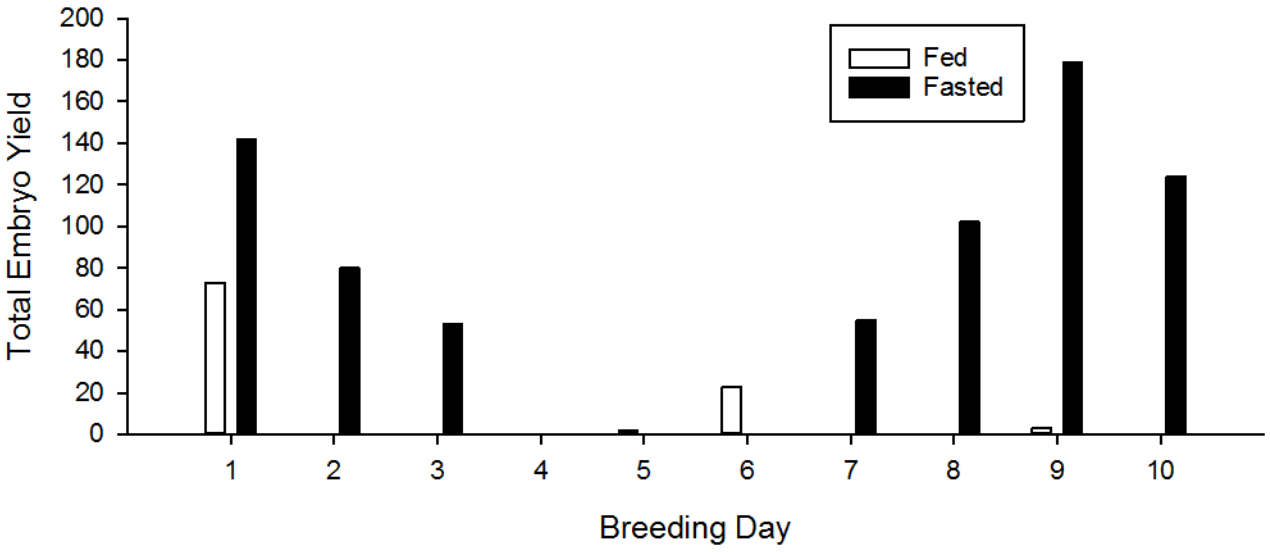
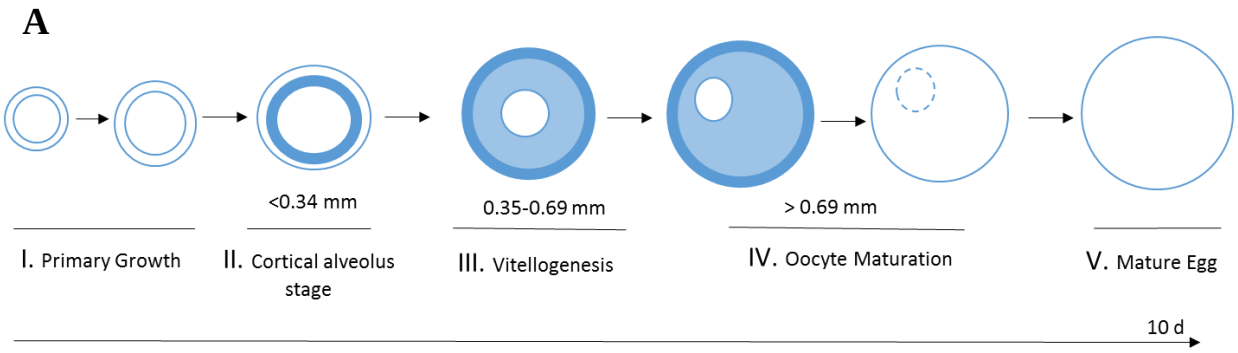


Figure 2.3. Embryo cortisol content after fasting stressor.

A) Oocyte maturation. Cortisol incorporation to the developing oocyte is thought to occur during vitellogenesis. **B) Daily embryo cortisol at 1 h post-fertilization of offspring yielded from fed females or 5 day fasted females.** Values represent mean ± SEM of cortisol. In (B), only the fasted group is shown daily as not data was available to run statistical tests on the fed group alone. Letters indicate significant effects (Kruskal-Wallis one-way ANOVA on Ranks, Dunn's post-hoc), asterisks indicate significant differences (Student's t-test).



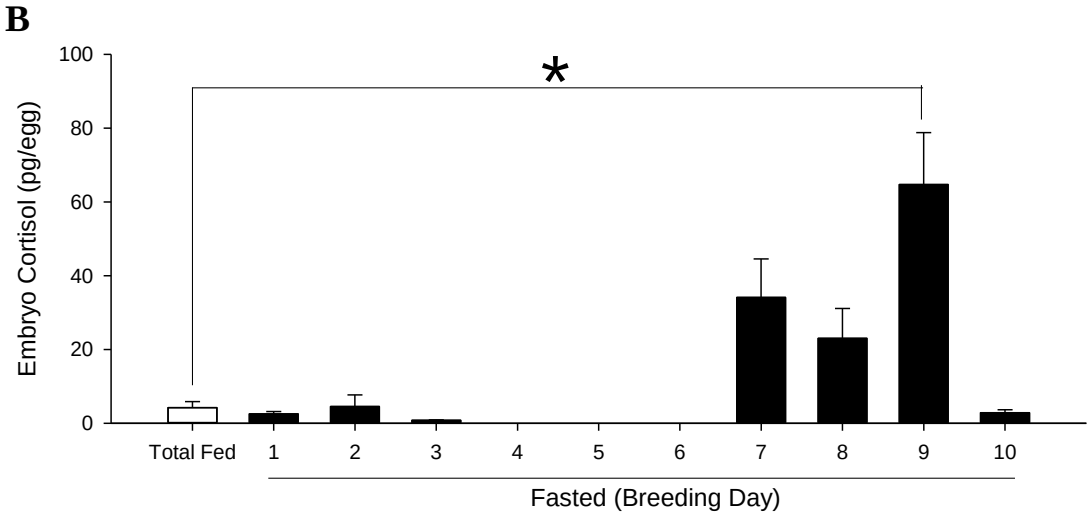


Figure 2.4. Maternal cortisol levels after consumption of cortisol-laced feed. **A) Maternal cortisol levels.** Whole-body cortisol content of adult female zebrafish following 5 days of feeding cortisol-laced food. **B) Maternal ovary cortisol levels.** Ovary cortisol content of adult female zebrafish following 5 days of feeding cortisol-laced food. Values represent means ± SEM. Asterisks indicate significant effects (one-way ANOVA, Holm-Sidak, P=0.002).

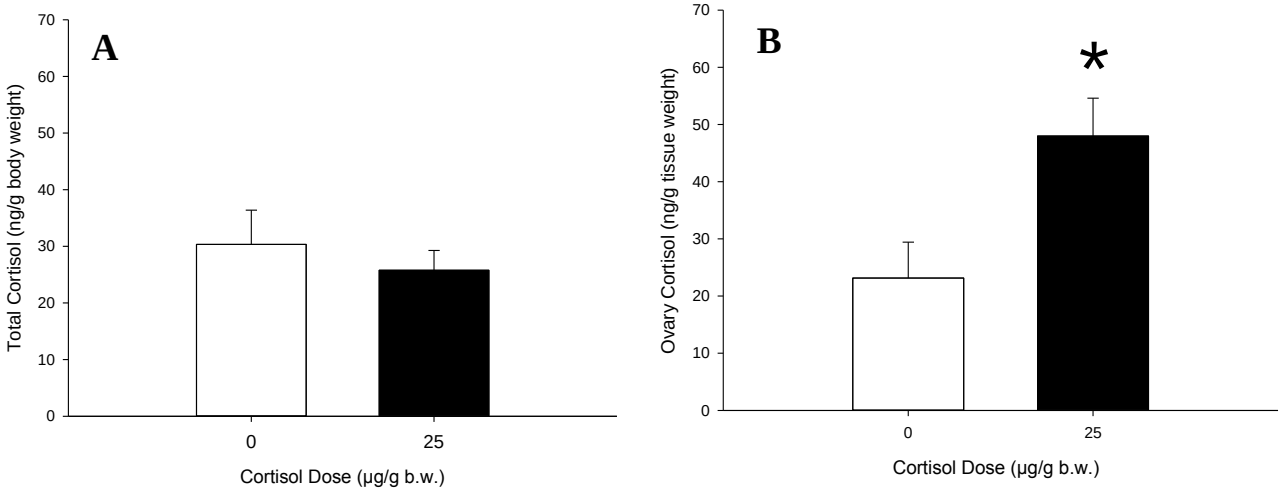


Figure 2.5. Daily embryo yield after consumption of cortisol-laced feed.

Embryos were collected at 1 h post-fertilization of offspring yielded from vehicle or cortisol treated females. Inset shows total daily embryo yield (n=10). Asterisks indicate significant differences (Student's t-test).

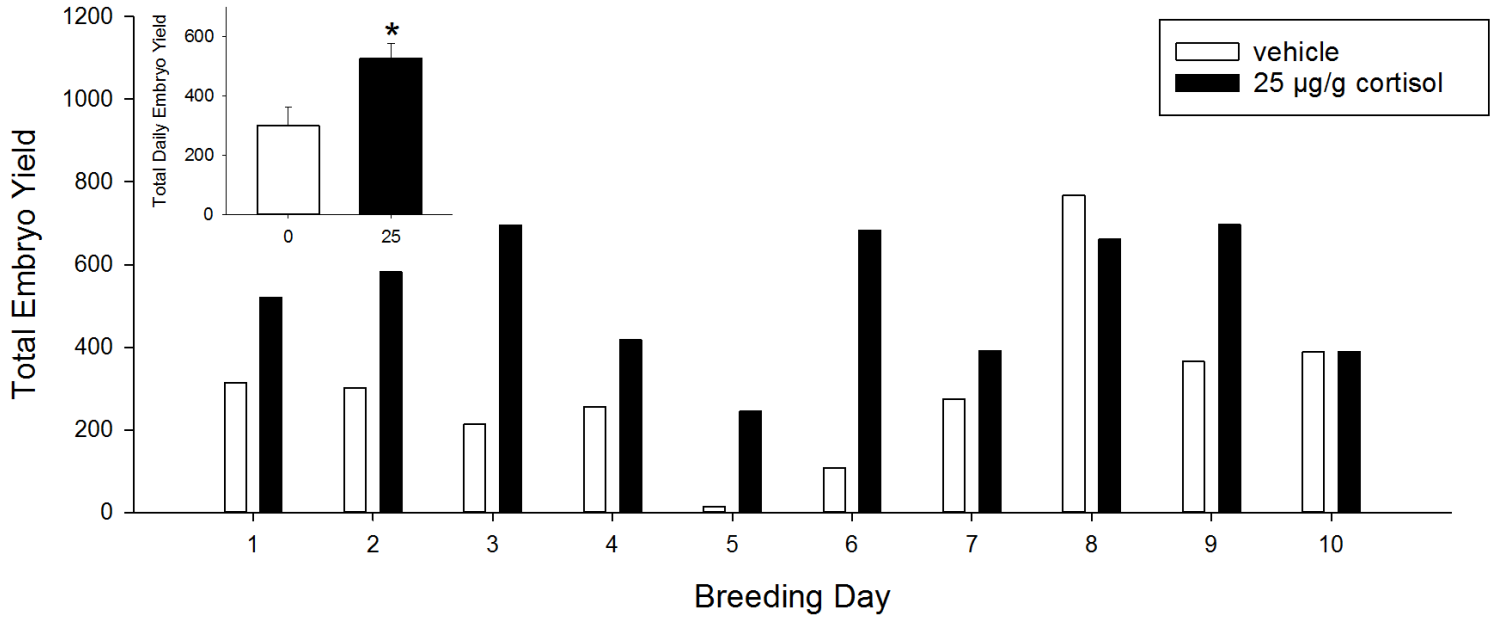


Figure 2.6. Embryo cortisol content after consumption of cortisol-laced feed.

Mean embryo cortisol at 1 h post-fertilization of offspring yielded from females following 5 days of feeding cortisol-laced food. Values represent either the daily mean ± SEM of cortisol from embryos collected over a breeding period of ten days during which fish (3F/2M) were bred every other day. A significant interaction was detected (2-way ANOVA, Holm-Sidak, P=0.002). Treatment effects within days are indicated by different lowercase letters.

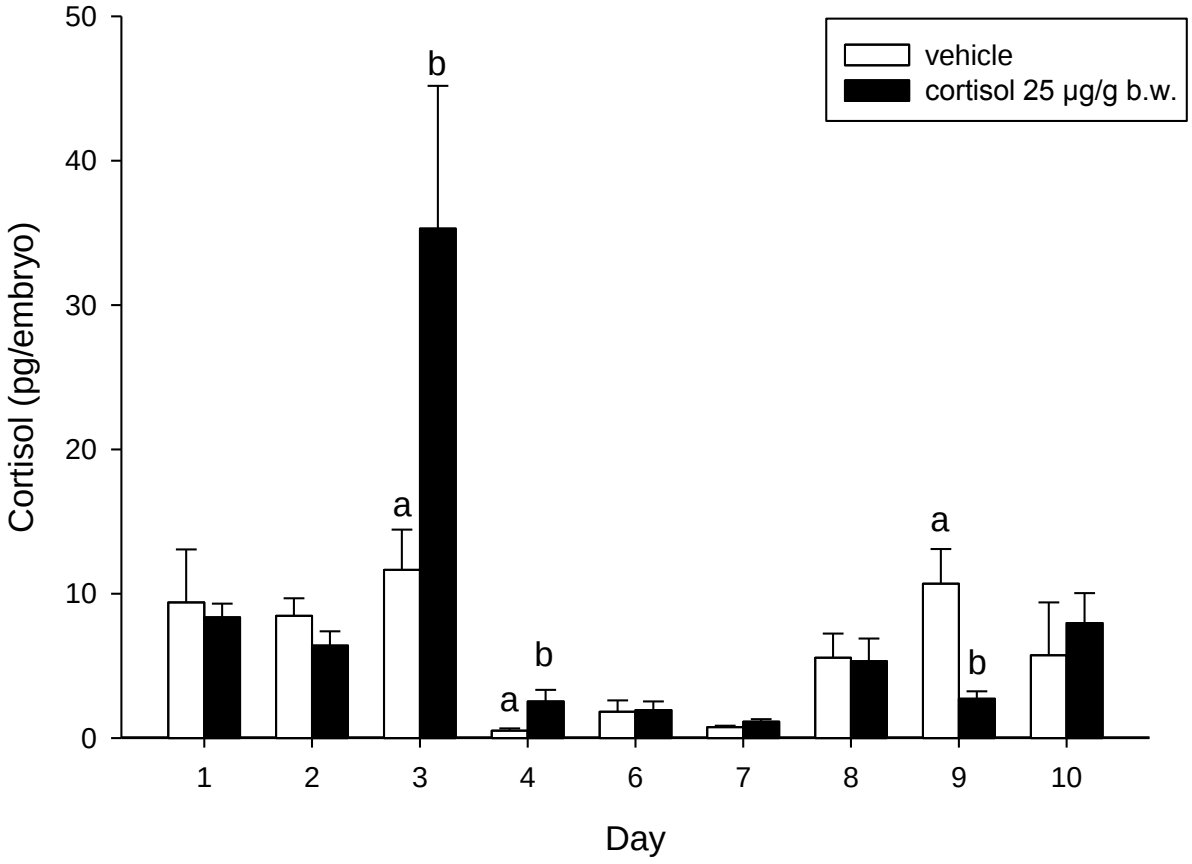


Figure 2.7. *11βhsd2* transcript abundance in ovarian follicles.

Control (0.01% ethanol; white bar); cortisol (100 ng/ml; red bar); cycloheximide (10 µg/ml; yellow bar); actinomycin-D (10 µg/ml; blue bar); cycloheximide + cortisol (orange bar); and actinomycin-D + cortisol (purple bars) treated ovarian follicle *11βhsd2* transcript levels. Follicles were treated for 4 h in L15 media. Data is shown as % control, mean ± standard error (SEM), n=4 fish. One-way repeated measures ANOVA (Holm-Sidak, $p<0.05$).

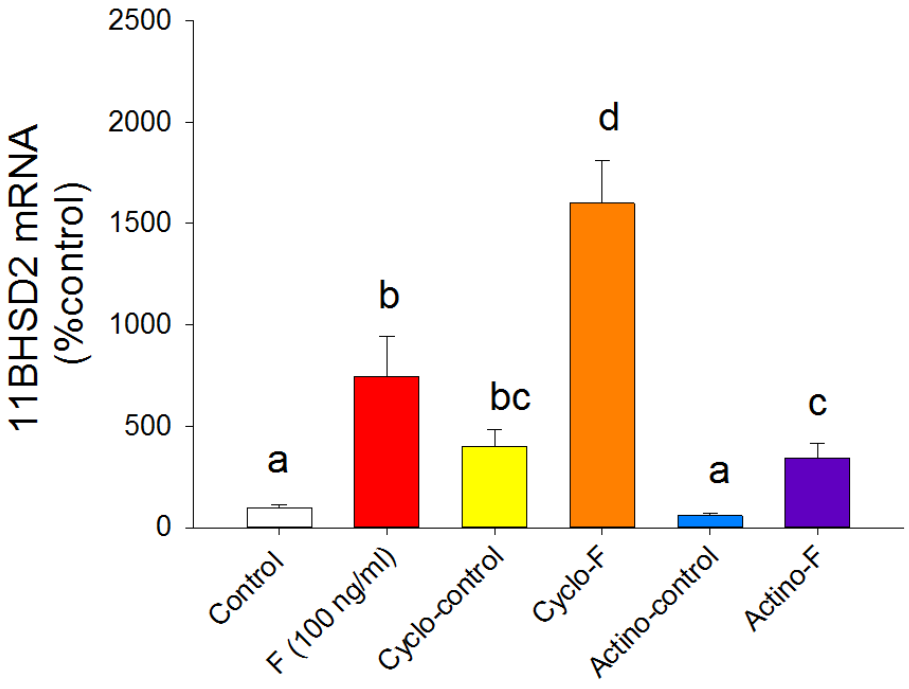
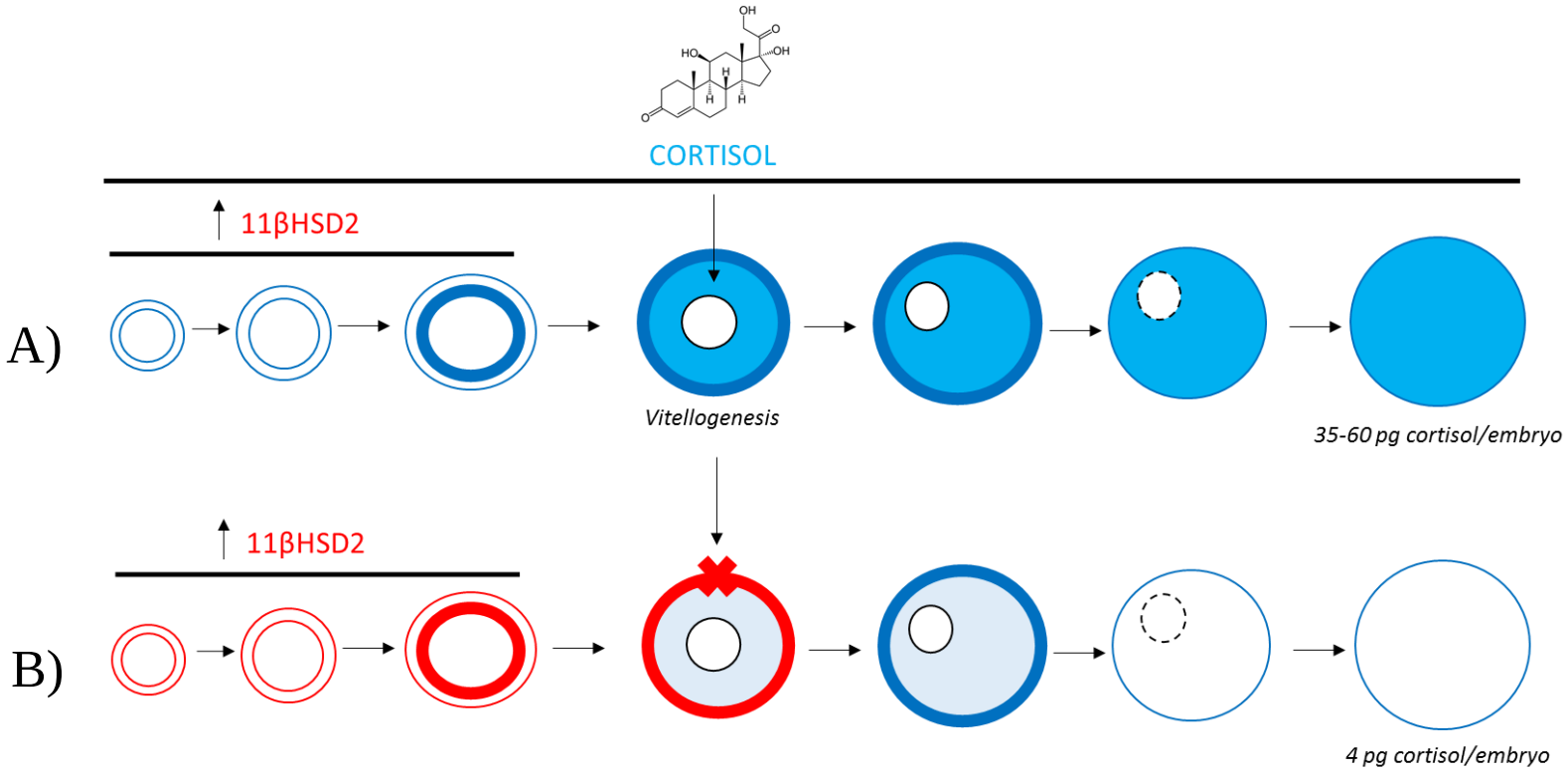


Figure 2.8. Proposed model of maternal deposition of cortisol into zebrafish oocytes.

Elevated maternal cortisol results in an incorporation of cortisol during oocyte vitellogenesis (A). Previtellogenic follicles (stages I and II) will simultaneously upregulate transcription of *11βhsd2* in the theca/granulosa cells surrounding the oocyte. When follicles reach the vitellogenesis stage of oocyte development cortisol is broken down in these layers, preventing harmful levels of cortisol from being incorporated into the yolk (B).



2.5 Discussion

This study demonstrates that maternal stress and the attendant rise in cortisol levels increases embryo yield in zebrafish. It further establishes that maternal cortisol is only transiently transferred to embryos due to a cortisol-induced upregulation of *11βhsd2* in zebrafish ovarian follicles. This restricts the accumulation of excess cortisol in the developing oocytes. The negative effects of elevated embryo cortisol content on early zebrafish development has been recently documented (Nesan and Vijayan 2012). However, we propose that the tight control of embryo cortisol levels, despite the stress-induced elevation of this hormone in the mothers, as a key adaptive strategy in regulating proper early developmental programming events in zebrafish.

We used fasting as a stressor in mothers, as this was previously shown to elevate cortisol levels in zebrafish (Ramsay et al. 2006). Our results support this finding as the fasted fish showed a 3-fold increase in whole body cortisol content. This was not the case when cortisol was fed to unstressed mothers to mimic a maternal stress scenario. Feeding of cortisol-spiked food did not affect whole body cortisol content. However, there were elevated cortisol levels in the ovary, unlike with fasting, suggesting differences in this steroid turnover and tissue compartmentalization with feeding this steroid. While a clear explanation for the lack of cortisol response is not known with exogenous feeding, one possibility may be the 12 h time-lag between the last feeding and sampling for cortisol measurement. The metabolic clearance of cortisol depends on many environmental factors, including ambient temperature and nutritional state, and is determined by the rate of tissue uptake and catabolism (Mommsen and Walsh 1988). In warm water (22-24˚C) fish such as tilapia the half-life for circulating cortisol was approximately 4 h (Ilan and

Yaron 1983), whereas in fingerling rainbow trout held at 11˚C cortisol levels returned to normal 8 h post stressors (Barton and Peter 1982). To our knowledge there have been no studies on cortisol turnover in zebrafish, but given the bolus route of administration, and the higher water temperature of 28.5˚C, it is likely that the steroid is rapidly cleared within 12 h in this species. The higher cortisol in the gonads may suggest preferential compartmentalization of this steroid into lipophilic tissues, such as the oocyte yolk. Clearly, further studies are needed to confirm that partitioning of cortisol into the oocytes in this species.

Interestingly, fasted mothers yielded more embryos, more frequently than the fed controls. While the embryo yield in general was less in this study, the fasted group consistently gave more embryos compared to the fed group. Increased fecundity has been shown previously in rainbow trout (*Oncorhynchus mykiss*) that were stressed early in vitellogenesis, compared to control fish or fish that were stressed late in oocyte maturation (Contreras-Sánchez et al. 1998). Most studies have focused on synchronously breeding salmonids, while little is known about stress effects on asynchronous breeders (Schreck 2010). Our results suggest that maternal stress, specifically elevation in cortisol levels, acts as a stimulus to increase spawning in the asynchronously breeding zebrafish. The increase in embryo yield that was seen in unstressed mothers fed cortisol further confirms a role for this steroid in regulating fecundity, while the mechanism(s) remains to be determined. The cortisol-induced increase in frequency and yield of spawning events may be an evolutionarily conserved pathway to ensure procreation during periods of prolonged stress. Indeed, there is evidence to support the hypothesis that cortisol may have a stimulatory effect on maturation. Treatment of yellow perch (*Perca flavescens*)

oocytes with the cortisol precursor 11-deoxycortisol effectively initiated germinal vesicle breakdown (GVBD) and induced ovulation. However even at high concentrations (1 μg/ml) it was not as effective as the maturation hormone 17α, 20β – dihydroxy-4-pregnen-3-one (17,20P) (Goetz and Theofan 1979). Similar results regarding oocyte maturation were observed in oocytes of Indian catfish (*Mystus vittatus*) treated with deoxycorticosterone, and hydrocortisone (Upadhyaya and Haider 1986). Therefore, we propose a synergistic role for cortisol with other pathways in the regulation of oocyte maturation in zebrafish. To our knowledge the effect of cortisol or cortisone on levels of 17,20P have not been studied in any species. Cortisol has minimal effects on modulating gonadal steroidogenesis, and therefore its effect on reproduction through the modulation of reproductive hormones would be negligible (Schreck 2010). While some studies have demonstrated that exogenously administered cortisol resulted in lowered serum testosterone and 17β-estradiol (*Oreochromis mossambicus*, Foo and Lam 1993), other studies in goldfish, carp and sparid failed to show cortisol modulation of testosterone and 17β-estradiol (Schreck 2010). Even when brown and rainbow trout were chronically stressed due to an intraperitoneal cortisol implant over 36 d, there were no changes in testosterone, 11-ketotestosterone, 17,20P, pituitary gonadotropin content or 17β-estradiol (Carragher et al. 1989). Through its glucocorticoid action, cortisol may be acting to increase energy during oogenesis however very little is known about the metabolic requirements of oocyte maturation (Boulekbache 1981), and therefore it is difficult to hypothesize how cortisol could be modulating energy pathways. The somatotropic axis is an alternative pathway that cortisol may be modulating to promote oocyte maturation. It is known that cortisol will increase growth hormone (GH) stimulation of insulin-like

growth factor 2 (IGF2) transcript abundance in the liver (coho salmon; Pierce et al. 2010, tilapia; Pierce et al. 2011). Both IGF2 and IGF1, the primary mediators of GH-promoting effects, are known to stimulate oocyte maturation in teleost species (Reinecke 2010). Further studies need to be completed to confirm how cortisol can increase fecundity in teleost fish.

It has long been maintained that cortisol is transferred to the developing oocyte during vitellogenesis and that this will have detrimental effects on the developing embryo (Nesan and Vijayan 2013b). In zebrafish, yolk bodies begin to form in stage III oocytes (0.34-0.69 mm; Figure 3A, Selman et al. 1993) and this is when steroid hormone incorporation is thought to occur (Mommsen et al. 1999). As oocytes are only sensitive to hormones during a particular stage of oogenesis, we proposed that there would be a delay in maximal embryo cortisol content after the onset of maternal stress. To test this, fish were bred for a total of 10 days, the length of time for a developing oocyte to progress from follicle to a mature egg in zebrafish (Clelland and Peng 2009). The transient increase in embryo cortisol content is seen only in embryos on day 9 after the fasting stressor, which supports the notion that maternal stress-mediated cortisol deposition is not a passive process and is actively regulated during oogenesis. As zebrafish are asynchronous breeders, previtellogenic oocytes may be accumulating this excess steroid and the time taken for these to mature and spawn corresponds with the transient increase in embryo cortisol content seen in this study. This notion is further supported by the transient elevation (day 9 after the first day of feeding spiked food) in embryo cortisol content seen also in cortisol-fed fish. However, the embryo cortisol content on day 10 was back to unstressed or control fed group levels, suggesting other control mechanisms

that limit cortisol deposition in this asynchronous breeder. Therefore we tested the hypothesis that there would be an upregulation of *11βhsd2* in ovarian follicles after exposure to cortisol, which would limit the accumulation of this steroid. Indeed, bioinformatics analysis revealed three putative glucocorticoid response elements (GREs) in the promoter region of zebrafish *11βhsd2* (Alderman and Vijayan 2012), suggesting autoregulation of this steroid content in oocytes. Here we show for the first time a 7-fold increase in transcript abundance of *11βhsd2* in response to cortisol exposure of zebrafish ovarian follicles. Transcriptional regulation of this enzyme was confirmed with the addition of actinomycin D, which attenuated the cortisol-mediated increase in *11βhsd2* transcript abundance. The addition of a translational inhibitor, cycloheximide, increased both the basal transcript levels, as well as cortisol-induced levels of *11βhsd2*, which suggests that not only is cortisol increasing transcript levels it is also increasing translation of this enzyme. Incubation of ovary homogenate with ^{3}H-cortisol for 4 h resulted in a total conversion of 85% to ^{3}H-cortisone and total cortisol conversion decreased 7.5-fold in the presence of the 11βHSD2 inhibitor, 18β-glycyrrhetinic acid (Alderman and Vijayan 2012). This suggests that the zebrafish ovary has the ability to quickly upregulate and clear excess cortisol and this may be a mechanism that limits the transfer of excess cortisol to the embryos even from stressed mothers. The function of 11βHSD2 in the ovary corresponds to its role in mammals, where high cortisol levels will upregulate levels of 11βHSD2 to reduce cortisol levels in tissues important in aldosterone signalling, a hormone absent in teleost fish (Mommsen et al. 1999). It is difficult to determine whether this is the case for other species, as there have only been a few studies which have examined the maternal transfer of cortisol, and even fewer that have

examined the spawning time course post stressor (Giesing et al. 2011, Stratholt et al. 1997).

Embryos exposed to stressed levels of cortisol will upregulate 11βHSD2 and break down cortisol, preventing yolk incorporation. Cortisol will enter the yolk during the small window it takes to upregulate sufficient amount of 11βHSD2. We postulate that this is why there is an increase in embryo cortisol at day nine, and not on any subsequent days. The theca and granulosa cells of isolated ovarian follicles can regulate levels of cortisol by increasing levels of 11βHSD2 as well as forming sulphated steroids in the early stages of oogenesis (Li et al. 2012). The present study supports the hypothesis that the theca and granulosa cells are regulating cortisol levels, as once cortisol is incorporated into the yolk there appears to be no further breakdown (Figure 8). If yolk-cortisol was metabolized we would expect to see similar results for each day that stressed mothers transferred cortisol to the embryo. However the results clearly show only a transient rise, suggesting that cortisol metabolism must occur upstream of its incorporation into the yolk. Further studies would need to be completed to confirm the oocyte stage of 11βHSD2 regulation and the cell type responsible for cortisol breakdown. In zebrafish embryos there are very low levels of *11βhsd2* transcripts transferred from the mother (Alsop and Vijayan 2008), and transcript abundance of this enzyme only increases post hatch. This further supports the hypothesis that basal levels of 11βHSD2 are too low to properly regulate cortisol levels post vitellogenesis.

A significant amount of work has examined the effect of excess glucocorticoids, mimicking maternal stress, on early developmental regulation in zebrafish (Hillegass et al. 2008, Nesan and Vijayan 2012, Pikulkaew et al. 2010). Our results underscore a tight

maternal control, excess cortisol induction of 11βHSD2, to effectively regulate and reduce excess cortisol incorporation in developing oocytes. This regulation will serve to increase the viability of the spawned embryos, as excess zygotic cortisol content leads to developmental dysfunction (Nesan and Vijayan 2013b). Regardless of embryo steroid regulation, cortisol also acts to increase fecundity and we propose that this steroid may have a stimulatory effect on maturation-inducing pathways in zebrafish, while the mechanism remains to be elucidated.

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2.7 Tables

Table 2.1. Primer sequences for qPCR (ovarian follicles).

Primer sequences, annealing temperatures, product sizes and references for oligonucleotide primers used in quantitative real-time PCR.

Gene	Primer Sequence	Amplicon (bp)	Annealing Temp (^{0}C)	Reference
11βhsd2	F: 5'TGCTGCTGGCTGTACTTCAC-3' R: 5'TGCATCCAACTTCTTTGCTG-3'	123	55	Alsop and Vijayan 2008
β-actin	F: 5' TGTCCCTGTATGCCTCTGGT-3' R: 5' AAGTCCAGACGGAGGATGG-3'	121	60	Alsop and Vijayan 2008

Table 2.2. Weight, K, GSI of fasted adult females.

Body weight, condition factor (K) and gonadosomatic index (GSI) of adult female zebrafish following a 5 day fasting stressor.

		Body Weight (mg)	Condition Factor (K)	Gonadosomatic Index GSI (%)
Treatment	**N**	**Mean ± SEM**	**Mean ± SEM**	**Mean ± SEM**
Fed	10	305 ± 39	0.93 ± 0.05	9.5 ± 1.0
Fasted	12	307 ± 27	1.31 ± 0.10	9.0 ± 1.3

Chapter Three: Excess zygotic cortisol content affects larval behaviour and the cortisol stress response in zebrafish

3.1 Introduction

Glucocorticoid signaling is important for vertebrate development, including the development of the brain and the endocrine stress response (Moisiadis and Matthews 2014). Abnormal levels of glucocorticoids during early development can alter developmental programming, often with detrimental effects (Moisiadis and Matthews 2014). While this has been explored in mammals, very little is known about the effect of elevations in zygotic cortisol content on teleost development. In teleosts, maternal cortisol transfer to the oocytes is the only source of steroid during early development, until hatch (Alsop and Vijayan 2008; Nesan and Vijayan 2013b). This maternal deposition of cortisol is essential for the developmental programming of zebrafish (*Danio rerio*) (Nesan and Vijayan 2013b). This initial maternal deposit of cortisol decreases until hatch, after which it begins to increase again through endogenous synthesis (current study, Alsop and Vijayan 2008). While the genes involved in the hypothalamus-pituitary-interrenal (HPI) axis activation are in place by 32 hours post fertilization (hpf), *de novo* synthesis of cortisol does not occur until 48 hpf (Alsop and Vijayan 2008, 2009a). However, the ability to mount a cortisol response to a stressor only appears by around 3 days post-fertilization (dpf; Alsop and Vijayan 2008, Alderman and Bernier 2009). If the mother is stressed and cortisol levels increase, this may be transferred to the developing oocyte via the yolk during vitellogenesis (Chapter 2). Abnormal increases in zygotic glucocorticoid level disrupt cardiac development and heart performance (Nesan and

Vijayan 2012), as well as the cortisol stress performance of zebrafish larvae (Nesan 2012).

The corticosteroid stress response in teleosts is mediated by the hypothalamus-pituitary-interrenal axis. Upon stressor perception, corticotropin releasing hormone (CRF) secretion from the hypothalamus results in release of adrenocorticotropic hormone (ACTH) into circulation (Vijayan et al. 2010). ACTH then acts on steroidogenic cells in the head kidney, resulting in the synthesis and release of cortisol into circulation (Vijayan et al. 2010). The cortisol response allows the animal to mobilize energy stores in order to cope with the metabolic costs of the stressor. Cortisol exerts its actions in the body via two cytosolic nuclear receptors, the glucocorticoid and mineralocorticoid receptors (GR and MR), as well as through nongenomic signalling pathways (Dindia et al. 2013). Glucocorticoid signalling through GR mediates the metabolic changes that allow the animal to both mobilize and replenish energy stores in order to cope with the stressor (Mommsen et al. 1999). If the animal does not cope adequately with stress, this may result in tertiary effects detrimental to fish reproduction, growth and immune function (Iwama 1998). Physiological stress is also known to modulate behavioural phenotypes, including social hierarchy, feeding activity and anxiety, many of which are well characterized and correlate with plasma cortisol level (Egan et al. 2009, Gilmour et al. 2005, Tudorache et al. 2014).

Fish behaviour has been explored from early larval stages to adults. The zebrafish larvae is well suited to behavioural analyses due to high offspring yield and the ability to generate high throughput data. As a result, many of the early behaviours are well characterized (Fero et al. 2011). The locomotor response to alternating periods of light

and dark is particularly interesting as it appears to be highly conserved, and has been seen in fish, mice and insects (Irons et al. 2010, MacPhail et al. 2009, Prober et al. 2006, Emran et al. 2007, Moorhouse et al. 1978). This assay provides information on locomotor activity, visual ability to detect light changes, and freezing behaviour (anxiety) in response to sudden light onset (Emran et al. 2008, MacPhail et al. 2009). Thigmotaxis is another behavioural assay to assess anxiety, commonly used in rodents (Simon et al. 1994) but adapted for use with zebrafish (Schnörr et al. 2012). Few studies to date have explored the links between physiological stress and larval behaviour in zebrafish (Tudorache et al. 2014, Wilson et al. 2013, Clark et al. 2012), while none have addressed the effects of increased maternal deposition of cortisol and its impact on larval behaviour. As the brain is essential is mediating both the response to acute stress, as well as coordinating behavioural responses, early neurogenesis is a potential target for altered developmental programming due to elevated cortisol deposition.

The zebrafish nervous system originates from the neuroectoderm, and factors including bone morphogenetic proteins (BMPs) and their antagonists (e.g. Noggin, Chordin) assist in neural specification (Schmidt et al. 2013). Neurogenesis begins during late gastrulation (10 hpf), as detected by the earliest proneural gene expression (Schmidt et al. 2013). This includes basic helix-loop-helix (bHLH) transcription factors, including the neurogenic differentiation (*neurod*) family, which promote differentiation of neurons in specific regions throughout the developing vertebrate brain (Wang et al. 2009, Schmidt et al. 2013, Mueller and Wullimann 2002). *Neurod4* is involved in primary neurogenesis in the midbrain, hindbrain and retina of zebrafish (Park et al. 2003, Wang et al. 2003). In terms of motor development, *islet* is one of the earliest markers of motor neurons (Eisen

1999) and regarding neuroendocrine function, *orthopedia* is a homeodomain transcription factor essential in the patterning of the hypothalamus and preoptic region, particularly in the development of specific populations of dopaminergic and neuroendocrine cells, including CRF neurons (Ryu et al. 2007, Fernandes et al. 2013). These are important developmental signals during neurogenesis, and are potential targets of disrupted glucocorticoid signalling in zebrafish (Nesan and Vijayan 2013b).

As maternal stress results in elevated levels of zygotic cortisol (seen in Chapter 2), and this maternal cortisol deposit is known to affect the developmental programming of the zebrafish (Nesan and Vijayan 2013), we hypothesized that stress-related larval behaviours and the physiological stress response are altered in response to elevated glucocorticoid deposition in the egg, and that this may be a result of disrupted neurogenesis. Embryos were injected with cortisol to mimic elevated maternal deposition and the stress and behavioural phenotypes were assessed after hatch at 4 dpf. Also, neurogenesis and associated transcription factors were assessed at 24 (neurogenesis), 36 and 48 (transcripts) hpf to determine the effects of elevations in zygotic cortisol on neurogenesis.

3.2 Materials and Methods

3.2.1 Animals

Zebrafish (Tupfel long fin strain) were held in a recirculating system in 10 L tanks (Pentair Aquatic Habitats, Apopka FL) on a 14:10 light cycle at 28 ºC. Water was maintained at 750 µS conductivity and pH 7.6. Fish were fed twice daily consisting of Zeigler adult zebrafish food in the morning (Pentair Aquatic Habitats, Apopka FL) and

live *Artemia* (San Francisco Bay Brand, Inc.) in the afternoon. For breeding, fish were set up the previous evening in breeding traps, and eggs were collected the next morning. Embryos were maintained in E3 embryo medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$, 0.1 ppm methylene blue as antifungal; Nusslein-Volhard and Dahm 2002, Burgess and Granato 2007) in 10 cm Petri dishes (60-100 embryos per dish), with daily renewal of embryo medium (approx. 50%) and removal of any dead embryos or larvae. No significant treatment effect on mortality was observed. This maintenance continued until embryos or larvae were required for experiments, to a maximum of 5 dpf. All protocols involved in the maintenance and experimental use of zebrafish were approved by the University of Calgary Animal Care Committee, and abide by the guidelines set out by the Canadian Council on Animal Care.

3.2.2 Microinjection of Cortisol

Injections were performed using a microinjection system and nitrogen gas (Narshige, Japan). Glass capillaries (Sutter Instruments, Novato, CA) were pulled into needles and trimmed prior to use. Cortisol (Sigma-Aldrich, St. Louis, MO, USA) solutions were prepared from ethanol stocks in sterile (0.2 µm filtered) water with 0.05% phenol red (Sigma-Aldrich, St. Louis, MO, USA) for visualization. On the day of injection, embryos were collected and maintained in system water until they were transferred to pre-warmed agarose injection trays. Following calibration (1 nL), pre-warmed cortisol solutions were delivered by microinjection into the yolk to mimic maternal deposition (vehicle control and 75 pg/egg). Injected fish were transferred into Petri dishes with E3 medium and raised as above. For cortisol analysis, samples were

collected at 1, 24, 48, and 96 hpf, and embryos or larvae were collected in pools of 20 (n=4-9).

3.2.3 Acute Stressor Challenge

Embryos were microinjected with either vehicle or cortisol as described above. At 4 dpf, control and cortisol-treated larvae were sampled quickly to assess resting cortisol (0 min) levels. Sampling consisted of quick water removal and the larvae (pools of 12 per 1.5 ml tube) were snap frozen on dry ice. The remaining larvae were gently swirled for 30 s, transferred back into Petri dishes and sampled as above at 5, 15, 30, 60 and 120 min post-stressor exposure (n=4-5 trials).

3.2.3.1 Cortisol ELISA

For cortisol quantification, pools of embryos or larvae (n=12 or 20) were collected and all water was removed prior to snap freezing on dry ice. These pools of embryos or larvae were then partially thawed on ice and homogenized in 120 or 200 µl, respectively, of 50 mM Tris buffer, pH 7.5 with added protease inhibitors (Roche Diagnostics, Laval, QC, CAN). Samples were sonicated on ice to homogeneity and stored at -80ºC until use.

Cortisol levels were measured using a competitive enzyme-linked immunosorbent assay (ELISA) based on the protocol of Yeh et al., (2013). Briefly, high binding 96 well plates (Immulon HB, VWR) were coated with 100 µl of cortisol monoclonal antibody (1.6 µg/ml; East Coast Bio, ME, USA) in phosphate buffered saline (1 x PBS; 10x stock: 1.37M NaCl; 27 M KCl, 18 mM KH_2PO4; Na_2HPO4), for 16 h at 4°C. The plate was then washed with PBS with 0.05% Tween 20 (PBST; 300 ul/well) and blocked with 0.1% bovine serum albumin (300 µl/well; BSA; Sigma, Oakville, ON, CAN) for 1 h at room

temperature. Standards comprised of cortisol (Sigma, H0888) serially diluted (0 ng/ml – 25 ng/ml) in PBS. Standards and samples (50 µl) were added to the wells in duplicate. Cortisol conjugated to horseradish peroxidase (1:160 dilution; East Coast Bio, ME, USA) diluted in PBS was immediately added to each well. Plates were incubated for 2 h, shaking, at room temperature. The plate was washed as described above, and the detection reagent was added (41 mM TMB and 8 mM TBABH in 200 mM potassium citrate, pH 4). After 1 hour the reaction was stopped with 1 M sulfuric acid. Wells were read at 450 nm using a microplate reader (VersaMax, Molecular Devices, CA, USA).

3.2.4 Behavioural Analysis

Embryos were injected with vehicle or cortisol as above and raised to 3 dpf. The larvae were transferred to clear multiwell plates (24 or 96) with lids (1 individual per well) and were allowed to settle overnight. Analyses were performed in an isolated room maintained at 28.5ºC. The movement of 4 dpf larvae was video captured and quantified using the ZebraBox infrared camera setup and the tracking extension of the ZebraLab software system (Viewpoint Life Sciences, Montreal, QC, CAN). In all behavioural protocols, the animal colour was set to black and the background-subtracted detection threshold was set to 20. This value represents a greyscale pixel intensity value, and any pixels darker than this threshold in the video are detected as the animal. The integration period (bin time) for movement data was set to 30 s. Data were processed and analyzed using Excel, and FastDataMonitor (Viewpoint Life Sciences, Montreal QC).

3.2.4.1 Activity in Light and Dark

For the light-dark response assay, 4 dpf larvae were subjected to short alternating periods of light and darkness, and their movement in response to these stimuli were

recorded. The protocol parameters consisted of alternating periods of light (500 lx, maximum light intensity) and dark every 7.5 minutes (450 s). This 15 minute cycle of light and dark was repeated four times for a total of 60 min. This was repeated over 3 trials (24 per treatment group per plate) for a total of 72 larvae per treatment.

Endpoints calculated per animal per 30 s bin (integration period), included total distance moved (mm), total duration in motion (s), and velocity (mm/s). These endpoints were used to calculate the sum, mean or area under the curve (distance x time) for each endpoint per animal.

3.2.4.2 Thigmotaxis

This behavioural assay is analogous to the open field test in rodents, used to assess anxiety by the tendency to remain close to the walls of the arena (Ahmad and Richardson 2013). To test this, 3 dpf injected larvae were transferred in 0.5 ml of E3 embryo medium into each well of a 24 well plate and allowed to settle as above. Larger wells allowed space for creating two distinct inner and outer zones with the tracking software, each with widths ≥ one larval body length. The parameters for this assay consisted of these zone designations, no light, and a total duration of 30 min. This assay is run in the dark to induce strong activity levels, thus encouraging the animal to fully explore the environment (Schnörr et al. 2012). This was repeated over 6 trials (8 per treatment group per plate) for a total of 48 larvae per treatment. The total distance moved (mm) over the entire duration of the 30 min assay was summed for each larvae, for both the inner and outer zones. Thigmotaxis was calculated as the total distance moved by the animal in the outer zone, as a percentage of the total distance moved by the animal in

total. This method corrects for individual differences in activity as recommended by Schnörr et al. (2012).

3.2.5 Neurogenesis

Embryos were injected with cortisol or vehicle as above. To assess neurogenesis, embryos were pulse-labelled with the thymidine analogue ethynyl deoxyuridine (EdU) at 24 hpf, raised until 5 dpf and the brain sectioned and immunostained to identify neurons born at 24 hpf (n = 5). Transcript abundances of neurogenic genes were also determined in injected larvae at 36 hpf, which were snap frozen in pools of 20 embryos (n = 7-9) for later qPCR analysis. Injected larvae at 36 and 48 hpf were also fixed and dehydrated in methanol for *in situ* hybridization for determination of spatial transcript abundance (n=4).

3.2.5.1 Quantification of Neurogenesis

Assessment of neurons born at 24 hpf was performed exactly as described in Kinch et al. (2015). A brief description of methods is as follows:

3.2.5.1.1 EdU Pulsing and Sampling.

Injected fish were pulsed-labelled with EdU at 24 h by collecting 20 embryos in a 1.5 ml tube, aspirating off excess embryo medium and replacing it with prewarmed EdU solution (10 mM, Molecular Probes C10338). Fish were allowed to incubate in this solution for 15 min on ice, then 15 min at room temperature. Fish were gently washed and returned to their Petri dishes. At 5 dpf, fish were sacrificed by fixation in 4% PFA (paraformaldehyde) for 1.5 h at room temperature (RT), washed in PBS (phosphate buffered saline, pH 7.4), and then dropped in 30% sucrose overnight at 4ºC for cryoprotection.

3.2.5.1.2 Sectioning and Immunohistochemistry

Cryoprotected fish were embedded in OCT (optimum cutting temperature) compound (VWR Scientific), snap frozen on dry ice and stored at -80 until sectioning. Transverse 10 µm sections were taken through the brain using the Leica CM 3050 S cryostat (Leica Microsystems, Wetzlar, Germany). Section positions were confirmed using the zebrafish brain atlas (Mueller and Wullimann 2005, see Figure 3.7). Antigen retrieval was performed using citrate buffer (10 mM trisodium citrate, pH 6.0, 0.5% Tween-20) and boiling for 20 min. Sections were permeabilized with 1% PBT (PBS + 1% Triton-X), blocked with 5% normal goat serum for 1 h at RT, then incubated overnight at 4ºC in primary α-HuC (1:400, Molecular Probes A21271). Secondary antibody was conjugated to Alexa Fluor 488 (1:400, Molecular Probes A11001) and incubation was for 2 h at RT in dark. Slides were stained with DAPI (1:1000, Molecular Probes D1308), and then the Click-iT reaction to detect EdU labelling was performed as per kit instructions (Molecular Probes C10338). Slides were mounted and imaged by fluorescence microscopy.

3.2.5.1.3 Analysis

Images were analysed using Fiji (Schindelin et al. 2012). Images were overlaid to produce a multichannel image, then the Cell Counter plugin was used to count labelled cells in the specified regions of selected sections (DAPI, blue; HuC, green; and EdU, red – see figures 3.8 and 3.9 for representative sections). Counts of neurons born at 24 h (DAPI, HuC and EdU-positive cells) were normalized to total number of neurons (HuC-positive cells) in that region.

3.2.5.2 Whole-Mount In-Situ Hybridization

3.2.5.2.1 Probe Synthesis

Riboprobe templates were obtained by PCR using 48 h larval cDNA as template, and gene-specific primers with added T7 sites (Table 3.3). After PCR purification, *in vitro* transcription was performed by combining 200 ng purified PCR product, 2 µl 10x DIG labelling dNTP mix (digoxygenin, Roche Diagnostics, Laval, QC, CAN), 4 µl 5x transcription buffer, 40 U RNase inhibitor (RiboLock, Thermo Scientific, Waltham, MA, USA), 40 U T7 RNA polymerase (Thermo Scientific, Waltham, MA, USA) and nuclease free water to 20 µl final volume. The reaction was incubated for 2 h at 37 ºC, volume was brought up to 50 µl with nuclease-free water and the riboprobe was purified using SigmaSpin columns (Sigma-Aldrich, St. Louis, MO, USA).

3.2.5.2.2 Hybridization and Staining

Whole mount in situ hybridization was performed as per the protocol of Kurrasch et al. (2009). Briefly, fish were fixed in 4% PFA, dehydrated in methanol, and stored at -20 until hybridization. Rehydration was in PBS-T (PBS + 0.1% Tween-20), followed by 30 min bleaching (0.5X SSC, 1% H2O2, 5% formamide) and permeabilization by proteinase K (36 hpf: 10 µg/ml x 30 min; 48 hpf 50 µg/ml x 8 min) and refixed. Larvae were prehybridized for 2 h at 70ºC, followed by overnight hybridization with probe (1:100). Following a series of washes at 70ºC, larvae were incubated in blocking buffer (5% sheep serum) for 1 h at RT. The anti-DIG antibody (Roche Diagnostics, Laval, QC, CAN) was diluted 1:1000 in blocking buffer and incubated overnight at 4 ºC. Larvae were thoroughly washed and then stained with NBT/BCIP solution in staining buffer for 40 minutes in the dark. The reaction was stopped by washing and refixation in 4% PFA. Fish

were transferred to 87% glycerol for clearing and imaged using a Nikon AZ-100 microscope.

3.2.6 Transcript Analysis by Quantitative Real-Time PCR

Total RNA was extracted from 36 hpf injected embryos (n=7-9 pools of 20 embryos). Briefly, samples were sonicated to homogeneity in Ribozol (Amresco, Solon, OH, USA). Chloroform was added and following phase separation, total RNA was precipitated from the aqueous phase by isopropanol. The RNA pellet was then washed multiple times with ice-cold 75% ethanol and the pellet was dissolved in nuclease-free water (Amresco, Solon, OH, USA) and quantified (SpectraDrop, Molecular Devices, Sunnyvale CA). 1 µg of RNA was DNase I-treated (Thermo Scientific, Waltham, MA, USA) and reverse transcribed to cDNA (High Capacity cDNA Reverse Transcription Kit, Applied Biosystems, Foster City CA) according to the manufacturer's protocol.

Gene-specific primers (Table 3.2) were used to amplify ~100 bp products in real-time quantitative PCR. A master mix contained (per sample) 1 µl cDNA template, 1 µl primer pair (10 µM), 16 µl 2X SYBR green supermix (BioRad) and 14 µl nuclease-free water. In each well, 10 µl of master mix was added and samples were run in triplicate. Amplification conditions were as follows: initial denaturation for 2 min at 94°C, 40 cycles of 20 s at 94°C, 20 s at annealing temperature (Table 3.2); 95°C for 1 min; 55°C for 1 min followed by melt curve analysis (55°C to 95°C at 0.5°C / 10 s). Threshold cycle values were calculated using CFX Manager detection software (BioRad). Relative transcript abundance was quantified using the MNE method, as described previously (Simon 2003, Wiseman et al. 2011). The housekeeping gene used was *β-actin*, as the transcript abundance was unchanged between treatments.

3.2.7 Statistics

Cortisol levels during embryogenesis and in response to stressor exposure were analysed by two-way ANOVA. A Tukey post hoc test was used to determine treatment and time effects. All other data were assessed by Student's t-tests (unpaired). Data were transformed where necessary to meet the normality and equal variance assumptions of parametric data, and if these assumptions could not be met, a non-parametric test was carried out (Mann Whitney U test). The significance level (α) was set to 0.05, and SigmaPlot 13 (Systat Software, Inc.) was used for all statistical analyses.

3.3 Results

3.3.1 Cortisol Developmental Profile

Cortisol levels in the vehicle-injected embryos dropped over the first 48 hpf, and then increased following hatching. A mean initial cortisol level of 4.1 ± 0.7 pg per embryo was present in the control embryos at 1 hpf, and this dropped significantly ($P = 0.028$) to 1.8 ± 0.5 pg / embryo by 48 hpf (Fig. 3.1). By 96 hpf (4 dpf), cortisol levels in the larvae increased significantly ($P < 0.001$) to a mean of 12.0 ± 2.3 pg / larvae. Embryos injected with cortisol showed significantly higher cortisol levels at 1 hpf (76.1 ± 5.7 pg/ embryo) compared to the controls. At 24 hpf, values in the cortisol-injected group were significantly higher than the controls ($P = 0.037$). By 48 hpf, the lowest point of the developmental cortisol profile, cortisol-injected values were no longer significantly different compared to the control ($P = 0.204$). At 96 hpf, cortisol levels had increased once again, and levels in the cortisol-injected group were significantly higher than controls ($P = 0.003$).

3.3.2 Stress Response

A significant interaction between time and treatment was detected (two way ANOVA, P = 0.017) in whole body cortisol levels in response to an acute stressor. Basal cortisol levels in the vehicle control, prior to stress, were 12.3 ± 2.4 pg per larvae, while the cortisol-injected group was higher (25.5 ± 6.9 pg/larvae, P = 0.050, Fig. 3.2). In the control group, cortisol levels rose significantly 5 min post-stressor (32.3 ± 3.8 pg/larvae, P = 0.035) and declined until 1 h post-stressor, returning to pre-stressor levels and remaining there until the last collection point at 2 h post-stressor (13.3 ± 2.3 pg / larvae, Fig. 3.2). In the cortisol-injected group, cortisol levels increased, though not significantly, over the course of the first 15 min in response to the acute stress challenge (0 vs. 5 min: P = 0.713, 0 vs. 15 min: P = 0.288, Fig. 3.2). Levels then dropped significantly 30 min post-stressor and remained low for the remaining 90 min. The time required to attain peak cortisol levels was 10 min slower in the cortisol-injected group (15 min, 1.8-fold increase from basal) compared to the vehicle controls (5 min, 2.6-fold increase from basal, Fig. 3.2).

3.3.3 Behaviour

At 4 dpf all larvae displayed elevated locomotor activity in the dark and decreased activity during periods of light (Fig. 3.3). Activity was approximately 6-fold lower in bright light than in the dark (Fig. 3.3). In the cortisol group, area under the curve (Fig, 3.4A), velocity (Fig. 3.4B), distance travelled (Fig. 3.4C, E) and time in motion (Fig. 3.4D, F) were all significantly elevated above control levels during the initial light period (L1), with the exception of velocity. The second light period displays a similar elevation to that observed in the first light period, but only in the latter half (L2L, Fig. 3.4),

although velocity is significantly elevated by cortisol microinjection over the entire second light period (Fig. 3.4B). This trend of elevated cortisol deposition increasing activity is observed during the initial half of the first dark period (D1E), but only in terms of duration in motion (Fig. 3.4C, E). No changes in activity due to cortisol microinjection is observed during any other dark period. If all light periods of the same type are combined for the whole 1 h protocol (Fig. 3.5), there are no changes in the dark. However there are overall changes in the light: the cortisol group has significantly higher area under the curve, distance travelled, and duration in motion upon exposure to light (Fig. 3.5). Thigmotaxis was significantly lower in the cortisol group than the controls (Fig. 3.6).

3.3.4 Neurogenesis

Neurogenesis at 24 hpf was not affected by elevated cortisol deposition in the early embryo (Fig. 3.8A), including the rostral hypothalamus (Hr, Fig. 3.8B), the posterior tuberculum (PT, Fig. 3.8C), and the dorsal thalamus (DT, Fig. 3.8D). However, neurogenesis was elevated by cortisol microinjection in both the pallium (P, Fig. 3.9B) and the preoptic region (Po, Fig. 3.9C).

3.3.5 Transcript Abundance

Transcript abundance of candidate genes involved in the stress response, development, and neurogenesis were assessed in the whole body at 36 hpf by qPCR (Table 3.4). Transcripts of the mineralocorticoid receptor (*mr*) were significantly downregulated at 36 hpf (Table 3.4). In addition, 11β hydroxysteroid dehydrogenase type 2 (*11βhsd2*) and corticotropin releasing factor (*crf*) transcripts displayed a trend of downregulation with P-values at or below 0.1 (Table 3.4).

Spatial distribution of key proneural transcripts in the intact larval (36 and 48 hpf) brain was assessed by whole-mount ISH (Figs. 3.10 – 3.11). Transcripts of neurogenic differentiation 4 (*neurod4*) were found in the eye (retina), midbrain, and in a segmented pattern in the hindbrain, at both 36 and 48 hpf, but with visibly reduced expression at 48 hpf in both groups (Fig. 3.10). The spatial transcript abundance of *neurod4* at 36 hpf appears to be stronger in the cortisol group, particularly in the retina but to a lesser extent along the midbrain and hindbrain regions (Fig. 3.10, 36 hpf). At 48 hours the cortisol group has a reduced retinal expression, but increased hindbrain expression relative to controls (Fig. 3.10). No significant changes in *neurod4* whole body transcript abundance were detected by qPCR (Table 3.4). Transcripts of orthopedia b (*otpb*) appear in the hindbrain, telencephalon, and ventral diencephalon at both 36 and 48 hpf (Fig. 3.11). While transcript abundance in the telencephalon decreases from 36 to 48 hpf, abundance in the hypothalamus and hindbrain increases over the same time period in both treatment groups (Fig. 3.11). Spatial transcript abundance of *otpb* at 36 hpf appears unchanged by cortisol microinjection (Fig. 3.11) and this is supported by qPCR (Table 3.4). At 48 hpf, expression of this gene appears higher with cortisol treatment (Fig. 3.11). No significant changes in *otpb* whole body transcript abundance were detected by qPCR (Table 3.4).

3.4 Figures

Figure 3.1. Cortisol levels during embryogenesis

Embryos were microinjected with either vehicle control or 75 pg of cortisol into the yolk immediately following fertilization, and were collected at 1, 24, 48, and 96 hpf. Values represent the means ± SEM, and n = 4-9 (pools of 20). A significant interaction was detected (two-way ANOVA, Tukey post hoc, $P < 0.001$) and significant changes within each treatment group are indicated by different lowercase letters, significant treatment effects within a time point are indicated by asterisks.

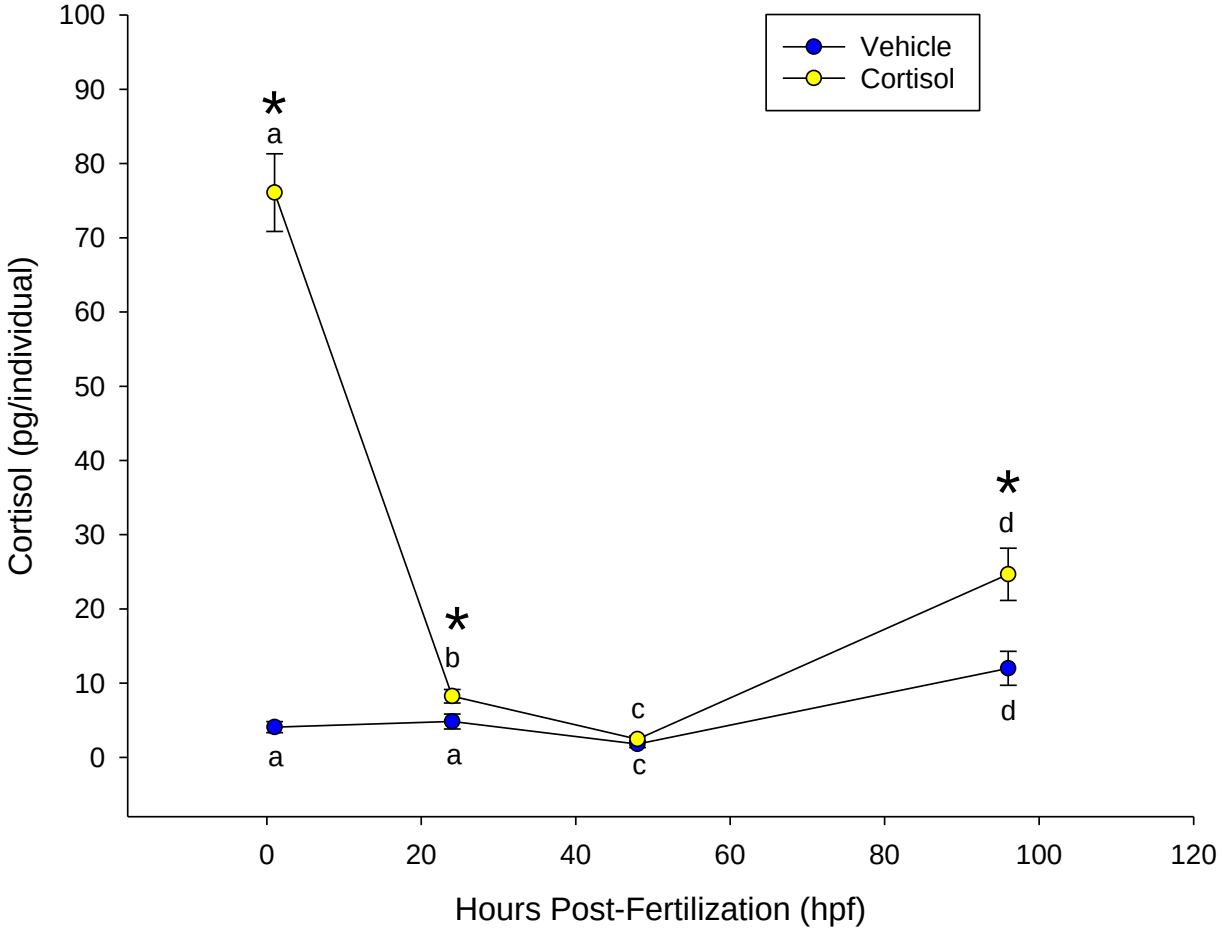


Figure 3.2. Cortisol response to a physical stressor at 4 dpf in larvae with altered cortisol deposition.

Embryos were microinjected with either vehicle control or 75 pg of cortisol into the yolk immediately following fertilization. At 4 dpf, a pre-stress timepoint was collected then fish were subjected to an acute physical stressor. Fish were allowed to recover and sampled at 5, 15, 30, 60 and 120 minutes post-stressor. Values represent the means ± SEM, and n = 4-5 (pools of 12). A significant interaction was detected (two-way ANOVA, P = 0.017) and time effects within treatment groups are indicated by different lowercase letters (Tukey post hoc).

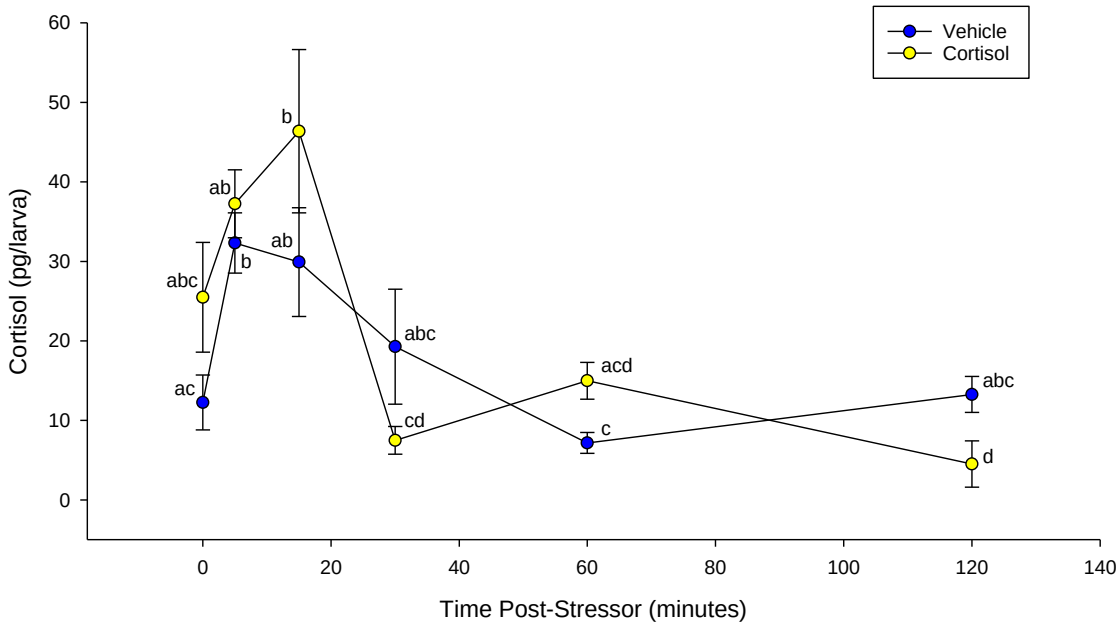


Figure 3.3. Activity in light and dark of 4 dpf larvae with altered cortisol deposition. Activity is expressed as total distance moved during each 30 s recording bin. The total recording period was 1 h with alternating light periods of 7.5 min each, indicated by the light and dark bars above the x-axis. Values represent means ± SEM, and n = 72. Inset figure represents cortisol values at 96 hpf (see Fig. 3.1).

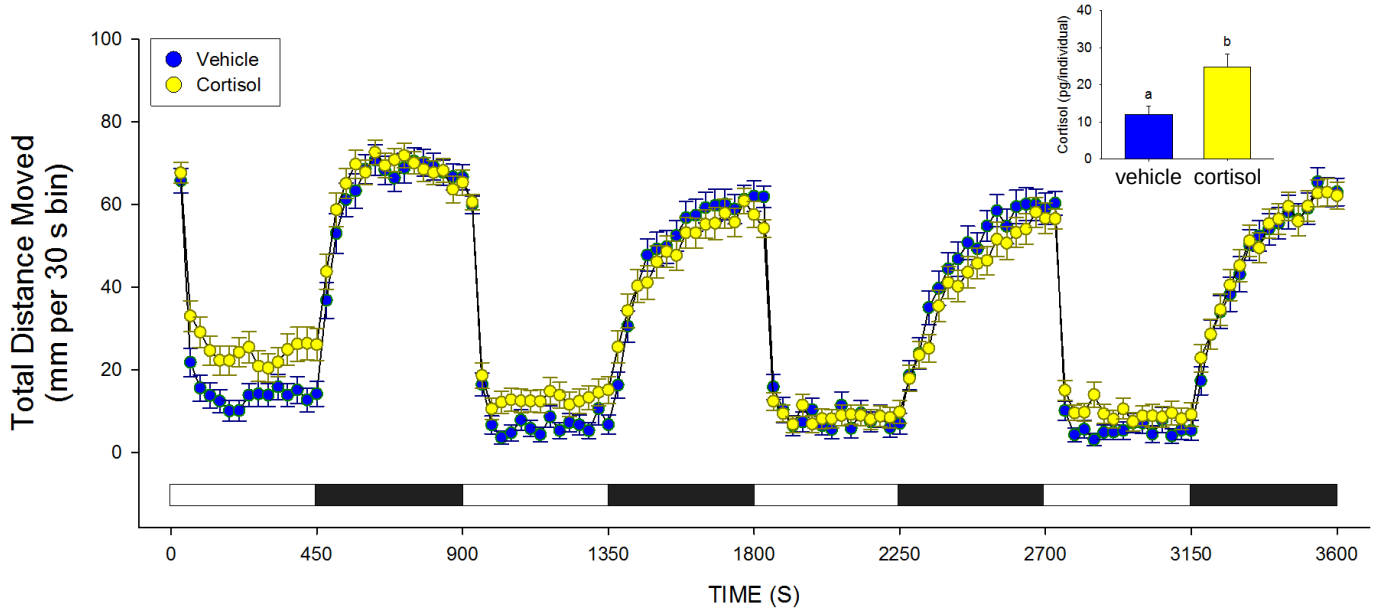
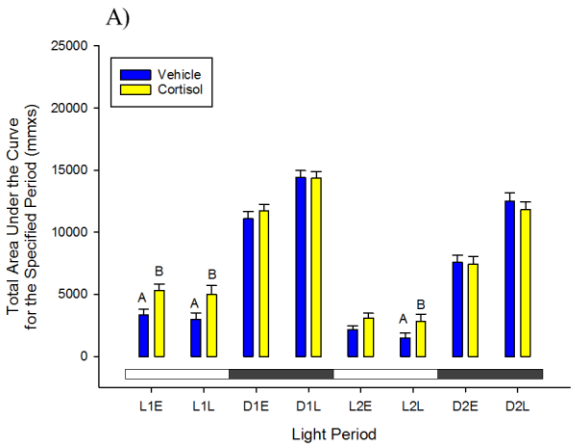
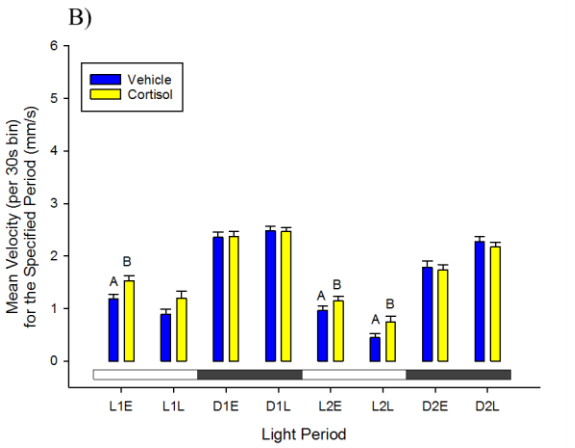
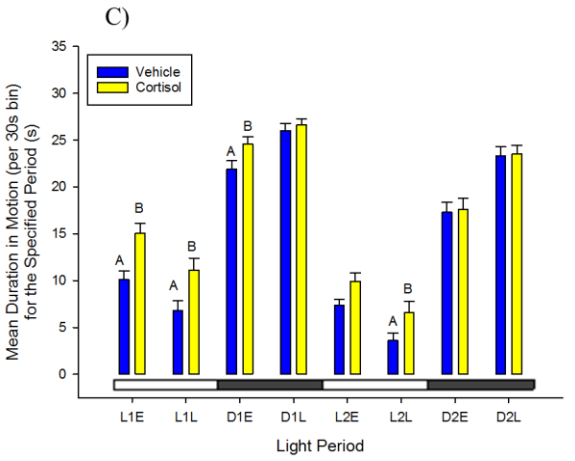


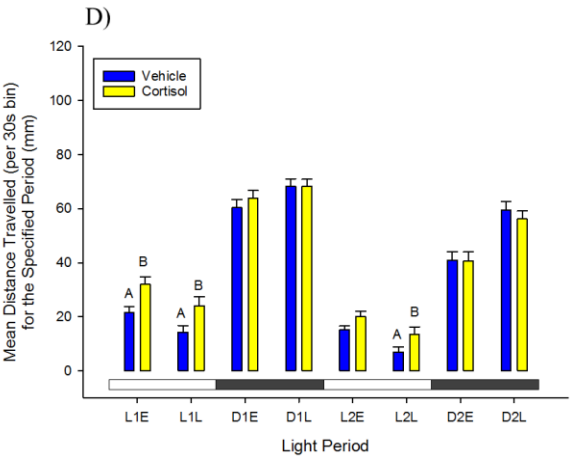
Figure 3.4. Activity in light and dark of 4 dpf larvae with altered cortisol deposition by staged (early/late) light period.

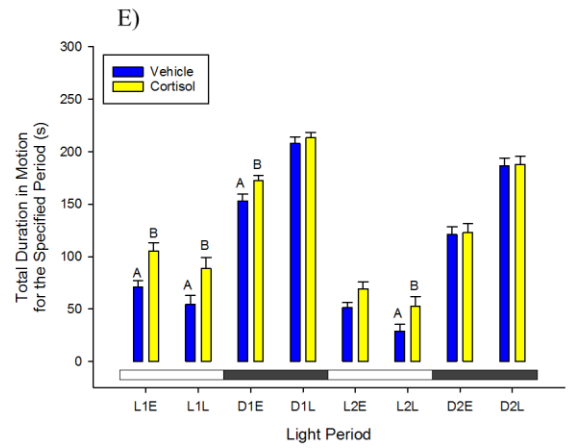
The first two light cycles (L1, D1, L2, D2) are indicated by the light and dark bars above the x-axis. These have been broken down into early (first 210 s) and late (last 240 s), indicated on the X-axis by 'E' or 'L'. Activity is expressed as (A) area under the curve, (B) mean velocity per 30s bin, (C) mean duration in motion per 30s bin, (D) mean distance travelled per 30s bin, (E) total duration in motion for the entire period, and (F) total distance travelled for the entire period. Different letters indicate significant differences (Student's t-test, unpaired). Values represent means ± SEM, and n = 72.


A)
Vehicle
Cortisol
Total Area Under the Curve for the Specified Period (mmxs)
25000
20000
15000
10000
5000
0
A
B
L1E
L1L
D1E
D1L
L2E
L2L
D2E
D2L
Light Period


B)
Vehicle
Cortisol
Mean Velocity (per 30s bin) for the Specified Period (mm/s)
6
5
4
3
2
1
0
A
B
L1E
L1L
D1E
D1L
L2E
L2L
D2E
D2L
Light Period


C)
Vehicle
Cortisol
Mean Duration in Motion (per 30s bin) for the Specified Period (s)
35
30
25
20
15
10
5
0
A
B
L1E
L1L
D1E
D1L
L2E
L2L
D2E
D2L
Light Period


D)
Vehicle
Cortisol
Mean Distance Travelled (per 30s bin) for the Specified Period (mm)
120
100
80
60
40
20
0
A
B
L1E
L1L
D1E
D1L
L2E
L2L
D2E
D2L
Light Period


E)
Vehicle
Cortisol
Total Duration in Motion for the Specified Period (s)
300
250
200
150
100
50
0
A
B
L1E
L1L
D1E
D1L
L2E
L2L
D2E
D2L
Light Period

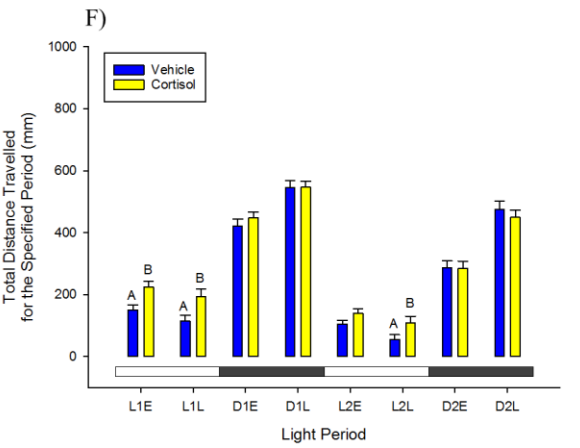
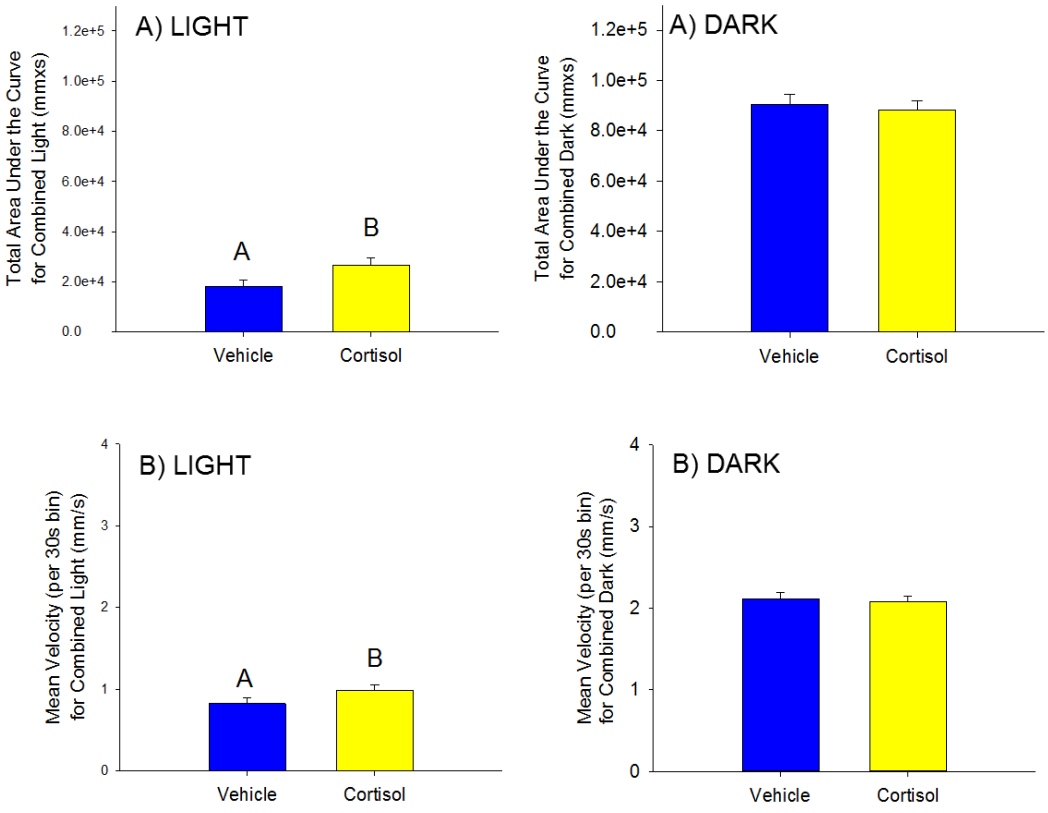
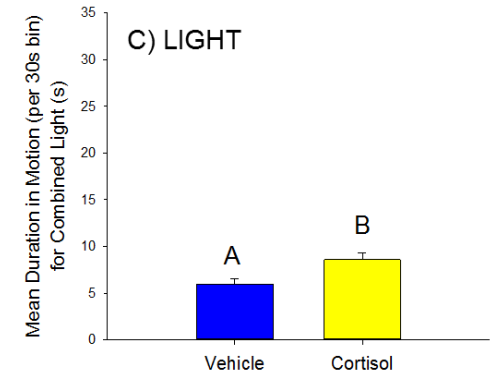
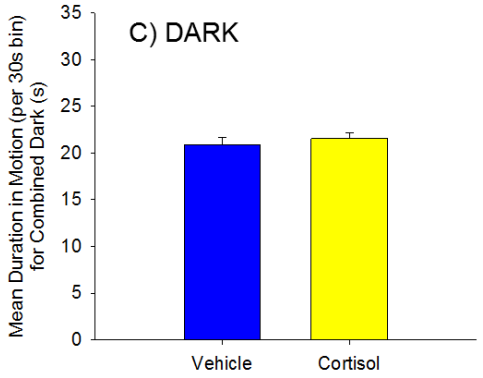

F)
Vehicle
Cortisol
Total Distance Travelled for the Specified Period (mm)
1000
800
600
400
200
0
A
B
L1E
L1L
D1E
D1L
L2E
L2L
D2E
D2L
Light Period

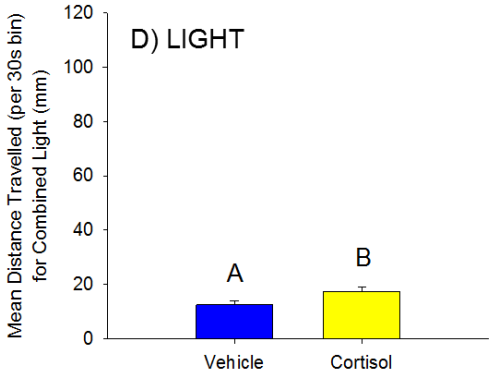
Figure 3.5. Activity in light and dark of 4 dpf larvae with altered cortisol deposition, with pooled light or dark periods.

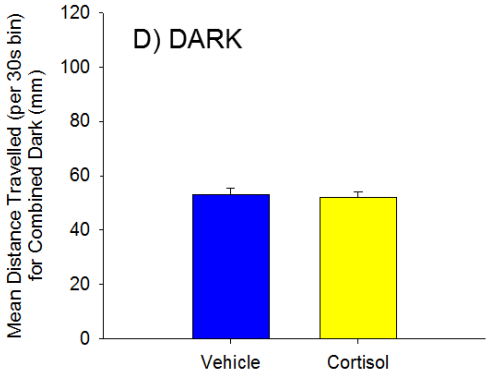
Activity is expressed as (A) area under the curve, (B) mean velocity per 30s bin, (C) mean duration in motion per 30s bin, (D) mean distance travelled per 30s bin, (E) total duration in motion for the entire period, and (F) total distance travelled for the entire period. The total recording period was 1 h with alternating light periods of 7.5 min each (pooled light or dark are 30 min). Different letters indicate significant differences (Student's t-test, unpaired). Values represent means ± SEM, and n=67-72.

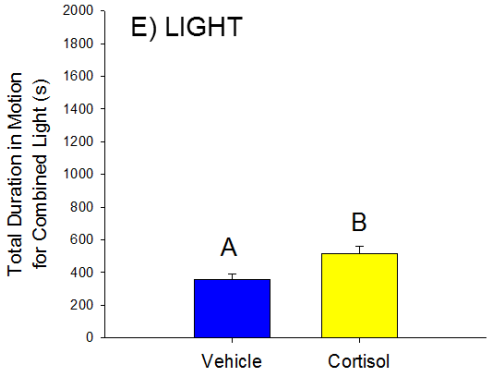


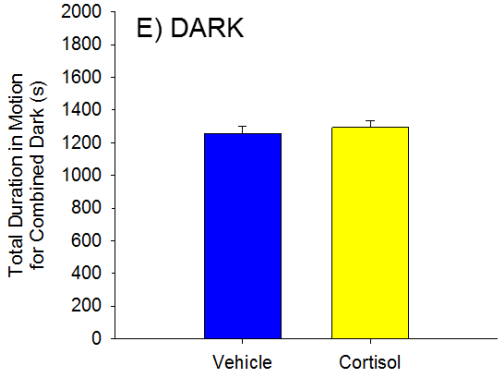

C) LIGHT
Mean Duration in Motion (per 30s bin) for Combined Light (s)
35
30
25
20
15
10
5
0
A
B
Vehicle
Cortisol

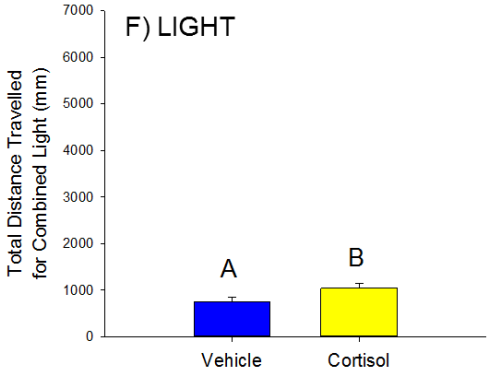

C) DARK
Mean Duration in Motion (per 30s bin) for Combined Dark (s)
35
30
25
20
15
10
5
0
Vehicle
Cortisol


D) LIGHT
Mean Distance Travelled (per 30s bin) for Combined Light (mm)
120
100
80
60
40
20
0
A
B
Vehicle
Cortisol


D) DARK
Mean Distance Travelled (per 30s bin) for Combined Dark (mm)
120
100
80
60
40
20
0
Vehicle
Cortisol


E) LIGHT
Total Duration in Motion for Combined Light (s)
2000
1800
1600
1400
1200
1000
800
600
400
200
0
A
B
Vehicle
Cortisol


E) DARK
Total Duration in Motion for Combined Dark (s)
2000
1800
1600
1400
1200
1000
800
600
400
200
0
Vehicle
Cortisol


F) LIGHT
Total Distance Travelled for Combined Light (mm)
7000
6000
5000
4000
3000
2000
1000
0
A
B
Vehicle
Cortisol

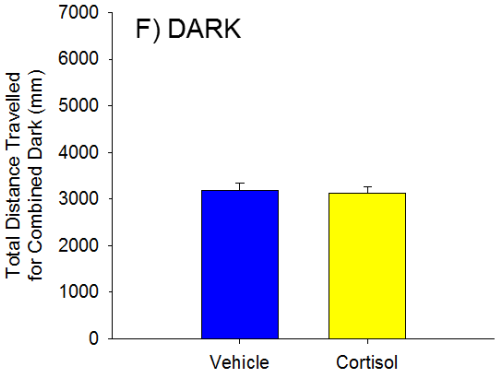

F) DARK
Total Distance Travelled for Combined Dark (mm)
7000
6000
5000
4000
3000
2000
1000
0
Vehicle
Cortisol

Figure 3.6. Thigmotaxis in 4 dpf larvae with altered cortisol deposition.

Activity of 4 dpf larvae was monitored in 24-well plates, and individual activity in the inner and outer zones of the well were recorded. Thigmotaxis is expressed as % of total distance travelled that occurred in the outer zone. Different letters indicate significant differences (Student's t-test, unpaired). Values represent means ± SEM, and n=48.

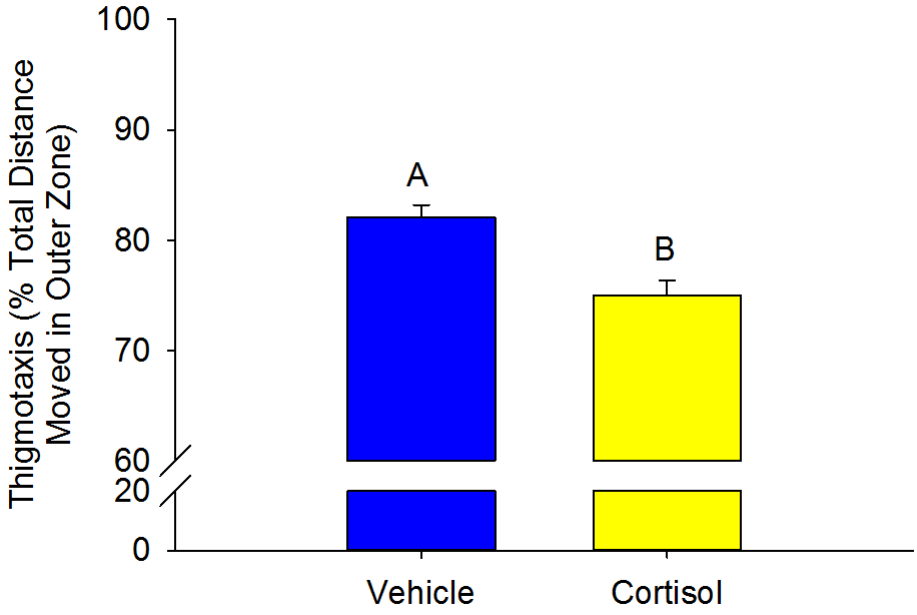


Figure 3.7. Regions in the 5 dpf zebrafish brain selected for quantification of neurogenesis in EdU-pulsed fish.

Selected regions indicated in red: P, pallium; Po, preoptic region; DT, dorsal thalamus; PT, posterior tuberculum (d/v, dorsal/ventral); H, hypothalamus. Selected transverse sections indicated by blue lines. See table 3.1 for full list of abbreviations. Adapted from Mueller and Wulliman (2005).

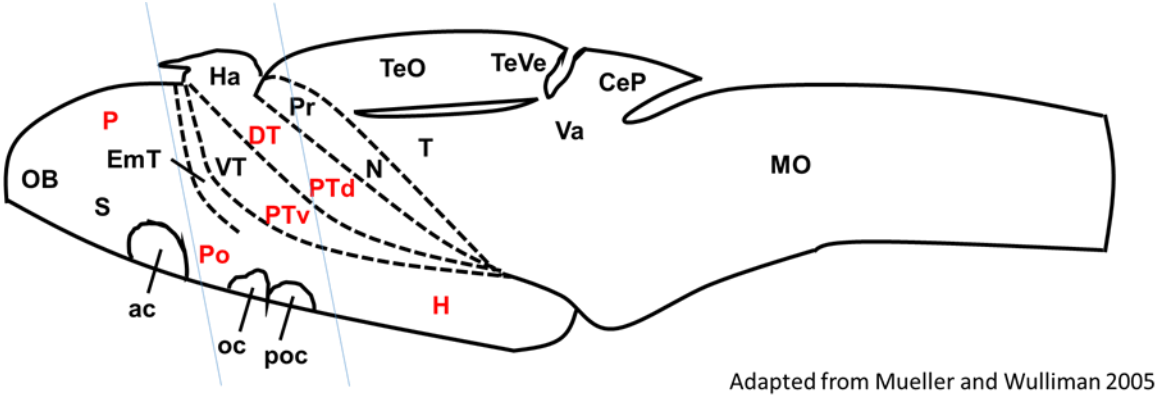
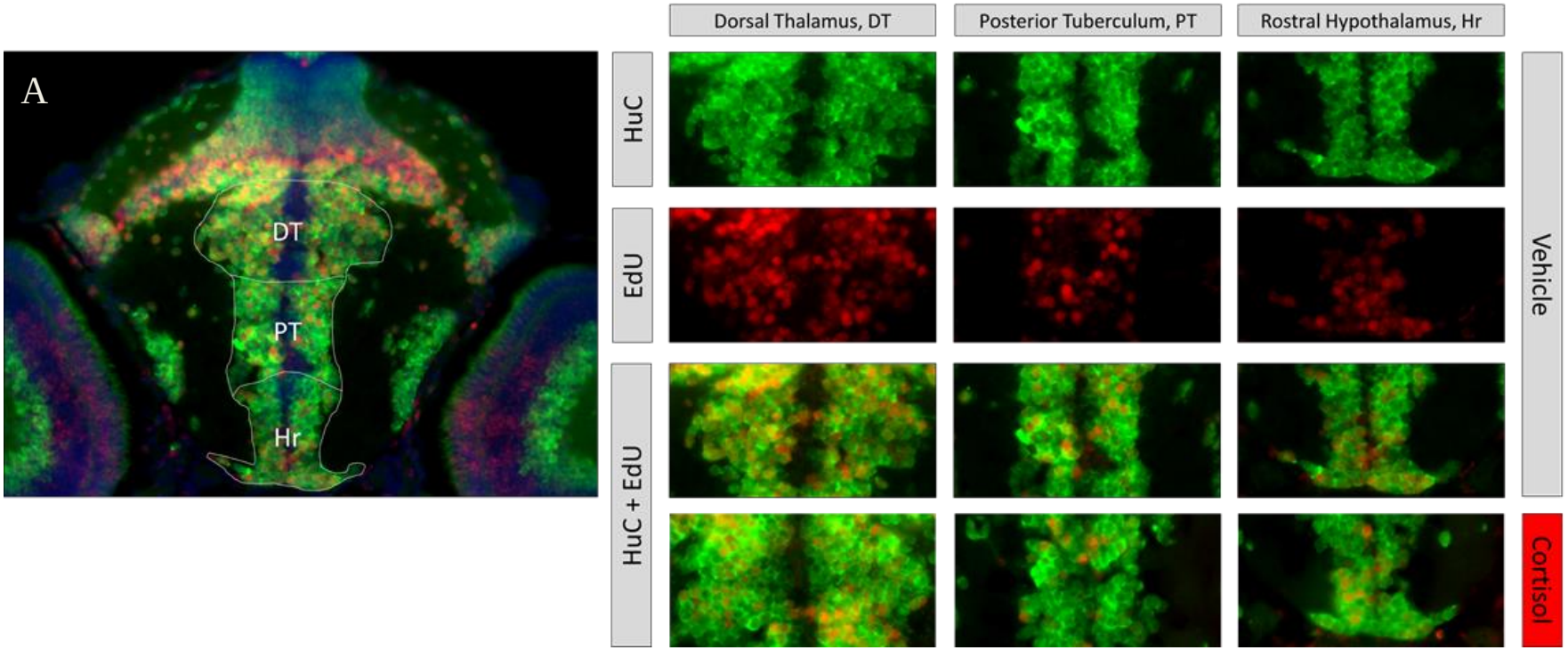
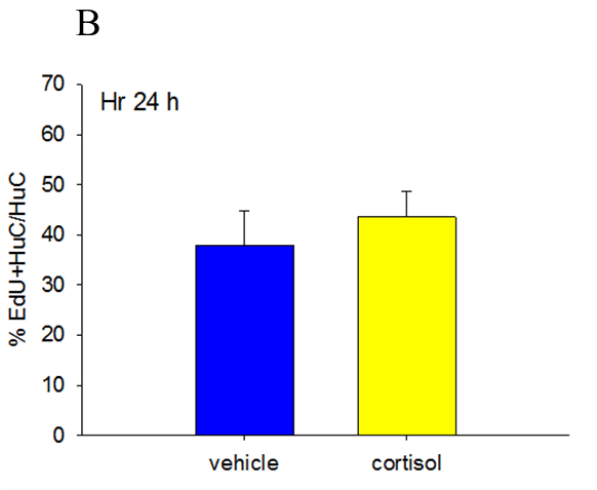
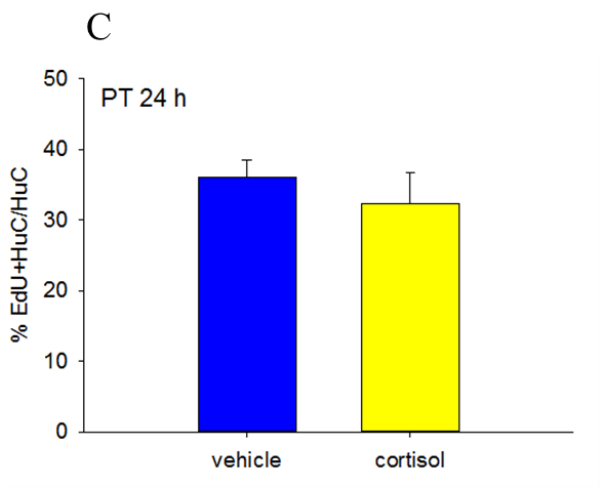


Figure 3.8. Neurogenesis in the rostral hypothalamus, posterior tuberculum, and dorsal thalamus in larvae with altered cortisol deposition.

Larvae were pulsed with EdU at 24 hpf and then raised until 5 dpf. A representative image as well as an overlay of staining is shown for each region quantified (control), with a representative image from the cortisol-treated group (A). Regions quantified are (B) rostral hypothalamus, (C) posterior tuberculum, and (D) dorsal thalamus. Red is EdU staining, green is HuC staining and blue is DAPI staining. Values represent means ± SEM, and different letters indicate significant differences (Student's t-test, unpaired, n=4-5).




B
Hr 24 h
% EdU+HuC/HuC
70
60
50
40
30
20
10
0
vehicle
cortisol


C
PT 24 h
% EdU+HuC/HuC
50
40
30
20
10
0
vehicle
cortisol

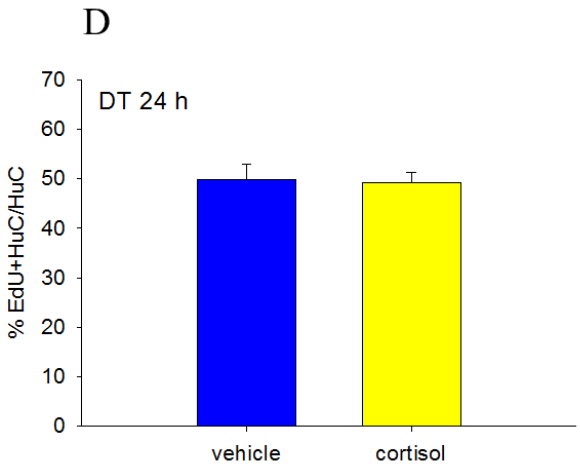
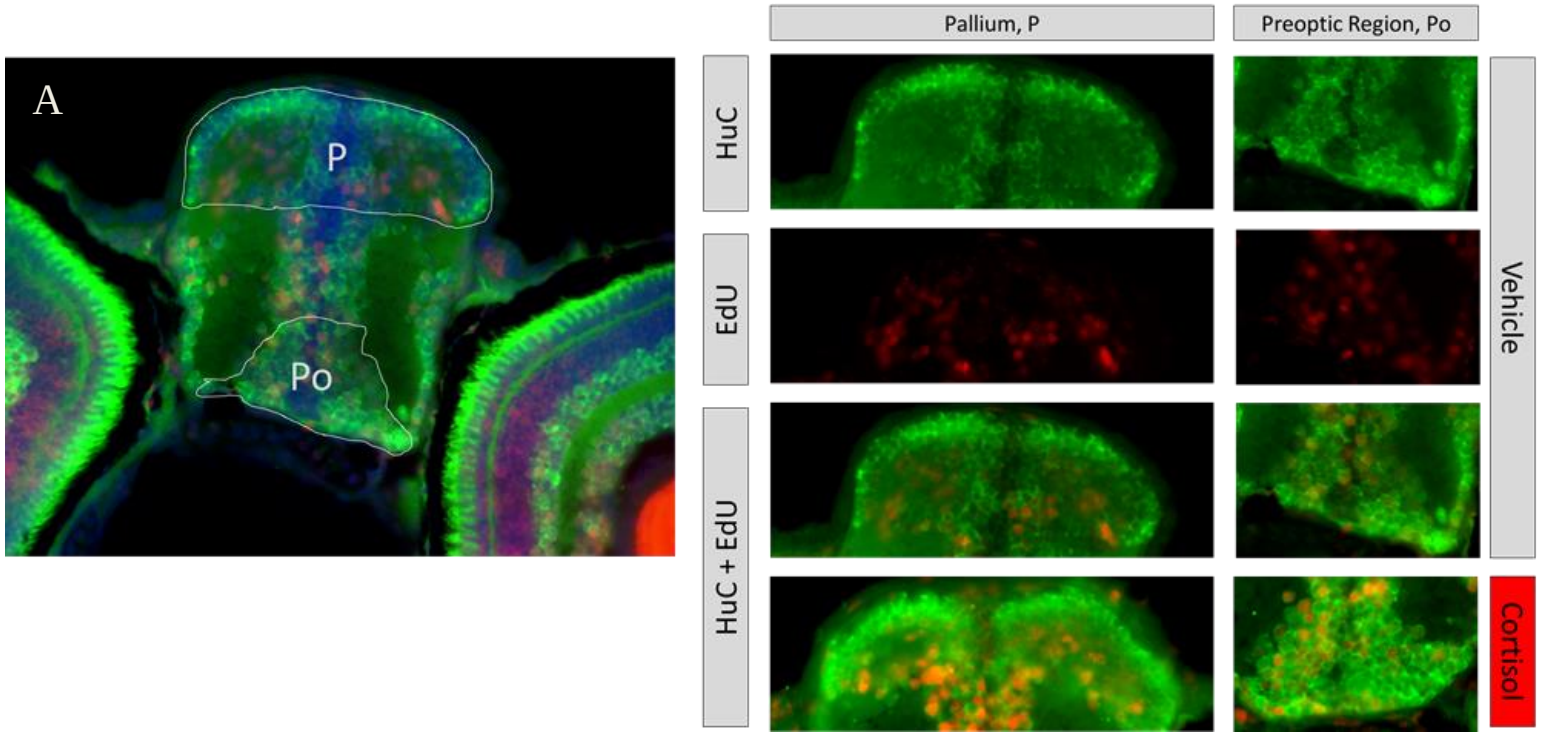
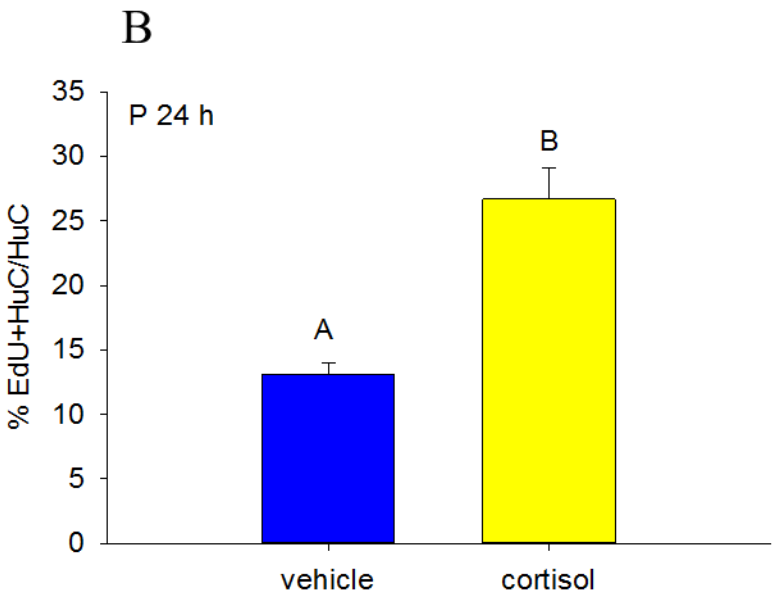

D
DT 24 h
% EdU+HuC/HuC
70
60
50
40
30
20
10
0
vehicle
cortisol

Figure 3.9. Neurogenesis in the pallium and preoptic region in larvae with altered cortisol deposition.

Larvae were pulsed with EdU at 24 hpf and then raised until 5 dpf. A representative image as well as an overlay of staining is shown for each region quantified (control), with a representative image from the cortisol-treated group (A). Regions quantified are (B) pallium and (C) preoptic region. Red is EdU staining, green is HuC staining and blue is DAPI staining. Values represent means ± SEM, and different letters indicate significant differences (Student's t-test, unpaired, n=3-5).




B
P 24 h
% EdU+HuC/HuC
35
30
25
20
15
10
5
0
A
B
vehicle
cortisol

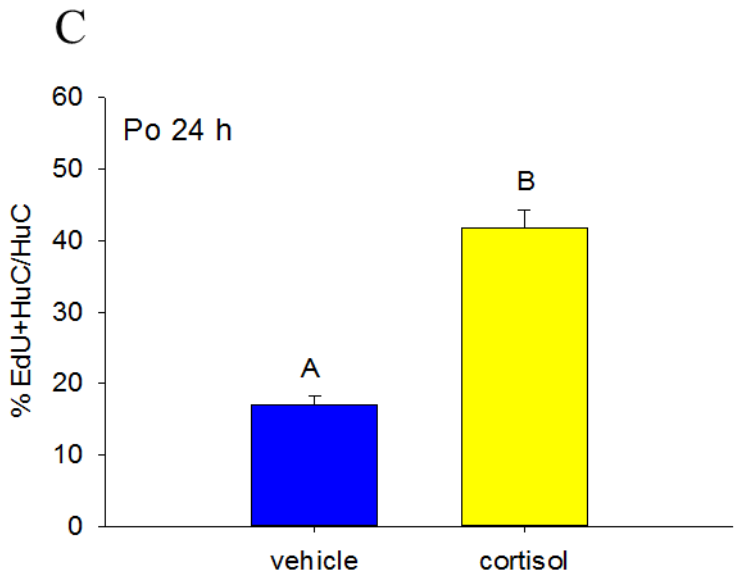

C
Po 24 h
% EdU+HuC/HuC
60
50
40
30
20
10
0
A
B
vehicle
cortisol

Figure 3.10. Transcript abundance of *neurod4* at 36 hpf and 48 hpf in zebrafish with altered cortisol deposition. Whole-mount *in situ* hybridization, representative images (L/R, lateral/dorsal) are shown for each treatment (vehicle: top, cortisol: bottom, 36 hpf: left, 48 hpf: right). Regions indicated on vehicle controls (H, hypothalamus; Ret, retina; MB, midbrain; HB, hindbrain).

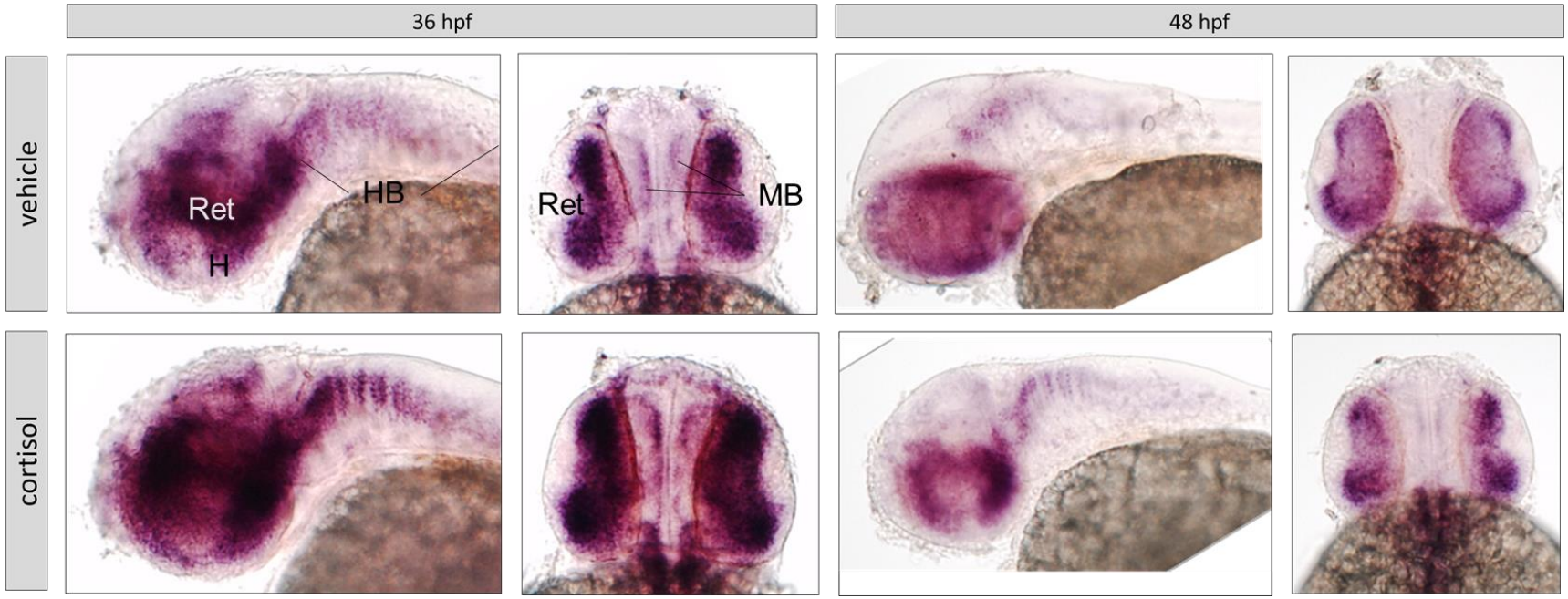
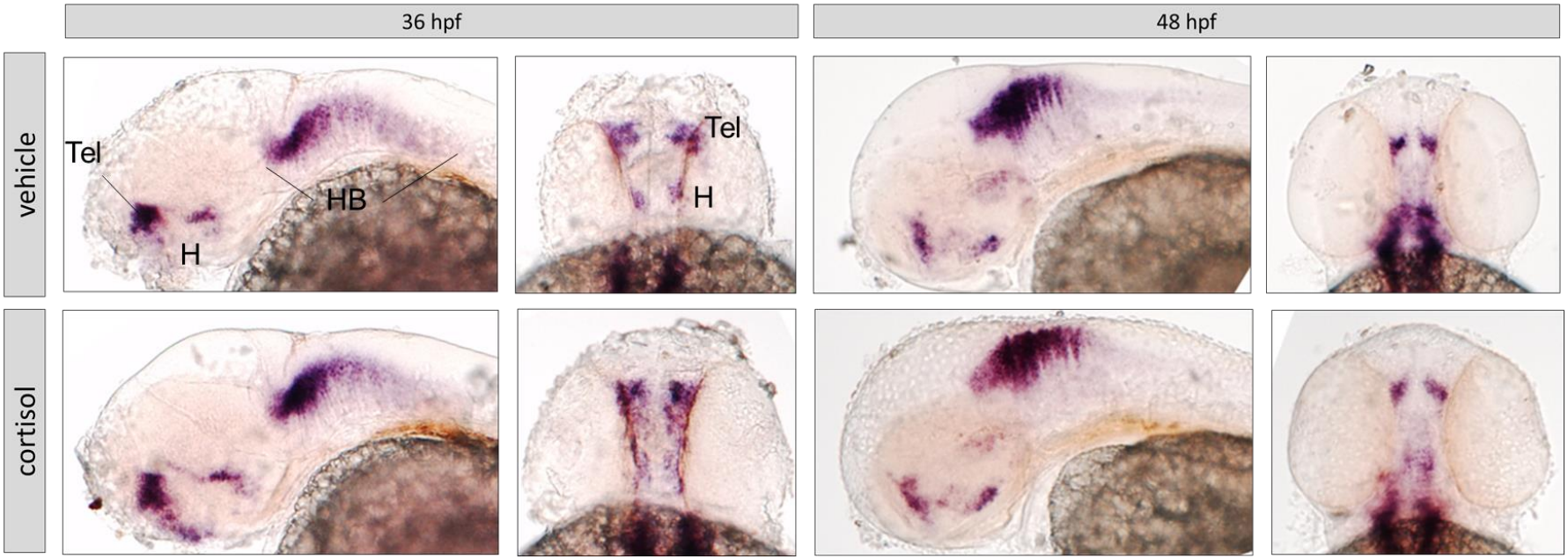


Figure 3.11. Transcript abundance of *otpb* at 36 hpf and 48 hpf in zebrafish with altered cortisol deposition.

Whole-mount *in situ* hybridization, representative images (L/R, lateral/dorsal) are shown for each treatment (vehicle: top, cortisol: bottom, 36 hpf: left, 48 hpf: right). Regions indicated on vehicle controls (H, hypothalamus; Tel, telencephalon; HB, hindbrain).



3.5 Discussion

Our results demonstrate for the first time that elevated zygotic cortisol levels, mimicking stressed levels of cortisol transfer from mother to offspring, disrupt the larval (96 hpf) cortisol response to a physical stressor in zebrafish. This abnormal cortisol deposition also resulted in disrupted behavioural patterns associated with decreased anxiety phenotypes, including the freezing response to bright light, and propensity to remain close to the walls of the arena (thigmotaxis). The current study is the first to demonstrate the effects of elevated embryonic cortisol deposition, analogous to prenatal stress, on neurogenesis in teleosts or any non-mammalian species. Peak levels of neurogenesis (24 hpf) as determined by the number of post-mitotic neurons born at this stage was higher in the pallium and preoptic area of embryos exposed to elevated cortisol levels.

The cortisol profile in our control zebrafish (Fig. 3.1) supports our earlier results showing a decrease in maternal deposits during embryogenesis and an increase in cortisol levels post-hatch in this species (Alsop and Vijayan 2008, 2009a; Nesan and Vijayan, 2013). The initial cortisol deposit in embryos from unstressed mothers is approximately 4 pg per individual and this cortisol is utilized during embryogenesis until hatch, which occurs at approximately 48 hpf. After this drop, cortisol synthesis begins *de novo*, preceded by increased transcript abundances of genes involved in the steroidogenic pathway (Alsop and Vijayan 2008; Nesan and Vijayan 2013b). The importance of maternal cortisol deposition, its utilization during embryogenesis until hatch and its subsequent biosynthesis, is conserved among fish species (Barry et al. 1995, Nechaev et al. 2006, Hwang et al. 1992, Stouthart et al. 1998). Indeed the maternal cortisol

deposition, acting through GR, is essential for zebrafish development (Nesan et al. 2012, Nesan and Vijayan 2012, 2013a, 2013b, Kumai et al. 2012, Pikulkaew et al. 2011).

Maternal stress and the attendant rise in cortisol levels may increase the deposition of this steroid to the oocytes (Chapter 2). To mimic this deposition, embryos were injected with 75 pg cortisol (Fig. 3.1), a level similar to that seen in embryos following a five-day fasting stressor in mothers (Chapter 2, Fig 2.3B). Microinjection of cortisol sustained significantly higher levels of this hormone only until 24 hpf (Fig. 3.1), and our results support the decrease of cortisol after microinjection seen previously in zebrafish (Nesan and Vijayan 2012). The return of all elevated cortisol deposits back to control levels by 48 hpf (hatch; Fig. 3.1) illustrates the importance of maintaining low cortisol levels during this developmental window. While the mechanism involved in this rapid clearance remains to be elucidated, the transcript abundance of *11βhsd2*, which converts cortisol to inactive cortisone, is low during early development in untreated embryos and increases post hatch coinciding with the increase in whole body cortisol content (Alsop and Vijayan 2008). It remains to be seen if excess cortisol may be upregulating this gene expression in early stages leading to the rapid clearance of cortisol as observed in zebrafish ovarian follicles (Chapter 2). At 96 hpf (4 dpf), the elevated cortisol levels in the injected fish are in agreement with an earlier study and suggest priming of the HPI axis (Nesan and Vijayan 2012). This led us to hypothesize that maternal stress, resulting in increased maternal cortisol deposition, disrupts HPI regulation in the offspring. To test this further, we challenged larvae with a physical stressor.

Elevated zygotic cortisol content disrupted the cortisol stressor response in the larvae at 96 hpf. The stressor-induced cortisol response was reduced in the cortisol group and is in agreement with a previous study showing a lack of significant stress response at 72 hpf due to excess cortisol deposition in the embryo (Nesan 2013). Therefore, in embryos with elevated maternal cortisol deposition, the ability to mount a cortisol response to stress at 4 dpf is compromised in terms of delayed onset, smaller magnitude of change and possibly negative feedback dysfunction. In other species, developmental exposure to elevated glucocorticoid generally enhances basal glucocorticoid levels in the offspring, as observed currently with zebrafish and also in rats (maternal dexamethasone, Levitt et al. 1996, maternal restraint stress, Green et al. 2011) and primates (endogenous maternal cortisol Pryce et al. 2011 [macaques]; Mustoe et al. 2014 [marmosets]), while bird eggs injected with corticosterone did not display alterations in basal offspring corticosterone levels (Hayward et al. 2006 [quail], Love and Williams 2008 [starling]). Studies of the effects on the stress response in these offspring are fewer, and the results are less clear with either no effect (rats/gestational dexamethasone, Hauser 2009; marmosets/gestational dexamethasone, Hauser et al. 2007), increases in responsiveness (rats/maternal restraint stress, Barbazanges et al. 1996; marmosets/maternal endogenous cortisol, Mustoe et al. 2014; quail/maternal cortisosterone implant, Hayward and Wingfield 2004) or decreases in responsiveness (quail/egg corticosterone injection, Hayward et al. 2006; starling/egg corticosterone injection, Love and Williams 2008). The mode of administration in the current study (yolk injection) is most similar to that used by Hayward et al. (2006), and Love and Williams (2008), where decreases in responsiveness were observed. Altogether, this suggests that species, type of

glucocorticoid (endogenous or synthetic), timing and mode of administration all appear to affect the programming effect on the stress response, while an elevation in basal cortisol is fairly well-conserved. It is possible that the elevated cortisol deposit in the mother is maladaptive, as the resulting disrupted cortisol response would not allow offspring to deal with physiological stressors in the same way as their counterparts from unstressed mothers. Whether or not this is the case remains to be conclusively determined in zebrafish. To further explore the ability of zebrafish larvae to cope with these challenges, we investigated their behavioural phenotype.

The locomotor response of zebrafish to light and dark conditions has been previously characterized (Burgess and Granato 2007, Emran et al. 2008, MacPhail et al. 2009, Irons et al. 2010). In the current study, the conserved pattern of transiently high and low activity in the dark and light, respectively, is clearly observed in both groups (Fig. 3.3, 3.5). The hyperactive response to sudden darkness is not mediated by the Mauthner neurons and has a longer latency period than the acoustic startle, and so is not likely a predator escape response (Burgess and Granato 2007). Therefore, it has been hypothesized that this hyperactivity triggered by sudden darkness during normal daylight hours is an attempt to seek the light that has been blocked so they can successfully feed (Burgess and Granato 2007). With the sudden onset of light, larvae will display a freeze response to this stimulus, interpreted as an initially aversive response that eventually dissipates with time (MacPhail et al. 2009). This same response pattern to light and dark has also been observed in locusts (Moorhouse et al. 1978) and mice (Hattar et al. 2003) and is known as masking by light, which occurs when light conditions that differ from the

normal circadian patterns override normal circadian patterns of activity (Hattar et al. 2003).

In zebrafish larvae with elevated zygotic cortisol, overall activity in the dark was not affected in any of the endpoints assessed: mean velocity, area under the curve, distance travelled, and time in motion (Fig. 3.5, dark). However, the cortisol-injected group had significantly higher distances travelled, time spent in motion and areas under the activity curve when all 4 light periods were combined together (Fig. 3.5). More detailed analysis (Fig. 3.4) suggests that most of these treatment effects occur in the initial light period, which suggests that the loss of treatment effect later in the assay may be due to habituation to the stimulus following an initially stronger response compared to controls. Apparent habituation may be confused with motor fatigue or sensory adaptation (Irons et al. 2010). Motor fatigue is unlikely in this case as larvae mount strong locomotor responses in the dark, and if the ability to perceive light was impaired beyond the normal rapid light adaptation, then activity levels in later cycles would likely differ from controls. This elevated locomotor activity in the initial light period suggests that the conserved aversive response to a novel light stimulus, displayed by a drastic reduction in movement (freezing), is impaired in zebrafish with elevated cortisol deposition. As freezing is an anxiety-related behaviour in zebrafish (Egan et al. 2009, Cachat et al. 2010) that can be induced by netting stress (8 dpf, Tudorache et al. 2014), the reduction of this response suggests a phenotype of decreased anxiety in these larvae.

This is further supported by significantly decreased thigmotaxis in the cortisol group (Fig. 3.6), another known measure of anxiety in zebrafish as well as rodent models (Schnörr et al. 2012, Ahmad and Richardson 2013, Simon et al. 1994, Treit and Fundytus

1988). Some studies in fish have shown behavioural changes due to developmental cortisol exposure or maternal stress. For instance, juvenile brown trout (*Salmo trutta*) exposed to cortisol at the egg stage (prior to fertilization) were more aggressive and had altered behaviour in a maze (Sloman et al. 2010), while offspring of predator-exposed stickleback had tighter shoaling behaviours (Giesing et al. 2011). However, neither study investigated anxiety. The effects of developmental glucocorticoid exposure in non-teleost species has been shown to affect anxiety-like behaviour although the results vary based on the timing of both treatment and age of offspring assessed (Moisiadis and Matthews 2014). For instance, prenatally stressed male guinea pigs displayed reduced anxiety as juveniles, while no effect was seen in adults (Kapoor and Matthews 2011). Increased anxiety of offspring has been observed in rats subjected to maternal dexamethasone (Welberg et al. 2001) or to prenatal restraint stress (Laloux et al. 2012, Zohar et al. 2011), but this can be a sex-specific process with males being more susceptible (Van den Hove et al. 2013, Markham et al. 2010). Birds injected with corticosterone into the yolk displayed either no effect (Rubolini et al. 2005) or increased fearfulness in chicks, though this could be modified by handling exposure (Janczak et al. 2006). While few studies address the effects of maternal cortisol effects on the behaviour of humans, synthetic glucocorticoid exposure during development may increase the risk of behavioural disorders (Van den Bergh et al. 2005, Moisiadis and Matthews 2014). Clearly, many parameters associated with developmental glucocorticoid elevation can affect later anxiety phenotypes rendering cross-species comparisons very difficult. To our knowledge, this is the first study to address this in a teleost model, and demonstrates that exogenous cortisol injected into the yolk immediately post-fertilization reduces anxiety-

related phenotypes at 4 dpf. Taken together, the altered cortisol response and anxiolytic phenotype suggest that elevated maternal cortisol deposition is modifying the physiological and behavioural responses to stress, potentially in response to a (simulated) stressful maternal environment. It remains to be determined whether the observed phenotypes due to elevated zygotic cortisol content in zebrafish are adaptive or maladaptive. While the mechanism behind these alterations are not clear, we hypothesized that this was due to alterations in brain development and investigated neurogenesis and associated transcripts.

The links between stress, glucocorticoids and neurogenesis are very well studied in mammals. Specifically, the links between depression, stress and decreased mammalian adult hippocampal neurogenesis has been of particular interest to research due to [1] this region being unique in its ability for neurogenesis in adults (Eriksson et al. 1998), [2] clinical relevance to the etiology of depressive disorders (Snyder et al. 2011), and [3] the association between increased stress and/or glucocorticoids and decreased adult hippocampal neurogenesis (Schoenfeld and Gould 2013). Although glucocorticoids and stress do play an essential role in early brain development, comparatively less is known about the relationships between glucocorticoids and early developmental (as opposed to adult) neurogenesis. While endogenous levels of glucocorticoids are essential for normal maturation of the brain, increased levels in mammals have been shown to reduce brain growth, increase cell death of progenitors, neurons and glial cells, and delay maturation in the developing brain, sometimes with lasting effects (Meyer 1983, Dunlop et al. 1997, Llorente et al. 2009, Noguchi et al. 2008, Uno et al. 1990). In neonatal offspring of rats exposed to prenatal stress, neurogenesis as measured by BrdU incorporation was reduced

in the nucleus accumbens and the hippocampus, although behaviour was not assessed (Kawamura et al. 2006). However, this observation is present in rats bred for high anxiety, but not low anxiety (Lucassen et al. 2009). Furthermore, mothers in the former strain fail to increase placental 11βHSD2 in response to acute stress despite having similar plasma corticosterone levels in response to stress, highlighting the importance of this enzyme in controlling transfer of stress hormone to the fetus (Lucassen et al. 2009). Another prenatal restraint stress study in rats found decreases in cell proliferation in selected regions of the rostral forebrain at postnatal day (P) 1, but not P2, 5, 8 and 15, and experiencing an increase in cerebellar proliferation at P8 (Van den Hove et al. 2006). That study also investigated the response to maternal deprivation stressor, observing an increased corticosterone response at P1, but a decreased response at P8 (Van den Hove et al. 2006). This again emphasizes the importance of the window of assessment, as differential effects were observed within a span of 8 days in the same experiment.

Teleost brain structures are well established and fairly conserved with respect to other vertebrates (Wullimann and Mueller 2004). A noteworthy difference in zebrafish is that they display much more widespread capability throughout the adult brain for neurogenesis (Kizil et al. 2012), while markers and signalling during early neurogenesis appear to be generally conserved in vertebrates (Schmidt et al. 2010). In the present study neurogenesis did not increase in the dorsal thalamus, posterior tuberculum and the rostral hypothalamus at 24 hpf, when peak neurogenesis is occurring (Kinch et al. 2015). However, neurogenesis in the pallium and the preoptic area at the same time point was higher. Altered early neurogenesis has been linked to behavioural phenotypes, as seen with early exposure to bisphenol A (BPA) in mice (Itoh et al. 2012) and zebrafish (Kinch

et al. 2015). The forebrain is thought to be a key target for prenatal stress in mammalian models, as it includes the limbic system, which regulates HPA function and behaviour (Moisiadis and Matthews 2014). The corresponding region in larval fish includes the pallial region, subregions of which in the adult are homologous to the mammalian hippocampus, isocortex, basolateral amygdala and and piriform cortex (Mueller 2012). The hippocampus and amygdala can centrally modulate HPA activity in mammals, though this has not been established in fish (Gunnar and Quevedo 2007). An increase in neurogenesis with cortisol was also seen in the preoptic area (POA), where CRF secreting neurons that project to the pituitary are found (Bernier et al. 2004). We hypothesize that altered neurogenesis in the POA may contribute to the altered larval stress performance seen with excess zygotic cortisol deposition (Nesan 2013, chapter 3), and this may involve altered programming of the brain by excess cortisol during early development.

This increase in neurogenesis is in contrast to what has been generally observed in rats exposed to prenatal stress (Kawamura et al. 2006, Van den Hove et al. 2006 – exception of the cerebellar increase at P8). However, one study comparing mild and severe prenatal stress in rats showed increased neonatal hippocampal neurogenesis in the offspring of mothers subjected to mild restraint stress (Fujioka et al. 2006). They also showed that aldosterone increases neurogenesis while dexamethasone inhibits, and this was confirmed with the respective receptor antagonists (Fujioka et al. 2006, Fischer et al. 2002). This suggests that the effects seen with neurogenesis may be occurring through an MR-mediated mechanism (Fujioka et al. 2006). In the current study, *mr* transcript abundance was significantly downregulated at 36 hpf, possibly suggesting increased turnover of this receptor. The potentially important role of MR during zebrafish

development has been suggested previously (Alsop and Vijayan 2009a). Furthermore, *mr* is highly expressed in the teleost brain, although little is known of its role in this tissue (Sturm et al. 2005). Transcriptomic analysis with *gr* morpholino knockdown predicted increased neuron proliferation at 36 hpf (Nesan and Vijayan 2013a), also implicating GR in the control of early neurogenesis in the zebrafish. Although transcript abundance of proneural genes *neurod4, otpb, and islet1* were not significantly changed by increased zygotic cortisol using qPCR, there appears to be a higher spatial abundance of *neurod4* and *optb* in the cortisol group by whole-mount *in situ* hybridization. However, a molecular mechanism leading to the altered neurogenesis remains to be elucidated.

Overall, excess zygotic cortisol levels mimicking elevated maternal deposition modified the larval cortisol response to a physical stressor. Also, larvae raised from the excess zygotic cortisol group had a reduced freezing response to light, indicating reduced anxiety. Thigmotaxis further confirmed a reduced anxiety behaviour in larvae raised from embryos with excess zygotic cortisol content. We also demonstrate for the first time that increased cortisol deposition in the embryo increases neurogenesis in select regions, including the hippocampal analogue in fish and the preoptic region. Together, the results suggest that abnormal cortisol levels in the embryo, mimicking a maternal stress scenario, affects the behavioural and physiological stress phenotype of larval zebrafish. We hypothesize that elevated neurogenesis may underlie the behavioural and stress phenotypes seen in the larvae of cortisol-treated embryos. The precise mechanism by which neurogenesis might be linked to stress and behaviour is not clear, though possibilities may include disrupted synaptogenesis or dysregulation of neurotransmitter

and neuropeptide levels. Also, whether the changes observed are adaptive or maladaptive for progeny development remains to be determined.

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3.7 Tables

Table 3.1. List of abbreviations for brain regions.

Neurogenesis (EdU) - Immunofluorescence			
ac	anterior commissure	poc	postoptic commissure
DT	dorsal thalamus	Pr	pretectum
EmT	eminentia thalami	PTd	dorsal part of posterior tuberculum
H	hypothalamus	PTv	ventral part of posterior tuberculum
MO	medulla oblongata	S	subpallium
OB	olfactory bulb	T	midbrain tegmentum
oc	optic chiasma	TeO	tectum opticum
P	pallium	TeVe	tectal ventricle
Po	preoptic region	VT	ventral thalamus (prethalamus)

***In Situ* Hybridization**	
H	hypothalamus
HB	hindbrain
MB	midbrain
Ret	retina
Tel	telencephalon

Table 3.2. Primer sequences for qPCR (36 h, neurogenesis).

Primer sequences, annealing temperatures, product sizes and references for oligonucleotide primers used in quantitative real-time PCR.

Gene	Primer Sequence	Amplicon (bp)	Annealing Temp (^{0}C)	Reference
11βhsd2	F: 5'- TGCTGCTGGCTGTACTTCAC -3' R: 5'- TGCATCCAACTTCTTTGCTG -3'	123	60	Alsop and Vijayan 2008
bmp2a	F: 5′- AGGCTGGAATGACTGGATTG-3′ R: 5′- TTGCTGTTCACCGAGTTCAC -3′	140	55	Nesan et al. 2012
bmp2b	F: 5′- CTGAAAACGATGACCCGAAC-3′ R: 5′- TCGTATCGTGTTTGCTCTGC -3′	88	55	Nesan et al. 2012
bmp4	F: 5'- CTTTGAGACCCGTTTTACCG -3' R: 5'- TTTGTCGAGAGGTGATGCAG -3'	147	57	Nesan et al. 2012
gr	F: 5'- ACAGCTTCTTCCAGCCTCAG -3' R: 5'- CCGGTGTTCTCCTGTTTGAT-3'	116	60	Alsop and Vijayan 2008
islet1	F: 5'-AGACCTTTACAAATGGCAGCAGA -3' R: 5'-CGGACGCGGGTTGTTTTCTC -3'	95	62	-
mr	F: 5'- CCCATTGAGGACCAAATCAC -3' R: 5'- AGTAGAGCATTTGGGCGTTG -3'	106	60	Alsop and Vijayan 2008
neurod4	F: 5'- CAGGTGCTACAATAACAGATCAC -3' R: 5'- AGTCTTCGTCCATCCATCCAAG -3'	100	60	-
otpb	F: 5'-GTTGTGACTCCAGCAGTGCG-3' R: 5'-TTCATCCAGTGGAGTTGAAGACCG-3'	105	62	-
β-actin	F: 5'- -TGTCCCTGTATGCCTCTGGT -3' R: 5'- -AAGTCCAGACGGAGGATGG -3'	121	60	Alsop and Vijayan 2008

Table 3.3. Primer sequences for riboprobe synthesis.

Primer sequences and product sizes for oligonucleotide primers used in template generation by PCR prior to riboprobe synthesis for neurogenic differentiation 4 (*neurod4*, NM_170762.1) and orthopedia b (*otpb*, NM_131100.1). T7 RNA polymerase binding sites are indicated in italics.

Gene (amplicon)	**Probe**	**Primers (5' to 3')**
neurod4 (923 bp)	sense	F: *TAATACGACTCACTATAGGG*CAGGTGCTACAATAACAGATCACA
		R: GTAGTGAGTCGGATGAGGCG
	antisense	F: CAGGTGCTACAATAACAGATCACA
		R: *TAATACGACTCACTATAGGG*GTAGTGAGTCGGATGAGGCG
otpb (537 bp)	sense	F: *TAATACGACTCACTATAGGG*TCAACTCCACTGGATGAAGGATG
		R: AGACGGGAACTGAGGCAAAC
	antisense	F: TCAACTCCACTGGATGAAGGATG
		R: *TAATACGACTCACTATAGGG*AGACGGGAACTGAGGCAAAC

Table 3.4 Transcript abundance at 36 hpf in larvae with altered cortisol deposition. Transcript abundance was measured by real-time qPCR, and genes were normalized to abundance of β-actin as this was unchanged between treatments. Significant changes detected by Student's t-test (unpaired) are indicated in bold. Values represent means ± SEM of fold change in the cortisol-injected group relative to vehicle control group, and n=7-9.

Gene	**Transcript Abundance** (mean ± SEM fold change with cortisol relative to vehicle control)	**P-value**
11βhsd2	0.733±0.085	0.0615
gr	0.851±0.075	0.102
mr	**0.766±0.067**	**0.0418**
crf	0.779±0.078	0.100
bmp2a	0.952±0.091	0.687
bmp2b	0.874±0.095	0.312
bmp4	0.941±0.237	0.281
islet1	0.834±0.098	0.178
neurod4	0.904±0.184	0.697
otpb	0.810±0.118	0.230

Chapter Four: Larval exposure to excess cortisol increases locomotor activity in zebrafish

4.1 Introduction

Stressor exposure in early life and the associated elevation in glucocorticoid levels may also alter the physiology and behaviour of the animal (Lupien et al. 2009, Anisman et al. 1998) but little is known in fishes. For instance, in rodents, separation from the mother is frequently employed as an early life stressor and has been associated with altered anxiety and learning behaviours (Anisman et al. 1998). This stressor has also been linked to elevated corticosteroid levels and GR signaling in rodents (Rice et al. 2008, Schmidt et al. 2010). However, little is known about the phenotypic effects of elevated cortisol levels after the commencement of the stress response in teleosts.

In zebrafish (*Danio rerio*), all the molecular components required for a functional HPI axis in the embryonic zebrafish are in place by 32 hours post fertilization (hpf), including corticotropin releasing factor (CRF) which is located in the hypothalamus and telencephalon (Chandrasekar et al. 2007). CRF acts on the anterior pituitary to release adrenocorticotropic hormone (ACTH), which is formed from the precursor protein proopiomelanocortin (POMC), transcripts of which are detectable by 18-20 hpf (Liu et al. 2003). Steroidogenic enzymes are detectable by 28 hpf and the melanocortin 2 receptor (MC2R) is detectable by 32 hpf (To et al. 2007, Chai et al. 2003). It is not until 40 hours later that the ability to endogenously raise cortisol levels in response to stress occurs, and this lag time may be analogous to the stress hyporesponsive period (SHRP) observed in other species (Nesan and Vijayan 2013b, Alsop and Vijayan 2009a, Schmidt 2010,

Levine 2001). Cortisol signalling in fish can occur through the glucocorticoid receptor (GR), the mineralocorticoid receptor (MR) or nongenomically (Dindia et al. 2013). The enzyme 11β-hydroxysteroid dehydrogenase type 2 (11βHSD2) assists in lowering cortisol levels by conversion to biologically inactive cortisone (Alderman and Vijayan 2012). Three day old zebrafish larvae can significantly elevate cortisol levels following either a physical stressor (Alsop and Vijayan 2008) or exposure to an osmotic stressor (Alderman and Bernier 2009). Any potential effect of this early larval cortisol stress response on behavioural and physiological performances are yet unknown in fish.

Glucocorticoid signalling is known to alter behaviour in several vertebrate taxa. In rodent models, glucocorticoids can affect locomotor activity, anxiety and aggression through mechanisms including GR signalling, modulation of dopaminergic activity and possibly nongenomic pathways (Boyle et al. 2006, Tronche et al. 1999, Roberts et al. 1995, Mikics et al. 2004). Corticosteroids also affect activity in birds (Lynn et al. 2003, Breuner and Wingfield 2000, Silverin 1997), and amphibians (Lowry et al. 1996). In teleost hierarchies, subordinate fish have elevated cortisol levels and reduced swimming activity (Gilmour et al. 2005, Øverli et al. 1998). In fact, administration of exogenous cortisol to trout significantly increases the chances of an individual in a size-matched pair becoming subordinate (DiBattista et al. 2005) and socially stressed subordinate fish often display decreased locomotor activity (Gilmour et al. 2005). Additionally, trout have been bred into high and low cortisol responder strains, demonstrating that this trait is heritable (Pottinger and Carrick 2001). Administration of cortisol to adult charr has been shown to increase locomotor activity (Øverli et al. 2002). Elevated CRF levels have been strongly linked to behaviour including elevated locomotor activity in many vertebrate species

(Lowry and Moore 2006, Backstrom and Winberg 2013). Altogether, this suggests that stress and the attendant rise in cortisol levels play a role in the development of subordinate behaviours in fish.

This study aims to determine the effects of elevated cortisol level, mimicking an acute early cortisol stress response, on the locomotory behaviour and anxiety of larval zebrafish. Specifically, the hypothesis tested is that elevated cortisol levels at around the time of the onset of the stress response will alter larval behaviours of anxiety and activity in response to light stimuli, representing brief disruptions in normal circadian light patterns, and that these effects are mediated by the glucocorticoid receptor. To test this, 3 dpf larvae were exposed to waterborne cortisol and the glucocorticoid receptor antagonist RU-486 for 24 h. Thigmotaxis, the anxious tendency of the larvae to remain in the perimeter of the arena, as well as the conserved locomotor response to alternating light and dark periods were assessed to determine modulation of behaviour by cortisol in the larvae. Also, the transcript abundances of *gr*, *mr*, *crf* and *11βhsd2* were determined in response to acute cortisol exposure to shed light on the molecular mechanisms regulating cortisol signalling in zebrafish larvae.

4.2 Materials and Methods

4.2.1 Animals

Zebrafish (Tupfel long fin strain) were held in a recirculating system (Pentair Aquatic Habitats, Apopka, FL, USA) and housed in 10 L tanks (14:10 light:dark). Water was maintained at 28 ºC, 750 µS conductivity, and pH 7.6. Fish were fed twice daily, receiving Zeigler adult zebrafish food in the morning (Pentair Aquatic Habitats, Apopka,

FL, USA) and live *Artemia* (San Francisco Bay Brand, Inc.) in the afternoon. Fish were bred by transfer to breeding traps the night before, and eggs were collected the following morning. Eggs were maintained in E3 embryo medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$, 0.1 ppm methylene blue as antifungal; Nusslein-Volhard and Dahm 2002, Burgess and Granato 2007). Larvae were held at 28.5 ºC in 10 cm Petri dishes with daily partial (~50%) embryo medium renewal until transfer to multiwell plates. All protocols involved in the maintenance and experimental use of zebrafish were approved by the University of Calgary Animal Care Committee, and abide by the guidelines set out by the Canadian Council on Animal Care.

4.2.2 Exposure

Fish were raised to 3 dpf and transferred to 96 well, 24 or 6 well plates in 200 µl, 500 µl or 4 ml volumes, respectively, of E3 embryo medium as above. Larvae were then treated via waterborne exposure with either vehicle (0.05% ethanol) control, 10 µg/ml cortisol (27.59 µM equivalent) with and without 1.25 µM of the GR antagonist RU-486 (sufficient to induce and block, respectively, GR transactivation in larval zebrafish; Benato et al. 2014). Plates were covered with a plastic lid and maintained at 28.5 ºC overnight (24 h).

4.2.3 Sample Processing

For cortisol quantification, pools of embryos or larvae (n=6 pools of 12 larvae) were collected at 4 dpf, after 24 h of exposure. Larvae were rinsed with E3 embryo medium and all water was removed prior to snap freezing on dry ice. For cortisol analysis, pools of larvae were partially thawed on ice and homogenized in 200 µl of 50 mM Tris buffer, pH 7.5 with added protease inhibitors (Roche Diagnostics, Laval, QC,

CAN). Samples were sonicated on ice to homogeneity and stored at -80 until use. For RNA analysis, snap frozen samples (n=6 pools of 20 larvae) were sonicated in Ribozol (Amresco, Solon, OH, USA) on ice, and extracted as described below.

4.2.4 Behavioural Analysis

The activity of 4 dpf (24 h after exposure) larvae was assessed using the ZebraBox camera setup and the tracking extension of the ZebraLab software system (Viewpoint Life Sciences, Montreal QC). Analyses were performed in an isolated room maintained at 28.5 ºC. Larvae were acclimated to the room one hour prior to analysis. In all cases, plates were calibrated for size and background-subtracted, detection threshold was set at 20 (grayscale pixel intensity below which is detected as the animal; animal colour being set to black). Specific protocol parameters are indicated below. All tests were carried out in the early afternoon, between 4-8 h after the lights turn on during the timed light cycle. Endpoints measured per animal per 30 s bin included total distance moved (mm), total duration in motion (s), and velocity (mm/s). To tabulate these endpoints over several bins, e.g. a single light period, the sum, mean or area under the curve (indicated on graph) for these endpoints for each animal for the specified time period was determined. Thigmotaxis was presented as the percentage of the total distance travelled by the larvae that occurred in the outer zone during the entire test period, thus correcting for individual differences in activity as recommended by Schnörr et al. (2012). Data were processed and analyzed using Excel, FastDataMonitor (Viewpoint Life Sciences, Montreal QC) and SigmaPlot 13 (Systat Software, Inc.).

4.2.4.1 Activity in Light and Dark

For the light-dark response assay, the protocol parameters were one hour total duration, composed of alternating periods of light (maximum light intensity, 500 lx) and dark every 7.5 minutes (450 s). This was repeated over 6 trials (12 larvae/treatment group/plate) for a total of 72 larvae per treatment.

4.2.4.2 Thigmotaxis

Thigmotaxis is analogous to the open field test in rodents, used to assess anxiety by the tendency to remain close to the walls of the arena. To test this, the wells of a 24-well plate were separated, using the software, into two distinct inner and outer zones. The protocol parameters were 30 minutes total duration. This assay is run in the dark to induce strong activity levels, thus encouraging the larvae to fully explore the environment (Schnörr et al. 2012).

4.2.5 Cortisol ELISA

Cortisol levels were measured using a competitive enzyme-linked immunosorbent assay (ELISA) based on the protocol of Yeh et al. (2013). Briefly, high binding 96 well plates (Immulon HB, VWR) were coated with 100 µl of cortisol monoclonal antibody (1.6 µg/ml; East Coast Bio, ME, USA) in phosphate buffered saline (1 x PBS; 10x stock: 1.37M NaCl; 27 M KCl, 18 mM KH2PO4; Na2HPO4), for 16 h at 4°C. The plate was then washed with PBS with 0.05% Tween 20 (PBST; 300 µl/well) and blocked with 0.1% bovine serum albumin (300 µl/well; BSA; Sigma) for 1 h at room temperature. Standards comprised of cortisol (Sigma H0888) serially diluted (0 ng/ml – 25 ng/ml) in PBS and 50 µl of either standards or samples were added to the wells in duplicate. Cortisol conjugated to horseradish peroxidase (1:160 dilution; East Coast Bio, ME, USA)

diluted in PBS was immediately added to each well. Plates were incubated for 2 h, shaking, at room temperature. The plate was washed as described above, and the detection reagent was added (41 mM TMB and 8 mM TBABH in 200 mM potassium citrate, pH 4). After 1 hour the reaction was stopped with 1 M sulfuric acid. Wells were read at 450 nm using a microplate reader (VersaMax, Molecular Devices, CA, USA).

4.2.6 Transcript Analysis by Quantitative Real-Time PCR

Total RNA was extracted from n=6 pools of 20 larvae. Briefly, samples were sonicated in Ribozol (Amresco, Solon, OH, USA). Chloroform was mixed with the homogenate and following phase separation, total RNA was precipitated from the aqueous phase by isopropanol. The RNA pellet was then washed multiple times with ice-cold 75% ethanol and the pellet was dissolved in nuclease-free water (Amresco, Solon, OH, USA) and quantified (SpectraDrop, Molecular Devices, Sunnyvale, CA, USA). 1 µg of RNA was DNase I-treated (Thermo Scientific, Waltham, MA, USA) to remove genomic DNA contamination and reverse transcribed to cDNA (High Capacity cDNA Reverse Transcription Kit, Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol.

Gene-specific primers (Table 1) were used to amplify ~100 bp products in real-time quantitative PCR. Transcript levels of the enzyme 11βHSD2 were specifically used as a positive control, as this is a known glucocorticoid-responsive gene in fish (Alderman and Vijayan 2012). A master mix contained (per sample) 0.75 µl cDNA template, 0.75 µl primer pair (10 µM), 12 µl 2X SYBR green supermix (BioRad, Hercules, CA, USA) and 10.5 µl nuclease-free water. In each well, 10 µl of master mix was added and samples were run in duplicate. Amplification conditions were as follows: initial denaturation for 2

min at 94°C, 40 cycles of 20 s at 94°C, 20 s at annealing temperature (Table 1); 95°C for 1 min; 55°C for 1 min followed by melt curve analysis (55°C to 95°C at 0.5°C / 10 s). Threshold cycle values were calculated using CFX Manager detection software (BioRad, Hercules, CA, USA). Relative transcript abundance was quantified using the mean normalized expression (MNE) method, as described previously (Simon 2003, Wiseman et al. 2011). As the abundance was unchanged between treatments, *β-actin* was used as housekeeping gene for normalization.

4.2.7 Statistics

All data were analysed by one-way ANOVA, followed by a Tukey post hoc test to determine treatment effects. Data were transformed where necessary to meet the normality and equal variance assumptions of parametric data, and if these assumptions could not be met, a non-parametric test was carried out (Kruskal-Wallis one-way ANOVA). The significance level (α) was set to 0.05, and SigmaPlot 13 (Systat Software, Inc.) was used for all statistical analyses.

4.3 Results

4.3.1 Cortisol Levels

Zebrafish larvae (3 dpf) exposed to waterborne cortisol for 24 h showed a significantly higher whole body cortisol level (Fig. 4.1, P = 0.007). Co-treatment with the glucocorticoid receptor antagonist RU-486 did not result in a significant difference from the cortisol treated group (P = 0.618) (Fig. 4.1). Treatment with RU-486 alone had no significant effect on cortisol levels compared to the vehicle control (P = 0.200) (Fig. 4.1).

4.3.2 Behavioural Response to Light and Dark

In all groups subjected to alternating 7.5 min periods of light and dark, the locomotor activity of the fish was lower in the light and higher in the dark (Fig. 4.2). Activity, measured as velocity (Fig. 4.3A), mean distance travelled (Fig. 4.3B), or mean duration in motion (Fig. 4.3C), was compiled for all 4 light (Fig. 4.3, left side,) or dark (Fig. 4.3, right side) periods. Treatment with RU-486 alone did not significantly alter any one of these measures of activity, regardless of light condition, compared to the control group (Fig. 4.3). However, mean velocity and distance travelled in the dark of larvae treated with a combination of cortisol and RU-486 was not significantly higher than those treated with RU-486 control alone. In general, cortisol-treated fish showed significantly higher activity (velocity, distance travelled and duration in motion) regardless of light condition compared to the control group (Fig. 4.3). In the light, co-treatment with RU-486 partially brought down mean velocity, as values were not significantly different from either the cortisol or control groups (Fig. 4.3). In the dark, co-treatment with RU-486 did not significantly reduce the cortisol-induced elevations in duration in motion due to cortisol treatment (Fig. 4.3B). However, co-treatment with RU-486 did bring down cortisol-induced elevations in mean velocity and distance travelled values down so they were not significantly different from the RU486-only group, but were still significantly different from the vehicle control.

4.3.3 Thigmotaxis

Thigmotaxis was not significantly affected by exposure of larvae with either cortisol or the GR antagonist RU-486 alone (Fig. 4.4). However, co-treatment with both

cortisol and RU-486 significantly reduced thigmotaxis compared to control ($P = 0.035$), but not in comparison to cortisol or RU-486 groups (Fig. 4.4).

4.3.4 Transcript Abundance

Transcript levels of *11βhsd2* were used as a positive control for cortisol signaling through the glucocorticoid receptor, as well as the blockade of this pathway by RU-486. Cortisol exposure significantly increased *11βhsd2* transcript levels 4.3-fold compared to the vehicle control group ($P < 0.001$, Fig. 4.5A). This significant cortisol-induced upregulation was completely abolished by co-treatment with RU-486 (Fig. 4.5A). Transcript levels of *11βhsd2* in the vehicle control were not significantly different from the RU-486-treated larvae (Fig. 4.5A). Exposure of 3 dpf larvae to either cortisol or RU-486 alone or in combination did not significantly alter the transcript abundance of *crf* (Fig. 4.5B), *gr* (Fig. 4.5C) or *mr* (Fig. 4.5D) compared to the controls.

4.4 Figures

Figure 4.1. Cortisol levels in zebrafish at 4 dpf, following 24 h exposure.

Fish were raised in Petri dishes until 3 dpf, then transferred to 96 well plates (200 µl volume) and exposed to treatments (vehicle, 1.25 µM RU-486, 27.59 µM cortisol, or both in combination). Different letters indicate significant differences (Kruskal-Wallis one-way ANOVA on ranks $P < 0.001$). Values represent means ± SEM, (n=6 pools of 12 larvae).

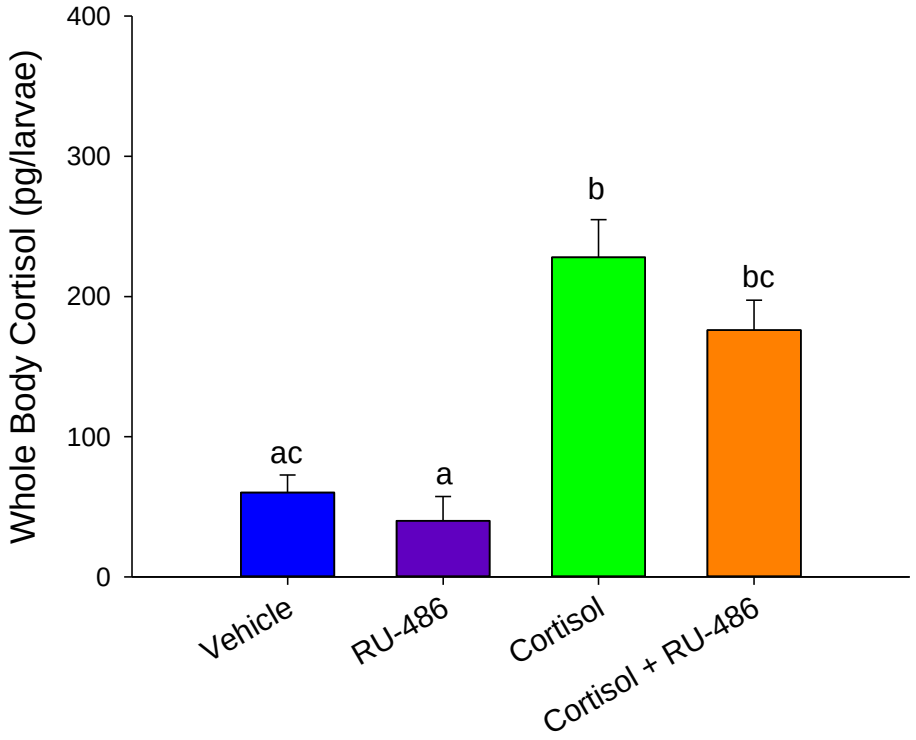


Figure 4.2. Activity in response to changes in light in 4 dpf larvae exposed to cortisol or RU-486 for 24 h.

Larvae at 3 dpf were treated with vehicle, 1.25 µM RU-486, 27.59 µM cortisol, or both in combination. Activity is expressed as total distance moved per 30 s recording bin. The total recording period was 1 h with alternating light periods of 7.5 min each, indicated by the light and dark bars above the x-axis. Values represent means ± SEM, and n=70-72.

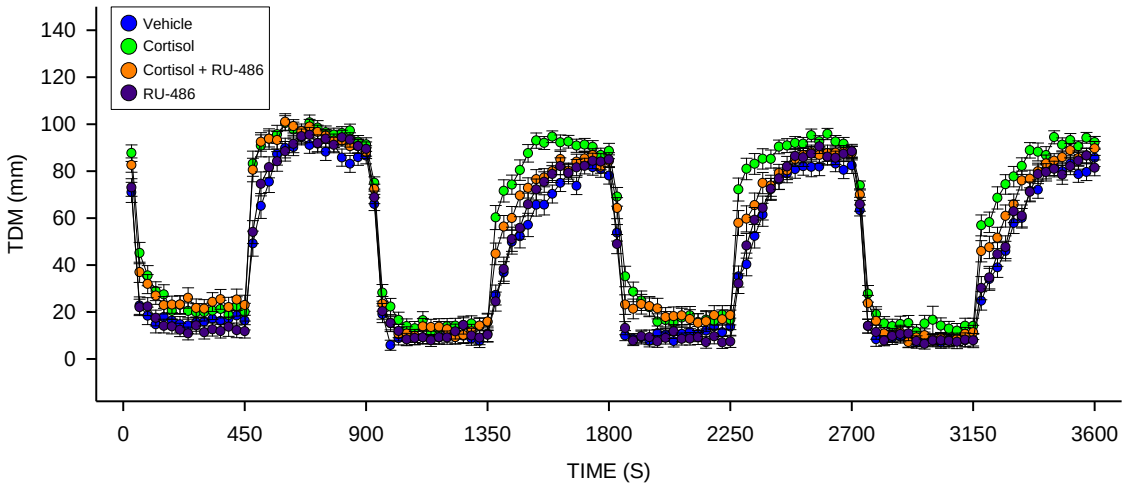
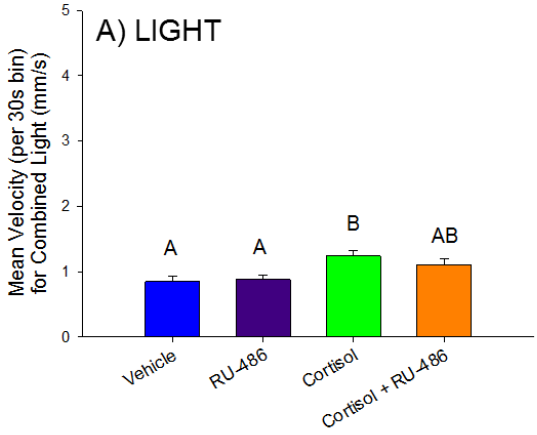
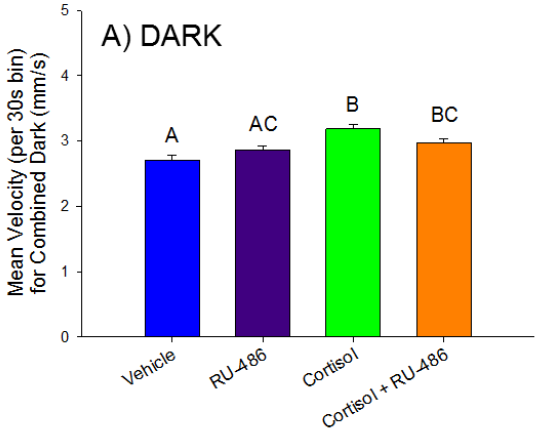
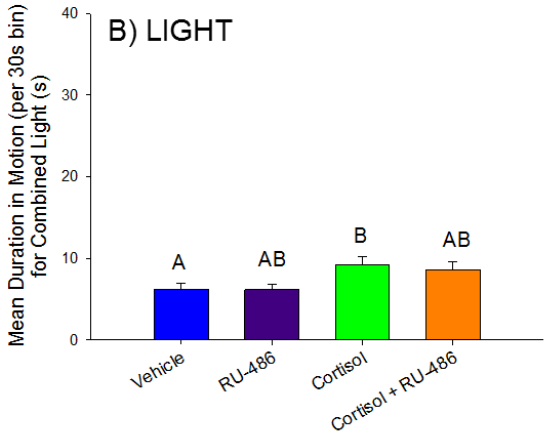


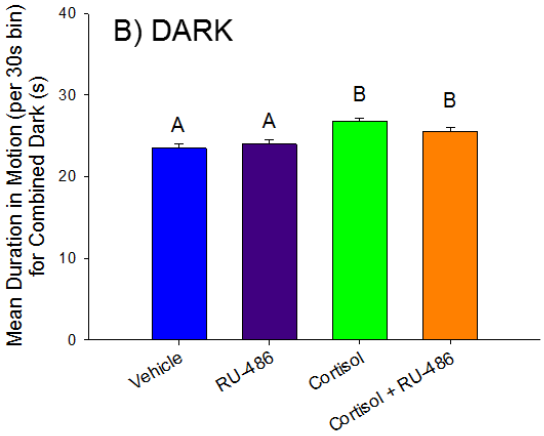
Figure 4.3. Activity in combined light (A) or dark (B) periods in 4 dpf larvae exposed to cortisol or RU-486 for 24 h.

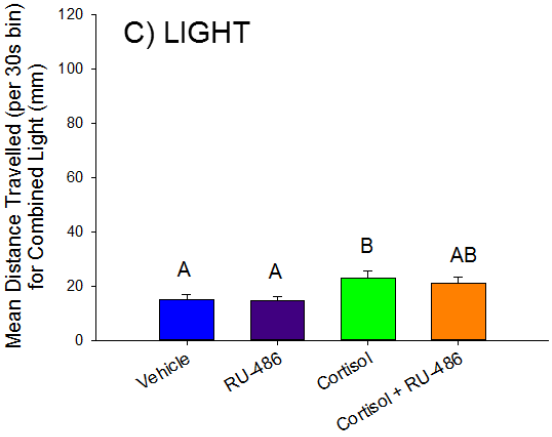
Larvae at 3 dpf were treated with vehicle, 1.25 µM RU-486, 27.59 µM cortisol, or both in combination. Activity is expressed as total distance moved per 30 s recording bin. The total recording period was 1 h with alternating light periods of 7.5 min each, indicated by the light and dark bars above the x-axis. Different letters indicate significant differences (one-way ANOVA). Values represent means ± SEM, and n=70-72.


A) LIGHT
Mean Velocity (per 30s bin) for Combined Light (mm/s)
0
1
2
3
4
5
A
A
B
AB
Vehicle
RU-486
Cortisol
Cortisol + RU-486


A) DARK
Mean Velocity (per 30s bin) for Combined Dark (mm/s)
0
1
2
3
4
5
A
AC
B
BC
Vehicle
RU-486
Cortisol
Cortisol + RU-486


B) LIGHT
Mean Duration in Motion (per 30s bin) for Combined Light (s)
0
10
20
30
40
A
AB
B
AB
Vehicle
RU-486
Cortisol
Cortisol + RU-486


B) DARK
Mean Duration in Motion (per 30s bin) for Combined Dark (s)
0
10
20
30
40
A
A
B
B
Vehicle
RU-486
Cortisol
Cortisol + RU-486


C) LIGHT
Mean Distance Travelled (per 30s bin) for Combined Light (mm)
0
20
40
60
80
100
120
A
A
B
AB
Vehicle
RU-486
Cortisol
Cortisol + RU-486

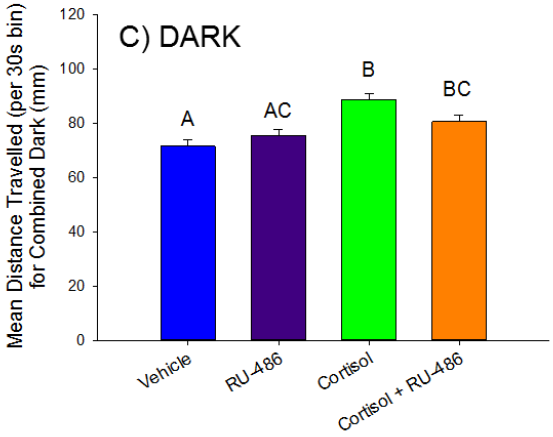

C) DARK
Mean Distance Travelled (per 30s bin) for Combined Dark (mm)
0
20
40
60
80
100
120
A
AC
B
BC
Vehicle
RU-486
Cortisol
Cortisol + RU-486

Figure 4.4. Thigmotaxis in 4 dpf larvae exposed to cortisol or RU-486 for 24 h. Larvae at 3 dpf were treated with vehicle, 1.25 µM RU-486, 27.59 µM cortisol, or both in combination. Activity of 4 dpf larvae was monitored in 24-well plates, and individual activity in the inner and outer zones of the well were recorded. Thigmotaxis is expressed as % of total distance travelled that occurred in the outer zone. Different letters indicate significant differences (one-way ANOVA). Values represent means ± SEM, and n=40.

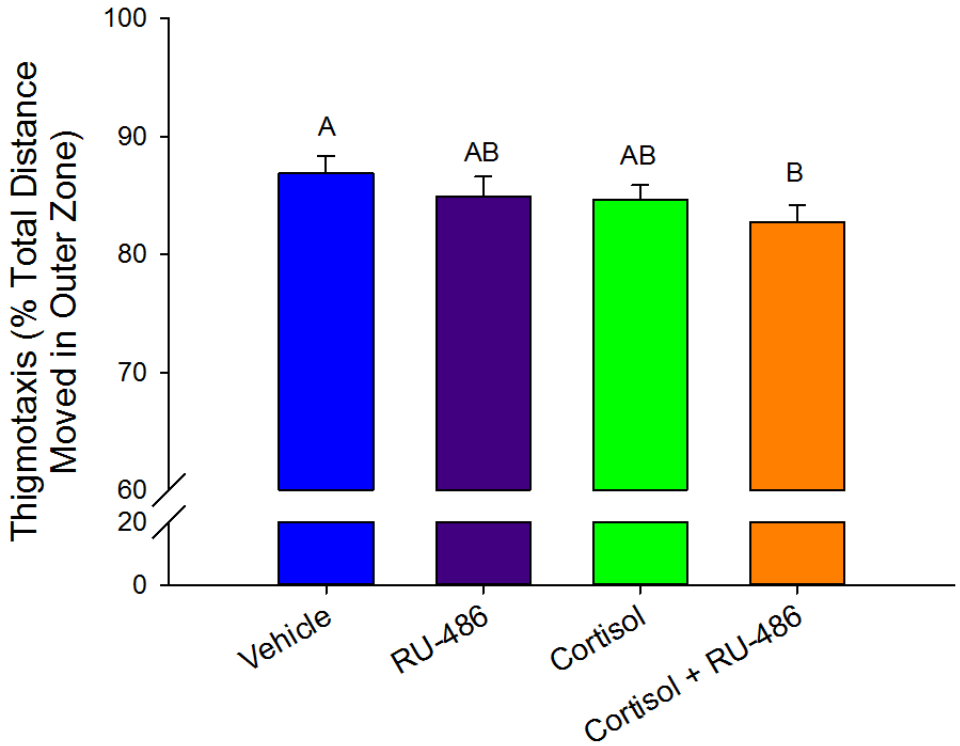
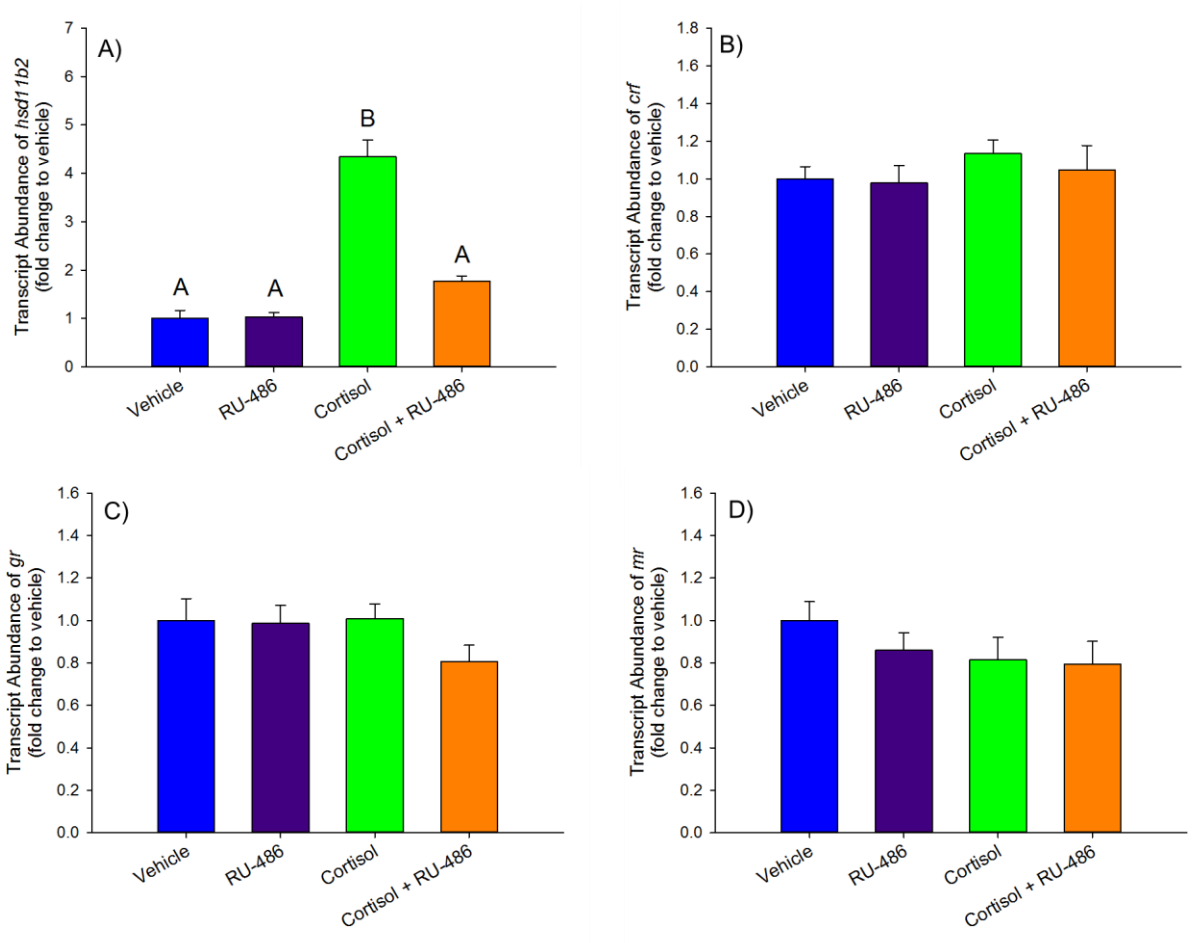


Figure 4.5. Transcript abundance in 4 dpf larvae exposed to cortisol or RU-486 for 24 h.

Larvae at 3 dpf were treated with vehicle, 1.25 µM RU-486, 27.59 µM cortisol, or both in combination. Transcript abundance of (A) *11βhsd2*, (B) *crf*, (C) *gr*, and (D) *mr* was determined by qPCR. Transcript abundance for each gene was normalized to that of *β-actin* as this housekeeping gene did not vary significantly between treatment groups. Values are fold changes relative to the mean vehicle control and represent means ± SEM, with n=6. Different letters indicate significant differences (one-way ANOVA).



4.5 Discussion

This study demonstrates for the first time that cortisol elevation, mimicking a stress response, increases locomotor activity in larval zebrafish. To date, studies addressing the effects of cortisol on fish development have only examined altered glucocorticoid signaling during the pre-hatch phase, when they are unable to elicit a stress response, and its functional role during embryogenesis (Nesan et al. 2012, Nesan and Vijayan 2012a, Nesan and Vijayan 2013a). No study in teleosts has investigated the effects of elevated glucocorticoid exposure, at a time when the stress axis becomes functional, on larval behaviour. The results from the present study suggest that elevated cortisol levels will lead to increased swimming activity in larval zebrafish.

The waterborne cortisol exposure led to elevated whole body steroid levels that were ~ 5-fold higher than those observed following our swirling stressor (see chapter 2), and approximately 10-fold higher than observed with heat stress in 5 dpf larvae (+8ºC, 10 min) (Yeh et al. 2013). Although the variability of cortisol levels in larval zebrafish in response to different stressors is not well known, differences in the magnitude of cortisol response to stressors is not uncommon in other fish species (Gamperl et al. 1994). Treatment with the GR antagonist RU-486 did not have a significant effect on larval cortisol levels in the current study. Typically in mammals and fish, treatment with RU-486 can impair the GR-mediated negative feedback response of cortisol, resulting in elevated circulating levels of this hormone (Reddy et al. 1995, Baulieu 1996, Chu et al. 2001). This was not observed in the present study and may be due to the already high levels of whole body cortisol associated with the waterborne exposure. Indeed, RU-486 was effective at inhibiting GR-signaling and this was confirmed by the abolishment of

cortisol-induced *11βhsd2* transcript levels by the GR antagonist (Fig. 4.5). *11βhsd2* has several putative glucocorticoid response elements (GREs) in its promoter region and is regulated by cortisol (Alderman and Vijayan 2012; chapter 2). Transcript levels of *11βhsd2* remain low until hatching in zebrafish, and increases as initial endogenous cortisol synthesis commences (Alsop and Vijayan 2008). Our results suggest that the endogenous elevation in cortisol levels during early development may play a role in the upregulation of *11βhsd2*, and underscores an important role for this enzyme in protecting the larvae from excess cortisol.

In the current study, cortisol elevated larval locomotor activity irrespective of the light or dark periods. Burgess and Granato (2007) suggests that the increased movement in the dark periods is a transient response to obstruction of light during the circadian daytime (e.g. by debris). They hypothesized that the increased activity is an attempt to seek the obstructed light that would normally be present during the day. The freezing behaviour during the light periods is a decrease in activity in response to sudden light onset, which is an aversive stimulus (MacPhail et al. 2009). While an increase in activity during only the light period (when larvae are expected to freeze in response to a novel or aversive stimulus) would suggest an anxiolytic effect (e.g. chapter 3), cortisol elevation also elevated activity levels in the dark, which does not suggest an anxiolytic effect. Rather, cortisol exposure is elevating all measures of activity (velocity, duration in motion, total distance moved) in the same manner, irrespective of timing and of light condition (Fig. 3, Fig. A.1). This pattern suggests that this cortisol treatment post-hatch is not anxiolytic. This is supported by the lack of change observed in thigmotaxis despite elevation in cortisol levels. Thigmotaxis, the propensity of an animal to remain close to

the walls of their environment, is a known measure of anxiety that has been validated in zebrafish (Schnörr et al. 2012, Colwill and Creton 2011). Altogether, the overall increase in larval activity irrespective of light or timing, as well as unaltered thigmotaxis in larvae with elevated cortisol levels suggests that acute elevation in glucocorticoid levels increases locomotor activity, but does not lead to an anxiety phenotype in zebrafish larvae.

Elevated cortisol levels do affect behaviour in other animals, including locomotor activity in fishes (Øverli et al. 1998, 2002). Cortisol elevation due to a social stress led to a reduced locomotor activity in juvenile arctic charr species (Øverli et al. 1998). Also, activity in juvenile rainbow trout fed cortisol-treated food was increased in response to a conspecific intruder in the short-term (1.5 h), but not long-term (48.5 h) (Øverli et al., 2002). Additionally, experiments in rodents and birds also show that glucocorticoids can have positive effects on activity. Rats injected with corticosterone exhibited elevated exploratory activity in a novel environment (Sandi et al. 1996). Sparrows fed corticosterone exhibited increased (dose-dependent) perch hopping activity (Breuner and Wingfield 2000).

Increased activity may be due to increased metabolic rate, as cortisol treatment results in higher metabolic rate in fish (eel (*Anguilla japonica*): Chan and Woo 1978; steelhead (*Oncorhynchus mykiss*): Barton and Schreck 1987; cutthroat trout (*Oncorhynchus clarki clarki*): Morgan and Iwama 1996). Consequently, cortisol also mobilizes energy substrate stores to cope with the increased metabolic demand (Vijayan et al. 2010). While the physiological underpinnings of enhanced swim performance are better characterized in adults (Gore and Burggren 2012), the increase in metabolic

capacity and substrate availability is a potential route by which cortisol may increase swimming performance in larvae. Therefore, given the known effects of cortisol on fish (juvenile) metabolism (Chan and Woo 1978, Barton and Schreck 1987), and the links between enhanced metabolic rate and swim performance (Gore and Burggren 2012), cortisol may be elevating swim activity in 96 hpf larvae through increased metabolic rate and energy mobilization.

While the proposed pathways by which cortisol modifies behaviour are not clear, some options have been proposed. For instance, the positive effect of corticosterone on locomotor activity in rats was not inhibited by either corticosteroid antagonist or the protein synthesis inhibitor cycloheximide (Sandi et al. 1996). As these effects had rapid (< 20 min) onset, and were not blocked by either corticosteroid receptor antagonist, the authors suggested a nongenomic glucocorticoid effect. The current study did not test for nongenomic effects, so further investigation would be required to confirm such a mechanism. Behaviour in rodents has also been shown to be mediated genomically through the corticosteroid receptors, specifically the mineralocorticoid receptor (MR). MR antagonist injection in the hippocampus increases locomotor behaviours in rats (Smythe et al. 1997, Bitran et al. 1998).

While comparable studies in fish are lacking, MR is present at high levels in the teleost brain (Sturm et al. 2005), and in juvenile trout, intraperitoneal cortisol implants altered dopaminergic and serotonergic activity in a region-specific manner, and this was not abolished by RU-486 (DiBattista et al. 2005). Corticosterone infusion in rats resulted in elevated locomotor activity in the short term (2 h), and this effect was abolished by lesion of the dopaminergic terminals in the nucleus accumbens (Piazza et al. 1996), also

implicating brain monoaminergic activity in the glucocorticoid-mediated locomotor response. Changes in transcript abundance are not a contributing factor as *gr* or *mr* transcript abundances were not altered. Given that the GR antagonist did not abolish the effects of cortisol on behaviour in the current study, and the known role of MR in altering rodent behaviour, we propose that cortisol may be acting either through the mineralocorticoid receptor (MR) or nongenomically by yet unknown receptors, and this remains to be tested.

The increased larval locomotor activity in response to elevated cortisol levels may be an adaptive response to evade stressors (Colwill and Creton 2011). Although, it has also been shown that when stressors are compounded or if the stress is not corrected, changes in locomotor activity can result in inappropriate reactions to threats such as predators (Schreck et al. 1981). Interestingly, the behavioural phenotype in larvae subjected to early developmental programming effects of elevated cortisol signalling (chapter 3: reduced anxiety) is distinct from the behavioural phenotype due to increased cortisol levels in the larvae post-hatch (increased locomotor activity, no effect on anxiety). Developmental programming effects can be seen as a response to a stressful maternal environment, programming the development of behaviour accordingly (e.g. reduced anxiety response). However, the effects of larval cortisol exposure on locomotory behaviour may be a response to a stressful larval environment, allowing the larvae to cope with more immediate threats (e.g. increasing activity to evade the stressful environment).

Overall, cortisol elevation at 4 dpf significantly altered behavioural phenotypes in terms of locomotor activity, but did not affect anxiety level. A key finding from this

study is that the locomotor activity of larvae is increased in response to cortisol elevation post-hatch. This suggests that stressor-induced cortisol elevation is adaptive and will allow the animal to evade the stressor by increasing the locomotor activity. This phenotype does not appear to be modulated by GR signalling, given that RU-486 did not offset the effect of cortisol. We propose that genomic and/or nongenomic MR signalling may be playing a role in the cortisol-mediated behavioural phenotype. Another important finding is that the cortisol-mediated increase in activity post-hatch is distinct from the anxiolytic phenotype observed with elevated zygotic cortisol deposition, suggesting that the window during which larvae see elevated cortisol levels during development results in different behavioural phenotypes.

4.6 Acknowledgements

This study was supported by the Natural Sciences and Engineering Research Council (NSERC) of Canada Discovery grant to MMV. CB was the recipient Alberta Innovates Technology Futures (AITF) master's scholarship.

4.7 Tables

Table 4.1. Primer sequences for qPCR (4 dpf, cortisol elevation).

Primers Used for Real-Time Quantitative PCR. Sequences, annealing temperatures, amplicon sizes and references are indicated.

Gene	Primer Sequence	Amplicon (bp)	Annealing Temp (^{0}C)	Reference
gr	F: 5'- ACAGCTTCTTCCAGCCTCAG -3' R: 5'- CCGGTGTTCTCCTGTTTGAT-3'	116	60	Alsop and Vijayan 2008
mr	F: 5'- CCCATTGAGGACCAAATCAC -3' R: 5'- AGTAGAGCATTTGGGCGTTG -3'	106	60	Alsop and Vijayan 2008
crf	F: 5'-CACCGCCGTATGAATGTAGA -3' R: 5'- GAAGTACTCCTCCCCCAAGC-3'	113	60	Nesan 2013
11βhsd2	F: 5'- TGCTGCTGGCTGTACTTCAC -3' R: 5'- TGCATCCAACTTCTTTGCTG -3'	123	55	Alsop and Vijayan 2008
β-actin	F: 5'- TGTCCCTGTATGCCTCTGGT -3' R: 5'- AAGTCCAGACGGAGGATGG -3'	121	60	Alsop and Vijayan 2008

Chapter Five: General Conclusions

5.1 Summary of Findings

Glucocorticoids play an essential physiological role in teleosts, allowing the animal to cope with stressor insults and regain homeostasis (Vijayan et al. 2010). Glucocorticoids also play an important role during development in zebrafish, with disrupted GR signaling resulting in a variety of developmental defects (Nesan and Vijayan 2013b). The current study demonstrates for the first time that maternal stress transfers excess cortisol to the oocyte, and this leads to alterations in the developmental programming events in zebrafish. The findings are summarized in Figure 5.1.

The first objective was to determine if maternal cortisol elevation can be transferred to the ovary and embryo, and how this is regulated. **Chapter 2** demonstrates that maternal transfer of cortisol occurs in zebrafish. This was tested by both endogenously (fasting) and exogenously (feeding) elevating cortisol levels in female zebrafish. The results clearly show that stress-induced cortisol levels are transferred to the embryos, but only transiently. This transfer corresponds with oocyte development in zebrafish. The results indicate for the first time a protective role for ovarian follicles in regulating excess cortisol deposition by activating follicular 11βHSD2, a key enzyme involved in cortisol breakdown, in response to excess cortisol stimulation.

The second objective was to determine if this elevated cortisol deposition from the mother alters the larval stress response and behaviour in the offspring, and if this may be due to disrupted neurogenesis. In **chapter 3**, this was tested by microinjection of cortisol into the yolk to mimic maternal deposition, and demonstrated that this elevated

zygotic cortisol content disrupts the larval stress response, is behaviourally anxiolytic, and disrupts neurogenesis at 24 hpf. As the affected regions are homologous to the mammalian limbic system and the preoptic region, this suggests a role for disrupted neurogenesis in the observed altered phenotypes of stress and anxiety.

The final objective was to determine how stress-mediated glucocorticoid signaling post-hatch alters larval behaviour. This was tested in **chapter 4**, by elevating cortisol levels in the larval zebrafish. This study demonstrated for the first time that cortisol treatment, mimicking a stressor-induced elevation, increases locomotor activity in the larval fish, with no effect on anxiety-related behaviour. The increased activity phenotype in larvae with elevated cortisol (mimicking stress) is distinct from the anxiolytic effect of elevated zygotic cortisol content, mimicking maternal deposition.

Overall, maternal stress may influence the developmental trajectory of the offspring and this is mediated by the stress steroid cortisol. Increased cortisol deposition in the embryos alters the larval stress response, reduces anxiety, and increases neurogenesis in a region-specific manner. However, the results suggest that excess stress steroid transfer from the mother to the embryo is tightly regulated in zebrafish and this is controlled by cortisol-induced upregulation of 11βHSD2, a key enzyme that breaks down cortisol to its inactive metabolite cortisone. We hypothesize that altered stress and behavioural responses are the outcome of developmental dysfunction associated with increased maternal cortisol deposition, and this may involve changes in neurogenesis. The behavioural phenotype seen in response to elevated cortisol levels post-hatch is distinct from that seen with elevated zygotic cortisol content, supporting the hypothesis that excess maternal transfer of cortisol affects developmental programming events in

zebrafish. Whether the phenotypes observed are adaptive or maladaptive to growth and survival remains to be determined.

5.2 Future Directions

The transfer of maternal cortisol is demonstrated *in vivo* in chapter 2, and *in vitro* analyses suggest that follicles upregulate transcript levels of *11βhsd2*. Future studies would serve to further confirm and clarify the localization and role of this enzyme in regulating hormone transfer throughout oogenesis. Given that the timing of cortisol transfer has been shown (chapter 2), studies in which oocytes, yielded during the window of elevated deposition, are collected and raised could confirm the effects seen in studies mimicking maternal cortisol deposition (chapter 3). Another path for future study would be to include the apparently positive effects of increased maternal glucocorticoid on embryo yield.

It would be interesting to further explore the effects of elevated cortisol deposition on neurogenesis across multiple time points, and investigate a role for the corticosteroid receptors, particularly MR, in mediating these changes in the brain. This line of research may lead to a mechanism linking excess zygotic cortisol levels on stress and behaviour phenotypes.

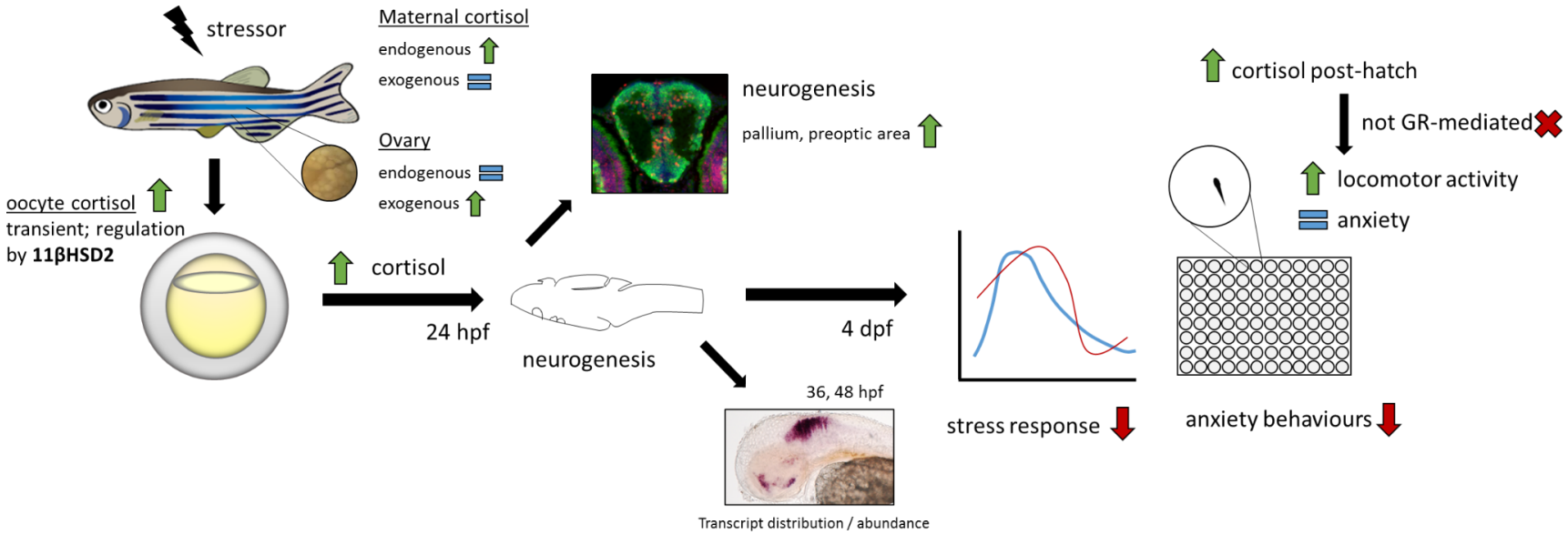
Altogether, this study suggests that cortisol is transferred from the mother to the embryo in zebrafish, and this can alter offspring neurogenesis, as well as their stress and behavioural phenotype. This altered phenotype may modify the way in which these offspring interact with and respond to their environment, potentially beyond the larval stage. Consequently, examining the long-term and multigenerational effects of elevated

zygotic cortisol deposition would be valuable in determining if the developmental programming changes leading to altered larval behaviour, physiology and neurogenesis involves epigenetic mechanisms and are heritable.

5.3 Figures

Figure 5.1: Schematic overview of thesis objectives.

Findings from this thesis include: (1) maternal cortisol is transferred from the mother to the oocyte, but only transiently, and this is regulated by 11βHSD2 [chapter 2, top left], (2) elevated zygotic cortisol increases neurogenesis, and disrupts the stress response and the behaviour of the offspring [chapter 3, center-right] and (3) elevated larval cortisol increases swimming activity [chapter 4, top right]. Effects indicated by coloured symbols.



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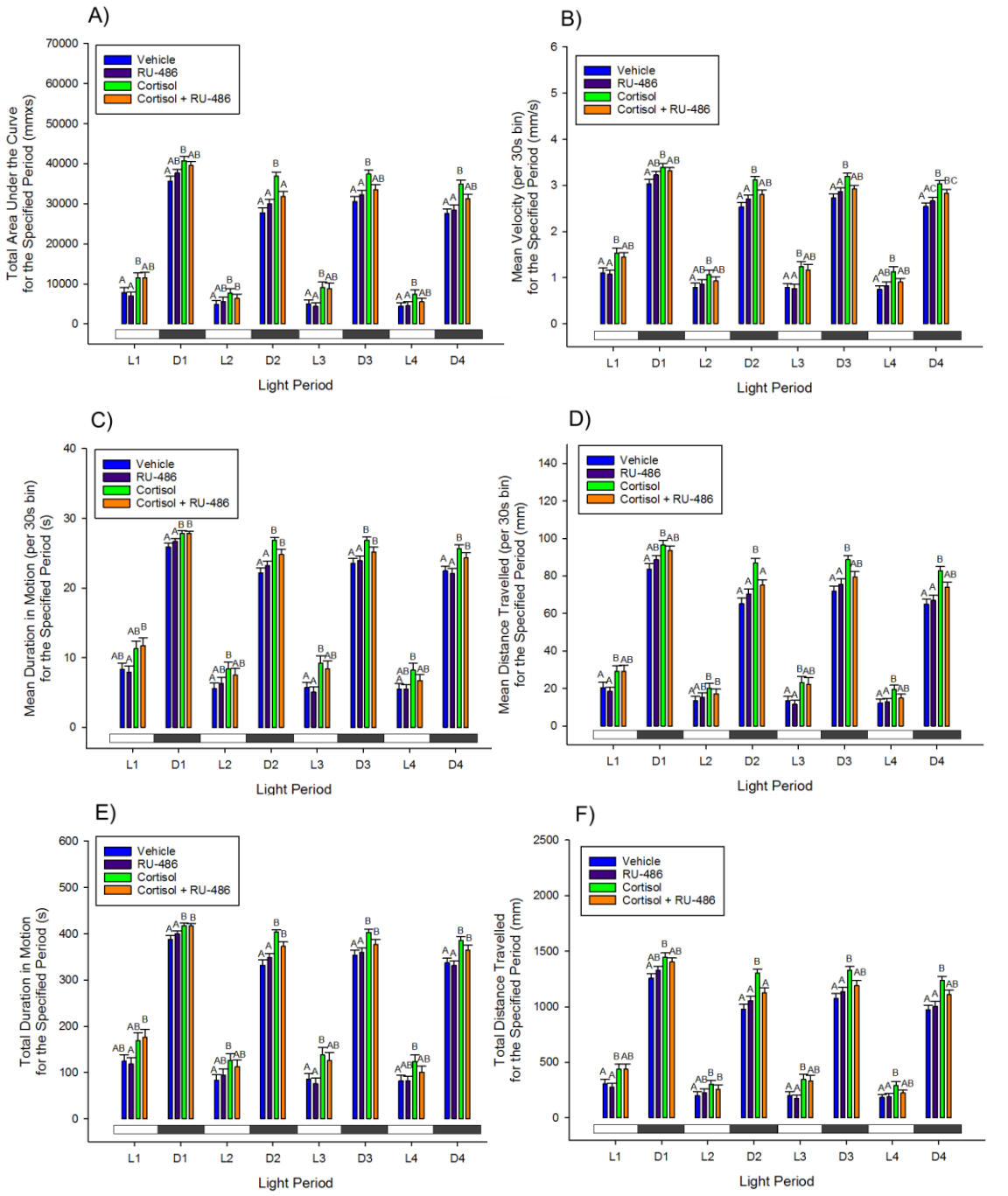
APPENDIX A: SUPPLEMENTARY FIGURES

Figure A.1. Activity by light period in response to changes in light in 4 dpf larvae exposed to cortisol or RU-486 for 24 h.

Larvae at 3 dpf were treated with vehicle, 1.25 µM RU-486, 27.59 µM cortisol, or both in combination. Light cycles of 7.5 min each are indicated by the light and dark bars above the x-axis (L, light and D, dark; four of each). Activity is expressed as (A) area under the curve, (B) mean velocity per 30s bin, (C) mean duration in motion per 30s bin, (D) mean distance travelled per 30s bin, (E) total duration in motion for the entire period, and (F) total distance travelled for the entire period. Different letters indicate significant differences (one-way ANOVA). Values represent means ± SEM, and n=70-72.


A)
Total Area Under the Curve for the Specified Period (mmxs)
Vehicle
RU-486
Cortisol
Cortisol + RU-486
L1 D1 L2 D2 L3 D3 L4 D4
Light Period
B)
Mean Velocity (per 30s bin) for the Specified Period (mm/s)
C)
Mean Duration in Motion (per 30s bin) for the Specified Period (s)
D)
Mean Distance Travelled (per 30s bin) for the Specified Period (mm)
E)
Total Duration in Motion for the Specified Period (s)
F)
Total Distance Travelled for the Specified Period (mm)