

**Early-life exposure to the antidepressant fluoxetine
induces a male-specific transgenerational disruption
of the stress axis and exploratory behavior in
adult zebrafish, *Danio rerio***

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ABSTRACT

Selective serotonin (5-HT) reuptake inhibitors (SSRIs) particularly fluoxetine (FLX, Prozac®), are often the first-line of pharmacological treatment for affective disorders in pregnant women. Given that SSRIs readily cross the placenta, a fetus from a SSRI-treated pregnant woman is potentially at risk from the disruptive effects of the SSRIs-induced 5-HT actions during this highly plastic stage of development. One of the prominent roles of 5-HT that we will explore here is its involvement in the development and programming of the stress axis. Pharmaceuticals including FLX and other SSRIs reach aquatic ecosystems through sewage release, so fish may also be inadvertently exposed. We investigated the premise that early-life exposure to FLX induces a transgenerational disruption of the stress axis using the zebrafish (ZF) *Danio rerio*, as a research model to encompass both the environmental and human health concerns. The FLX concentrations studied were environmentally relevant ($0.54 \mu\text{g}\cdot\text{L}^{-1}$) or comparable to concentrations detected in the cord blood of FLX-treated pregnant women ($54 \mu\text{g}\cdot\text{L}^{-1}$).

Exposure to FLX during the first 6 days of life induced a reduction of whole-body cortisol levels in adult ZF (filial generation 0; F_0), an effect that persisted across 4 consecutive generations without diminution, even though the descendants (F_1 to F_4) were not directly exposed to FLX. This effect was more pronounced and persistent in males than females. The *in vivo* cortisol response of the interrenal cells (the fish ‘adrenal’) to an intraperitoneal injection of adrenocorticotrophic hormone was also reduced in the F_0 and F_3 FLX-exposed males. RNA sequencing of the F_0 and F_3 male whole kidney containing the interrenal cells detected an array of differentially expressed genes (>500) altered by FLX treatment. Enrichment analysis of these genes revealed that early FLX exposure significantly modified numerous canonical pathways (>40) including key pathways related to steroidogenesis. These findings provide further insights on the underlying mechanism

of the transgenerational disruption induced by FLX. We also showed that altered cortisol levels were linked to reduced exploratory behavior in adult males from the F₀ to F₂ FLX lineage. In contrast, females were susceptible to the effects of FLX-induced hypocortisolism during a different window of development. Exposure to FLX in the sex differentiation period (15 to 42 days post-fertilization) increased exploratory behavior in the adult females. Transcriptional profile of selected steroidogenic genes in the whole-larvae from the F₀ varied in magnitude and direction in both treatments, despite the same low cortisol phenotype induced by both concentrations. We also found an up-regulation in the transcript levels of steroidogenic-related genes and a down-regulation of a gene involved in the inactivation of cortisol in the F₃ larvae ancestrally exposed to the human-relevant concentration. These findings on the transcript levels of the selected genes in the larvae from F₀ and F₃ suggest that the larvae adopted specific coping mechanism(s) to the disruptive effects of FLX depending on the exposure concentration and the filial generation. The pigmentation patterns in some of the descendants of the exposed fish (F₁ to F₃) were reduced by the 6-day early-FLX treatment. In response to a 6-day embryonic exposure to a second antidepressant, venlafaxine, the F₄ adult females that were ancestrally exposed (in the F₀) to the human-relevant FLX concentration displayed an intensified reduction of cortisol levels. Therefore, FLX exposure of the great-great-grandparents (F₀) permanently and most likely epigenetically shaped the response of future generations to other antidepressants. Collectively, our data are cause for concern, given the high-prescription rates of FLX to pregnant women and the potential long-term negative impacts on humans and aquatic organisms exposed to ever rising levels of SSRIs.

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LIST OF ABBREVIATIONS

(p)CREB	phospho-CREB
11-KT	11-ketotestosterone
11 β -HSD1	11 β -hydroxysteroid dehydrogenase 1
11 β -HSD2	11 β -hydroxysteroid dehydrogenase 2
17,20 lyase	17 α -hydroxylase
17-OH pregnenolone	17-hydroxy pregnenolone
17-OH progesterone	17-hydroxy progesterone
3 β -HSD	3 β -hydroxysteroid dehydrogenase
5-HT	serotonin / 5-hydroxytryptamine
5-HT _{1A}	serotonin receptors 1A
5-HTT	serotonin transporter
AADC	aromatic L-amino acid decarboxylase
ACTH	adrenocorticotrophic hormone
adra1	adrenoceptor alpha 1
adra2	adrenoceptor alpha 2
adrb2a	adrenoceptor beta 2 surface a
ANOVA	analysis of variance
AT	automated tracking
BPA	bisphenol A
CAs	catecholamines
CpG	cytosine-phosphodiester-guanine
CREB	cAMP response element binding protein
CRF	corticotropin-releasing factor
CRF-BP	corticotropin-releasing factor receptor binding protein
CRFR	corticotropin-releasing factor receptor
CRFR1	corticotropin-releasing factor receptor type 1
CRFR2	corticotropin-releasing factor receptor type 2
CRFR3	corticotropin-releasing factor receptor type 3
CTR	control
CV	coefficient of variation
CYP17	17 α -hydroxylase
CYP17A1	cytochrome P450 family 17 subfamily A polypeptide 1
CYP19A1A	cytochrome P450 family 19 subfamily A polypeptide 1a
CYP21A2	21-hydroxylase / cytochrome P450 family 21 subfamily A polypeptide 2

DEGs	differentially expressed genes
DNA	deoxyribonucleic acid
dpf	days post-fertilization
DRN	dorsal raphe nucleus
E ₂	17 β -estradiol
ELISA	enzyme-linked immunosorbent assay
F	filial
F ₋₁	parents of the F ₀ generation
Fig.	figure
FLX	fluoxetine
GC	glucocorticoid
GPCR	G protein-coupled receptor
GR	glucocorticoid receptor
GR-alpha	glucocorticoid receptor alpha-isoform
GR-beta	glucocorticoid receptor beta-isoform
GREs	glucocorticoid responsive elements
HBP	halogen-binding pocket
HFL	HIGH-FLX lineage
HPA	hypothalamic-pituitary-adrenal
hpf	hours post-fertilization
HPI	hypothalamic-pituitary-interrenal
hsd11b2	hydroxysteroid (11-beta) dehydrogenase 2
hsd3b2	hydroxy-delta-5-steroid dehydrogenase 3 beta- and steroid delta-isomerase 2
IMM	inner mitochondrial membrane
ip	intraperitoneal
LBD	ligand-binding domain
LFL	LOW-FLX lineage
MAOIs	monoamine oxidase inhibitors
MBD2b	methyl-CpG binding domain 2b
MC2R	melanocortin type 2 receptor
min	minutes
mpf	months post-fertilization
mRNA	messenger RNA
MT	manual tracking
NGFI-A	nerve growth factor-inducible protein A
nr3c1	nuclear receptor subfamily 3 group C member 1

nr5a1a	nuclear receptor subfamily 5 group A member 1a
NTT	novel tank diving test
OMM	outer mitochondrial membrane
P450 _{scc}	P450 side-chain cleavage
PCA	principal component analysis
PCs	principal components
PKA	protein kinase A
POA	preoptic area
POMC	pro-opiomelanocortin
PVN	paraventricular nucleus
qRT-PCR	quantitative real-time polymerase chain reaction
RNA	ribonucleic acid
SSRI	selective serotonin reuptake inhibitor
StAR	steroidogenic acute regulatory
TCAs	tricyclic antidepressants
TPH	tryptophan hydroxylase
VEN	venlafaxine
wpf	weeks post-fertilization
ZF	zebrafish

CHAPTER 1: General Introduction

1.1. Rationale for the study

Depressive episodes are a major health burden in pregnancy and postpartum periods (Meltzer-Brody et al., 2018), with an estimated prevalence rate between 7 and 15% of pregnant women experiencing this condition (Engelstad et al., 2014; Oberlander et al., 2006). Due to the high risks associated with depression to pregnant women and the numerous adverse effects to the neonate (Susser et al., 2016), antidepressants, especially fluoxetine (FLX, Prozac®) from the selective serotonin (5-hydroxytryptamine; 5-HT) reuptake inhibitor (SSRI) family, are prescribed to treat this affective disorder (Latendresse et al., 2017; Locher et al., 2017; Man et al., 2017; Morkem et al., 2017; Sarginson et al., 2017). Members of the SSRI family inhibit the 5-HT transporter located on 5-HT neurons, augmenting the magnitude and duration of 5-HT actions within the synapse (Alwan et al., 2016). Serotonin (Fig. 1.2) is involved in regulating mood as well as many other physiological and behavioral processes (Mossner and Lesch, 1998). During the critical window of brain development, 5-HT induces epigenetic programming of the stress axis in addition to its involvement as a neurotransmitter (Andrews and Matthews, 2004). Given that SSRIs can cross the placental barrier, fetuses from SSRIs-treated pregnant women are at risk from the disruptive effects of SSRIs-induced 5-HT actions (Ewing et al., 2015; Kaihola et al., 2016; Loughhead et al., 2006; Rampono et al., 2009). Clinical studies have provided evidence of the physiological disruption of the stress axis in children following prenatal exposure to SSRI medications via maternal treatment (Oberlander et al., 2002; 2008; 2005).

The ever-increasing rates of prescriptions have unfortunately led to an escalation in environmental discharges of SSRIs compounds. Fluoxetine is one of the antidepressants with the most frequent detection rate from waste water treatment plants with concentrations as high as

929 ng·L⁻¹ (Arnnok et al., 2017; Bueno et al., 2007; Comber et al., 2018). Pharmaceuticals are designed to target enzymes, receptors or other cellular macromolecules, which may be evolutionally conserved across vertebrates (Gunnarsson et al., 2008). The pseudo-persistence or continuous release of FLX into the environment, concomitantly with the conservation of SSRI targets across vertebrates and of the biochemical pathway and functions of 5-HT (Mennigen et al., 2011), render aquatic organisms such as fish, a target for the possible unintended effects of this drug. Studies on fish have reported that exposure to FLX during adulthood induces endocrine disruptive effects in many biological endpoints including reproduction, stress, behavior, and feeding, among others (Abreu et al., 2014; Craig et al., 2014; Mennigen et al., 2009; 2008; 2011; Saaristo et al., 2017; Wolkers et al., 2017).

Even though evidence for SSRI-induced disruption of the stress axis exists following prenatal exposure in humans and short-term effects in fish, there remains little focus on the long-term consequences manifested in adulthood or even in future generations of early exposures to these drugs. Therefore, during my Ph.D. project, I used the model fish *Danio rerio* or the zebrafish (ZF) to better understand both the environmental and human health concerns of early-life FLX exposure, as the ZF is considered a suitable model in stress research (Steenbergen et al., 2011) and other human-related brain disorders (Howe et al., 2013; Kalueff et al., 2014).

To further assess the implications of high FLX prescriptions summarized in this section, I examine the null hypothesis that early-life FLX exposure does not induce transgenerational disruption of the stress axis, which is addressed by means of diverse experiments described in the subsequent chapters of this thesis. This first general chapter expands on key concepts introduced in this rationale, while reviewing the literature on which the experimental work is based.

1.2. Depression

Depression is a commonly occurring mental disorder considered to be a leading cause of disability worldwide (Spijker et al., 2004; Ustun et al., 2004; Wong and Licinio, 2001). The symptoms of this disorder, especially when long-lasting, are associated with reduced quality of life and in some cases suicide (Kessler and Bromet, 2013; Sullivan et al., 2000). The lifetime prevalence of this disorder between 2001 and 2003 was estimated to be 16.6% (± 0.5) among adult respondents in the U.S.A. (Kessler et al., 2005) and 11.3% in 2012 in Canada (Pearson et al., 2013). These estimates vary substantially across countries as a result of the different socio-demographics attributed to each culture with the main factors being gender, age and marital status (Kessler and Bromet, 2013). Cross-national studies on depression reported women having twice the prevalence of suffering from depression than men (Piccinelli and Wilkinson, 2000; Van de Velde et al., 2010). The incidence of developing a depressive episode is at its highest during childbearing, with an estimate of 7 to 15% of pregnant women experiencing this condition (Engelstad et al., 2014; Oberlander et al., 2006).

Depression is best conceptualized as a complex interaction between environmental factors and genetic susceptibility (Sullivan et al., 2000). However, experimental and clinical evidence supports the monoamine hypothesis as the main underlying mechanism of this disorder (Albert et al., 2012; Krishnan and Nestler, 2008; Wong and Licinio, 2001). This hypothesis originally postulated a deficit in the catecholamines norepinephrine and dopamine in the brain of depressed individuals (Schildkraut, 1965) but was subsequently revised and refined to include insufficiency in 5-HT neurotransmission (Fig. 1.1A) (Albert et al., 2012; Coppen, 1967).

Over the years, different pharmacological treatments targeting the enhancement of either one or a combination of the monoamine neurotransmitters have been developed. The first

antidepressants to be used as treatments for the regulation of depression were introduced in the 1950s. These antidepressants belonged to the family of the monoamine oxidase inhibitors (MAOIs) and the tricyclic antidepressants (TCAs) (López-Muñoz and Alamo, 2009). Prior to these drug treatments, therapies included electroconvulsive therapy, insulin coma and deep sleep therapy, but these were too coarse and extremely dangerous for the patient. Hence, the clinical introduction of the MAOIs and TCAs antidepressants revolutionized the fields of psychiatry and psychopharmacology with respect to the treatment of depression. Albeit the success of these treatments at increasing the availability of the target monoamines at the synaptic cleft, their biochemical mechanism of action was very broad and led to potential toxic side effects (Blier and de Montigny, 1999; Hiemke and Hartter, 2000; Khawam et al., 2006). Nonetheless, these discoveries were of great scientific value to the development of the next generation of antidepressants (López-Muñoz and Alamo, 2009).

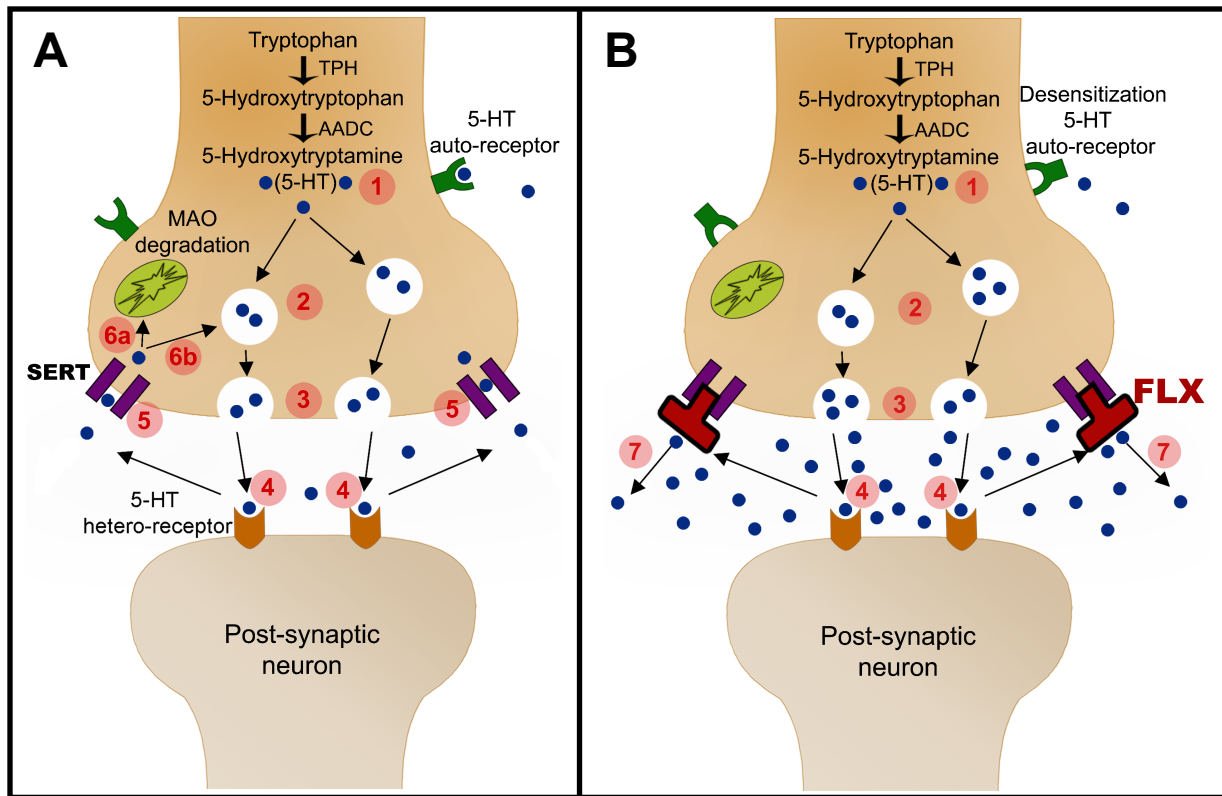


Fig. 1.1. Serotonin synthesis, release and reuptake from a serotonergic neuron in (A) a depressed patient and (B) a FLX-treated patient. Blue dots (●) represent 5-HT. Numbers in brackets refer to both panels: [1] 5-HT synthesis from the precursor tryptophan by tryptophan hydroxylase (TPH) and aromatic L-amino acid decarboxylase (AADC); [2] Storage of 5-HT into vesicles for transportation; [3] 5-HT is released into the synaptic cleft upon melding of the vesicle with the cell membrane; [4] 5-HT receptor on the post-synaptic neuron is activated upon 5-HT binding; [5] 5-HT is transported back into the 5-HT neuron via the transporter SERT; [6a] 5-HT is recycled back into vesicles for further usage; [6b] 5-HT is metabolized by the monoamine oxidase (MAO) enzyme; [7] FLX binding to SERT inhibits 5-HT reuptake.

1.3. The Selective Serotonin Reuptake Inhibitor (SSRI) family

The inter-individual variability and the relatively low therapeutic safety of these early antidepressant drugs were the main contributors to the research endeavors to continue to generate new antidepressants (Bondy, 2005; Ryan et al., 2005). The clinical introduction of MAOIs and TCAs as pharmacological treatments for depression opened the way to gain a better understanding of the pathophysiology of this disorder (López-Muñoz and Alamo, 2009) and ultimately contributed to the development of the SSRI family of antidepressants. Soon after their introduction in the late 1980s, SSRIs became the most widely prescribed pharmacotherapies for depression. To this date, SSRIs are the first-line of pharmacological treatment for affective disorders, particularly in pregnant women and children in several countries (Latendresse et al., 2017; Locher et al., 2017; Man et al., 2017; Morkem et al., 2017; Sarginson et al., 2017). Their popularity is attributed to their high level of tolerability (Barbey and Roose, 1998), supposed few side effects (Kaiholola et al., 2016), efficacy at treating depression and most importantly their therapeutic safety compared to all previous antidepressants (Morrison et al., 2004).

Fluoxetine was the first successful SSRI to be developed for the treatment of neuropsychiatric disorders including depression, obsessive-compulsive disorders, eating disorders, social phobia, and panic disorders, among others (Davis et al., 2016; López-Muñoz and Alamo, 2009). Fluoxetine was originally manufactured by the Eli Lilly Co. (U.S.A.) under the name Prozac® (registered in 1985). Soon after the success of Prozac®, other SSRIs were developed and introduced to the market. These include fluvoxamine (Luvox®), paroxetine (Paxil®), sertraline (Zoloft®), citalopram (Celexa®) and escitalopram (Lexapro®) (Henry et al., 2004; Hiemke and Hartter, 2000; Susser et al., 2016) (Fig. 1.2). These six SSRIs have similar pharmacodynamic

properties while being distinct in their pharmacokinetic actions, including drug-drug interactions (Catterson and Preskorn, 1996; Hiemke and Hartter, 2000).

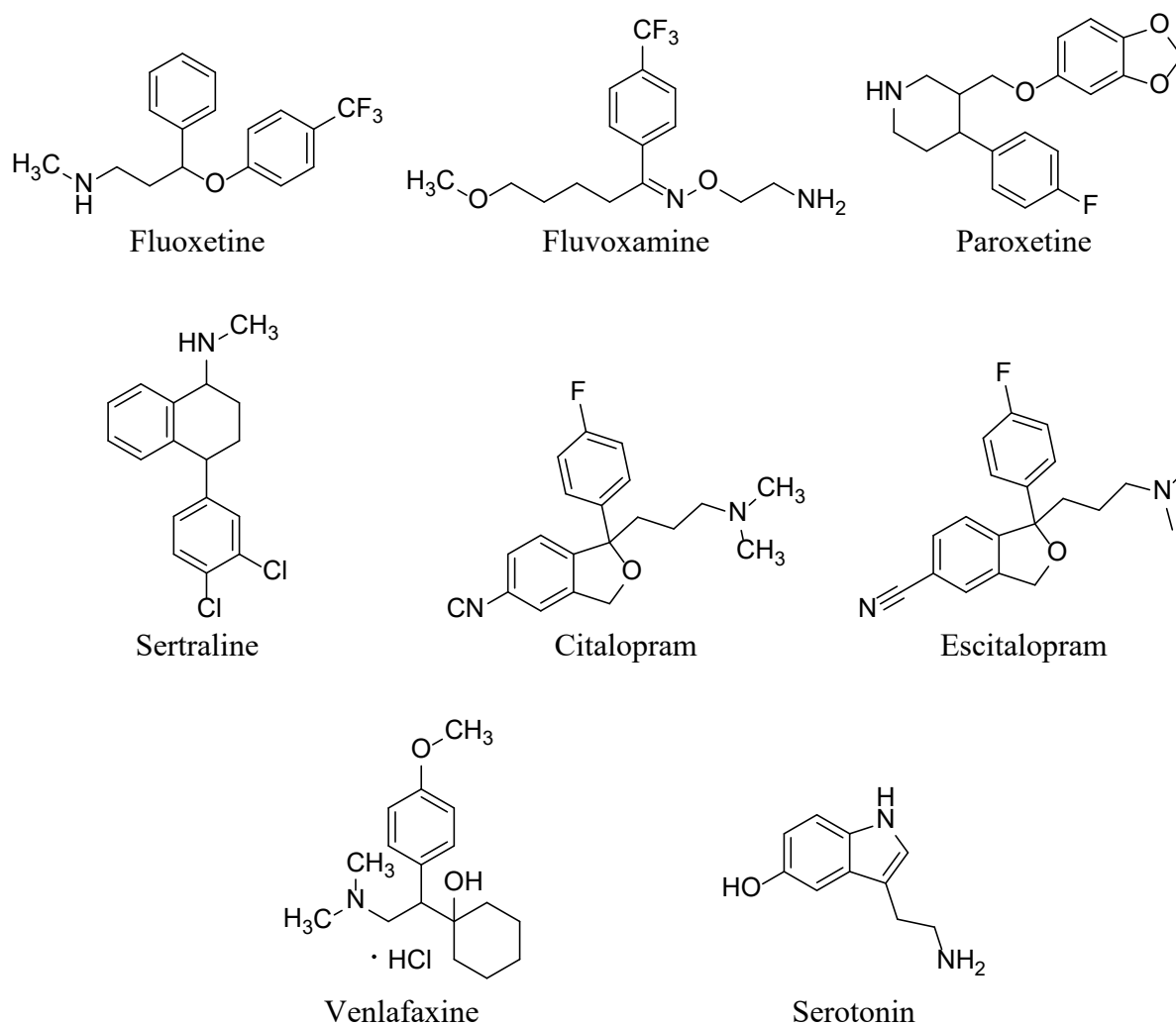


Fig. 1.2. Chemical structures of the SSRI family, venlafaxine (from the serotonin-norepinephrine reuptake inhibitor family) and serotonin.

1.3.1. SSRI mechanism of action

Selective serotonin reuptake inhibitors alleviate depressive symptoms by selectively and strongly blocking the 5-HT reuptake transporter, SERT or also known as 5-HTT (5-HT transporter). This inhibition of the 5-HT reuptake from the synaptic cleft results in the increase of 5-HT levels at the post-synaptic receptor sites, consequently enhancing its signaling effects (Brooks et al., 2003; Quick, 2003) (Fig. 1.1*B*). The specificity of the SSRI family relies on the interaction between the halogen elements (F and Cl – see Fig. 1.2) of their chemical structure with the halogen-binding pocket of SERT (Davis et al., 2016; Tavoulari et al., 2009; Zhou et al., 2009), which is represented in Fig. 1.3*A* for R-FLX and Fig. 1.3*B* for S-FLX using the halogen-binding pocket of LeuT, a bacterial SERT homolog. Since the chemical structure of FLX has a chiral center, two different enantiomers of FLX exist, R-FLX and S-FLX. Both enantiomers have similar potency at binding to SERT thereby inhibiting 5-HT reuptake (Cârcu-Dobrin et al., 2017).

Even though SERT inhibition follows immediate initiation of SSRI treatment, there is a 14- to 21-day delay in clinical improvements (Castro et al., 2003). The release of 5-HT within the synaptic cleft is regulated by a negative feedback mechanism controlled by the 5-HT auto-receptor 1A (5-HT_{1A}) located primarily in the dorsal raphe nucleus (DRN) which contains about 50 to 60% of 5-HT neurons in the brain of humans (Piñeyro and Blier, 1999). Additionally, 5-HT neurons from the DRN are associated with a wide range of physiological and behavioral functions including mood regulation (Ansorge et al., 2007; Forchetti and Meek, 1981). The lack of an immediate therapeutic effectiveness of the SSRIs and other antidepressant families that target the increase in the magnitude and duration of 5-HT action within the synapse, is due to the time required for adaptive changes to occur to the pre-synaptic 5-HT_{1A}. Chronic SSRI treatment leads to the functional desensitization of the soma and dendritic 5-HT_{1A} auto-receptors in the DRN,

consequently decreasing the effectiveness of the negative feedback mediated by the extracellular 5-HT (Fig. 1.1*B*) (Castro et al., 2003; Hervás et al., 2001; Le Poul et al., 2000; Le Poul et al., 1995).

In the mature brain of vertebrates, 5-HT modulates many physiological, cognitive and behavioral functions including learning, mood, anxiety, stress, aggressiveness, appetite, reproductive and endocrine activity, among many (Mossner and Lesch, 1998). During neurodevelopment however, 5-HT also acts as a neurotrophic factor by regulating cell division, migration, growth cone elongation, differentiation, synaptogenesis, myelination, and dendritic pruning (Gaspar et al., 2003; Haydon et al., 1987; Lauder, 1990). Additionally, studies using rats revealed that overstimulation of 5-HT during brain development is implicated in the programming of the hypothalamic-pituitary-adrenal (HPA) axis, commonly known as the stress axis (Andrews and Matthews, 2004; Laplante et al., 2002). Fetal programming as a concept emerged in the 1980s describes the process whereby early neonatal exposure to stimuli or insults during critical developmental windows may modify the organization of gene expression patterns increasing the predisposition of the fetus to long-lasting changes in physiological functions (Sarr et al., 2012).

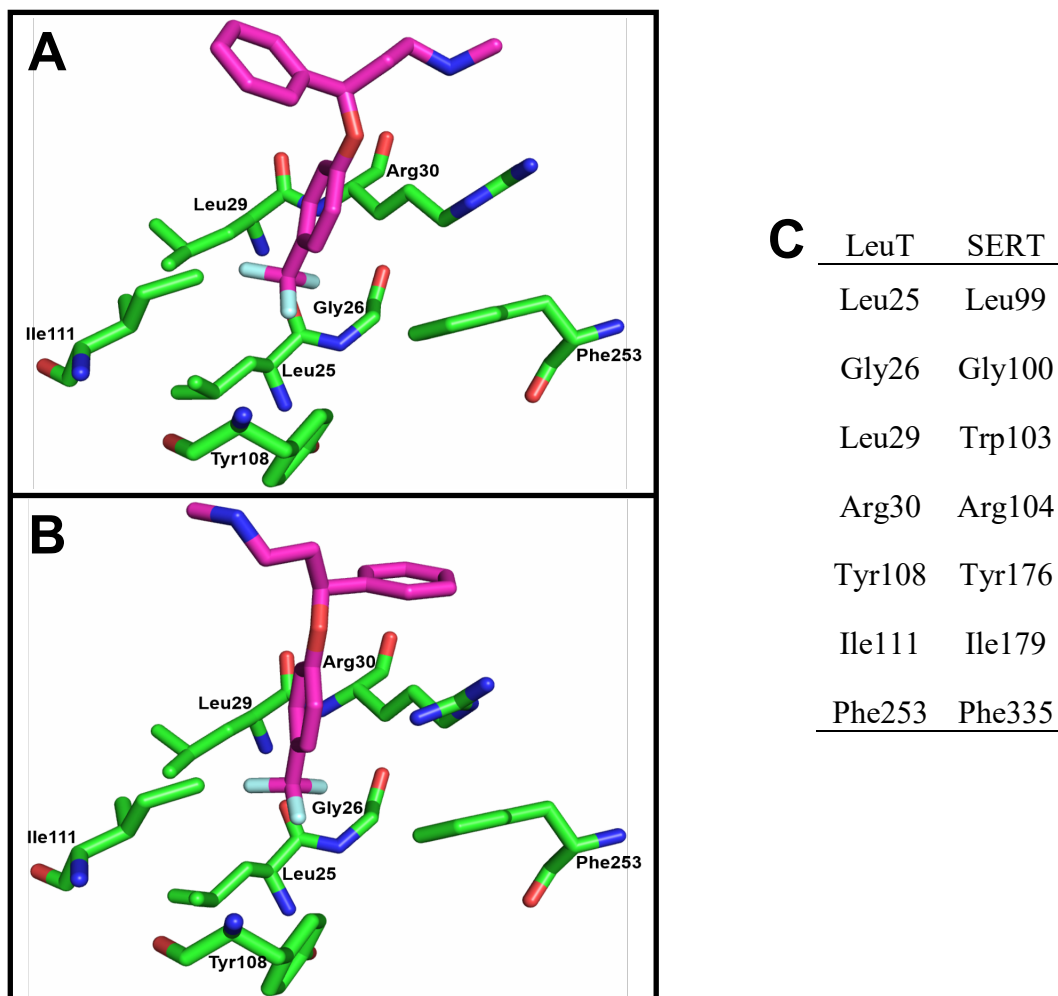


Fig. 1.3. Representation of (A) R-FLX and (B) S-FLX in the halogen-binding pocket (HBP) of LeuT, a bacterial SERT homolog. FLX is shown in pink while the residues from the HBP are shown in green. The residues from the HBP are labeled with 3-letter amino acid code at the C α . Structures were extracted from the Protein Data Bank using the accession codes 3GWV and 3GWW. Molecular graphics were rendered with Pymol (DeLano Scientific). (C) Comparison of the residues in the HBP from LeuT and human SERT. Data were obtained from Zhou et al. (2009).

1.3.2. Maternal transfer of SSRIs in mammals

Studies in rodents have shown that the parent compound and metabolites of the SSRI family drugs readily cross the placenta (Ewing et al., 2015). For instance, FLX plasma levels in newborn rats reach 83% of that of the treated mother (Olivier et al., 2011). The placental transfer of these drugs was also reported in humans. Following prenatal FLX treatment, infant plasma FLX concentration at delivery was found to be 65 to 91% of the concentration measured in the mother's plasma (Heikkinen et al., 2003; Kim et al., 2006). The *in utero* SSRI exposure levels were reported to be strongly correlated with the mother's FLX plasma availability (Kim et al., 2006). Through the alteration of 5-HT signaling, members of the SSRI family have the potential to disrupt the development of many 5-HT-dependent processes including the programming of the HPA axis in the exposed fetus. As a result, prenatal exposure to SSRI may exert long-lasting neurobiological and behavioral consequences on the offspring.

Evidence on the HPA axis programming by SSRI exposure was reported in both human and animal models. Neurodevelopmental exposure to FLX for 28 days, reduced basal circulating glucocorticoid (GC) levels (corticosterone for rodents) in adolescent rats. Interestingly, the long-term consequences on the stress axis were only observed in males (Pawluski et al., 2012), suggesting a sex difference in SSRI sensitivity. Adverse effects of SSRI treatment on the stress axis are also observed in humans. Prenatal SSRIs exposure, more specifically FLX, paroxetine, sertraline and citalopram, reduced the early evening basal cortisol levels in 3-month-old children (Oberlander et al., 2008). Since the HPA axis is the major regulator of homeostasis in the body (Honour, 1994), long-lasting alterations of the HPA axis may potentially affect any endocrine, metabolic, and behavioral processes associated with the restoration of homeostasis.

1.4. The stress axis

Stress is generally defined as any real or perceived, intrinsic or extrinsic stimuli (i.e. the stressor) that disrupt the homeostasis or internal balance of the body (Amir-Zilberstein et al., 2012; Stephens and Wand, 2012). Maintenance of homeostasis in the presence of any stressor triggers the activation of a complex range of responses known collectively as the stress response (Amir-Zilberstein et al., 2012), which is divided in three phases, primary, secondary (Barton, 2002) and tertiary (Wendelaar Bonga, 1997). The primary phase includes the immediate sympathetic-adrenal-medullary system activation that leads to the release of catecholamines (CAs, epinephrine and norepinephrine) (Mostl and Palme, 2002). A concomitant physiological primary stress response is the neuroendocrine activation of the hypothalamic-pituitary-adrenal (HPA) axis in mammals, birds and reptiles and due to convergent evolution, the HP-interrenal (I) axis in fish and amphibians (Dores and Garcia, 2015). The activation of the HPA/I axis results in the secretion of glucocorticoids (GCs) into the bloodstream (Harris and Carr, 2016). Cortisol is the primary GC in humans, fish and many other vertebrates, while corticosterone is the predominant GC in most rodents, birds, amphibians and reptiles (Ramot et al., 2017; Tsachaki et al., 2017).

The secondary phase refers to the immediate changes to tissue and blood constituents as a result of the release of CAs and GCs. They are mainly associated with the physiological adjustments in metabolism, ion balance as well as cardiovascular, immune and respiratory responses. The main function of this secondary response is to re-allocate energy within and between the tissues of an organism to meet the added demands imposed by the induced stress. The process of activating all of these biologically adaptive responses that ultimately result in the maintenance of homeostasis is known as allostasis (McEwen, 2005). However, when the allostatic systems are hyperactivated or fail to adequately respond to a stressor, it could be harmful for the

body. This cost of adaptation that may ultimately result in pathophysiology, and in extreme cases death, is defined as the allostatic load (i.e. the aftermath from being ‘stressed out’). The changes in performance that occur as a result of the allostatic load are components of the tertiary phase. These include alterations in reproductive capacity, behaviors, swimming performance (for fish), growth and disease resistance, among others.

To better understand and correlate the potential negative effects of the allostatic load with their inducers, the stress response is generally assessed using changes in either GC or CA levels. The short half-life of circulating CAs (< 10 min) during a stressful event makes CAs a poor marker of the stress response (Wendelaar Bonga, 1997). Alternatively, the constant release of GCs under resting conditions and their relatively long-lasting half-life in blood plasma upon activation by a stressor (~66 min) (Jung et al., 2014), renders GCs a better indicator of the magnitude of the stress response in vertebrates (Barton et al., 1980; Djordjevic et al., 2003; Fulkerson and Jamieson, 1982; Jentoft et al., 2005; Smyth et al., 1998).

1.4.1. Primary endocrine modulation of the HPA/I axis

The onset of a stress response starts with the perception of the stressor by multiple neural circuits (Harris and Carr, 2016), whose major outputs feed into the hypophysiotropic neurons in the paraventricular nucleus (PVN) of the hypothalamus in mammals (Dinan, 1996) or the preoptic area (POA) (Doyon et al., 2005; Lowry and Moore, 2006) and basal hypothalamus of teleost fish (Winberg et al., 1997). The key hypophysiotropic neurons responding to a stressor are the corticotropin-releasing factor (CRF) neurons, capable of *de novo* synthesis and release of CRF, a 41-amino acid neuropeptide (Harris and Carr, 2016; Vale et al., 1981). In mammals, hypothalamic CRF is released from the nerve endings of the CRF-containing neurons into the capillary beds at

the median eminence, where it is carried through the portal blood into the capillaries of the anterior pituitary (referred as the hypothalamic-hypophyseal portal system). Teleosts lack this portal system, instead CRF is directly released into the anterior pituitary (Dores and Garcia, 2015). The activation of CRF receptors (CRFRs) located on the corticotropes of the anterior pituitary gland, results in the production and release of the adrenocorticotrophic hormone (ACTH) into the peripheral blood stream. The CRF receptors belong to the class B subtype of G protein-coupled receptors (GPCRs), which are primarily linked to the activation of the G_s-adenylate cyclase signaling system (Bale and Vale, 2004). Two distinct CRFRs have been characterized in vertebrates, CRFR type 1 (CRFR1) and CRFR type 2 (CRFR2) (Alderman and Bernier, 2007; Alderman and Bernier, 2009; Lowry and Moore, 2006). A third CRFR (CRFR3) was also identified but only in a diploid catfish species, *Ameiurus nebulosus*. However, CRFR1 is the primary receptor that mediates the HPA/I axis activation (Bale et al., 2002). The CRF signal is further modulated by a soluble 37-kDa N-linked glycoprotein, the CRF-binding protein (CRF-BP). The binding of CRF to a CRF-BP forms a dimer complex, thereby reducing the levels of bioactive CRF (Bale and Vale, 2004). The CRF-BP is highly expressed in the pituitary, though it is also found co-localized with CRF in other CRF-active areas (Huising et al., 2007; Smith et al., 2016).

Adrenocorticotrophic hormone is released upon CRF stimulation, and is a bioactive peptide derived from the proteolytic cleavage of the prohormone pro-opiomelanocortin (POMC) by prohormone convertases 1 and 2 (Costa et al., 2004; Takahashi and Mizusawa, 2013). Once released into the circulation, ACTH binds to the melanocortin type 2 receptor (MC2R), an ACTH-specific receptor predominately expressed on the plasma membrane of interrenal cells in fish or adrenocortical cells in mammals (Dores and Garcia, 2015; Kilianova et al., 2006). The MC2R is a GPCR that stimulates the cyclic adenosine monophosphate (cAMP) signaling cascade through the

activation of a G_s-protein (Malik et al., 2015). The complete functionality and activation of MC2R require the presence of a melanocortin-2 receptor accessory protein (MRAP) (Dores and Liang, 2014; Malik et al., 2015). Although, two distinct MRAPs, MRAP1 and MPRA2, have been identified in teleosts and tetrapods, MRAP1 was found to be the most efficient at inducing the activation of MC2R. The ACTH-MC2R-MRAP interaction ultimately leads to the synthesis and secretion of the GCs, cortisol and corticosterone, into the bloodstream. A schematic representation of the primary endocrine modulation of the HPI axis in teleosts is illustrated in Fig. 1.4.

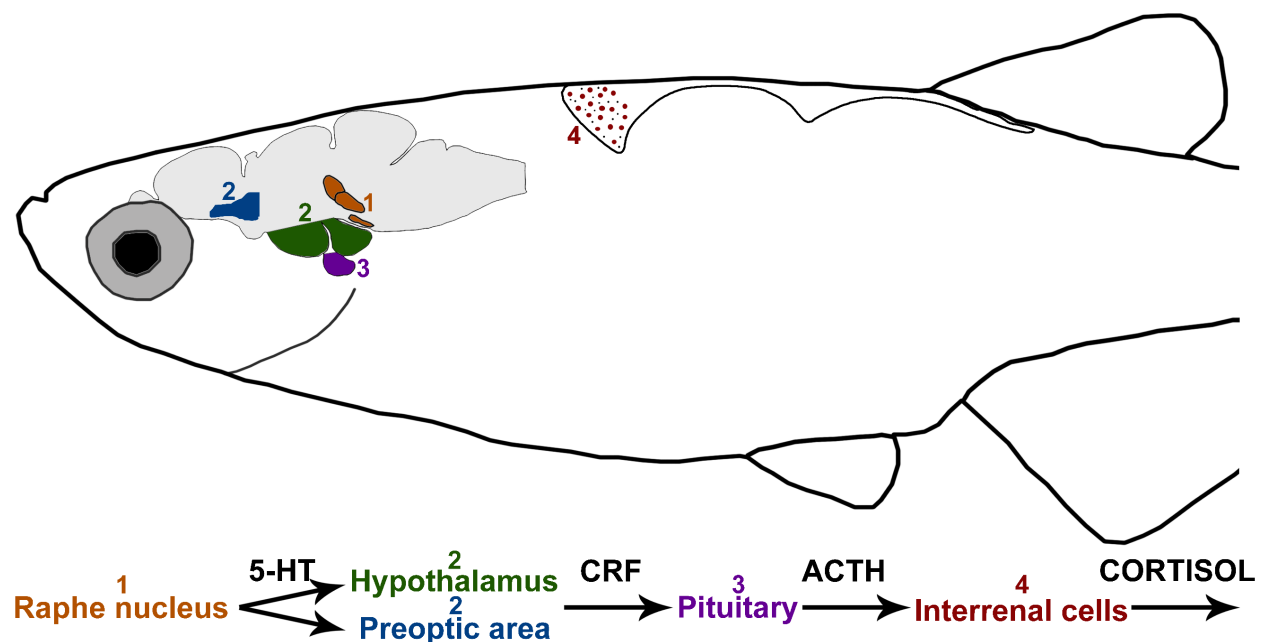


Fig. 1.4. Overview of the primary endocrine modulation of the teleost HPI axis. The perception of a stressor induces 5-HT synthesis from the serotonergic neurons in the raphe nucleus (1). 5-HT is subsequently released into the hypothalamus and POA (2) to trigger the *de novo* synthesis of CRF. In teleosts, CRF-containing neurons directly innervate the anterior pituitary where CRF is released. The activation of CRFR1 following CRF binding, stimulates the synthesis and release of ACTH from the corticotropes of the anterior pituitary (3) into the circulation. Following ACTH binding to MC2R, its specific receptor located on the interrenal cells (4), cortisol is synthesized and released into the bloodstream.

1.4.2. Glucocorticoid synthesis

The synthesis of GCs (Fig. 1.5) by steroidogenic cells of the adrenal gland in mammals or interrenal cells in teleosts is a dynamic process catalyzed by a series of enzymes where cholesterol is the primary substrate. This process is activated upon the binding of ACTH to its specific receptor, MC2R, with the assistance of MRAP (Turcu and Auchus, 2015). Through cAMP/protein kinase A (PKA) signaling, ACTH stimulates cholesterol acquisition from external sources and/or through *de novo* synthesis (Shen et al., 2016). The first and rate-limiting enzyme reaction of GC biosynthesis takes place in the inner membrane of the mitochondria (IMM), where cholesterol is catalyzed to pregnenolone by the P450 side-chain cleavage enzyme (CYP11A or P450_{scc}) in mammals. Studies in ZF identified two CYP11A enzymes sharing 80% identity at the amino acid level, CYP11A1 and CYP11A2 (Goldstone et al., 2010). For many years, evidence suggested that ZF CYP11A1 was the paralog involved in steroidogenesis (Hu et al., 2004; To et al., 2007); however, it was recently reported that ZF CYP11A1 is only required for gastrulation, whereas the ZF CYP11A2 expression is induced following the development of the interrenal primordium of the larvae. The paralog ZF CYP11A2 is now considered the functional equivalent of the mammalian CYP11A1 (Parajes et al., 2013).

The trafficking of cholesterol from the outer mitochondrial membrane (OMM) into the IMM for its catalysis into pregnenolone is carried out by the Steroidogenic Acute Regulatory (StAR) protein. Pregnenolone is subsequently exported to the endoplasmic reticulum where it is metabolized by 17 α -hydroxylase (CYP17 or 17,20 lyase) and 3 β -hydroxysteroid dehydrogenase (3 β -HSD) into 17-hydroxy progesterone (17-OH progesterone) (Payne and Hales, 2004). In humans and other mammals, the enzyme involved in catalyzing the 17 α -hydroxylation of pregnenolone and progesterone is CYP17A1 (Miller, 2017). However, in teleosts, two isozymes

of CYP17 are present, CYP17A1 and CYP17A2. The teleost CYP17A1 isozyme shares 49% amino acids identity with its homolog CYP17A2. Both forms were found to efficiently catalyze the 17 α -hydroxylation reaction in the *de novo* synthesis of GCs (Pallan et al., 2015). The second enzyme involved in this multiple step reaction, 3 β -HSD, was identified as two forms in humans, 3 β -HSD type 1 and 3 β -HSD type 2. They both share similar enzymology but differ in their tissue distribution. Type 2 (3 β -HSD2) is highly expressed in the adrenal gland, hence it is the isozyme involved in GC biosynthesis in humans (Miller, 2013). The same isoforms were also characterized in ZF, but in contrast to humans, they differ in their physiological functions with 3 β -HSD1 (or 3 β -HSD) being the isoform involved in the steroidogenesis pathway of ZF (Lin et al., 2015).

Following the series of reactions that yield 17-OH progesterone, 21-hydroxylase (CYP21A2) converts this intermediate to 11-deoxycortisol. The mammalian CYP21A2 and the teleost CYP21A2 were found to be functional homologs (Midzak and Papadopoulos, 2016; Wilson et al., 2013). Lastly, 11-deoxycortisol is mobilized to the IMM, where it is catalyzed by 11 β -hydroxylase (CYP11B1) to the final product, cortisol. In some vertebrates including rodents, steroidogenic cells lack the CYP17 lyase, resulting in different hydroxylation reactions that produces corticosterone rather than cortisol as their dominant GC (Turcu and Auchus, 2015).

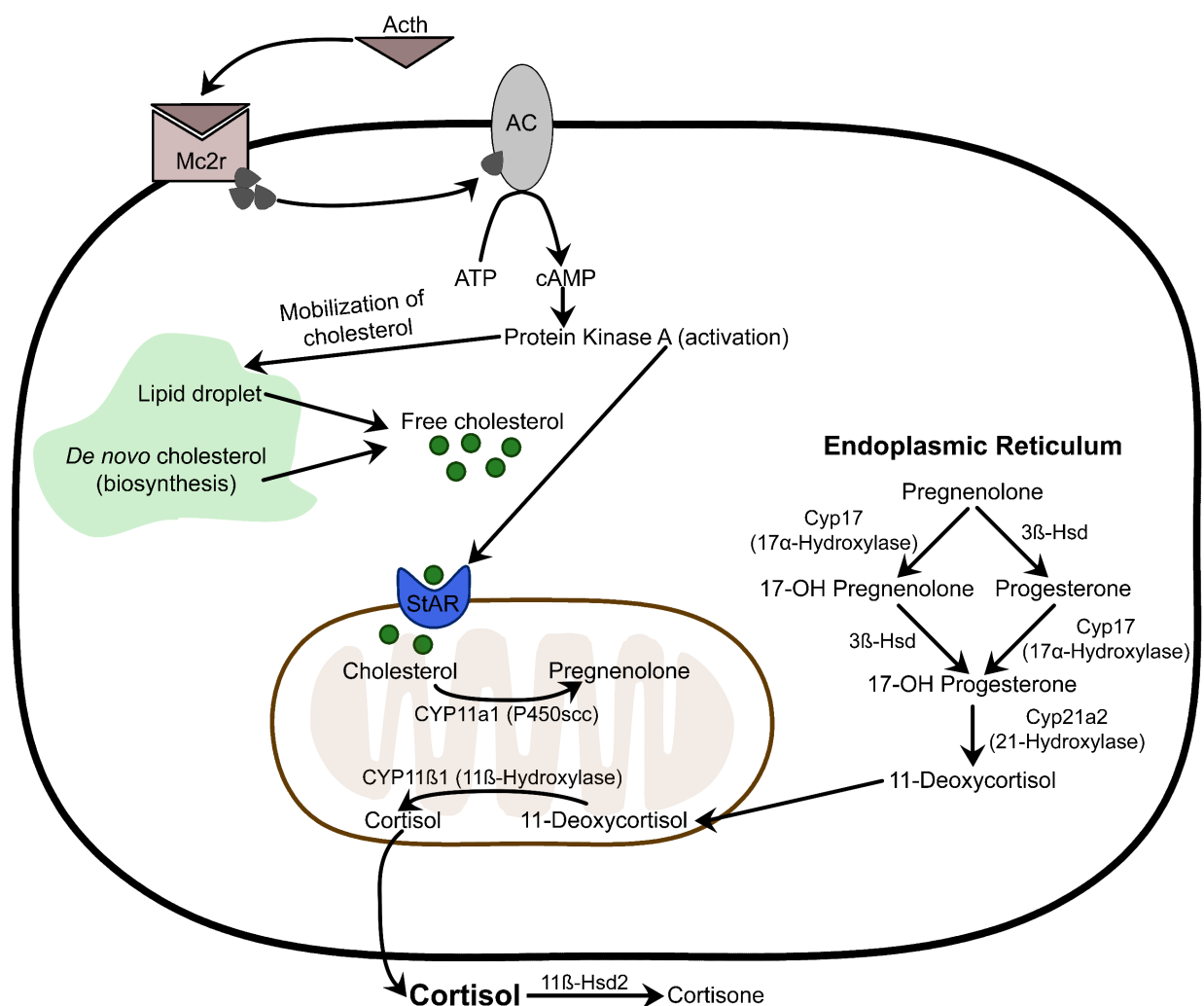


Fig. 1.5. A schematic representation of cortisol synthesis in the interrenal cell of ZF. The cellular mechanisms that underlie steroidogenesis are triggered by the binding of ACTH to its specific receptor, MC2R. This is followed by the activation of second messengers, which through the cAMP/PKA signaling cascade stimulate three different processes: 1) Acquisition of free cholesterol either by *de novo* synthesis or by cellular import. 2) Stimulation of a very rapid transcription of StAR as well as its phosphorylation results in the mobilization of free cholesterol from the OMM into the IMM. 3) Activation of steroidogenic enzymes leading to the synthesis and secretion of cortisol. Cortisol can be cell-dependent deactivated by the enzyme 11β-HSD2 into cortisone.

1.4.3. Glucocorticoid transport and mechanism of action

Once released into the circulation, free cortisol and corticosterone bind to the glucocorticoid receptor (GR) to exert their actions of mediating the tissue-level stress response (Oakley and Cidlowski, 2013). Prior to activation by ligand, the GR resides in the cytoplasm surrounded by a large multi-complex structure of chaperons and immunophilins, the function of which is to increase ligand-binding affinity and to prevent any other GR activity (Weikum et al., 2017). Activation of the GR releases the bound chaperones leading to the translocation of the GR-ligand complex into the nucleus. Once in the nucleus, they interact as homodimers with specific glucocorticoid responsive elements (GREs) within promoter regions to stimulate or inhibit the transcription of many GC-responsive genes. Two main mechanisms by which GR binds to GREs are described (Oakley and Cidlowski, 2013; Tiwari et al., 2017). The first involves cooperative binding where binding of the first GR monomer expedites the sequential binding of the second monomer. Thus, in this model homodimerization of GR occurs in the nucleus. The second scenario relies on the binding of the preformed homodimer of GR to the GRE, and homodimerization may occur either in the cytoplasm or nucleus. Research continues to better understand the significance of the different locations for GR dimerization and whether this may impact the regulation of the target genes (Oakley and Cidlowski, 2013; Tiwari et al., 2017). Two GR isoforms are reported in humans and ZF, GR alpha-isoform (GR-alpha) and the GR beta-isoform (GR-beta) (Chatzopoulou et al., 2015; Oakley and Cidlowski, 2013). Despite the presence of these two variants, only GR-alpha is the canonical receptor that functions as a ligand-dependent transcription factor in both humans and ZF. Due to its small ligand-binding domain (LBD), GCs do not bind to the GR-beta but instead it exerts a dominant negative effect on the GR-alpha transcriptional activity (Chatzopoulou et al., 2017). The human GR-alpha shares 59.3% amino acids sequence similarity

to the ZF GR- α , and their DNA-binding domain and LBD sequence are highly conserved (Schaaf et al., 2009).

1.4.4. Local and negative feedback regulation of glucocorticoid synthesis

The activity of GCs in mammals and teleosts can be locally (cell-specific) regulated through the enzyme 11 β -hydroxysteroid dehydrogenase 2 (11 β -HSD2), which oxidizes the active 11 β -hydroxyl GC form, cortisol and corticosterone, into its inactive 11-keto form, cortisone and 11-dehydrocorticosterone, respectively (Wilson et al., 2013). Another important local regulatory mechanism observed in mammals is the ability of the target cell to re-activate the 11-keto form (e.g., cortisone) back to its active form via reduction with the enzyme 11 β -hydroxysteroid dehydrogenase 1 (11 β -HSD1). In contrast, fish lack a homologue of this enzyme, as a result, cortisone cannot be recycled in ZF (Baker, 2010; Miller and Auchus, 2011; Wilson et al., 2013).

The plasma availability of GCs to protect the organism from prolonged elevated GC levels is further regulated by a negative feedback mechanism at the HPA/I axis. This negative feedback is exerted by cortisol and corticosterone at the hypophysiotropic neurons and corticotropes to down-regulate the synthesis and secretion of CRF and ACTH in teleosts and tetrapods. This negative feedback is primarily mediated by the binding of GCs to GR (Dores and Garcia, 2015; Herman et al., 2012).

1.4.5. Serotonergic modulation of HPA/I axis programming

There is significant evidence to support the hypothesis that fetal life events permanently affect the structure and/or function of different biological processes in an individual (Antonelli, 2014; Reissland and Kisilevsky, 2016). During brain development, the stress axis is highly

susceptible to any physiological alterations induced either by internal or external stimuli, leading to permanent modifications of the HPA axis response (Matthews, 2002). These long-term modifications, known as the programming of the HPA axis, involve epigenetic changes to DNA and histones of genes associated with the activation of the stress axis.

An *in vitro* assay using hippocampal neurons revealed the direct involvement of 5-HT signaling on the up-regulation of GR expression, consequently, affecting the stress response (Mitchell et al., 1990b). These functional interactions and mechanisms were later validated and further developed by a series of elegant studies that examined the effects of maternal care of rats on the stress response of the adult offspring (Meaney et al., 1994; 2000; 2007; Mitchell et al., 1992; Smythe et al., 1994; Weaver et al., 2004; 2005; 2007; 2014). These studies attempted to unravel the underlying mechanism(s) of the different GR expression patterns observed in the pups that were highly groomed/licked over the first week of lactation compared to the low groomed/licked pups. It is important to note that the first postnatal week of rats is equivalent to the 3rd trimester in humans in terms of the neurodevelopment of the brain (Clancy et al., 2007). Concomitantly with the high hippocampal 5-HT and GR levels, the highly groomed pups also displayed a decrease in CRF levels in the PVN in addition to low corticosterone levels in response to a stressor as adults. These effects were not detected in the rats that were reared with low grooming (Caldji et al., 1998; Francis et al., 1999; Liu et al., 1997).

The alteration of the HPA axis responsivity in the adult rat offspring induced by the variation in the amount of postnatal maternal care was attributed to an epigenetic programming of GR expression in the hippocampus (Weaver et al., 2014). Hippocampal GR regulates CRF expression via a negative feedback mechanism operated by GC binding (Tasker and Herman, 2011; Zhang et al., 2013). The main target of this epigenetic mechanism controlled by 5-HT is the

nerve growth factor-inducible protein A (NGFI-A) binding site located on the exon 1₇ (1_F in humans) promoter of the rat *NR3C1*, the gene that encodes GR (Weaver et al., 2014). The *NR3C1* gene is highly conserved between humans, rodents as well as ZF (Schaaf et al., 2009; Turner et al., 2006).

High maternal care during the first week of lactation (also brain developmental period on rodents) stimulates the release of 5-HT from the raphe nucleus, which through the subsequent binding to the GPCR, 5-HT₇, triggers the cAMP signaling cascade. Upon the activation of PKA, the cAMP response element binding protein (CREB) is phosphorylated (phospho-CREB; (p)CREB) and through its transcription factor action, induces the expression of NGFI-A. High levels of NGFI-A are associated with an increase in CREB binding protein (CBP) levels, which was found to be a histone acetyl transferase. The NGFI-A transcriptional factor recruits CBP and the methyl-cytosine-phosphodiester-guanine binding domain 2b (MBD2b) protein to the exon 1₇ of the GR promoter. At this site, CBP increases acetylation on histone H3 lysine 9 which is associated with transcriptionally active chromatin. This process is followed by the MBD2b induced DNA demethylation of a 5'-cytosine-phosphodiester-guanine (CpG) dinucleotides located on the NGFI-A binding site. The remodeling of chromatin and DNA demethylation facilitates the steady binding of the transcriptional factor NGFI-A to the exon 1₇ GR promoter. This interaction initiates GR transcription in the hippocampus (Buschdorf and Meaney, 2015; Weaver et al., 2005; Weaver et al., 2007; Weaver et al., 2014). High levels of hippocampal GR reduce the HPA axis tone to a stressor. The activation of GR in the hippocampus via a descending pathway on hypothalamic neurons is associated with an indirect negative feedback mechanism of the stress axis, thereby decreasing CRF levels, eventually leading to the down-regulation of GCs (Denver, 2009; Makino et al., 2002). The demethylation pattern of exon 1₇ GR promoter from the offspring

with high mother-interaction, was found to persist to adulthood (Francis et al., 1999; Liu et al., 1997). These studies indicate that 5-HT can permanently influence and modulate the epigenetic state of GR, thereby altering the stress response of the organism (Fig. 1.6).

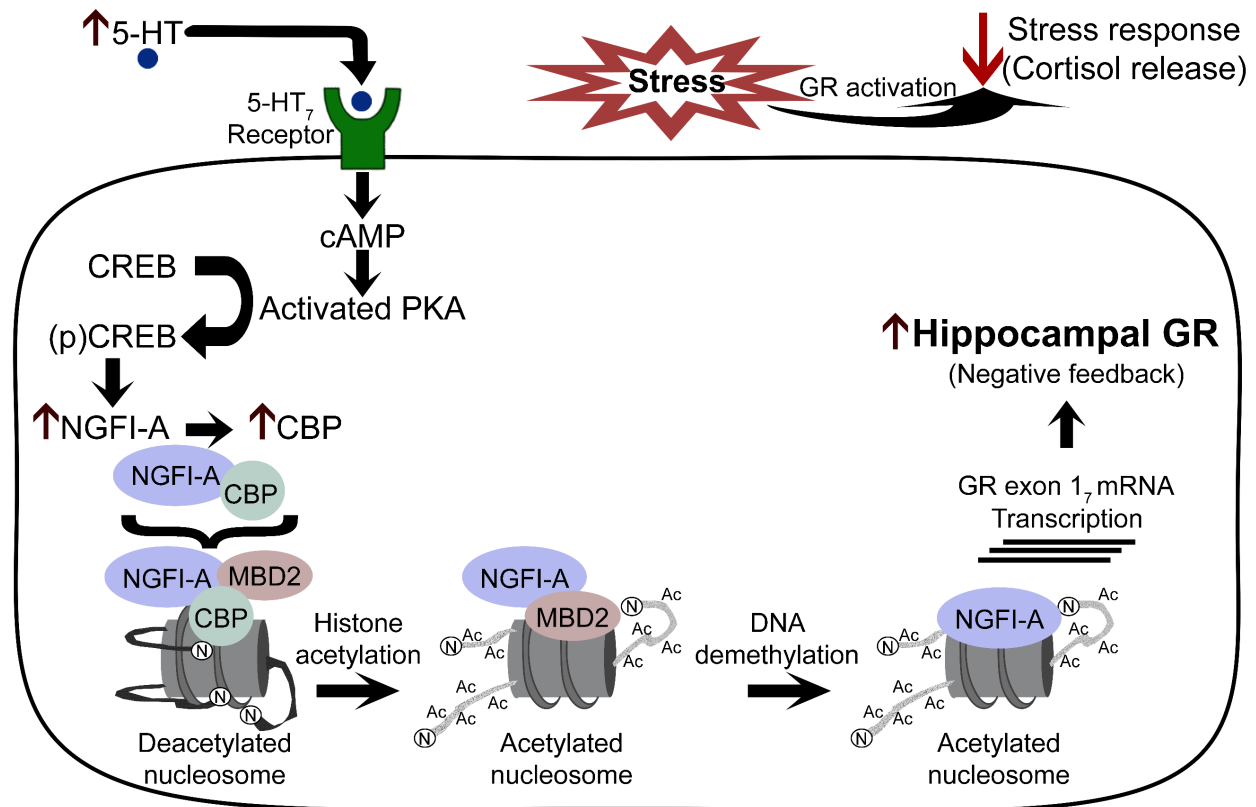


Fig. 1.6. The epigenetic mechanism involved in the programming of the HPA axis in rats through 5-HT signaling. The binding of 5-HT to its receptor 5-HT₇, induces the cAMP signaling cascade, which leads to an increase in the transcriptional factor NGFI-A and the histone acetylase, CBP. The recruitment of CBP and MBD2 by NGFI-A to the exon 17 GR promoter triggers its acetylation and DNA demethylation. The remodeling of chromatin and DNA demethylation facilitates the interaction of NGFI-A to the exon 17 GR promoter, ultimately inducing GR transcription. The increase of hippocampal GR enhances the negative feedback sensitivity, consequently, decreasing the HPA axis response.

1.5. Pharmaceuticals in the environment

The use of pharmaceuticals in recent decades attributed to a variety of factors including population growth, affordability, population ageing (in some countries), advances in pharmacological science and easier access to medication, is increasing over the years. This has become a topic of concern with regards to the environment (Boxall et al., 2012; Corcoran et al., 2010; Jelic et al., 2011; Mennigen et al., 2011; Oakes et al., 2010). As a result of their high demand, pharmaceuticals are constantly developed, and the ever-increasing rates of prescriptions have led to an escalation in environmental discharges of these compounds. The main routes of entry of these pharmaceuticals into the environment are via wastewater associated with domestic, manufacturers and hospital discharges following human excretion or improper disposal (Comber et al., 2018; Kasprzyk-Hordern et al., 2008). Pharmaceuticals not completely degraded in the sewage treatment plants are discharged in treated effluents, eventually reaching surface waters, consequently resulting in contamination of the aquatic environment (Bound and Voulvoulis, 2005; Fent et al., 2006). The active ingredients and metabolites of numerous pharmaceuticals are detected throughout the environment including sewage, surface waters, soil, sediments and sludge. Traces of active ingredients are also detected in drinking water in some countries (Brooks et al., 2009; Kasprzyk-Hordern et al., 2008; Lees et al., 2016; Zorita et al., 2009). The groups of pharmaceuticals with worldwide detection rates include non-steroidal anti-inflammatory drugs, analgesics, beta blockers, antibiotics, antiepileptic drugs, natural hormones, contraceptives, psychiatric drugs, anti-hypertensive drugs, chemotherapy drugs and blood lipid/cholesterol regulators (Comber et al., 2018; Corcoran et al., 2010; Yang et al., 2017b).

Pharmaceuticals are chemicals primarily designed to prevent and treat human and animal diseases by altering physiological functions through the regulation of specific biological targets,

such as receptors and enzymes (Corcoran et al., 2010; Jelic et al., 2011). Many of the targets and mechanisms by which certain pharmaceuticals exert their actions are moderately to highly conserved across vertebrates (Gunnarsson et al., 2008; Tanoue et al., 2015). As a result, some pharmaceuticals are potentially hazardous compounds considering that they are biologically active, ubiquitous and pseudo-persistent due to their continuous release into the environment. Thus, many aquatic organisms are chronically exposed to the active ingredients of many different drugs, consequently triggering unintended effects that ultimately may lead to endocrine disruptive functions (Boxall et al., 2012; Mennigen et al., 2011; Niemuth et al., 2015; Tyler et al., 1998; van der Ven et al., 2006). Another factor that influences the uptake of these compounds and their bioaccumulation in non-target species is their physicochemical profile, including hydrophobicity and ionic behaviors (Chen et al., 2017).

Several studies have reported a range of ecotoxicological effects of commonly detected pharmaceuticals, in addition with their occurrences in various water sources. One of the major pharmaceutical families detected in the aquatic environment throughout the world is the antidepressants. In the U.S.A. alone, antidepressant consumption increased 400% from 1988 to 2008 according to the National Center for Health Statistics (Arnnok et al., 2017). A study by Metcalfe et al. (2010) reported a total concentration of $3.2 \mu\text{g}\cdot\text{L}^{-1}$ of different measurable antidepressants and their respected metabolites detected at the site of wastewater discharge in the Grand River in southern Ontario, Canada in the summer of 2007. Fluoxetine is the most frequently detected antidepressant in many aquatic environments, with measured concentrations up to $929 \text{ ng}\cdot\text{L}^{-1}$ (Bueno et al., 2007; Comber et al., 2018; Fong and Ford, 2014; Kolpin et al., 2002; Metcalfe et al., 2003a; Metcalfe et al., 2003b). The significant presence of FLX in diverse aquatic ecosystems, its high prescription rates (Mesquita et al., 2011) and its high acute toxicity to non-

target organisms (Fent et al., 2006) render FLX one of the most studied drugs worldwide (Boseley, 2008).

Despite the fact that FLX is exclusively designed to treat psychotic disorders in humans, its main target, SERT, is highly conserved across vertebrates including fish (Mennigen et al., 2011). Therefore, persistent release of this drug may pose a threat leading to endocrine disruptions in these non-target aquatic organisms.

1.6. Thesis outline

Given the strong evidence that over-stimulation of 5-HT during early-life induces epigenetic modifications on the stress axis (Caldji et al., 1998; Francis et al., 1999; Weaver et al., 2014), our primary goal during my Ph.D. project was to investigate if embryonic exposure to SSRI treatment during a critical neurodevelopmental window, had the potential to alter the lifespan stress response of the offspring. We also examined if these phenotypic changes were transmitted to future generations and their possible implications on the fitness of the affected organism. Therefore, using ZF as a model, I investigated the effects of early-life exposure to FLX on the stress axis. The results obtained from the series of experiments gathered throughout this doctoral research are described in the following chapters:

Chapter 2: Early-life exposure to fluoxetine induces transgenerational disruption of the stress axis and impairs exploratory behavior in adult male zebrafish

Chapter 3: Physiological disruptions and transgenerational alterations in the expression of stress-related genes in zebrafish larvae following exposure to fluoxetine

Chapter 4: Fluoxetine exposure during sexual development results in sex- and time-specific effects on the exploratory behavior of adult zebrafish

Chapter 5: Developmental and reproductive outcomes of zebrafish following an early-life exposure to fluoxetine

Chapter 6: Venlafaxine exposure in F₄ embryos intensifies the reduced cortisol phenotype in the adult females and altered sex steroids levels in adult zebrafish

The thesis is organized as series of distinct chapters that will form the basis for independent publications. Necessarily, these chapters have slightly different formats, and there is a level of redundancy in their introductions.

CHAPTER 2: Early-life exposure to fluoxetine induces transgenerational disruption of the stress axis and impairs exploratory behavior in adult male zebrafish

2.1. Statement of contribution

Marilyn Vera Chang, designed the study, developed the methodology, conducted the majority of the experiments and analyses and prepared the manuscript. Rémi Gagné (Environment Health Centre of Health Canada), conducted the RNA Sequencing and all the analyses in between until obtaining the two-separate generalized linear models of the differentially expressed genes and helped with the manuscript revision. Antony St-Jacques (University of Ottawa), wrote the automated tracking script and the other scripts to compute the behavioral metrics. Dr. Christopher Martyniuk (University of Florida), helped with bioinformatics analysis and manuscript editing. Dr. Carole Yauk (Environment Health Centre of Health Canada), helped with the RNA Sequencing experimental design and manuscript editing. Dr. Thomas Moon, helped with the design of the study and manuscript editing. Dr. Vance Trudeau, helped with the design of the study and manuscript editing.

2.2. Introduction

Pregnancy and the postpartum period are accompanied by an increase in vulnerability to depression and anxiety (Meltzer-Brody et al., 2018). Psychiatric disorders such as these during pregnancy are associated with preterm delivery and numerous adverse neonatal outcomes, including impairments in cognitive and physical abilities and increased risk to develop neuropsychiatric disorders (Huizink et al., 2004; 2003; Susser et al., 2016; Van den Bergh et al., 2008). A variety of pharmacological agents exist today as treatment for these affective disorders. The selective serotonin (5-hydroxytryptamine; 5-HT) reuptake inhibitor (SSRI) family of

antidepressants, especially fluoxetine (FLX), the active ingredient in the well-known drugs such as Prozac®, is generally the first-line of pharmacological treatment of such disorders for pregnant women in several countries (Latendresse et al., 2017; Man et al., 2017). The SSRIs exert their therapeutic actions by enhancing serotonergic neurotransmission through inhibition of 5-HT reuptake transporters on pre-synaptic neurons (Wong et al., 1995).

Given that SSRIs are transferred from the treated mother to the fetus through the placenta, concerns have been raised regarding the neurobehavioral outcomes of prenatal SSRI exposure during developmentally-sensitive periods (Ewing et al., 2015; Kaihola et al., 2016; Loughhead et al., 2006; Rampono et al., 2009). Critically, during brain development, 5-HT acts as a neurotrophic factor regulating neuronal proliferation, differentiation, migration, and synaptogenesis (Badenhorst et al., 2017; Kroeze et al., 2016) in addition to its prominent role in the programming of the stress axis, also known as the hypothalamic-pituitary-adrenal (HPA) axis in mammals (Andrews and Matthews, 2004; Oberlander, 2012), which is highly plastic during development (Heim and Binder, 2012; Loman et al., 2010). Furthermore, dysregulation in the physiological response of the HPA axis (changes in cortisol levels) in children and in adolescent rodents follows prenatal exposure to SSRI medications via maternal treatment (Oberlander et al., 2002; 2008; 2005; Pawluski et al., 2012). Even though evidence exists for SSRI-induced disruption of the HPA axis following prenatal exposure, there remains little focus on the long-term consequences manifested in adulthood or even in future generations of these early exposures.

Here, we report on the transgenerational disruption of the stress response and of behaviors following early developmental FLX exposure to concentrations within the lower range detected in the cord blood of FLX-treated pregnant women ($54 \mu\text{g}\cdot\text{L}^{-1}$) (Hendrick et al., 2003; Kim et al., 2006; Rampono et al., 2004) and to an environmentally relevant concentration ($0.54 \mu\text{g}\cdot\text{L}^{-1}$)

(Mennigen et al., 2010b). We also provide insights on the potential mechanisms underlying the observed effects by means of global transcriptional analysis. We used zebrafish (ZF) *Danio rerio* as an amenable model system to determine the effects of FLX, since embryos develop external to the mother and can be directly exposed to specific concentrations of the studied chemical. Additionally, ZF have high physiological and genetic homologies to humans and the ease of genetic manipulations have rendered ZF a suitable model in stress research (Steenbergen et al., 2011) and other human-related brain disorders (Howe et al., 2013; Kalueff et al., 2014).

This is the first study to demonstrate that a 6-day FLX exposure at a critical developmental stage permanently suppressed cortisol levels in ZF, an effect that persists for 3 generations in the unexposed descendants. We also show that altered cortisol levels are correlated to reduced exploratory behaviors in FLX-exposed animals. Finally, transcriptomic profiling of the whole kidney containing the interrenal cells revealed the transgenerational disruption of key pathways closely associated with cortisol synthesis in the filial generation 0 (F₀; exposed-fish) and the F₃ (three generations following exposure) adult fish. This is a cause for concern given the high-prescription rates of FLX to pregnant women and the potential long-term negative impacts on humans and aquatic organisms exposed to SSRIs.

2.3. Results

2.3.1. Cortisol response to an acute stressor is reduced by FLX

Our first objective was to determine whether early developmental exposure (during the 6 first days of life) to FLX induces disruptive transgenerational effects on the stress axis, also known as the hypothalamic-pituitary-interrenal (HPI) axis in teleosts. We examined whole-body cortisol levels as a stress response indicator in adult female and male ZF in the exposed F₀ generation and

the unexposed F₁ to F₃ following a brief, standardized net handling stressor. A two-way ANOVA was conducted to examine the effects of the stressor and FLX treatments on cortisol levels. In the control (CTR) and in each of the treatment groups (LFL, Low-FLX lineage, 0.54 µg·L⁻¹; and HFL, High-FLX lineage, 54 µg·L⁻¹), the cortisol response to the acute stressor was significantly elevated relative to basal (non-stressed) levels in males from each generation: F₀ ($F_{1,42} = 286.537$, $P < 0.001$), F₁ ($F_{1,48} = 965.475$, $P < 0.001$), F₂ ($F_{1,48} = 95.288$, $P < 0.001$) and F₃ ($F_{1,46} = 358.778$, $P < 0.001$). However, total cortisol levels were significantly reduced in the FLX lineages from F₀ (38 and 57% reduction for LFL and HFL, respectively; $F_{2,42} = 51.618$, $P < 0.001$), F₁ (34 and 37%; $F_{2,48} = 11.392$, $P < 0.001$), F₂ (42 and 56%; $F_{2,48} = 12.596$, $P < 0.001$) and F₃ (35 and 52%; $F_{2,46} = 47.778$, $P < 0.001$) compared to their matched CTR group (Fig. 2.1A). The total cortisol content at both basal levels and following stress significantly varied across the FLX treatments (interactions) within generations F₀ ($F_{2,42} = 5.917$, $P = 0.005$), F₁ ($F_{2,48} = 7.144$, $P = 0.002$) and F₃ ($F_{2,46} = 16.162$, $P < 0.001$). However, these interactions were not significant in the F₂ ($F_{2,48} = 0.003$, $P = 0.997$).

A two-way ANOVA also revealed that the females (Fig. 2.2A) in all treatments from generations F₀ ($F_{1,41} = 64.037$, $P < 0.001$), F₁ ($F_{1,46} = 149.006$, $P < 0.001$), F₂ ($F_{1,45} = 168.748$, $P < 0.001$) and F₃ ($F_{1,48} = 170.433$, $P < 0.001$) also exhibited a cortisol response to the acute stressor. Their total cortisol levels were also significantly decreased in the FLX lineages of generations F₀ (13 – 30% reduction for LFL and HFL, respectively; $F_{2,41} = 4.008$, $P = 0.026$), F₂ (26 and 30%; $F_{2,45} = 10.798$, $P < 0.001$) and F₃ (31 and 37%; $F_{2,48} = 8.755$, $P < 0.001$) relative to their matched CTR groups. Generation F₁ was not affected by FLX ($F_{2,46} = 0.118$, $P = 0.889$). The effects observed on the FLX lineages did not vary with the stressor [no interactions, F₀

($F_{2,41} = 0.933$, $P = 0.402$); F_1 ($F_{2,46} = 2.968$, $P = 0.061$); F_2 ($F_{2,45} = 0.408$, $P = 0.667$); F_3 ($F_{2,48} = 1.974$, $P = 0.150$).

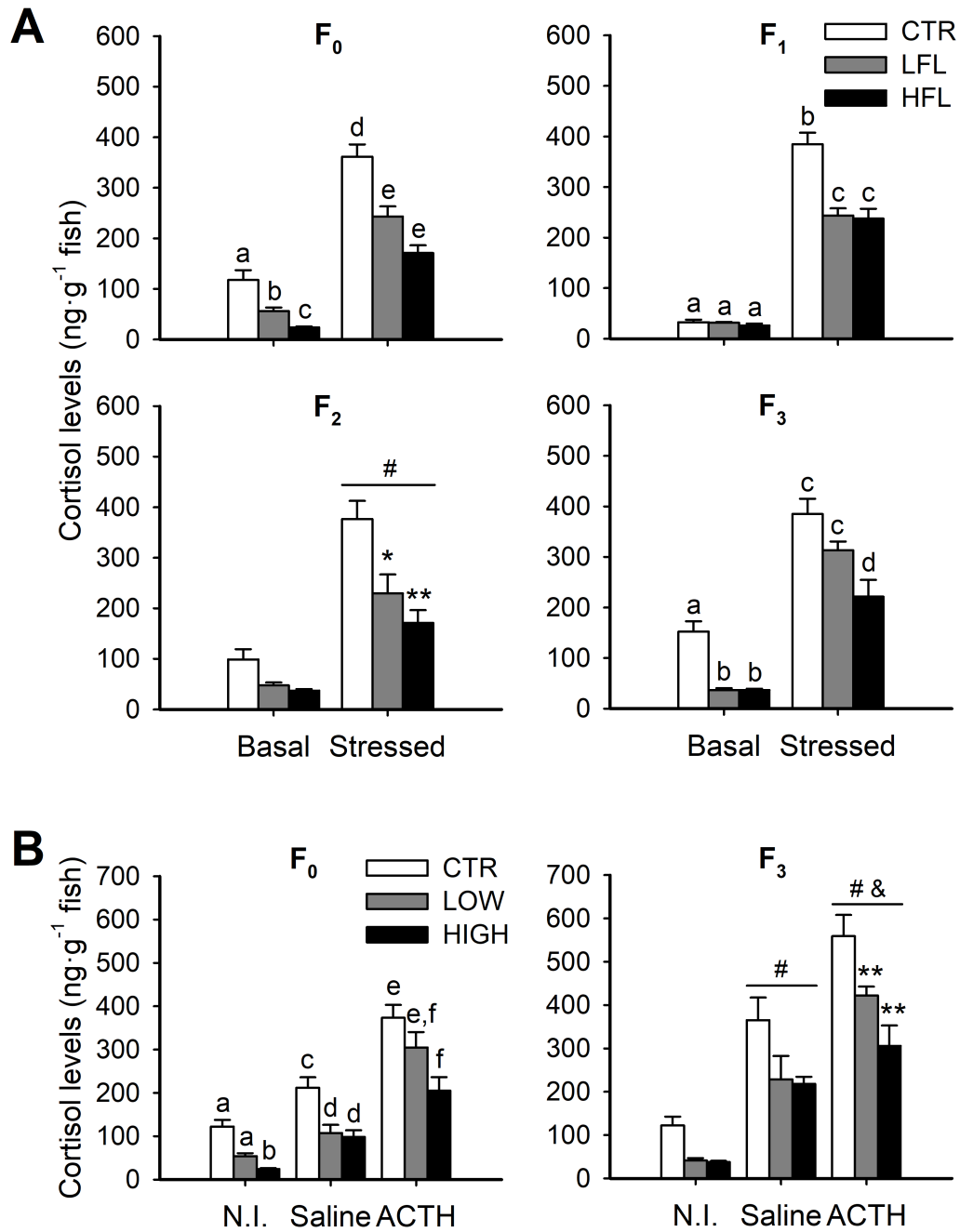


Fig. 2.1. Whole-body cortisol levels ($\text{ng} \cdot \text{g}^{-1}$ fish) in male adult ZF from the CTR and FLX lineages across generations. (A) Early developmental FLX exposure to the F_0 induced a transgenerational disruption of the stress axis manifested by a reduction of the basal and stress-induced total cortisol levels in adult males. The stress response was instigated following a standardized net handling stressor. The F_0 to F_3 denotes the different filial generations. $n = 7 - 10$ per group. (B) Male fish from the FLX lineages in the F_0 and F_3 generations exhibited a blunted cortisol production response following ip ACTH injection ($0.0625 \text{ IU} \cdot \text{g}^{-1}$ fish). $n = 8 - 17$ per group. N.I. = non-injected group. For panel A and B, the data are presented as mean $\pm$ SEM and analyzed by two-way ANOVA (on ranks for A, F_2 ; B, F_3), $P < 0.05$. The letters represent statistical difference when interactions are present. $^{\#}P < 0.001$ compared with the basal group for panel A, and compared to the N.I group for panel B; $^{\&}P < 0.001$ compared with the saline group. The asterisks (*) represent significant difference in the FLX group compared to the CTR: $^*P = 0.004$ and $^{**}P < 0.001$.

2.3.2. Cortisol response to an intraperitoneal (ip) injection of adrenocorticotrophic hormone (ACTH) is reduced by FLX

The decreased basal and net stress-induced cortisol production in FLX-exposed male ZF led us to predict a reduced responsiveness of the steroidogenic interrenal cells (equivalent to the cortical cells in the mammalian adrenal). We therefore administered ACTH to determine its impact on basal and stimulated cortisol production in the F_0 and F_3 generations. To control for handling/injection stress, control conditions included both non-injected and saline-injected groups. A two-way ANOVA was performed to assess the effects of the injection and the FLX concentrations on cortisol levels. The expected increase in total cortisol levels across the injected groups (non-injected, saline and ACTH groups) was observed in both the F_0 ($F_{2,96} = 157.436$, $P < 0.001$) and F_3 ($F_{2,91} = 154.809$, $P < 0.001$). However, the interrenal cells of the males from the FLX lineages had a significantly attenuated response in each injection group compared to the CTR lineage in the F_0 ($F_{2,96} = 28.441$, $P < 0.001$) and F_3 ($F_{2,91} = 22.345$, $P < 0.001$) generations (Fig. 2.1B). In the F_0 , the response of the interrenal cells to the treatments on the synthesis of cortisol

significantly varied across the FLX lineages (interaction, $F_{4,96} = 3.121$, $P = 0.018$). However, these interactions were not observed in the F_3 ($F_{4,91} = 1.493$, $P = 0.211$).

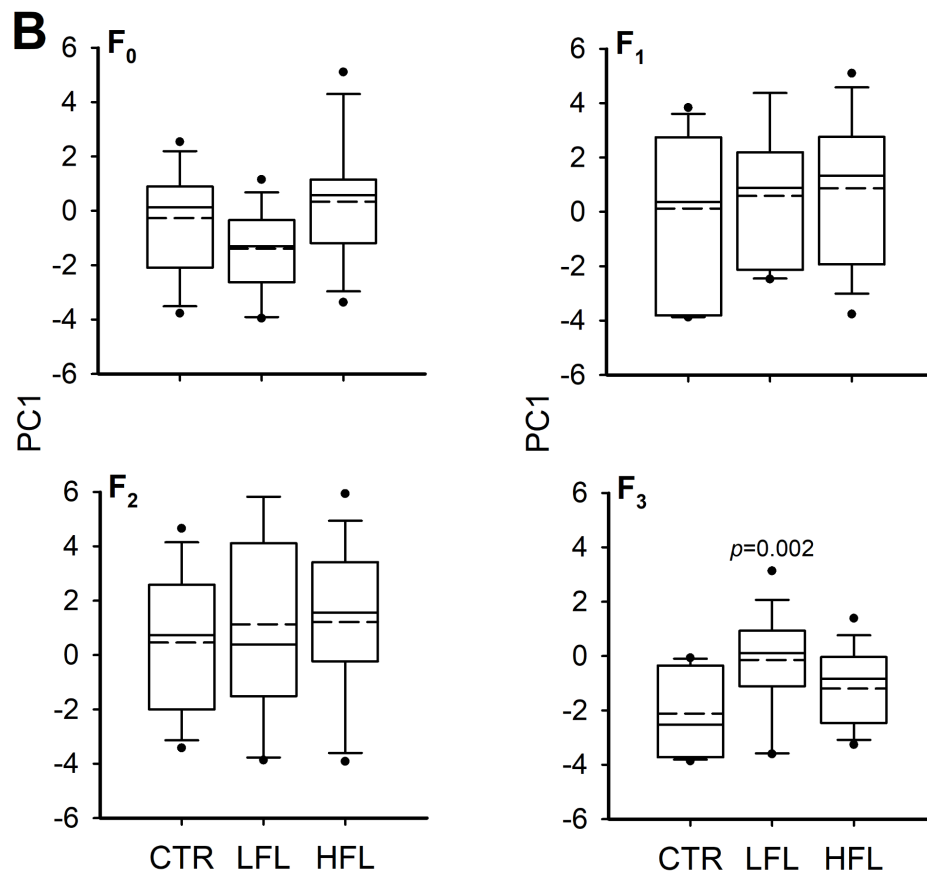
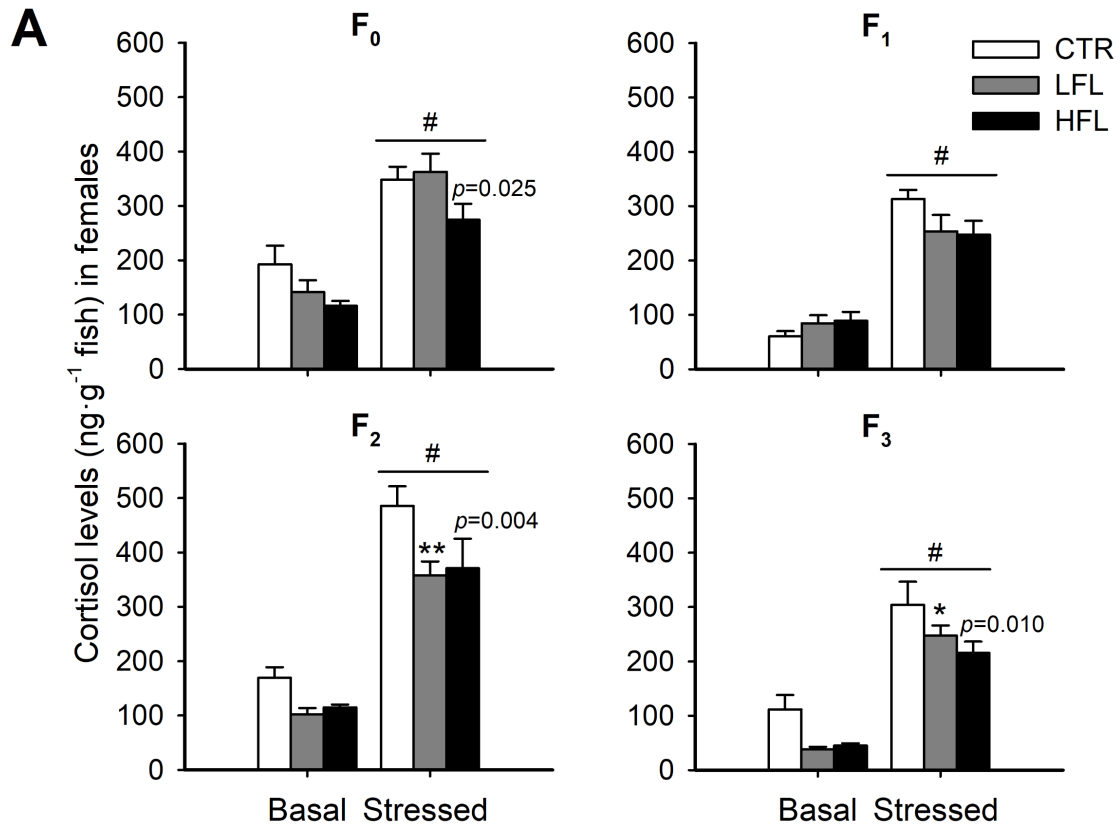


Fig. 2.2. Whole-body cortisol levels and behavioral analysis of adult female ZF from the CTR and FLX lineages. (A) Early-life FLX exposure to the F₀ reduced the basal and the net stress-induced total cortisol levels of the F₀, F₂ and F₃ generations. $n = 6 - 10$ biological replicates per group. Data are presented as mean $\pm$ SEM and analyzed by two-way ANOVA (on ranks for F₂ and F₃). $^{\#}P < 0.001$ compared with the basal group. The asterisks (*) or P -values shown above the bars represent significant difference in the FLX group compared to the CTR: $*P = 0.012$ and $**P < 0.001$. (B) FLX did not trigger any transgenerational alterations in female behavior following the novel tank diving test (NTT). PCA was used to extract components comprised of linear combinations of 10 different behavioral metrics, which are related to the locomotor and exploratory behaviors of the fish. PC1 accounts for 59% of the variability of the dataset. $n = 13 - 17$ fish per group in each generation. Behavioral data are represented in box-and-whisker plots showing the upper and lower quartiles and range (box), median value (solid line), mean value (dashed line) and the 10th and 90th percentiles of the data (whiskers); all data outside the range of the whiskers are presented as individual data points. P -values shown above the bars represent significant differences compared to the CTR analyzed by Student's t -test (or Mann-Whitney U Test for HFL – F₁ and F₃).

2.3.3. Behavioral responses to novelty are reduced by FLX

Since disruption of the stress axis can elicit many behavioral alterations (Haller et al., 1998; Makara and Haller, 2001), we investigated whether the blunted total cortisol levels produced by the females and males from the FLX lineages are linked to an altered behavioral response to novel environments. We conducted the novel tank diving test (Levin et al., 2007) and tracked the locomotor and exploratory activities of each individual fish by video recordings analyzed using a validated in-house automated tracking (AT) Python script. We performed principal component analysis (PCA) on 10 different behavioral metrics (Table 2.6) obtained from the AT. Separate PCAs were performed on the female and male data sets. PCA yielded a single component (PC1) that strongly loaded most of the behavioral metrics (Table 2.1) and explained 51% and 59% of the behavioral variance for males and females, respectively. The two variables that did not robustly

contribute to PC1 were maximum speed and total distance traveled. Positive scores were associated with high exploratory and locomotor activities.

The locomotor and exploratory behaviors in males from the LFL group in F_0 ($t(26) = 1.955, P = 0.061$), F_1 ($t(28) = -0.407, P = 0.687$), F_2 ($t(25) = 1.746, P = 0.093$) and F_3 ($U = 105, P = 0.983$) were not altered. This is in marked contrast to males in the HFL, where PC1 scores revealed that their exploratory and locomotor activities upon the novel tank diving paradigm were significantly reduced in the F_0 ($U = 53, P = 0.025$), F_1 ($t(25) = 2.837, P = 0.009$) and F_2 ($t(25) = 2.298, P = 0.030$) generations compared to their matched CTR lineage (Fig. 2.3A). There was no significant difference in the behavior of the F_3 from the HFL ($t(26) = 1.430, P = 0.165$).

The females (Fig. 2.2B) from the LFL group in F_0 ($t(27) = 1.761, P = 0.090$), F_1 ($t(27) = -0.447, P = 0.659$) and F_2 ($t(29) = -0.623, P = 0.538$) did not show any alterations in their behaviors. The females from the HFL were also unaffected [F_0 ($t(26) = 0.759, P = 0.455$); F_1 ($U = 88, P = 0.497$); F_2 ($t(27) = 0.745, P = 0.463$) and F_3 ($U = 68, P = 0.071$)]. Only the F_3 ($t(31) = -3.464, P = 0.002$) from the LFL exhibited a significant increase in the locomotor and exploratory behaviors compared to the CTR.

Table 2.1. Loadings and contributions of the behavioral metrics to PC1

Behavioral metrics	Females		Males	
	Loadings	Contribution (%)	Loadings	Contribution (%)
Latency middle third	-0.295	8.7	-0.317	10.0
Latency top half	-0.358	12.8	-0.350	12.3
Latency top third	-0.358	12.8	-0.370	13.7
Transitions	0.374	14.0	0.373	13.9
Time middle third	0.362	13.1	0.385	14.8
Time top third	0.297	8.8	0.282	7.9
Distance middle third	0.375	14.0	0.393	15.5
Distance top third	0.332	11.1	0.308	9.5
Total distance	0.213	4.5	0.129	1.7
Max speed	-0.032	0.1	0.082	0.7

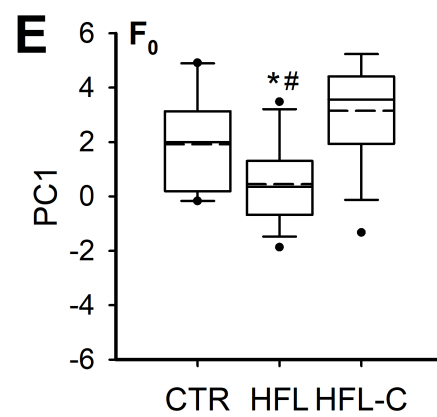
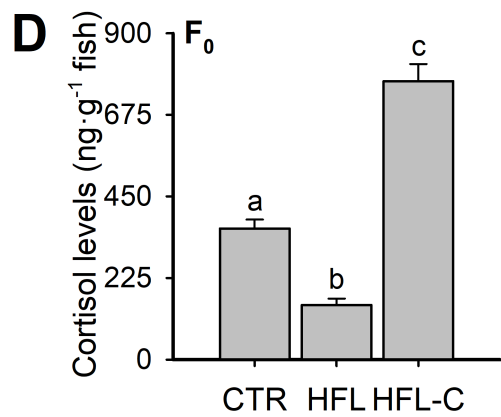
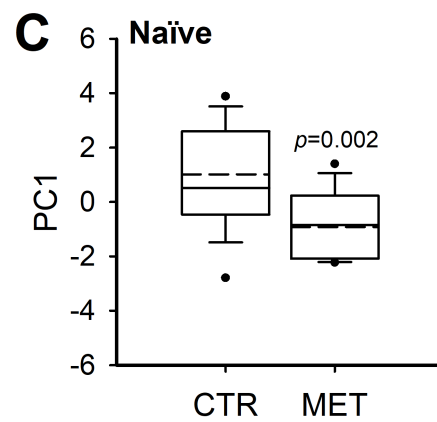
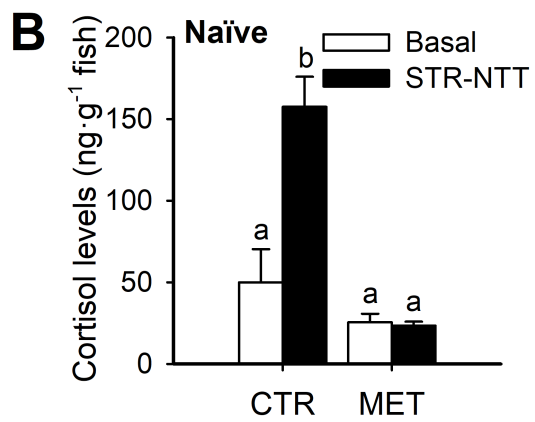
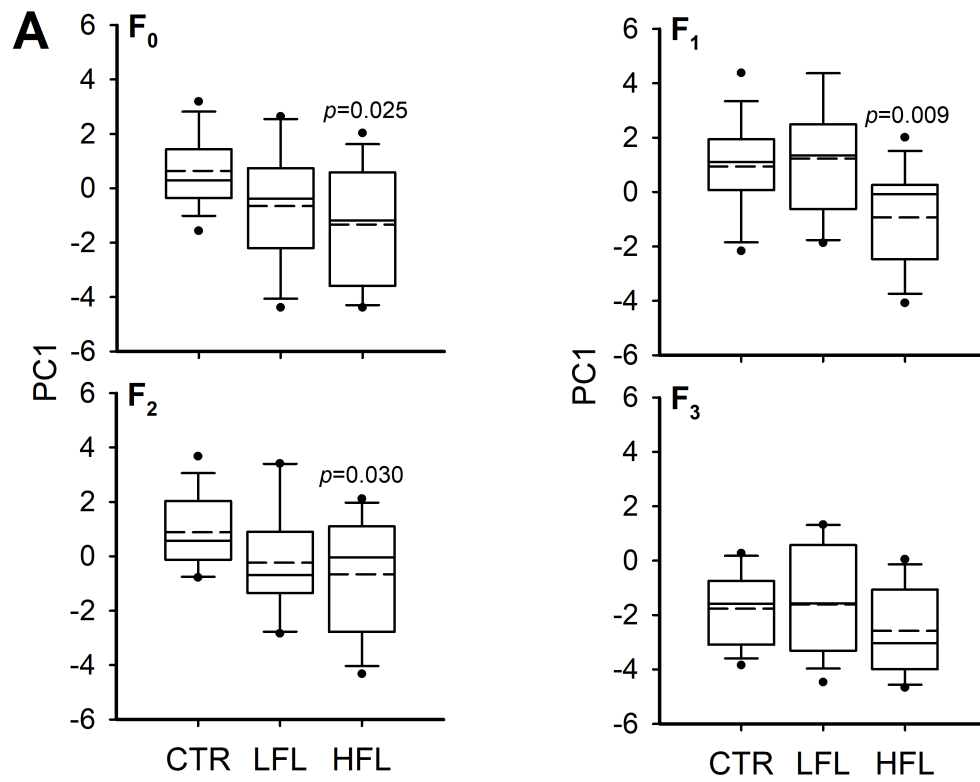


Fig. 2.3. The reduced cortisol levels in males from the HFL decreased their locomotor and exploratory behaviors. (A) PC1 scores representing the locomotor and exploratory behavioral responses to the novel tank diving test (NTT) of the CTR and FLX lineages. PCA was used to reduce the dimensionality of the data set comprised of 10 behavioral metrics. PC1 accounted for 51% of the behavioral variance of which positive weights are associated with high exploratory and locomotor activities. $n = 13 - 16$ biological replicates per group in each generation. (B) Metirapone (MET) exposure inhibited the total cortisol production in naïve adult males subjected to the NTT. STR-NTT, stressed levels following the NTT; $n = 4$ and 16 fish for basal and novel tank diving test groups, respectively. Analyzed by two-way ANOVA on ranks. (C) Pharmacological cortisol reduction in naïve males with MET recapitulated the transgenerational inherited behavioral phenotype observed in the males from the HFL. $n = 15$ fish in each group. (D) Whole-body cortisol levels (stressed) of males from the F_0 following the NTT. $n = 8 - 18$ fish in each group. Analyzed by one-way ANOVA. (E) Cortisol (C) supplementation to the F_0 male adult fish from the HFL (HFL-C) rescued their locomotor and exploratory behaviors. $n = 13 - 17$ fish in each group. Data (A, C and E) are presented in box plots showing the median (solid line), the mean (dashed line), the interquartile range (box) and the whiskers embracing data within the 10th and 90th percentiles; all data outside the range of the whiskers are presented as individual data points. The asterisk (* $P = 0.035$) or P -values shown above the bars represent the significant difference within treatment compared to the CTR. The number sign ($^{\#}P < 0.001$) identifies statistical differences between HFL and HFL-C. Behavioral data were analyzed using Student's t -test (or Mann-Whitney U Test for panel A, HFL F_0 and LFL F_3). Whole-body cortisol levels (B and D) are expressed as mean $\pm$ SEM; $P < 0.05$.

2.3.4. Blunted cortisol levels are responsible for the reduced locomotor and exploratory behaviors in FLX-treated males

The observed association between the FLX-induced decreases in cortisol and behaviors in males across several generations led us to the hypothesis that cortisol regulates locomotor and exploratory behaviors. To test this hypothesis, we assessed the behavioral responses of naïve male fish (from a clean population) to the novel tank diving test following treatment with metirapone, an 11 β -hydroxylase inhibitor which blocks cortisol synthesis. A two-way ANOVA conducted to investigate the effects of metirapone treatment and the novel tank diving test on cortisol levels

revealed that metyrapone significantly decreased whole-body cortisol levels of the naïve fish ($F_{1,36} = 20.724$, $P < 0.001$; Fig. 2.3B), but no effects were observed with the novel tank diving test ($F_{1,36} = 3.300$, $P = 0.078$). However, there was a statistically significant interaction between the effects of the novel tank diving test and the metyrapone treatment on total cortisol levels ($F_{1,36} = 4.660$, $P = 0.038$). Indeed, the locomotor and exploratory activities of the males treated with metyrapone were significantly decreased during the novel tank diving test ($t(28) = 3.389$, $P = 0.002$; Fig. 2.3C).

We then examined whether cortisol supplementation to the males from the HFL F_0 could rescue their impaired locomotor and exploratory activities. Cortisol treatment of the HFL males in the F_0 significantly increased their total cortisol levels ($F_{2,33} = 93.779$, $P < 0.001$; Fig. 2.3D) in addition to their locomotor and exploratory behaviors ($t(28) = -4.157$, $P < 0.001$) compared to the males from the untreated HFL treatment group (Fig. 2.3E). Cortisol exposure normalized the behavioral response of the HFL males since no significant difference was found between the HFL males supplemented with cortisol and the males from the CTR ($t(28) = -1.788$, $P = 0.085$). As previously shown (Fig. 2.3A), the behavioral phenotype of the males from the HFL without cortisol treatment was confirmed to be reduced when compared to the CTR ($t(24) = 2.235$, $P = 0.035$; Fig. 2.3E).

2.3.5. Transcriptomic profiling of male ZF kidney confirms impaired steroidogenesis

We next tested whether impaired cortisol production caused by early-life exposure to FLX was associated with shifts in steroidogenesis-related transcriptional profiles. In teleosts, steroidogenic interrenal cells homologous to the steroidogenic cells of the mammalian adrenal cortex are located mainly within the head kidney, interspersed and scattered amongst other cell

types including chromaffin, hematopoietic and epithelial cells (Flik et al., 2006). The entire kidney was used since the head kidney in ZF and many other teleosts is not a well-defined, compact organ as in mammalian species (Flik et al., 2006). The RNA sequencing (RNA-Seq) analysis identified 17098 and 16864 unique transcripts expressed in the whole kidney in the F₀ and F₃, respectively. Of these, the expression of 596, 917, 1424 and 3544 genes with an average fold change of 9 (Fig. S2.1) was significantly ($P < 0.05$) altered by FLX in F₀ LFL, F₀ HFL, F₃ LFL and F₃ HFL, respectively. The number of unique and shared differentially expressed genes (DEGs) among the two treatments in the F₀ and F₃ generations is displayed as a Venn diagram (Fig. 2.4).

Ingenuity Pathway Analysis (IPA) performed with each of the four DEGs lists revealed 42 to 275 significant ($P < 0.05$) canonical pathways altered by FLX. Of these, a total of 30 pathways were identified in common across at least 3 of the FLX-treated groups. The top 5 most significantly enriched canonical pathways in each group (Table 2.2 and Table S2.14 for the description of the DEGs in each pathway) were associated with immune functions (antiproliferative role of the transducer of ERBB2 (TOB) in T cell signaling), cellular signaling (calcium signaling, ERK/MAPK signaling, integrin linked kinase (ILK) signaling, integrin signaling, pancreatic adenocarcinoma signaling and paxillin signaling), molecular trafficking (caveolar-mediated endocytosis signaling), protein synthesis (eukaryotic initiation factor 2 (EIF2) signaling), cellular structure (epithelial adherens junction signaling and remodeling of epithelial adherens junctions), energy metabolism (mitochondrial dysfunction and sirtuin signaling pathway) and transcriptional activation (retinoic acid receptor (RAR) activation and thyroid receptor/retinoic X receptor (TR/RXR) activation). Strikingly, alterations in genes associated with mitochondrial dysfunction observed in the FLX lineages of F₀ and F₃ are likely related to the cortisol disruption of the treated males since the primary site of cortisol biosynthesis is the mitochondria.

We further investigated the most prominent overrepresented canonical pathways centered on steroidogenesis (Table 2.3 and Table S2.15 for the description of the DEGs in each pathway). Through the activation of *Gas* and cAMP-mediated signaling, ACTH stimulates the synthesis of cortisol in the interrenal cells (Malik et al., 2015). These two cell signaling pathways were altered in the LFL F₀ and HFL F₃. IPA also revealed a significant alteration in the signaling of the glucocorticoid receptor (GR) in both FLX lineages of the F₃. Circadian rhythm signaling was another pathway disrupted by FLX, which was solely identified in both FLX lineages of the F₃. To strengthen biological interpretation of the data, enrichment analysis of the four DEGs listed was also performed using another leading commercial software, Pathway Studio. Similar enriched pathways were uncovered with Pathway Studio (Table S2.8) compared to IPA. An interesting unique pathway revealed by this analysis was the biosynthesis of cholesterol, the universal precursor for steroid synthesis. This pathway was significantly down-regulated in the HFL from both generations and in the LFL from the F₃ generation (Table S2.8).

To gain insights into the biological processes and functions enriched in the kidney of the FLX-treated fish in the generation F₀ and F₃, we performed functional clustering analysis with the IPA software on the DEGs. The enriched functional categories ($P < 0.05$) commonly affected by FLX in all 4 groups were mainly related to lipid metabolism, transport of molecules, carbohydrate metabolism, cellular function and maintenance, protein synthesis, inflammatory responses and tissue morphology. Among these, we focused on the biological processes and functions associated with lipid metabolism and transport of molecules for their importance to cortisol production (Table 2.4). Many cholesterol-related processes including its synthesis and metabolism were affected by FLX. The synthesis of steroids and specifically glucocorticoids was also detected as a target of

disruption by FLX. IPA also uncovered many metabolic enzymes to be disrupted (enzymopathy) in the LFL and HFL of the F₃.

We performed additional quantitative real-time PCR (qRT-PCR) analysis on a set of 10 genes (Table 2.5) that showed significantly different expression levels in at least one of the four DEGs lists. These genes were selected because they are key genes associated with either cholesterol or steroid synthesis. Comparison of expression levels of all the 10 genes examined determined by RNA-Seq and qRT-PCR revealed a 68% agreement in the direction and magnitude of change across all groups (LFL and HFL from F₀ and F₃). However, considering only conditions in which the genes were identified as differentially expressed (P -value < 0.05) by RNA-Seq, there was 92% concordance between the RNA-Seq and qRT-PCR results.

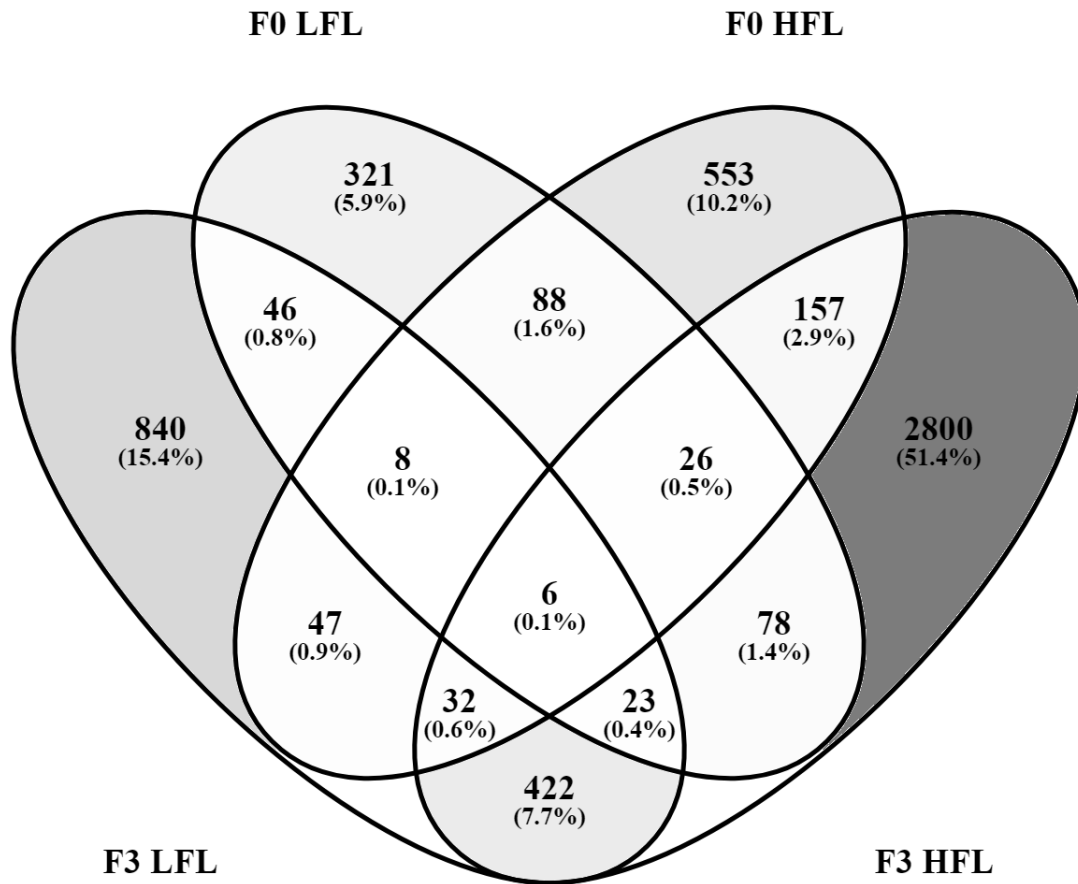


Fig. 2.4. Venn diagram illustrating the number of shared and uniquely differentially expressed genes ($P < 0.05$) in the male kidney among FLX lineages in the F₀ and F₃ generations. The two FLX lineages in F₃ shared the most differentially expressed genes and the HFL group in F₃ displayed the most uniquely differentially expressed genes (Venn diagram created using Oliveros (2007-2015)).

Table 2.2. *P*-values of the top significant canonical pathways in the kidney of males from the F₀ and F₃ FLX lineages compared to controls

Canonical pathways	F ₀		F ₃	
	LFL	HFL	LFL	HFL
Antiproliferative Role of TOB in T Cell Signaling	6.3×10⁻³	2.3×10 ⁻²	NA	3.2×10 ⁻²
Calcium Signaling	NS	2.8×10 ⁻²	1.3×10⁻³	3.1×10 ⁻³
Caveolar-mediated Endocytosis Signaling	NS	1.4×10⁻³	1.7×10 ⁻²	1.5×10 ⁻²
EIF2 Signaling	5.6×10⁻³	NS	2.0×10 ⁻²	1.0×10 ⁻⁴
Epithelial Adherens Junction Signaling	NS	7.4×10⁻⁴	4.9×10 ⁻²	5.2×10 ⁻³
ERK/MAPK Signaling	NS	2.1×10 ⁻²	1.9×10 ⁻²	4.0×10⁻⁶
ILK Signaling	NS	8.7×10⁻⁴	4.7×10⁻⁴	1.7×10⁻⁵
Integrin Signaling	NA	3.6×10 ⁻²	1.9×10 ⁻²	4.7×10⁻⁷
Mitochondrial Dysfunction	3.9×10⁻³	NS	5.0×10⁻¹¹	1.4×10 ⁻³
Pancreatic Adenocarcinoma Signaling	3.2×10 ⁻²	7.8×10 ⁻³	NS	1.6×10⁻⁵
Paxillin Signaling	NS	1.5×10⁻³	1.0×10 ⁻²	3.2×10 ⁻⁴
RAR Activation	5.2×10⁻⁴	5.8×10 ⁻³	NS	1.9×10 ⁻³
Remodeling of Epithelial Adherens Junctions	1.9×10 ⁻²	1.2×10⁻³	1.5×10 ⁻²	1.7×10 ⁻³
Sirtuin Signaling Pathway	1.1×10⁻²	2.2×10 ⁻²	3.2×10⁻¹³	4.8×10⁻⁷
TR/RXR Activation	NA	2.3×10 ⁻³	1.1×10⁻³	2.8×10 ⁻⁴

NA, not available; pathway not detected.

NS, not significant.

P-values in bold represent the top 5 significant canonical pathways in each of the treatments.

Table 2.3. *P*-values of key cortisol-related canonical pathways in the kidney of males from the F₀ and F₃ FLX lineages

Canonical pathways	F ₀		F ₃	
	LFL	HFL	LFL	HFL
Biosynthesis of cholesterol†	NA	<1.0×10 ⁻³	1.1×10 ⁻²	2.0×10 ⁻³
cAMP-mediated signaling	1.9×10 ⁻²	NA	NA	2.4×10 ⁻²
Circadian Rhythm Signaling	NS	NS	3.7×10 ⁻²	3.5×10 ⁻³
Gαs Signaling	2.3×10 ⁻²	NA	NA	1.5×10 ⁻²
Glucocorticoid Receptor Signaling	NS	NS	3.2×10 ⁻²	1.3×10 ⁻⁷

NA, not available; pathway no detected.

NS, not significant.

† This enriched pathway was obtained following gene set enrichment analysis in Pathway Studio.

Table 2.4. *P*-values of significantly enriched key cortisol-related biological pathways and functions in the kidney of males from the F₀ and F₃ FLX lineages

Enriched pathways and functions	F ₀		F ₃	
	LFL	HFL	LFL	HFL
Concentration of cholesterol	NA	6.1×10 ⁻⁵ (-0.809)	NA	2.2×10 ⁻⁷ (0.561)
Enzymopathy	NA	NA	2.2×10 ⁻¹¹	7.0×10 ⁻¹⁰ (0.568)
Metabolism of cholesterol	NA	5.1×10 ⁻⁴ (-0.555)	NA	NA
Quantity of steroid	NA	2.4×10 ⁻⁵ (-0.805)	NA	4.6×10 ⁻⁷ (1.119)
Steroid metabolism	NA	1.7×10 ⁻⁵ (0.923)	4.4×10 ⁻⁵ (1.437)	NA
Synthesis of glucocorticoid	5.1×10 ⁻³	NA	NA	NA
Synthesis of steroid	NA	NA	9.9×10 ⁻⁶ (3.107)	NA
Transport of molecule	NA	2.5×10 ⁻⁷ (1.412)	7.0×10 ⁻⁹ (1.637)	8.0×10 ⁻¹⁹ (-4.015)
Uptake of cholesterol	NA	2.4×10 ⁻⁴ (-1.095)	NA	NA

Z-scores are presented in brackets below the *P*-values when available. A positive Z-score indicates a predicted activation, whereas a negative Z-score indicates a predicted inactivation of the enriched pathway.

NA, not available; pathway not detected.

Table 2.5. qRT-PCR analysis (fold change) of selected genes

Gene symbol	F ₀ LFL		F ₀ HFL		F ₃ LFL		F ₃ HFL	
	RNA-Seq	qRT-PCR	RNA-Seq	qRT-PCR	RNA-Seq	qRT-PCR	RNA-Seq	qRT-PCR
<i>ctgfa</i>	-1.49*	-2.35*	1.05	-3.17*	-1.35	-1.45	-1.06	-2.03
<i>cyp11a1</i>	4.07*	1.13	-1.58	3.27	-1.82	-2.01*	1.32	-2.38
<i>dhcr7</i>	-1.27	1.36	-1.04	-1.25	-1.10	-1.84	-5.28*	-1.92
<i>lcat</i>	1.13	-1.08	1.37	-1.44	-1.58	-1.27	-215.03*	-1.93
<i>lss</i>	-1.05	-1.37	-2.25	-1.33	-2.65	-2.14	-13.29*	-4.75*
<i>namptb</i>	-1.12	-1.07	1.63*	-1.37	1.03	-1.55	-12.94*	-2.67*
<i>pmvk</i>	1.25	1.23	1.03	-1.27	-1.50	-1.14	-4.26*	-3.73*
<i>rb1</i>	-1.04	-1.46	-1.27	-2.25*	-1.10	1.24	-9.96*	-1.92*
<i>star</i>	1.16	8.42	1.03	26.54*	29.30*	218.58*	5.81	19.98
<i>sts</i>	-1.03	1.07	1.22	-1.29	-1.53	-1.15	-4.51*	-1.78

**P*-value < 0.05

2.4. Discussion

Our findings revealed that exposure to human physiological and environmentally relevant doses of FLX during a critical period of brain development reduces both basal and stress-induced total cortisol levels in adult ZF across generations. Strikingly, the magnitude of this attenuation is not consistent between the basal and stress conditions. The FLX-induced cortisol reduction is more pronounced following a stressor, hence FLX treatment also dampened the magnitude of the stress axis reactivity. The stress response is critical to the organism as it is the driving force for its adaptation and survival to changes (Buschdorf and Meaney, 2015). Its disruption can trigger the alteration of a set of neural, behavioral, endocrine, and molecular responses. Disruption of the stress response strongly contributes to the development of diverse psychological and behavioral phenotypes (Heim and Binder, 2012; Simpson et al., 2012; Zannas and West, 2014). For instance, chronic blunted cortisol levels have been associated with long-term detrimental effects to human health including burnouts, chronic fatigue, fibromyalgia, immune disorders, and post-traumatic stress disorder, among others (Bakusic et al., 2017; Demitrack and Crofford, 1998; Fries et al., 2005; Nijhof et al., 2014; Wichmann et al., 2017). Hypo-reactivity of the HPA axis is also observed in children prenatally exposed to SSRIs through maternal treatment (Davidson et al., 2009; Oberlander et al., 2008).

Attenuation of the cortisol response was more severe in males than females from the FLX lineages. The HFL was the most affected with 30 to 52% reductions in cortisol across generations compared to CTR males, whereas ZF males from the LFL displayed 0.4 to 40% reductions across generations. Conversely, the females in the FLX lineages experienced a wider range of variation in their stress response, from 37% reduction to a 55% increase compared to the CTR females. In humans and rodents, sex differences in the efficacy and toxicity of pharmacological treatments

have been extensively studied. Differences are generally attributable to sex-specific levels of sex steroids in addition to sex-based variability in pharmacokinetic parameters (Bigos et al., 2009; Gandhi et al., 2004; Soldin and Mattison, 2009; Ueno and Sato, 2012). A potential contributor to the marked differences in response to the developmental FLX exposure in our study could be 11-ketotestosterone (11-KT), the primary sex steroid in male fish (Fuzzen et al., 2011), which total levels were also significantly diminished by FLX in generations F₀, F₁ and F₃ (Fig. S2.2). Nonetheless, these results on the total sex steroid levels should be interpreted with caution as sex steroids, contrary to cortisol, are synthesized in many tissues (Balthazart and Ball, 2006; Li et al., 2015). Therefore, the total levels may not be indicative of the concentration of hormone to which target tissues are exposed. However, these findings on the male-specific disruption of the stress axis are consistent with a previous study on rats where exposure of the mother to FLX throughout lactation during the neural development stage of the pups displayed a reduction in serum corticosterone levels (Pawluski et al., 2012). Considering that sex is a potential source of response variation to the disruptive effects of FLX, it is critical to include both males and females in such studies.

Inadequate, excessive or prolonged disruption of cortisol levels are all implicated in the alteration of many critical biological processes including behavioral responses in fish (Nesan and Vijayan, 2013) and mammals (Myers et al., 2014; Stephens and Wand, 2012). Our assessment of the behavioral responses to novelty revealed a significant sex-specific decrease in the locomotor and exploratory activities in males from the HFL. The attenuation of these behaviors persisted to generation F₂ without diminution. Our findings of reduced locomotor and exploratory activities in ZF treated with FLX during a critical period of brain development coincide with studies performed on male rats (Ko et al., 2014; Lee and Lee, 2012), suggesting that the effects of FLX on ZF could

be cautiously extrapolated to mammals. The sexually dimorphic behavioral response to novelty observed in our FLX-lineage was also displayed in rats prenatally exposed to citalopram, another SSRI (Simpson et al., 2011). Our results are also consistent with two human studies where prenatal exposure to SSRIs through maternal treatment impaired gross motor and adaptive responses in 10-month-old infants (Hanley et al., 2013), and increased internalizing and anxious behaviors in 3- and 6-year-old children (Hanley et al., 2015).

The pharmacological inhibition of cortisol synthesis with metyrapone recapitulated the behavioral phenotype found in the males from the HFL group. Naïve male ZF exposed to metyrapone displayed reduced exploratory and locomotor activities. In support of this finding, supplementation of cortisol to the HFL F₀ males rescued their behavioral response to novelty. These results support the notion that the impaired behavioral response observed in our F₀ to F₂ males is the result of the blunted cortisol levels elicited by the developmental FLX exposure to the F₀. These observations are in agreement with those on chronic hypocortisolism in humans, where associated psychological disorders are linked to low movement (chronic fatigue, burnouts, etc.) (Bakusic et al., 2017; Demitrack and Crofford, 1998; Fries et al., 2005; Nijhof et al., 2014; Wichmann et al., 2017). Additionally, the examination of the cortisol levels in response to ACTH revealed a disruption in the capacity of the steroidogenic interrenal cells to synthesize cortisol in males from the FLX lineages, suggesting that the steroidogenic cells are one target of the transgenerational disruptive mechanisms induced by FLX.

To gain additional insights into the biological functions and canonical pathways transgenerationally-disrupted by early-life FLX exposure in the F₀, we analyzed transcriptomic profiles of male kidneys of the CTR and the two FLX lineages from the F₀ and F₃ generations. The ~4-fold increase in the number of DEGs observed in the F₃ compared to the F₀ from the HFL

suggests that the F₃ underwent adaptive mechanistic responses to cope with the disruptive effects of FLX, as only 9% of the total detected unique transcripts were found to differ between these two generations. This is consistent with the numerous other biological pathways and functions (>400) that were disrupted by FLX. Altered processes were related to kidney function, immune response, hematopoietic and epithelial cell processes, diverse adrenal pathways, among others (data not shown). Importantly, differential expression analysis revealed two key genes directly involved in steroidogenesis, *star* and *cyp11a1*.

Functional analysis of the DEGs confirmed the disruption of the steroidogenic process by FLX across the examined generations. It also revealed alterations in cholesterol-related pathways including its biosynthesis, uptake and metabolism. Cholesterol availability is crucial for steroid synthesis as it is the metabolic precursor for these enzymatic reactions (Midzak and Papadopoulos, 2016; Shen et al., 2016). Additionally, enrichment analysis revealed that the Gαs and cAMP-mediated signaling pathways, the activation of which is involved in stimulating cortisol biosynthesis upon the binding of ACTH (Malik et al., 2015), were significantly affected by FLX. Circadian rhythm signaling was also impaired by FLX. Interrenal cells exhibit a circadian pattern of cortisol secretion (Chan and Debono, 2010; Kalsbeek et al., 2012); therefore, disruption of this pattern may be shifting the basal cortisol levels that are subjected to robust daily variations (Chung et al., 2011). This may be associated with the blunted basal cortisol levels observed in the FLX lineages, since cortisol levels in each generation were measured at the same time of the day. Overall, the transcriptomic profile of the kidney uncovered key biological functions and pathways that are ultimately disrupted by early-life FLX exposure and that could explain the reduced ability of the interrenal cells to synthesize cortisol upon a stressor and ACTH induction.

Overstimulation of 5-HT signaling during a critical period of brain development has been shown to increase GR expression in the rat hippocampus (Mitchell et al., 1990a). It is also well-known that activation of GR in the hippocampus controls cortisol negative feedback regulation (Liu et al., 1997; Sapolsky et al., 1984; Zhang et al., 2013), thus attenuating the HPA response to stress. Alteration of GR expression induced by 5-HT is mediated by epigenetic mechanisms, and specifically methylation in the promoter (in the exon 1₇) of the gene encoding GR (Weaver et al., 2007; 2014). Epigenetic mechanisms are permanent alterations in gene expression and as a result of their stability, they are potentially transmitted to subsequent generations (Le Dantec et al., 2015; Nestler, 2016). Therefore, we should consider the possibility of an integrated disruptive action by FLX on this transgenerational phenotype where both 5-HT-induced epigenetic modifications and the disruption of the steroidogenic cells are involved in the blunted cortisol levels observed in the treated-males.

In conclusion, a 6-day FLX exposure during brain development to a concentration within the lower range of that detected in the cord blood of FLX-treated pregnant women (HFL) and to an environmentally relevant concentration (LFL) leads to a male-specific impairment of cortisol synthesis for at least 3 consecutive generations in ZF without diminution. These findings on the impairment of cortisol levels are consistent with the disrupted biological pathways and functions linked to steroidogenesis that were altered by FLX in the whole kidney containing interrenal cells. The impairment of the cortisol response in males elicited by FLX reduces the exploratory and locomotor activities in response to novelty for two subsequent generations. Given this evidence of the transgenerational effects of developmental FLX exposure on the stress axis and behavioral response, an investigation is required to determine whether these effects occur in humans because FLX is generally the first-line of pharmacological treatment in pregnant women suffering from

affective disorders (Latendresse et al., 2017; Locher et al., 2017; Man et al., 2017; Morkem et al., 2017; Sarginson et al., 2017). Finally, given that levels of FLX detected in the aquatic environment (Mennigen et al., 2010a) also reduced the stress response over the 3 generations, our findings highlight potential risks to natural populations of fish.

2.5. Materials and methods

2.5.1. Transgenerational studies and in vivo exposures

All procedures conducted in this study were approved by the University of Ottawa Animal Care Protocol Review Committee and are in compliance with the guidelines of the Canadian Council on Animal Care for the use of animals in research. Adult ZF of the AB strain were obtained from Big Al's Aquarium in Ottawa, Ontario and allowed to acclimate for 4 weeks prior to generating the parents of the F_0 generation (F_{-1}). The sex ratio of the F_{-1} (~1F:1M) ensured our founder fish (F_0) originated from a balanced population without any genetic predisposition to a certain sex. The fish in the generation F_{-1} were subsequently bred to produce the F_0 which was used to generate the CTR and the two FLX-exposed lineages for the F_1 to F_3 generations (see Fig. 2.5).

To model human fetal exposures, one group of F_0 embryos was exposed to $54 \mu\text{g}\cdot\text{L}^{-1}$ FLX (HFL; High-FLX lineage), which is within the range ($41.3 - 78.3 \mu\text{g}\cdot\text{L}^{-1}$) detected in the cord blood of FLX-treated pregnant women (Hendrick et al., 2003; Kim et al., 2006; Rampono et al., 2004). To model exposures of wild fish, another group of F_0 embryos was exposed to $0.54 \mu\text{g}\cdot\text{L}^{-1}$ FLX (LFL; Low-FLX lineage), which is environmentally relevant to fish exposed to pharmaceutical pollutants released from sewage treatment plants (Mennigen et al., 2010a). We have previously studied the short-term effects of both concentrations on the reproductive and

metabolic physiology of adult goldfish *Carassius auratus* (Mennigen et al., 2010a; 2011) and ZF (Craig et al., 2014). All experimental fish in each generation were mated at 24 ± 2 weeks post-fertilization (wpf). Only virgin fish were used in this experiment to avoid potential confounding effects brought about by fish with different breeding experiences on the assessment of their reproductive fitness. Fifteen breeding pairs were randomly chosen to generate founders (F_0) and all subsequent generations ($F_1 - F_3$). Pairs in F_0 to F_3 that did not spawn at trial 1 were provided a second opportunity with a different mate randomly chosen from non-spawning individuals within the same lineage to eliminate the possibility of mate preference, which has been extensively studied in ZF (Pyron, 2003; Spence et al., 2008; Uusi-Heikkilä et al., 2012). Sex ratios, reproductive fitness, condition factor and developmental outcomes of the descendants were monitored throughout the study; however, no significant changes were observed (Chapter 5).

Mating pairs were set up in the late afternoon in crossing cages (Aquatic Habitats) with a plastic divider that separated the female from the male and left undisturbed until the following morning. The crossing cages were composed of a 1-L holding tank (crossing cage) and an additional container that was inserted inside the cage. The bottom of this inner-container was perforated with small holes which allowed eggs to fall down into the cage and be protected from predation. On the day of the spawning, the inner-container which held the fish was transferred to a new cage containing fresh system water. Extra care was taken to prevent any unnecessary stress of the fish during the transfer. The pairs were then allowed to spawn for 1 h 45 min between 09h00 and 11h00. Eggs were immediately collected, submerged in 0.0075% bleach for 2 min, rinsed and counted. All fertilized eggs were kept, yielding a minimum population of 150 adult fish (at 20 wpf) per lineage in each generation. At 3 hours post-fertilization (hpf), embryos were randomly assigned to Petri dishes containing either embryo medium alone (embryos in: F_{-1} , CTR F_0 , and F_1 to F_3) or

supplied with one of the two FLX (Sigma-Aldrich) concentrations (embryos in F₀ - FLX lineages). The exposure of the F₀ embryos (CTR and FLX lineages) was performed in glass Petri dishes from 3 hpf to 6 days post-fertilization (dpf) during the critical period of ZF brain development (Mueller and Wullmann, 2016). The F₁ to F₃ embryos from each lineage were distributed in plastic Petri dishes and labeled with the number assigned to their parents to monitor for any embryonic or larval developmental effect resulting from a specific mating pair. Embryos from all the generations were reared in Petri dishes until 6 dpf at a maximum density of 1 embryo·mL⁻¹ and maintained at 28 °C without feeding to allow complete yolk sac absorption. Embryo medium was renewed daily and the appropriate concentrations of FLX replaced. Mortality and hatching were monitored daily during these 6 days and the surviving larvae from the same lineage were randomly distributed and transferred to a temperature-controlled ZF facility and reared in 1-L tanks containing heated (28.5 ± 0.2 °C), aerated, dechloraminated City of Ottawa tap water (hereby referred to as system water) at a density of 50 larvae·L⁻¹ as per recommendation by Matthews et al. (2002). At this point, larvae were fed three times per day with Zebrafish Management Ltd. fry food diet of the appropriate size and according to their developmental stage and from 60 dpf until adulthood, fish were fed twice daily with No.1 crumble-Zeigler (Aquatic Habitats). The commercial feed in all stages from 16 dpf was supplemented with live *Artemia* nauplii (Artemia International LLC) once per day. The water was changed and the debris was cleaned three times a week. At 30 dpf, juvenile fish were transferred into 3- and 10-L tanks (Aquatic Habitats) supplied with flow-through system water. To avoid high rearing density-induced masculinization and to reduce any effect on reproductive performance, adult fish were housed at a density of 5 fish·L⁻¹ as suggested in the literature (Castranova et al., 2011; Matthews et al., 2002). Larvae and adult ZF were maintained under a 14 h light:10 h dark photoperiod. In addition to temperature, water quality parameters

including pH (7 – 7.5), conductivity (120 – 150 μ S), ammonium nitrite and ammonium nitrate were checked periodically to ensure that they were within the appropriate range (Lawrence, 2007; Ribas and Piferrer, 2014). Fish from the same lineage in each generation were randomly mixed every month to avoid formation of social hierarchies and to reduce potential tank effects. All subsequent experimental testing described in this study was performed at 6 months post-fertilization (mpf). Therefore, at approximately 5 mpf, fish were separated by sex to minimize any handling stress during experiments and to prevent fish from spawning in the tanks.

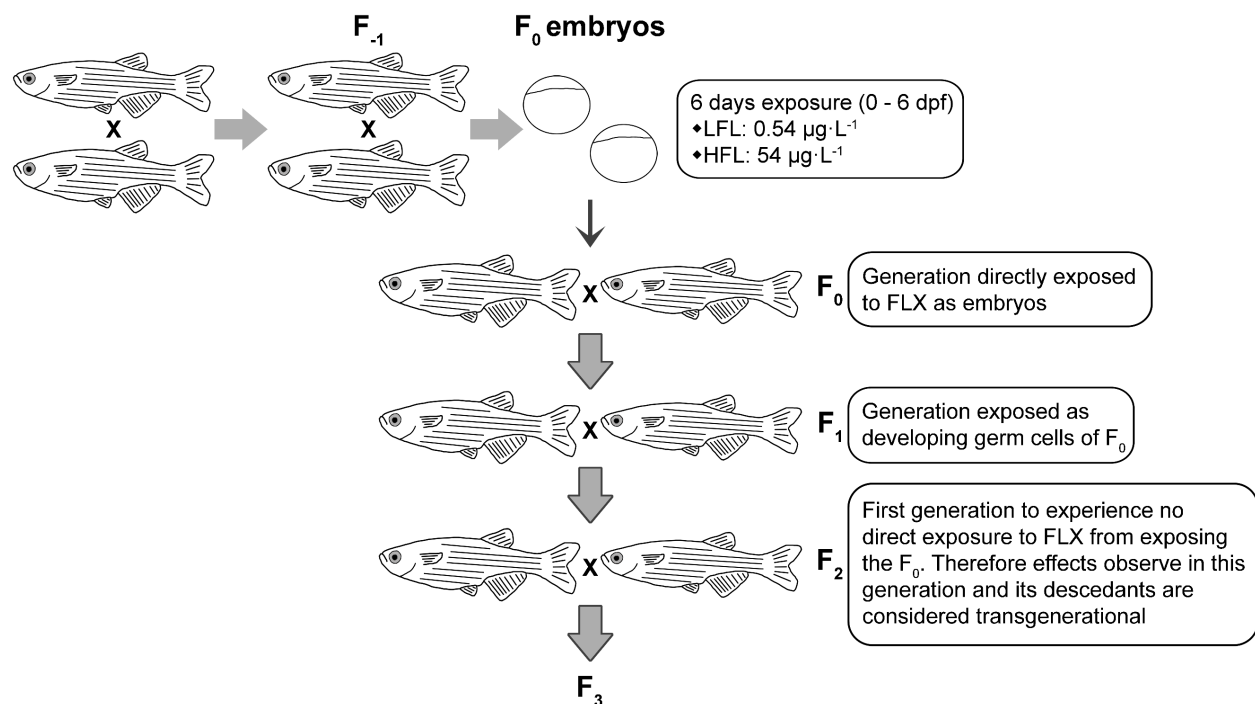


Fig. 2.5. Schematic representation of the ZF mating process to generate the CTR and the two FLX lineages. Adult ZF of the AB strain were mated to generate the F_{-1} , which was subsequently bred to produce the F_0 . Exposure of F_0 as embryos to one of the two FLX concentrations generated the FLX lineages for the F_1 to F_3 generations. The F_1 was exposed to FLX as developing germ cells when the F_0 embryos were being exposed. Therefore, only the effects observed in the F_2 and its descendants are considered transgenerational (Aluru, 2017) since they have not experienced a direct exposure to FLX.

2.5.2. Acute stress experiment and sampling

Females and males arbitrarily chosen from the same lineage were subjected to the standardized net stressor as described by Ramsay et al. (2009). Prior to the test, the fish were transferred to the experimental room and allowed to acclimate for one week to the 3-L tanks wrapped in black plastic and supplied with flow-through system water. The darkened tanks were used to prevent any unnecessary stress brought on by the presence of the experimenter while the animals were handled for the stress study. It is important to note that the lids of the tanks were not covered, so these fish still experienced the 14 h light:10 h dark photoperiod. During this period, fish were fed normally until one day prior to the experiment. At the end of this period, a group of fish was immediately sacrificed (Basal group) and the rest underwent the net stressor prior to sampling (Stressed group). In all experiments, fish were sacrificed by submerging them in ice-cold water to avoid any confounding effects of anesthetics (Wilson et al., 2009). Fish were weighed, immediately snap-frozen in liquid nitrogen and stored at -80°C for further whole-body hormone analysis. The acute stressor was performed in each generation at the same time of the day (between 09h15 and 10h30).

2.5.3. Intraperitoneal (ip) injection of ACTH

Adult (6 mpf) male ZF from the F_0 and F_3 generations were intraperitoneally injected with a single ip dose of porcine ACTH (Sigma-Aldrich). The ACTH concentration of $0.0625\text{ IU}\cdot\text{g}^{-1}\text{ BW}$ was estimated by conducting a prior dose-response experiment from which the minimal stimulatory ACTH concentration that triggered the maximum synthesis of cortisol was selected. Prior to the ip injection, fish randomly picked from each lineage (CTR and the two FLX) were anesthetized in a $60\text{ }\mu\text{M}$ ethyl 3-aminobenzoate methanesulfonate salt (tricaine; Sigma-Aldrich)

solution (Westerfield, 2000), weighed and immediately placed on a sponge saturated with cold water. The injection procedure was conducted in such a way as to guarantee that the animal did not spend more than 20 s out of water. To control for the stress induced by the ip injection protocol, a group of fish from the CTR and FLX lineages were either not injected (unstressed group) or injected with the vehicle only, Ringer's solution (ZF saline; saline group) (Westerfield, 2000). The total injection volume of either ACTH or Ringer's solution was $10 \mu\text{L} \cdot \text{g}^{-1}$ BW. After the injection, the animals were individually placed in a tank with clean water ($28 \pm 1^\circ\text{C}$) and 30 min following their recovery from the anesthesia, the fish were sacrificed. All the animals recovered in less than 1 min, and no mortalities occurred from the injection. Injections were performed between 09h30 and 12h30.

2.5.4. Metirapone exposure

Adult ZF (8 mpf) of the AB strain bred in-house from a clean population were exposed in system water to $325 \mu\text{M}$ metirapone (Adooq Bioscience) for one week. Effective metirapone dose and treatment period were estimated using a pilot study to determine levels that inhibit cortisol production in ZF. Fish were placed in 5-L glass tanks at a density of $5 \text{ fish} \cdot \text{L}^{-1}$, provided with adequate aeration and exposed to either system water containing metirapone or DMSO vehicle (0.02% v/v). The experiment was designed as static renewal, where 100% of the water was replaced daily 1 h after feeding (including either metirapone or DMSO). Ammonia, nitrate and nitrites were measured, however, no differences were found between treatments (data not shown). At the end of the one-week exposure, 5 fish in the metirapone and DMSO treatments were immediately sacrificed to assess unstressed cortisol levels. The remaining fish from both treatments were subjected to the novel tank diving test (see 2.5.7) prior to being terminally

anesthetized, weighed, snap-frozen in liquid nitrogen and stored at -80°C for further whole-body cortisol assessment.

2.5.5. Cortisol supplementation experiment

Male adult ZF (5 mpf) from the HFL group in the F_0 were exposed in system water to $50\text{ mg}\cdot\text{L}^{-1}$ hydrocortisone (Sigma-Aldrich) using the same experimental design as the metyrapone *in vivo* exposure (see 2.5.4). The *in vivo* hydrocortisone exposure was performed over a period of 4 days to allow the fish to physiologically adjust to the new cortisol levels. Prior to the experiment, new F_0 fish were generated and their cortisol levels and behaviors were examined to ensure both F_0 displayed the same effects following FLX treatment as embryos. The adult fish in the CTR lineage from the F_0 were only exposed to the DMSO vehicle (0.0625% v/v). An initial pilot study conducted revealed the highest accumulation of cortisol in ZF occurred after 3 h of in tank exposure, and therefore 3 h into the 4th exposure day to hydrocortisone, the fish underwent the novel tank diving test (see 2.5.7). Fish were subsequently sacrificed, weighed, snap-frozen in liquid nitrogen and stored at -80°C for further cortisol measurements.

2.5.6. Whole-body lipid extraction and hormones quantification

Whole-body lipids were extracted using a protocol adapted from Folch et al. (1957). Briefly, individual fish were pulverized in liquid nitrogen using a mortar and pestle and homogenized in 15 mL $\text{CHCl}_3\text{:MeOH}$ (2:1 v/v). After 15 min of incubation at room temperature, 5 mL 2 M KCl buffered with 5 mM EDTA was added to the homogenate, vortexed and incubated for an additional 20 min. The organic phase was then transferred to a clean glass tube, evaporated to dryness under a stream of nitrogen while the tubes were heated at 45°C ; the lipid extract was

reconstituted in ethylene glycol monomethyl ether. Whole-body extraction efficiencies were determined by spiking homogenates with known amounts of the appropriate radioactive isotope (^{14}C or ^3H) and were 87% for cortisol, 85% for 17β -estradiol (E_2) and 89% for testosterone; values were not corrected for individual extraction efficiencies.

Total cortisol concentrations were assessed by using a ^{125}I radioimmunoassay kit (MP Biomedicals) according to the manufacturer's protocol followed by the estimate of the radioactive counts with a Wizard gamma counter (PerkinElmer). The intra- and the inter-assay coefficients of variation (CV) were calculated to be 4-8% and 7-15%, respectively.

Total 11-KT levels were estimated using a commercial enzyme-linked immunosorbent assay (ELISA) (Cayman Chemical) as per the manufacturer's protocol. The intra- and the inter-assay CV were 4-12% and 5-18%, respectively. Total E_2 and testosterone levels were determined by using commercially available ELISA kits (TECO Diagnostic) according to the manufacturer's instructions. The intra- and the inter-assay CV for these ELISA assays were calculated to be, for E_2 5-8% and 7-13%, respectively and for testosterone 7-9% and 9-19%, respectively. For all ELISA assays, absorbance values were estimated using a microplate spectrophotometer (SpectraMAX Plus 384; Molecular Devices).

2.5.7. Behavioral experiments and analyses

The novel tank diving test adapted from Levin et al. (2007) was used to assess locomotor and exploratory activities of the fish evoked by their habituation response to novelty (Rosemberg et al., 2011; Wong et al., 2010). Adult females and males from each lineage in generations F_0 to F_3 were placed in separate 3-L tanks ($16 \text{ fish} \cdot \text{tank}^{-1}$) and housed in the testing room one week prior to the experiment. The illumination, light cycle, temperature and water conditions of the testing

room were similar to those of the main ZF facility. All behavioral testing in each generation was performed over a 3-day period, between 09h30 and 14h30 on a stable surface with all environmental distractions kept to a minimum. At the end of the acclimatization period, each fish was individually placed in the trapezoid-shaped test tank (outside dimensions: 22.5 cm along the bottom $\times$ 28 cm at the top $\times$ 15 cm high $\times$ 7 cm width; Aquatic Habitats) filled with system water and their behavioral activity recorded from the front for 6 min in the absence of the camera operator. The test tank water was replaced with clean system water after each individual test. To enable a more efficient quantification of the ZF behavioral activities, the trapezoidal test tank underwent two different horizontal virtual divisions (Fig. 2.6), the first one was divided into two and the second into three equal zones. Videos were analyzed every 30 frames $\cdot$ s⁻¹ for a total of 10,800 frames using an AT Python script. A Python script was also used to calculate the behavioral metrics (Table 2.6) assessed in this study. Briefly, the AT works by first converting the video file into still frames at a user defined frame per second rate, and subsequently segmenting the target object (fish) from the background in each frame of the video file. The segmentation procedure subtracts the background and identifies the fish by searching for an elliptical shape. The algorithm uses the position (coordinates) of the fish from the previous frames to eliminate moving objects with similar shapes including water droplets and feces. In case of possible conflicts with coordinates from the fish with other moving objects, the algorithm red-flags the specific frame and allows the user to manually select the target object. A ruler is included in the video recording for calibration. Tracking began with the fish positioned at the bottom of the tank after the first 90 frames of the video by which the fish was allowed to recover from the stress of being released from the net, and proceeded to the end of the video (10,800 frames). The script generates a video with the tracking patterns by displaying a dot on the tracked target to allow the user to verify that

the tracked target is indeed the fish. Prior to the use of the AT algorithm on our experimental fish, the AT was validated (see Section S2.6.1) to ensure reliable results.

Table 2.6. Behavioral metrics of the locomotor and exploratory behaviors following the novel tank diving test computed using an automated tracking Python script

Behavioral metrics	Units	Description
Latency middle third	s	Delay before entering the middle third of the tank
Latency top half	s	Delay before entering the top half of the tank
Latency top third	s	Delay before entering the top third of the tank
Transitions		Number of times the fish crossed into the top half of the tank
Time middle third	s	Total time spent in the middle third of the tank
Time top third	s	Total time spent in the top third of the tank
Distance middle third	cm	Total distance spent in the middle third of the tank
Distance top third	cm	Total distance spent in the top third of the tank
Total distance	cm	Total distance traveled around the tank
Max speed	cm·s ⁻¹	Maximum speed reached by the fish

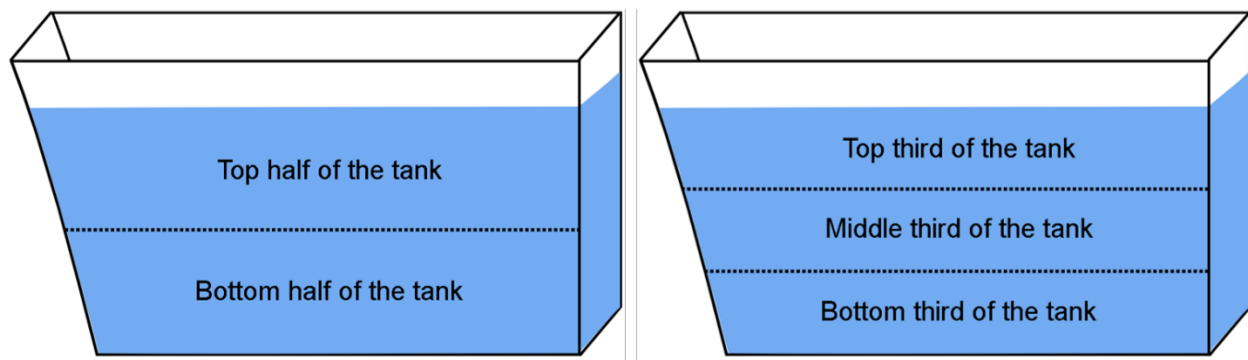


Fig. 2.6. The horizontal virtual divisions of the novel tank diving test used to analyze the locomotor and exploratory behaviors of the fish. The left panel represents the divisions with two zones referred to a bottom half and top half of the tank. The right panel shows the three virtual divisions referred to as bottom third, middle third and top third of the tank.

2.5.8. Transcriptomics

Whole-transcriptome sequencing (RNA-Seq) was performed on adult male kidneys of the F₀ and F₃ generations that were individually collected, flash frozen on dry ice and stored at -80°C . Total RNA was extracted using the RNeasy Plus Micro kit (Qiagen) and its integrity assessed using an Agilent Bioanalyzer 2100. The biological replicates sequenced in each lineage ranged from 5 to 8 individual males. Stranded mRNA libraries were built using the TruSeq Stranded mRNA library Prep kit for NeoPrep (Illumina) with 50 ng of total RNA. Libraries were pooled and sequenced on the NextSeq 500 with a 75 cycle flowcell, from which an average of 36 million reads per sample were obtained. Differential gene expression analyses were performed as follows: BCL files were converted for FASTQ files with BCL2FASTQ from Illumina and then CutAdapt (Martin, 2011) was used to trim adapters and polyGs tails. The STAR (Dobin et al., 2013) aligner was used to map the reads on ZF (GRCz10 Ref 86) from Ensembl and the quality assessment/quality control of the libraries was evaluated with Qorts (Hartley and Mullikin, 2015). Approximately 91.0% of the reads could be mapped to the genome, of which 12% mapped reads

were outside of annotated genes and 1% could not be unambiguously assigned to one unique transcript. DEGs were identified from the remaining ~28.5 million reads per sample using the edgeR package (Robinson et al., 2010) and the normalization procedure used in TMM (Robinson and Oshlack, 2010). Biological outliers have a global effect on the differential analysis, reducing the robustness of the study. Therefore, to adjust for the influence of outliers on the DEGs, a model was generated using the function to estimate dispersion “estimateGLMRobustDisp”, which consists of assigning a weight to each observation and the observations that strongly deviate from the model fit are assigned lower weights. This approach dampens the influence of outliers on the differential analysis while maintaining the robustness of the study (Zhou et al., 2014). Finally, two separate generalized linear models of the DEGs were built for F_0 and F_3 to perform the analysis. Statistical significance was evaluated using the criteria of $P\text{-value} < 0.05$.

2.5.9. Gene Network and Pathway analyses

DEGs were further analyzed for biological relevance using the IPA software (Ingenuity Systems). The corresponding human orthologs of the ZF genes from the DEG dataset were identified in the Ingenuity’s Knowledge Base using HomoloGene as the reference database. A total of 11,176 (63%) probes were successfully mapped by IPA and for duplicated probes, the highest differential expression value was used for downstream analysis. Right-tailed Fisher’s exact tests were used to compute P -values to determine the probability that each enriched biological function and canonical pathway (i.e. well-characterized metabolic and cell signaling pathways) assigned to that data set might be due to random chance. A $P\text{-value} < 0.05$ was considered statistically significant.

The Gene Set Enrichment Analysis algorithm was also applied to the data set using Pathway Studio 10.0 (Ariadne) operating with the ResNet 11.0 database (Mammals). The corresponding human homologs of the ZF genes were identified using Ensembl (<http://www.ensembl.org>). If a human homolog could not be identified, the gene was not included in the pathway analysis. A total of 13,303 (74%) unigenes were successfully mapped in Pathway Studio and for duplicated probes, the option of “best *P*-value, highest magnitude fold change” was used. A Kolmogorov–Smirnov test with 1000 permutations was performed to determine whether specific gene sets were preferentially regulated compared to the background reference probability distribution. The gene set categories examined for enrichment included curated Ariadne cell processes, cell signaling and receptor signaling pathways. The enrichment *P*-value for a gene-set was set at $P < 0.05$.

2.5.10. Gene expression analysis of cortisol- and lipid-related genes by qRT-PCR

qRT-PCR was used to measure the relative expression levels of 10 genes. cDNA was synthesized using the QuantiTect Reverse Transcription kit (Qiagen) according to the manufacturer’s protocol. qRT-PCR was performed using the Rotor-Gene SYBR Green PCR kit (Qiagen) and the thermal cycling conducted in a Rotor-Gene Q real-time PCR cycler (Qiagen). Primers (Table 3.1) were designed using the online software Primer-BLAST (Ye et al., 2012) and validated by sequencing their amplicon to verify target specificity and by determining their efficiency ($100 \pm 10\%$, $R^2 > 0.98$). Melting curve analysis was conducted at the end of the qRT-PCR protocol between 60 to 95 °C with an interval of 0.5 °C. Samples were run in technical triplicates and their absolute abundance was calculated based on standard curves using Rotor-Gene

Q Series Software 2.0.3 (Qiagen). The mRNA absolute abundance was normalized using the NORMA-GENE algorithm (Heckmann et al., 2011).

Table 2.7. List of primer sets (in alphabetical order) used to conduct qRT-PCR on male ZF kidney from the F₀ and F₃ generations

Gene symbol	Gene name	Primer sequence (5'→3')	GenBank ID
<i>ctgfa</i>	connective tissue growth factor a	F: GAAAGTGCCTGGGAAGTGCT R: TCCTCCTCTCTGTAAGCTGCTA	NM_001015041.2
<i>cyp11a1</i>	cytochrome P450 family 11 subfamily A polypeptide 1	F: CATTCCAGCAGGGACTTTAG R: CAGCGAGAGGGACAGTAT	NM_152953.2
<i>dhcr7</i>	7-dehydrocholesterol reductase	F: ATGACCTCTGGGTTTTGGGG R: TCCACAGGCCAAACAGTACG	NM_201330.1
<i>lcat</i>	lecithin-cholesterol acyltransferase	F: TGTGGGACGACACCAGAAAC R: AGTCCTACCCCATACAGGCA	NM_001324407.1
<i>lss</i>	lanosterol synthase (2,3-oxidosqualene-lanosterol cyclase)	F: AGAGTGCCTTACACAAGCCC R: GGGAAAGCCTCCCTTGTTC	NM_001083567.1
<i>namptb</i>	nicotinamide phosphoribosyltransferase b	F: AGTGCTTCCGTCATACCTGC R: CACTCCAACCCTCATCGCTC	NM_212668.2
<i>pmvk</i>	phosphomevalonate kinase	F: GAGTCCGAGTGTGGTTTGG R: TGACCGATGACAGAAGCTCG	NM_001083579.1
<i>rb1</i>	retinoblastoma 1	F: AGTCGCCCTACAATTCGGTG R: CAGGACGGGGTTTGCTTTTG	NM_001077780.1
<i>star</i>	steroidogenic acute regulatory protein	F: AGCCCTTGTTCAAGTCAGACG R: TGGCAAAGTGGAGGTGACAG	NM_131663.1
<i>sts</i>	steroid sulfatase (microsomal), isozyme S	F: GGCATGTTTCCCACTCA R: CTGTTGGGTCTTTGGATAGG	XM_005168397.4

2.5.11. Statistical analyses

Statistical analyses were conducted using SigmaPlot 11.0 (Systat Software, Inc.). Data sets were examined for normality and homoscedasticity prior to hypothesis testing. Cortisol data were expressed as the mean $\pm$ standard error of the mean (SEM) and analyzed using two-way analysis of variance (ANOVA), except for Fig. 2.3D where the analysis was conducted with a one-way ANOVA. Box-Cox (Box and Cox, 1964) transformation was applied when the cortisol data were not normally distributed. Alternatively, ANOVA on ranks indicated in each graph where applicable, were used when the distribution of the data was non-Gaussian even after undergoing transformations. The behavioral data set (PC1 scores) was presented in box plots and analyzed using the Student's *t*-test or Mann-Whitney *U* Test (for non-normally distributed data). Significance was set at $P < 0.05$. To compare significance within the groups, the analysis was followed by a Tukey's post-hoc test.

2.6. Supporting information

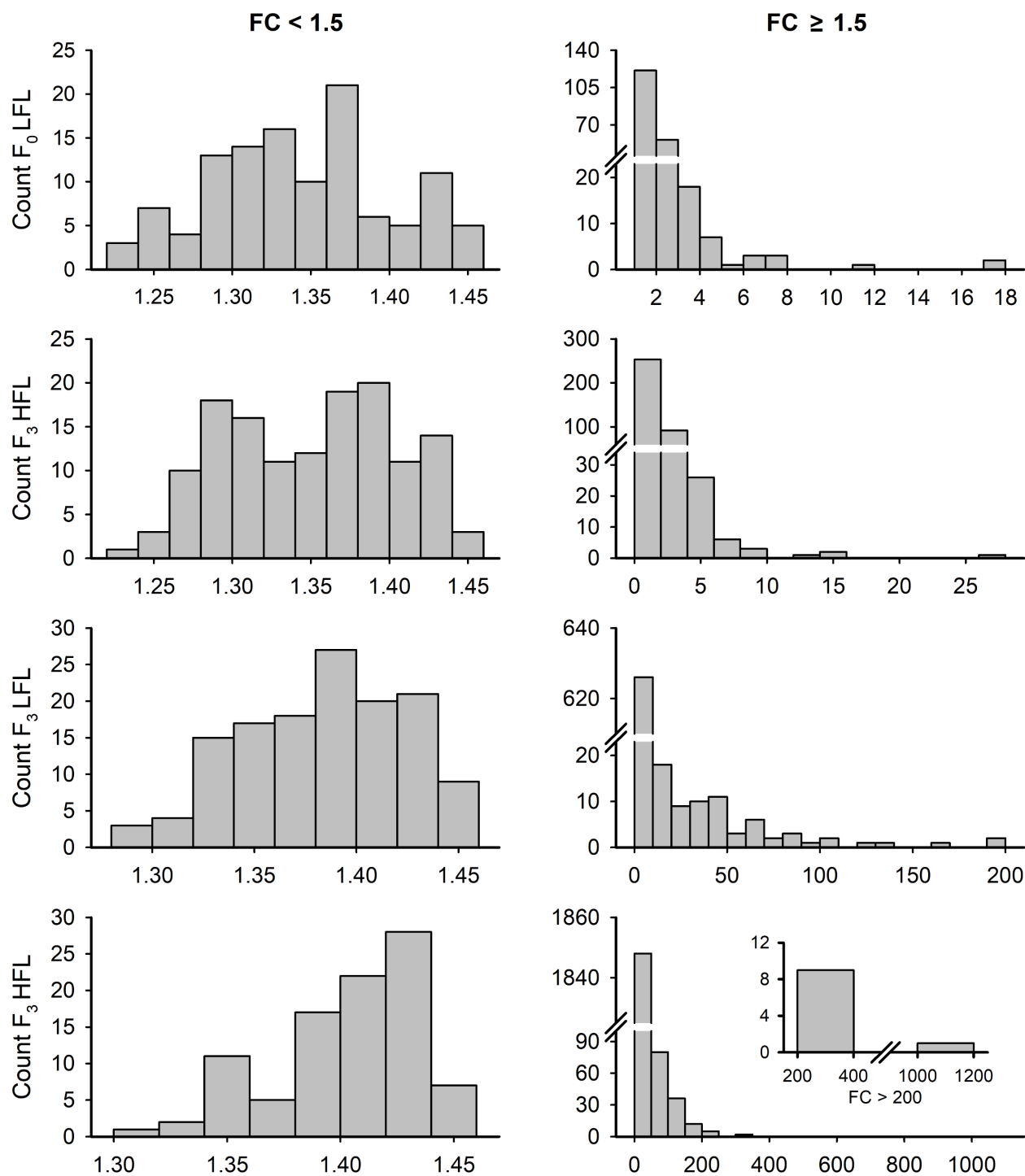


Fig. S2.1. Distribution of the DEGs according to their fold change (FC) across the FLX lineages (LFL, Low-FLX lineage; HFL, High-FLX lineage) from the F₀ and F₃ generations.

Table S2.8. Median fold change of enriched pathways following analysis using Pathway Studio ($P < 0.05$)

Enriched pathways	F ₀		F ₃	
	LFL	HFL	LFL	HFL
Biosynthesis of cholesterol	NA	0.48	0.49	0.19
Cholesterol catabolism	NA	2.73	NA	NA
Cholesterol export	2.14	NA	NA	0.42
Steroidogenesis	0.50	NA	2.05	0.38

NA, not available; pathway no detected.

Fig. S2.2. Effects of exposing the F₀ to FLX on sex steroid levels of ZF across generations. The data from total testosterone levels (*Left*), 11-ketotestosterone (*Middle*) and 17 β -estradiol levels (*Right*) are displayed as the mean $\pm$ SEM. $n = 12 - 19$ fish per group, two-way ANOVA (on ranks for: Testosterone in the F₃; 11-ketotestosterone in the F₁; 17 β -estradiol in the F₀, F₁ and F₃), $P < 0.05$. The letters represent statistical difference when interactions are present. P -values shown above the bars represent significant differences compared to the females (within sex). The asterisks (*) represent significant difference within FLX treatments: * $P = 0.05$ compared to CTR, ** $P < 0.01$ compared to CTR, *** $P < 0.001$ compared to CTR and # $P = 0.019$ compared to LFL.

Table S2.9. Two-way ANOVA on the effects of sex and FLX treatment on total testosterone levels in ZF from the CTR and FLX lineages

Generations	Variables	DF (Residual)	F	P -value
F ₀	SEX	1 (88)	50.673	<0.001
	FLX	2 (88)	0.0332	0.967
	SEX $\times$ FLX	2 (88)	2.107	0.128
F ₁	SEX	1 (90)	3.882	0.052
	FLX	2 (90)	2.899	0.060
	SEX $\times$ FLX	2 (90)	2.540	0.084
F ₂	SEX	1 (89)	12.302	<0.001
	FLX	2 (89)	5.925	0.004
	SEX $\times$ FLX	2 (89)	17.964	<0.001
F ₃	SEX	1 (98)	46.446	<0.001
	FLX	2 (98)	9.936	<0.001
	SEX $\times$ FLX	2 (98)	31.049	<0.001

DF, degrees of freedom

P -values in bold were determined by two-way ANOVA on ranks.

Table S2.10. Two-way ANOVA on the effects of sex and FLX treatment on total 11-ketotestosterone levels in ZF from the CTR and FLX lineages

Generations	Variables	DF (Residual)	F	<i>P</i> -value
F ₀	SEX	1 (86)	3276.212	<0.001
	FLX	2 (86)	4.918	0.010
	SEX × FLX	2 (86)	2.042	0.136
F ₁	SEX	1 (89)	329.461	<0.001
	FLX	2 (89)	11.749	<0.001
	SEX × FLX	2 (89)	0.632	0.532
F ₂	SEX	1 (87)	5052.299	<0.001
	FLX	2 (87)	1.808	0.170
	SEX × FLX	2 (87)	0.620	0.540
F ₃	SEX	1 (99)	5562.604	<0.001
	FLX	2 (99)	1.463	0.236
	SEX × FLX	2 (99)	7.718	<0.001

DF, degrees of freedom

P-values in bold were determined by two-way ANOVA on ranks.

Table S2.11. Two-way ANOVA on the effects of sex and FLX treatment on total 17 β -estradiol levels in ZF from the CTR and FLX lineages

Generations	Variables	DF (Residual)	F	<i>P</i> -value
F ₀	SEX	1 (90)	168.127	<0.001
	FLX	2 (90)	4.635	0.012
	SEX $\times$ FLX	2 (90)	0.184	0.832
F ₁	SEX	1 (92)	81.004	<0.001
	FLX	2 (92)	12.744	<0.001
	SEX $\times$ FLX	2 (92)	5.506	0.006
F ₂	SEX	1 (87)	485.495	<0.001
	FLX	2 (87)	13.305	<0.001
	SEX $\times$ FLX	2 (87)	5.845	0.004
F ₃	SEX	1 (99)	278.596	<0.001
	FLX	2 (99)	4.201	0.018
	SEX $\times$ FLX	2 (99)	0.246	0.783

DF, degrees of freedom

P-values in bold were determined by two-way ANOVA on ranks.

2.6.1. Validation of the AT script

Thirty-one adult ZF fish of the AB strain, 16 females and 15 males, were used for the validation procedure of the AT algorithm. The 31 fish were subjected to the novel tank diving test and recorded for 6 min as described in section 2.5.7. The videos were then tracked using our developed AT algorithm and for comparison and data validation, the videos were also manually tracked using Logger Pro 3.13 (Vernier Software & Technology). Logger Pro is a data-collection and analysis software that allows for a frame by frame video analysis.

Tracking was conducted every 30 frames·s⁻¹ beginning after the first 90 frames following the release of the fish from the net and with the fish positioned at the bottom of the tank, and proceeded to the end of the video (10,800 frames; 6 min). The videos were first cropped using a Python script to ensure consistency of the video content for both tracking algorithms, the AT and the manual tracking (MT). Eighteen behavioral metrics were computed from the data (coordinates) acquired from the AT and MT (Table S2.12).

To validate the AT algorithm, the percent difference of each of the 18-behavioral metrics acquired from the AT and MT was calculated (Table S2.12). The percent difference between both tracking systems ranged from 0.2 to 8.0%, with the highest variability observed in the maximum speed parameter. To reduce the dimensionality of the data and to further analyze these two data sets, PCA was performed using the 18-behavioral metrics obtained from the AT and MT. Female and male data sets were collectively analyzed for PC analysis. PC1 and PC2 strongly loaded most of the behavioral metrics and explained 56% and 23% of the behavioral variance, respectively (Table S2.13; Fig. S2.3A). Statistical analysis on PC1 and PC2 scores showed no significant difference between the data sets obtained from the AT and MT in neither females (Fig. S2.3B) nor males (Fig. S2.3C).

Table S2.12. The percent differences of the complete list of behavioral metrics computed from the automated tracking (AT) Python script compared to the manual tracking conducted using Logger Pro.

Behavioral metrics	Units	Description	% Difference
Latency middle third	s	Delay before entering the middle third of the tank	5.3
Latency top half	s	Delay before entering the top half of the tank	1.7
Latency top third	s	Delay before entering the top third of the tank	0.9
Transitions		Number of times the fish crossed into the top half of the tank	4.0
Time bottom third	s	Total time spent in the bottom third of the tank	0.3
Time middle third	s	Total time spent in the middle third of the tank	2.3
Time top third	s	Total time spent in the top third of the tank	4.9
Time bottom half	s	Total time spent in the bottom half of the tank	0.2
Time top half	s	Total time spent in the top half of the tank	2.4
Distance bottom third	cm	Total distance spent in the bottom third of the tank	3.4
Distance middle third	cm	Total distance spent in the middle third of the tank	4.6
Distance top third	cm	Total distance spent in the top third of the tank	6.7
Distance bottom half	cm	Total distance spent in the bottom half of the tank	3.4
Distance top half	cm	Total distance spent in the top half of the tank	6.0
Total distance	cm	Total distance traveled around the tank	3.5
Entry duration	s	Average time the fish spent in the top half of the tank	3.9
Mean speed	cm·s ⁻¹	Average speed of the fish	2.8
Max speed	cm·s ⁻¹	Maximum speed reached by the fish	8.0

Table S2.13. Loadings and contributions of the behavioral metrics to PC1 and PC2

Behavioral metrics	PC1		PC2	
	Loadings	Contribution (%)	Loadings	Contribution (%)
Latency middle third	-0.167	2.8	-0.303	9.2
Latency top half	-0.194	3.8	-0.233	5.4
Latency top third	-0.209	4.4	-0.115	1.3
Transitions	0.284	8.1	0.003	0.0
Time bottom third	-0.3	9.0	0.125	1.6
Time middle third	0.292	8.5	-0.072	0.5
Time top third	0.273	7.4	-0.206	4.2
Time bottom half	-0.291	8.5	0.172	3.0
Time top half	0.291	8.4	-0.172	3.0
Distance bottom third	0.054	0.3	0.453	20.5
Distance middle third	0.293	8.6	-0.017	0.0
Distance top third	0.264	7.0	-0.175	3.0
Distance bottom half	0.124	1.5	0.431	18.5
Distance top half	0.291	8.4	-0.137	1.9
Total distance	0.218	4.8	0.322	10.3
Entry duration	0.191	3.6	-0.206	4.3
Mean speed	0.22	4.8	0.319	10.2
Max speed	0.035	0.1	0.172	3.0

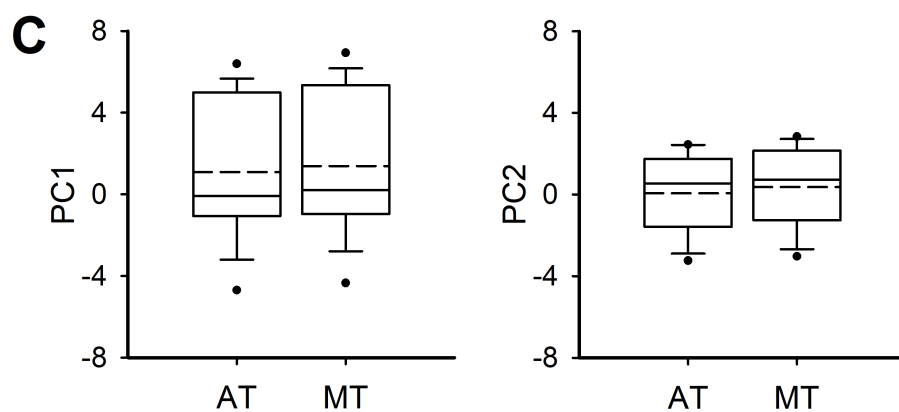
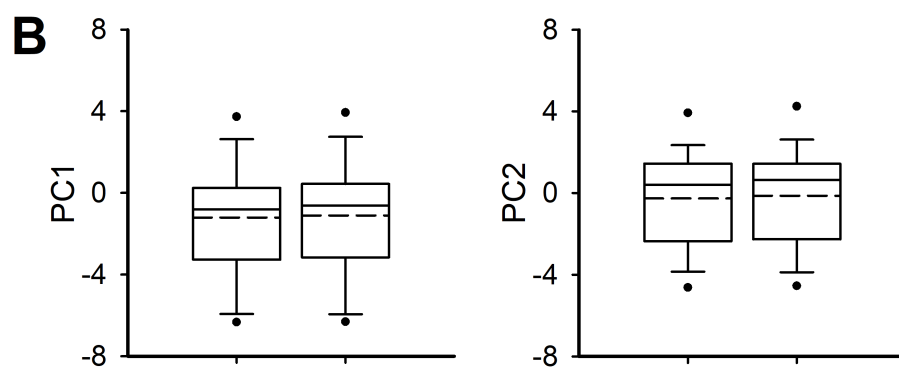
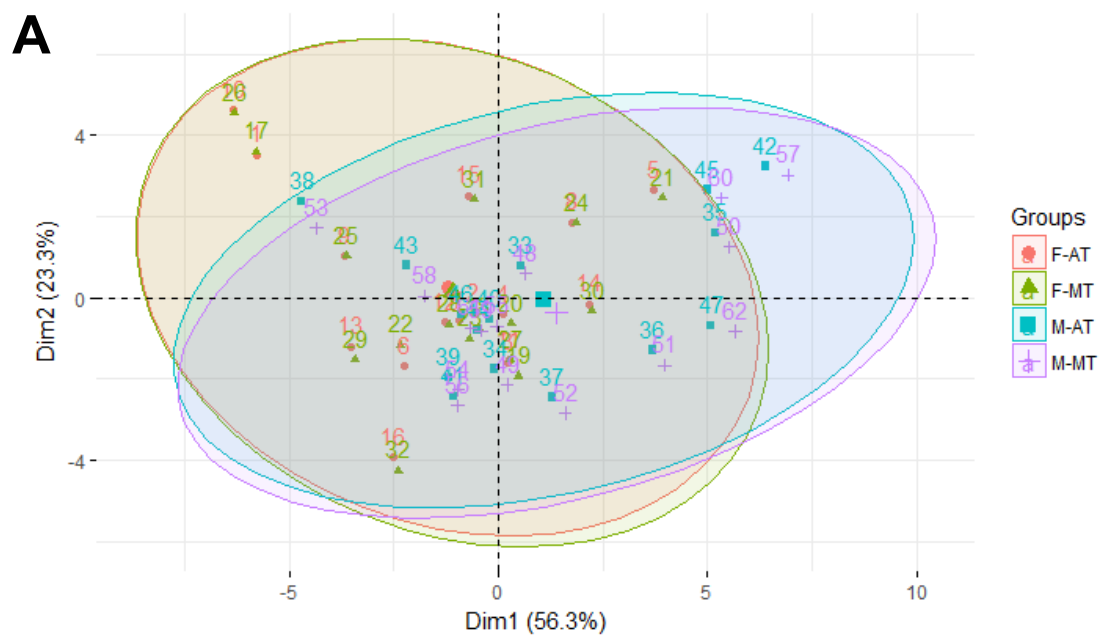


Fig. S2.3. Principal component analysis of the 18-behavioral metrics generated from tracking the behavioral response of adult ZF fish to the novel tank diving test using the AT and MT algorithm. (A) Venn diagram illustrating PC1 (Dim1) and PC2 (Dim2) of the behavioral response of the females and males acquired from the two-different tracking software. (B) Box plot representation of the PC1 (*Left*) and PC2 (*Right*) of the females behavioral response. (C) Box plot representation of the PC1 (*Left*) and PC2 (*Right*) of the males behavioral response. $n = 16$ females and 15 males.

2.6.2. Differentially expressed genes in each pathway

Table S2.14. Differentially expressed genes of the top significant canonical pathways in the kidney of males from the F₀ and F₃ FLX lineages compared to controls

Canonical pathways	F ₀		F ₃	
	LFL	HFL	LFL	HFL
Antiproliferative Role of TOB in T Cell Signaling	TGFB1, TOB1, CDKN1B	CCNA2, CCNE2, CDK2	NA	RB1, TGFB1, CUL1, SMAD4, TOB1, CDKN1B
Calcium Signaling	NS	MYH4, CALR, MYL6, RYR3, TPM1, TPM4, ACTC1, ACTA1, EP300	MYH4, TNNI2, TNNC2, ATP2B1, TNNT3, NFATC4, MYL1, ATP2A1, EP300, HDAC6, CASQ1, RYR3, ATF4, TPM4, ACTC1, ATP2B4	PRKACB, MYH4, TNNT1, CAMK4, CAMK1D, ATP2B1, GRIA1, Tpm1, CABIN1, ATP2A1, EP300, CAMK2D, NFAT5, MAPK3, RYR3, TPM4, CHRNA5, ACTA1, GRIN3B, HDAC4, CREB3, CHP1, CREBBP, PPP3CC, MYL9, NFATC2, SLC8A1,

				CAMKK2, CAMK2G
Caveolar-mediated Endocytosis Signaling	NS	ITGA11, HLA-B, FLOT1, ACTG2, ACTC1, ACTA1, EGFR	ITGA2B, INS, HLA-B, ITGA6, ACTC1, ACTG1, ITGAL	ITGB1, ITGAE, RAB5C, ITGAM, FLNC, HLA- A, ITGA11, HLA-B, COPE, ITGAV, COPG2, INSR, ACTA1
EIF2 Signaling	MYCN, PTBP1, EIF3F, EIF5B, RPL37, ACTC1, ACTA1, RPL13A, EIF3M	NS	ATF3, RPL7L1, EIF2S1, HSPA5, XIAP, VEGFA, FGFR4, EIF4A1, INS, EIF5, ATF4, AGO1, ACTC1, EIF3L, EIF1AY	RPL11, RAF1, RPS27, PIK3R1, PIK3R5, HRAS, Rpl22l1, HSPA5, CCND1, MYC, VEGFA, SHC1, RPL14, PPP1CC, KL, FGFR4, MAPK3, RPL21, IRS2, ACTA1, PIK3C2B, NRAS, GRB2, FGFR1, RPL30, RPL37A, XIAP, FAU, EIF4A3, AGO3, EIF4A1, RPS27A, RPL5, RPS15A, RPS25, INSR, EIF2AK2, AGO4, EIF3K
Epithelial Adherens Junction Signaling	NS	MYH4, MYL6, ACTG2, ACTN4, TUBB, PARD3, ACTC1, ACTA1, CLIP1,	MYH4, MAGI1, TUBB4B, RHOA, RAC1, Actn3, TUBA4A, ACTC1,	EPN3, TUBA1B, MYH4, ACTR2, TGFB1, NRAS, ARPC1B, TUBB4B, ARPC5L,

		TUBB2B, EGFR	ACTG1, MYL1	FGFR1, HRAS, ACVR2B, TUBB2B, MYL9, CTNNA2, ACTR3, TUBB4A, MAGI2, NOTCH1, ACTA1, ARPC4, ACVR2A, FARP2, CTNND1
				PRKACB, RAC2, RAF1, PIK3R1, SRF, PIK3R5, HRAS, TLN1, EP300, BRAF, MYC, PTK2, SHC1, PPP1CC, KL, MOS, FGFR4, MAPK3, IRS2, PLA2G12A, ITGB1, PIK3C2B, NRAS, PAK6, GRB2, YWHAB, FGFR1, VRK2, PLA2G4C, CREB3, CREBBP, STAT3, MKNK2, DOCK1, PPP2CB, PLA2G4A, ARAF, DUSP1, PRKCD, DUSP4
ERK/MAPK Signaling	NS	MYC, TLN2, PAK6, PPP2R3A, DUSP1, DUSP4, PIK3CB, IRS2, EIF4E, EP300	PPP2CA, VRK2, PLA2G4C, RAC1, MKNK2, EP300, FOS, PPP1R3D, ELF3, PPP1R10, ETS2, PRKCD, FGFR4, ATF4	
ILK Signaling	NS	MYH4, MYL6, EP300, MYC, PPP2R3A, IRS2, PIK3CB,	MYH4, PPP2CA, RHOC, Actn3, RICTOR, RHOH, MYL1,	MAP2K6, MYH4, PIK3R1, PIK3R5, GSK3A, CCND1, PDGFC,

Integrin Signaling

NA

ILKAP,
ACTG2,
ACTN4,
NOS2,
ACTC1,
ACTA1

EP300,
VEGFA,
FOS, RHOG,
FGFR4,
RHOA,
ATF4,
KRT18,
ACTG1,
ACTC1,
MMP9

EP300, MYC,
PTK2,
VEGFA,
RHOG, JUN,
KL, RHOT1,
FGFR4,
MAPK3,
ILKAP, IRS2,
NOS2,
ACTA1,
TESK1, DSP,
ITGB1,
PIK3C2B,
RHOC, GRB2,
FGFR1,
CREB3,
CREBBP,
FERMT2,
VIM, MYL9,
DOCK1,
PPP2CB,
RHOF, FLNC,
MMP9

RAC2, RAF1,
ARPC1B,
PIK3R1,
PIKFYVE,
PIK3R5,
HRAS, TLN1,
NCK1, BRAF,
PTK2, ITGAE,

ITGA2B,
MPRIIP,
RHOC,
RAC1,
Actn3,
ITGA6,
TTN,
RHOH,
ITGAL,
ARF6,
RHOG,
RHOA,
FGFR4,
ACTC1,
ACTG1

TLN2,
PAK6,
ITGA11,
ILKAP,
PIK3CB,
IRS2,
ACTG2,
ACTN4,
ACTC1,
ACTA1

SHC1,
TSPAN3,
ARF6, RHOG,
ACTR3, KL,
ITGA11,
RHOT1,
ARF4,
FGFR4,
MAPK3,
ITGAV,
ILKAP, IRS2,
ACTA1,
CAPN10,
CAPN5,
ITGB1,
PIK3C2B,
ACTR2,
NRAS, PAK6,
ARPC5L,
RHOC, GRB2,
FGFR1,
MYL9,
DOCK1,
RHOF,

				ITGAM, TSPAN1, CTTN, ARPC4
				HSD17B10, PSENEN, MT- CO2, VDAC2, NDUFS1, MT- CYB, MT- CO3, NDUFB6, MT-ND2, OGDH, MT- CO1, COX6B1, COX17, MT- ATP6, CPT1A, UCP2, ATP5A1, LRRK2, MT- ND4, VDAC3, APP, GSR, MT-ND1, MT- ND5, NDUFV2, UQCRC2, CYB5A, MT- ND3, UQCRC1
Mitochondrial Dysfunction	NDUFA4, ATP5C1, PRDX5, COX7A2, COX7A2L, VDAC1, VDAC3, VDAC2	NS	NDUFA4, FURIN, SDHB, ATP5H, ATP5D, ATP5L, VDAC2, PDHA1, ATP5F1, COX4I1, NDUFS4, ATP5J, ATP5G2, NDUFV1, NDUFB4, COX17, UCP2, ATP5A1, COX7A2L, VDAC3, ATP5C1, NDUFS8, ATP5B, CAT, CYC1, UQCRC2, COX5A, MT-ND3	
Pancreatic Adenocarcinoma Signaling	NAPEPLD, TGFB1, TYK2, CDKN1B, MMP9	PLD2, SUV39H1, PIK3CB, IRS2, BIRC5, PLD1, CDK2, EGFR	NS	RAF1, PLD2, TGFB1, SUV39H1, PIK3R1, PIK3R5, CDK4, PDGFC, CCND1, VEGFA, RB1, E2F6, KL, FGFR4, MAPK3, SMAD4, IRS2, ERBB2, PIK3C2B, GRB2, FGFR1, TYK2, STAT3, BCL2L1, CDKN1B,

				NOTCH1, MMP9
				ITGB1, PIK3C2B, NRAS, PAK6, GRB2, FGFR1, PIK3R1, PIK3R5, HRAS, TLN1, NCK1, MAPK11, ITGAE, PTK2, DOCK1, ARF6, ITGAM, ITGA11, KL, FGFR4, ITGAV, IRS2, ACTA1
Paxillin Signaling	NS	TLN2, PAK6, ITGA11, PIK3CB, IRS2, ACTG2, ACTN4, ACTC1, ACTA1	ARF6, ITGA2B, FGFR4, CSK, RAC1, Actn3, ITGA6, ACTC1, ACTG1, ITGAL	PRKACB, ARID1A, PIK3R1, MAPK11, EP300, VEGFA, LRAT, JUN, RARA, RDH16, RBP2, SMAD4, NR2F6, MAPKAPK2, SMAD1, RDH13, ZBTB16, ADCY2, NRIP2, ADCY3, CREBBP, ADCY6, SMARCD3, ADCY9, RBP7, SMARCA2, DUSP1, PRKCD, NCOR2, SMARCC1, HLTF
RAR Activation	ADCY9, RDH10, TGFB1, PRKAR1B, SMARCC2, RDH12, RDH13, ZBTB16, RBP4, GTF2H3	DUSP1, NRIP2, ALDH1A2, PIK3CB, NRIP1, SMAD1, PNPLA4, SMARCD3, RBP4, EP300, GTF2H3	NS	TUBA1B, ACTR2, RAB5C,
Remodeling of Epithelial Adherens Junctions	ACTC1, ACTG1,	ACTG2, ACTN4, TUBB,	NME1, ARF6, TUBB4B,	

Sirtuin Signaling Pathway

ACTA1, CLIP1	ACTC1, ACTA1, CLIP1, TUBB2B	Actn3, TUBA4A, ACTC1, ACTG1	ARPC1B, ARPC5L, TUBB4B, TUBB2B, ARF6, CTNNA2, ACTR3, TUBB4A, HGS, ACTA1, ARPC4, CTNND1
			TUBA1B, GABARAPL2, SUV39H1, ARG2, TOMM70, LDHB, VDAC2, MYC, NDUFS1, ATG9A, MAPK3, ZBTB14, PRKAA1, MT-ND2, PGK1, PFKFB3, CPT1A, HSF1, ATP5A1, PGAM2, STAT3, PCK1, AGTRAP, TIMM23B, TIMM17A, UQCRC2, MT-ND3, EPO, GADD45B, TIMM10, JUN, PCK2, MT-CYB, FOXO3, NDUFB6, NOS2, NFE2L2, PRKDC, MT- ATP6, EPAS1, UCP2, GLS, NQO1, MAPK6, G6PD, GLUD1, MT- ND4, SIRT3,
NDUFA4, ATP5C1, MYCN, HSF1, ATG4A, GLUD1, VDAC1, VDAC3, LDHB, VDAC2	NDUFA4, SIRT5, HSF1, SUV39H1, NDUFB3, ABCA1, MYC, MT- ND5, ATG4A, TSPO, HIST1H1T, SDHD, NOS2	NDUFA4, FOXO4, SDHB, ATP5D, TP73, PAM16, ARG2, LDHB, VDAC2, PDHA1, XPC, PRKAA1, MAP1LC3A, NFE2L2, ATP5F1, NDUFS4, PGK1, ATP5J, PRKDC, NDUFV1, NDUFB4, SLC25A4, UCP2, NDRG1, LDHD, ATP5A1, TUBA4A, SMARCA5, PGAM2, BAX, PCK1, VDAC3, ATP5C1, ATP5B, NDUFS8, UQCRC2, CYC1, MT- ND3, ATG101, LDHA, SLC25A5	

				BAX, VDAC3, APP, MT-ND5, MT-ND1, NDUFV2, GABARAP
				PIK3C2B, CAMK4, UCP2, GPS2, GRB2, PIK3R1, FGFR1, PIK3R5, PCK1, NCOA4, ATP2A1, EP300, SLC16A3, UCP3, COL6A3, KL, FGFR4, IRS2, G6PC, NCOR2, THRB
TR/RXR Activation	NA	ENO1, PIK3CB, G6PC, IRS2, THRB, FGA, EP300, NCOA4	F10, UCP3, UCP2, COL6A3, FGFR4, NCOR1, PCK1, THRB, DIO2, EP300, ATP2A1	

NA, not available; pathway not detected.

NS, not significant.

Table S2.15. Differentially expressed genes of key cortisol-related canonical pathways in the kidney of males from the F₀ and F₃ FLX lineages

Canonical pathways	F ₀		F ₃	
	LFL	HFL	LFL	HFL
Biosynthesis of cholesterol†	NA	CEL, LSS, SOAT2, SOAT1, SH3D19, MVD, CYP51A1, HSD17B7P2, HMGCR, MICAL2, MSMO1, NSDHL, MVK, TM7SF2, FDPS, VDR, MICAL3, FDFT1, STS, MICAL1, IDI1, HMGCS2, HMGCS2, COQ6, SC5D, DHCR7, PMVK, HMGCL, GGPS1, DHCR24	MSMO1, MICAL1, LSS, CYP51A1, SH3D19, VDR, CEL, GGPS1, MVK, DHCR24, MICAL3, SC5D, STS, PMVK, FDFT1, SOAT2, HMGCS2, HMGCS2, HMGCR, FDPS, SOAT1, MVD, DHCR7, HMGCL, MICAL2, TM7SF2, IDI1, NSDHL, COQ6, HSD17B7P2	VDR, LSS, SOAT1, DHCR7, MVK, STS, PMVK, SC5D, MICAL1, MICAL2, CYP51A1, CEL, DHCR24, FDFT1, MICAL3, HSD17B7P2, NSDHL, SH3D19, MVD, HMGCR, TM7SF2, SOAT2, MSMO1, HMGCS2, HMGCS2, IDI1, FDPS, COQ6, HMGCL, GGPS1
				ENPP6, PRKACB, RAF1, CAMK4, CAMK1D, PDE3A, EP300, BRAF, CAMK2D, MAPK3, GRM6, PDE4D, PTGER4, ADCY2, CREB3, ADCY3, CREBBP, ADCY6,
cAMP-mediated signaling	ADCY9, NAPEPLD, AVPR2, RAP1GAP, VIPR2, PRKAR1B, DUSP4, DRD3	NA	NA	

				RGS4, AKAP6, STAT3, PPP3CC, ADCY9, AVPR2, LPAR1, VIPR1, DUSP1, PDE5A, PDE8B, DUSP4, CAMK2G
Circadian Rhythm Signaling	NS	NS	NR1D1, CSNK1D, ATF4, EP300	PER3, GRIN3B, NR1D1, CRY2, CREBBP, CREB3, CRY1, PER2, EP300
Gas Signaling	ADCY9, AVPR2, VIPR2, RYSR3, PRKAR1B	NA	NA	PRKACB, ADCY2, CREBBP, CREB3, ADCY3, GNB5, ADCY6, EP300, GNB1, BRAF, ADCY9, AVPR2, ADD3, VIPR1, MAPK3, RYSR3, ADD1, PTGER4
Glucocorticoid Receptor Signaling	NS	NS	MAP3K14, VCAM1, KRT17, RAC1, PCK1, NFATC4, HSPA5, PTGES3, HSPA1L, EP300, FOS, HSPA4, FGFR4, PRKAA1,	PRKACB, RAF1, TGFB1, POLR2D, PIK3R1, PBX1, HRAS, HSPA5, HSPA4, IKBKB, IKBKG, FGFR4, MAPK3,

NCOR1, KRT18, PLAU, JAK3, KRT15, TAB1	PRKAA1, IRS2, TAB1, MAP3K14, PIK3C2B, FGFR1, HSPA9, CREBBP, TAT, PCK1, STAT3, PPP3CC, SMARCD3, BCL2L1, TAF1, SMARCA2, DUSP1, KRT15, ARID1A, GTF2F2, PIK3R5, MAPK11, HSPA1L, EP300, SHC1, NFKBIA, JUN, NFAT5, PCK2, KL, FOXO3, CEBPA, SMAD4, NOS2, FKBP5, AGT, MAP2K7, VCAM1, NRAS, KRT17, GRB2, CHP1, TSG101, POLR2G, TAF6L, VIPR1, NFATC2, NCOR2, SMARCC1, HLTF
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NA, not available; pathway no detected.

NS, not significant.

† This enriched pathway was obtained following gene set enrichment analysis in Pathway Studio.

2.7. Acknowledgments

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CHAPTER 3: Physiological disruptions and transgenerational alterations in the expression of stress-related genes in zebrafish larvae following exposure to fluoxetine

3.1. Statement of contribution

Marilyn Vera Chang, designed the study, developed the methodology, conducted the experiments and analyses and prepared the manuscript. Dr. Thomas Moon, helped with the design of the study and manuscript editing. Dr. Vance Trudeau, helped with the design of the study and manuscript editing.

3.2. Introduction

Fluoxetine (FLX), the active ingredient of the widely prescribed antidepressant Prozac® (Mishra et al., 2017), was designed to elicit its therapeutic effects by increasing the extracellular serotonin (5-HT) concentrations in the central nervous system (Frazer, 2001; Wong et al., 1995). As a result of its widespread use in the treatment of mental disorders including anxiety, panic disorder, and depression (Croen et al., 2011; Grzeskowiak et al., 2012), FLX is frequently detected worldwide in aquatic environments (Comber et al., 2018; Metcalfe et al., 2003b; Yang et al., 2017b) at concentrations ranging from 1 to 929 ng·L⁻¹ (Bueno et al., 2007; Kolpin et al., 2002; Metcalfe et al., 2003a). The physicochemical properties and photolytic stability of FLX render this active chemical persistent in the aquatic environment (Kwon and Armbrust, 2006; Lees et al., 2016) with limited environmental degradation (Benfield et al., 1986; Brooks, 2014; Hiemke and Hartter, 2000). The phylogenetic conservation of the 5-HT system and pathways across vertebrates (Gunnarsson et al., 2008; Mennigen et al., 2011) has raised concerns regarding FLX adverse effects in non-target aquatic organisms including fish (Boxall et al., 2012; Kreke and Dietrich, 2008).

The serotonergic system in vertebrates regulates neuroendocrine pathways related to mood, reproduction, stress, behavior and social interactions, among others (Azmitia, 1999; Brooks et al., 2003; Mennigen et al., 2011). During brain development, 5-HT is also involved in the programming of the stress axis, also known as the hypothalamic-pituitary-interrenal (HPI) axis in teleosts or HP-adrenal (HPA) axis in mammals (Andrews and Matthews, 2004). The HPI/A axis is critical for an individual to deal with adverse conditions by releasing cortisol (Barton, 2002). The HPI/A axis is activated by any internal or external stimulus which induces the release of corticotropin-releasing factor (CRF) from the hypothalamus. This neuropeptide is the main regulator of the release of adrenocorticotrophic hormone (ACTH) from the anterior pituitary, which in turn induces the synthesis and release of cortisol from the interrenal cells in teleosts (Dores and Garcia, 2015; Mommsen et al., 1999) or from the adrenal cortex in mammals (Ramot et al., 2017).

Moreover, considering the susceptibility of the HPI/A axis to 5-HT overstimulation during neurodevelopment, FLX has the potential to shape and induce long-term alterations in the HPI/A response (Pawluski et al., 2012). In fact, we previously demonstrated that a 6-day early developmental exposure to FLX elicited a long-term disruption of the stress axis exhibited by a reduced cortisol production in adult zebrafish (ZF, *Danio rerio*) across 3 generations (Chapter 2). Therefore, here, we investigated whether this disruption in cortisol to generation 0 (F₀) larvae ZF can be induced by a shorter exposure period and whether FLX can alter the developmental timing of their HPI axis. We also evaluated the expression levels of key genes associated with the HPI axis at the end of the 6-day FLX exposure in the F₀ and compared this transcriptional profile with the larvae of the F₃ generation to better understand the transgenerational disruptive effects of FLX on the stress axis. The FLX concentrations used in our studies were environmentally relevant

(0.54 $\mu\text{g}\cdot\text{L}^{-1}$) and a concentration similar to that detected in the cord blood of pregnant women receiving FLX therapy (54 $\mu\text{g}\cdot\text{L}^{-1}$).

3.3. Materials and methods

3.3.1. Experimental animals

The control (CTR) and FLX lineage larvae from the F₀ and F₃ generations used in this study were generated as previously described in Chapter 2 section 2.5.1. Briefly, adult ZF of the AB strain were paired and allowed to interact and spawn for 1 h 45 min between 09h00 and 11h00. Fertilized eggs (F₀) were immediately collected and randomly assigned to glass petri dishes containing embryo medium alone (CTR) or supplemented with either one of two FLX (Sigma-Aldrich; Oakville, Ontario, Canada) concentrations, 0.54 and 54 $\mu\text{g}\cdot\text{L}^{-1}$, referred as LOW and HIGH treatments, respectively. At 3 hours post-fertilization (hpf), F₀ embryos were exposed for 6 days during a critical window of ZF brain development (Mueller and Wullimann, 2016) and maintained at 28 °C with daily embryo medium and FLX renewal. Concentrated stock solutions of FLX were prepared in the same water that was used for the embryo medium preparation, therefore, there was no need for vehicle control. Henceforth, no further chemical exposures were conducted in this study.

Each subsequent generation (F₁, F₂, and F₃) was produced from randomly selecting and mating 15 pairs (at 6 months post-fertilization, mpf) from each lineage (Fig. 3.1). Embryos from the F₁ to F₃ were collected over a 4-day period. Feeding of the larvae started at 6 days post-fertilization (dpf) with either Zebrafish Management Ltd. (Winchester, England) fry food diet or with No.1 crumble-Zeigler (Aquatic Habitats; Apopka, Florida) according to their developmental stages. The commercial feed in all stages from 16 dpf was supplemented with live *Artemia* nauplii

(Artemia International LLC; Fairview, Texas) once a day. Fish were maintained in 28.5 °C aerated, dechloraminated City of Ottawa tap water under a 14 h light:10 h dark photoperiod. All procedures were approved by the University of Ottawa Protocol Review Committee and are in agreement with the Canadian Council on Animal Care guidelines for the use of animals in research and teaching.

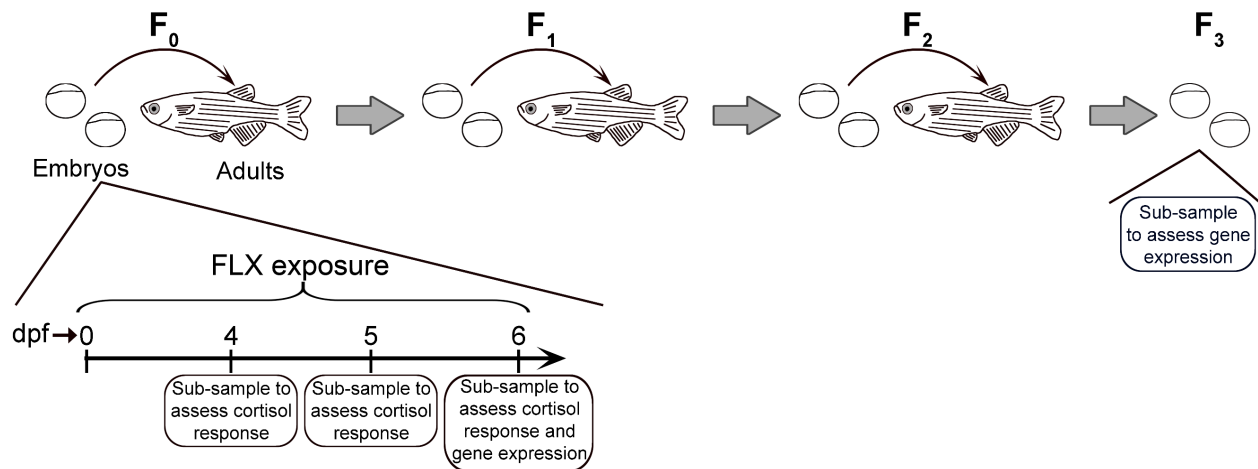


Fig. 3.1. A schematic representation of the generation of the CTR and FLX lineages and experimental design. The two FLX lineages were produced following a 6-day exposure of the F₀. A sub-sample of the F₀ larvae from the CTR and the two FLX treatments were collected at 4, 5 and 6 days of exposure to assess the HPI axis response to a standardized swirling stressor. At the end of the 6-day exposure, larvae were also collected to examine the expression levels of stress-related genes. The expression levels of the same genes were also quantified in larvae from the F₃. The spawning to acquire the F₃ larvae happened over a 4-day period, therefore, their age at the time of the sampling varied from 6 to 9 dpf.

3.3.2. Acute stress experiment and sampling

F₀ larvae (pools of 33 – 36 animals) from the CTR and FLX treatments were sampled at 96, 120 and 144 hpf for the assessment of their basal and stressed-induced cortisol levels. Basal levels were measured in immediately frozen collected larvae. The stress protocol was that of Alsop and Vijayan (2008). This consists of vigorously swirling the larvae in a glass petri dish for 30 s,

followed by a 5 min post-stress at 28 °C, collection and flash freezing of the larvae on dry ice. All samples were stored at -80 °C until analysis.

3.3.3. Cortisol extraction and quantification

Total cortisol levels from the collected larvae were extracted using a protocol modified from Folch et al. (1957). Briefly, pooled larvae were sonicated in 7.5 mL CHCl₃:MeOH (2:1 v/v) and following a 15-min incubation at room temperature, 2.5 mL 2 M KCl buffered with 5 mM EDTA were added to the homogenate, vortexed and incubated for an additional 20 min. The organic phase was then transferred to a clean glass tube, evaporated to dryness under a nitrogen stream while the tubes were heated at 45 °C and finally, the extract was reconstituted in ethylene glycol monomethyl ether and stored at -80 °C until cortisol levels were assessed.

Total cortisol concentration was measured by using a ¹²⁵I radioimmunoassay kit (MP Biomedicals; Solon, Ohio) according to the manufacturer's protocol followed by the measurement of the radioactive counts with a Wizard2 automatic gamma counter (PerkinElmer; Downers Grove, Illinois). The intra- and the inter-assay coefficient of variation (CV) were calculated to be 4 – 8% and 7 – 15%, respectively.

3.3.4. RNA extraction, cDNA synthesis and qRT-PCR

Larvae (pools of 15) from the F₀ and F₃ generations were collected for transcript level analysis of 15 genes associated with the HPI axis using Real-Time qPCR (qRT-PCR). The F₀ larvae were sampled at the end of the FLX exposure (6 dpf). The F₃ larvae were sampled on the same day at an age that varied from 6 to 9 dpf since they were acquired over a 4-day period. Total RNA was extracted using the RNeasy Plus Mini kit (Qiagen; Toronto, Ontario, Canada) and its

integrity assessed using an Agilent Bioanalyzer 2100. cDNA was subsequently synthesized using the QuantiTect Reverse Transcription kit (Qiagen; Toronto, Ontario, Canada) according to manufacturer's protocol. The Rotor-Gene SYBR Green PCR kit (Qiagen; Toronto, Ontario, Canada) was used to perform the qRT-PCR thermal cycling which was conducted in a Rotor-Gene Q real-time PCR cycler (Qiagen; Toronto, Ontario, Canada) according to the manufacturer's protocol. Primers (Table 3.1) were designed using the online software Primer-BLAST (Ye et al., 2012) and synthesized by Integrated DNA Technologies. Prior to their use, primer sets were validated by sequencing their amplicon to verify target specificity and by determining their efficiency ($100 \pm 10\%$, $R^2 > 0.98$). Melting curve analysis was conducted at the end of the qRT-PCR protocol between 60 to 95 °C with 0.5 °C increments to ensure a single amplified product. Samples were run in technical triplicates and their absolute abundance was calculated based on standard curves using Rotor-Gene Q Series Software 2.0.3 (Qiagen; Hilden, Germany). Absolute mRNA abundance was normalized using the NORMA-GENE algorithm (Heckmann et al., 2011).

Table 3.1. List of primer sets (in alphabetical order) used to conduct qRT-PCR in 6 – 9 dpf larvae from the F₀ and F₃ generations in ZF

Gene symbol	GenBank ID	Primer sequence (5'→3')
<i>crf</i>	BC085458.1	F: GCCGATTTCCTAGATCTGAC R: TCTTTGGCTGATGGGTTCG
<i>crfbp</i>	NM_001003459.1	F: CTAAAGCGAGAGTTACCAGAGG R: GATAACGTCAGTAGGTTCGCC
<i>crfr1</i>	XM_691254.6	F: CTGGGCTAAGAAAGGGAACCTAC R: TGAAGAGGATGAATGCGACC
<i>crfr2</i>	XM_002667848.6	F: TCAGGGATTCTTTGTGTCGG R: GTGGGTGATGTGGGAATGG
<i>cyp17a1</i>	NM_212806.3	F: TAGACCCACCACAGACCTTTAC

		R: GCCACTTAAACTCTGCAGACC
<i>cyp21a2</i>	XM_021474355.1	F: GCACCCTCAGTACAGTGACC R: CGCAGTCGTAACATCTCACTG
<i>hsd11b2</i>	NM_212720.2	F: GCCTATAAGACAGGGCAGAGC R: TCCAGAAGGTTTGGGGACAAG
<i>hsd3b2</i>	NM_212797.1	F: TCAGAGACCCGGAGAAAAGA R: AAGGTGACATGAGAGCATGG
<i>mc2</i>	AY161848.1	F: ACGCTGTTATTGATCCGGCA R: ACCGCTTGCAGATCCTTGAA
<i>mr</i>	EF567113.1	F: ATGTGCCCCACTTCGGCTATT R: TGTTCCCTTGCGCTCCAATCT
<i>mrp2a</i>	XM_001342887.7	F: CACCGATATTCCATTGTGATCGGC R: GTGGGGCTCCTGTTTTGGTC
<i>mrp2b</i>	XM_005168521.4	F: TATTCAAACCGCAGCCAAGC R: TCAAAGACACGGGCTCCTC
<i>nr3c1</i>	NM_001020711.3	F: CTAAAGCTCTGCCAGGGTTCC R: CCTCCAGCCCAGTCCAAAAG
<i>ff1b</i>	NM_131794.1	F: GCCCAATGTACAAGCGAGAC R: TCCCGTTAGCCTCCAGTTTG
<i>star</i>	NM_131663.1	F: AGCCCTTGTTCAAGTCAGACG R: TGGCAAAGTGGAGGTGACAG

3.3.5. Statistical analyses

Statistical analyses were performed using SigmaPlot 11.0 (Systat Software, Inc.). Data were expressed as the mean $\pm$ SEM and analyzed using either two-way analysis of variance (ANOVA) for cortisol assessment or one-way ANOVA for gene expression analysis followed by Tukey's post-hoc test. Data sets were first examined for normality and homogeneity of variance prior to hypothesis testing. Box-Cox (Box and Cox, 1964) transformation was applied when data

were not normally distributed. Alternatively, ANOVA on ranks, indicated in the caption of each graph where applicable, was used for data that was non-Gaussian distributed even after undergoing transformations. The significance level was set at $\alpha < 0.05$. A Grubbs test (Grubbs and Beck, 1972) was employed for the statistical outlier evaluation and extreme outliers were eliminated; the criterion of only one outlier per group was followed.

3.4. Results

3.4.1. HPI axis assessment

We investigated whether the disruption of the stress axis by FLX is induced earlier than the previously studied 6-day exposure by estimating the basal and stress-induced cortisol levels of the F₀ larvae following 4 days (at 96 hpf) and 5 days (at 120 hpf) FLX exposures (Fig. 3.2). We also measured cortisol levels at the end of the treatment (144 hpf) to determine if the cortisol reduction observed in adults exposed to FLX from 0 to 6 dpf (Chapter 2) was also exhibited as larvae (Fig. 3.2). A two-way ANOVA was conducted to examine the effects of the swirling stressor and FLX treatments on cortisol levels at the three different developmental stages: 96, 120 and 144 hpf. At both, 96 hpf and 120 hpf, the larvae did not respond to the swirling stressor ($F_{1,42} = 0.0220$, $P = 0.883$ and $F_{1,42} = 0.202$, $P = 0.655$, respectively). However, their basal cortisol levels were significantly lowered by FLX ($F_{2,42} = 6.648$, $P = 0.003$ and $F_{2,42} = 6.809$, $P = 0.003$, respectively) and this effect was similar under both basal and stress-induced conditions (no interaction, $F_{2,42} = 0.0283$, $P = 0.972$ and $F_{2,42} = 0.120$, $P = 0.887$, respectively). The HPI axis response to the swirling stressor was only activated at 144 hpf ($F_{1,41} = 26.884$, $P < 0.001$). As predicted, FLX significantly blunted the larvae total cortisol levels at 144 hpf ($F_{2,41} = 7.819$, $P = 0.001$) with no interaction driven by the stressor ($F_{2,41} = 0.0633$, $P = 0.939$).

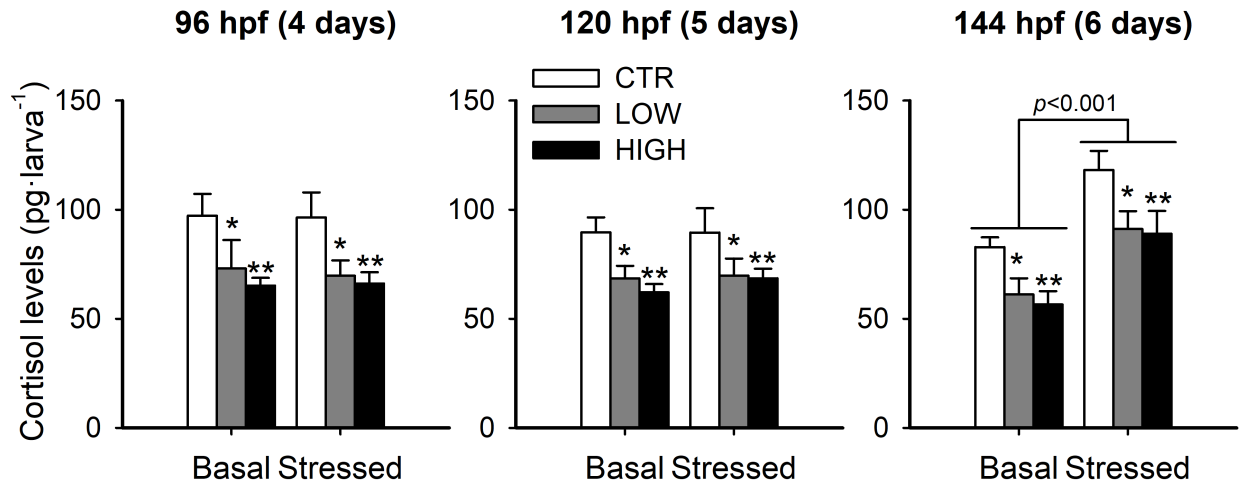


Fig. 3.2. The effects of FLX on cortisol levels of larvae ZF following exposure periods from 3 hpf to 96, 120 and 144 hpf. Embryos were collected and immediately exposed to one of the two concentrations of FLX; LOW, 0.54 and HIGH, 54 $\mu\text{g}\cdot\text{L}^{-1}$. At the end of each exposure period, larvae were subjected to a swirling stressor for 30 s and subsequently placed in a 28 °C incubator for 5 min before assessing cortisol levels. Data are expressed as mean $\pm$ SEM, $n = 7 - 8$ biological replicates (pools of 33 to 36 larvae) in each group, and analyzed by two-way ANOVA. Significant difference in the FLX group compared to the CTR are indicated by asterisks (*); ** $P < 0.005$ and * $P < 0.05$.

3.4.2. Expression analysis of selected genes associated with the HPI axis

We further evaluated the expression levels of genes related to the regulation of cortisol activity in the F_0 and F_3 larvae to gain general insights into the effects of FLX on the HPI axis. The transcript levels of *crf* and several genes involved in the regulation of CRF activity were unchanged in the F_0 (Fig. 3.3): *crf* ($F_{2,16} = 0.774$, $P = 0.478$), corticotropin-releasing hormone binding protein (*crfbp*; $F_{2,16} = 0.536$, $P = 0.595$) and corticotropin-releasing factor receptor type 2 (*crfr2*; $F_{2,15} = 0.423$, $P = 0.663$). In contrast, a one-way ANOVA revealed that *crfr1* levels were significantly different upon FLX exposure in the F_0 ($F_{2,16} = 8.197$, $P = 0.004$). A Tukey's post-hoc test showed that the *crfr1* expression levels were significantly reduced by 63% in the LOW

treatment compared to the CTR. Similar results were observed in the F₃ (Fig. 3.3) in which expression levels of CRF related genes were not altered by FLX: *crf* ($F_{2,13} = 0.0961$, $P = 0.909$), *crfbbp* ($F_{2,13} = 2.487$, $P = 0.122$) and *crfr2* ($F_{2,13} = 2.913$, $P = 0.090$). However, in contrast to F₀, *crfr1* gene expression levels were not different in the F₃ ($F_{2,13} = 2.964$, $P = 0.087$).

The transcript levels of nuclear receptor subfamily 5 group A member 1a, *nr5a1a* (*fflb*), the marker for interrenal development, did not change with FLX exposure in the F₀ ($F_{2,13} = 0.682$, $P = 0.523$), whereas a significant alteration was observed in the F₃ ($F_{2,12} = 16.766$, $P < 0.001$). A Tukey's post-hoc test revealed a significant increase of 526% in the *nr5a1a* (*fflb*) gene expression levels in the HIGH treatment compared to the CTR (Fig. 3.4). Furthermore, expression of the melanocortin type 2 receptor (*mc2r*), also known as the ACTH receptor, was significantly affected by FLX exposure in the F₀ ($F_{2,12} = 6.843$, $P = 0.010$) and F₃ ($F_{2,10} = 21.458$, $P < 0.001$) generations. In the F₀, the *mc2r* transcript levels were significantly increased by 162% in the LOW treatment, while in the F₃, a 480% increase was observed in the HIGH treatment compared to their respective CTR (Fig. 3.4). The complete functionality and activation of the MC2R require the presence of a melanocortin-2 receptor accessory protein (MRAP). The transcript abundance of *mrapp2a* was significantly altered by FLX in the F₀ ($F_{2,13} = 15.858$, $P < 0.001$) and F₃ ($F_{2,13} = 40.854$, $P < 0.001$) as determined by one-way ANOVA. The Tukey's post-hoc test revealed a 37% significant decrease in the *mrapp2a* gene expression levels in the HIGH treatment relative to the CTR in the F₀, whereas in the F₃, this transcript was significantly elevated by 63 and 173% in the LOW and HIGH treatment, respectively. Expression of *mrapp2b* was also found to significantly differ in the FLX-exposed larvae of both generations, F₀ ($H_2 = 11.368$, $P = 0.003$) and F₃ ($F_{2,12} = 11.531$, $P = 0.002$). In the F₀, the statistical effects were observed between the LOW and HIGH treated

larvae, while in the F₃, the HIGH treatment elicited an increase of 299% of the *mrp2b* transcript levels compared to the CTR (Fig. 3.4).

The expression levels of key genes involved in steroidogenesis were also investigated (Fig. 3.5). No significant effects upon FLX treatment to the F₀ were found in the transcript levels of steroidogenic acute regulatory (*star*) gene in F₀ ($F_{2,14} = 0.571$, $P = 0.578$) and F₃ ($F_{2,13} = 2.398$, $P = 0.130$) or of the hydroxy-delta-5-steroid dehydrogenase 3 beta- and steroid delta-isomerase 2 (*hsd3b2*) gene in F₀ ($F_{2,11} = 3.609$, $P = 0.062$). However, the cytochrome P450 family 17 subfamily A polypeptide 1 (*cyp17a1*; $F_{2,12} = 6.946$, $P = 0.010$) and cytochrome P450 family 21 subfamily A polypeptide 2 (*cyp21a2*; $F_{2,13} = 5.825$, $P = 0.016$) gene expression levels were significantly altered by FLX in the F₀. The LOW FLX treated larvae displayed a significant 298% increase in the *cyp17a1* transcript abundance compared to the CTR larvae and the HIGH FLX treated larvae had a 59% reduction in the *cyp21a2* transcript levels relative to the CTR. In the F₃, a statistical significant difference in the gene expression levels of *hsd3b2* ($F_{2,12} = 9.266$, $P = 0.004$), *cyp17a1* ($F_{2,12} = 5.576$, $P = 0.019$) and *cyp21a2* ($F_{2,8} = 9.638$, $P = 0.007$) were found across FLX treatments. A Tukey's post hoc test revealed that the alteration in the transcript levels were mainly exhibited by the larvae from the HIGH treatment in which 951, 264 and 396% elevation was observed in *hsd3b2*, *cyp17a1* and *cyp21a2* gene expression levels, respectively, compared to the CTR.

Cortisol activity is mediated by two receptors, the glucocorticoid receptor (GR), which is encoded by the nuclear receptor subfamily 3 group C member 1 (*nr3c1*) gene and by the mineralocorticoid receptor (MR), encoded by the *mr* gene (Fig. 3.6). Levels of *nr3c1* were not altered by FLX in any of the two generations, F₀ ($F_{2,16} = 2.174$, $P = 0.146$) or F₃ ($F_{2,13} = 1.648$, $P = 0.230$). However, *mr* transcript levels were significantly altered by FLX in the F₀

($F_{2,15} = 5.813$, $P = 0.014$) with a 38% decrease in the HIGH treatment compared to the CTR. In contrast, the *mr* transcript levels were unaffected in the F_3 ($F_{2,13} = 2.038$, $P = 0.170$). Cortisol is inactivated by the enzyme 11 β -hydroxysteroid dehydrogenase 2 (11 β -HSD2) encoded by the hydroxysteroid 11-beta dehydrogenase 2 (*hsd11b2*) gene; expression levels were found unaltered in the F_0 ($F_{2,16} = 2.221$, $P = 0.141$) but significantly affected in the F_3 ($F_{2,13} = 3.962$, $P = 0.045$) by FLX (Fig. 3.6). The effects in *hsd11b2* transcript abundance of the F_3 larvae were observed in the LOW treatment with a 143% increase compared to the CTR.

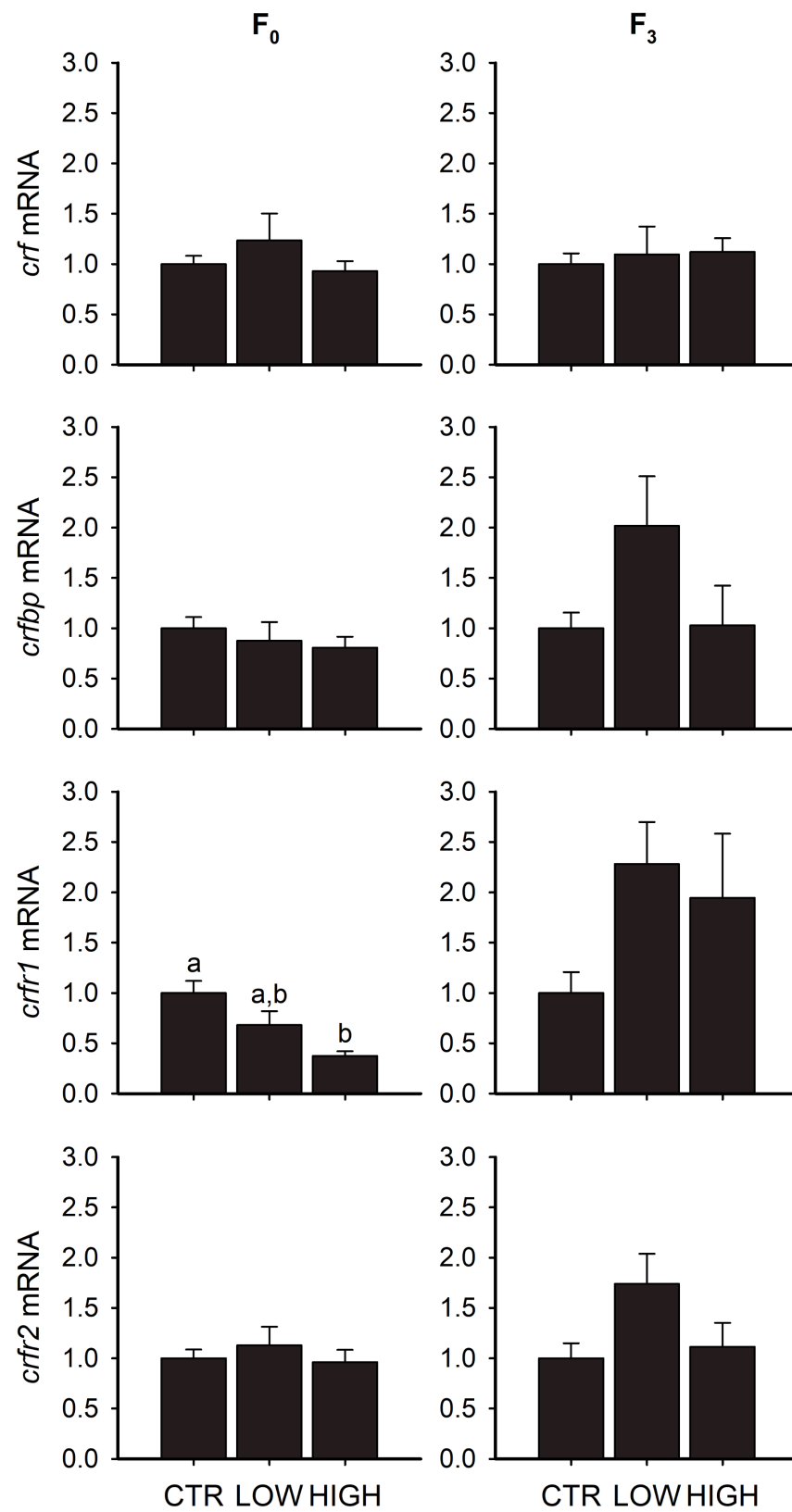


Fig. 3.3. Transcript levels of genes associated with corticotropin-releasing factor (CRF) activity regulation in 6 – 9 dpf ZF larvae from the CTR and FLX lineages in the generation F_0 (*Left*) and F_3 (*Right*). The FLX lineages were generated following a 6-day FLX exposure to the F_0 . Transcript levels were normalized to the CTR. Data are expressed as mean $\pm$ SEM and analyzed by one-way ANOVA, $P < 0.05$. $n = 5 - 7$ biological replicates per group in F_0 and $n = 4 - 6$ in F_3 . Each replicate consists of 15 pooled larvae.

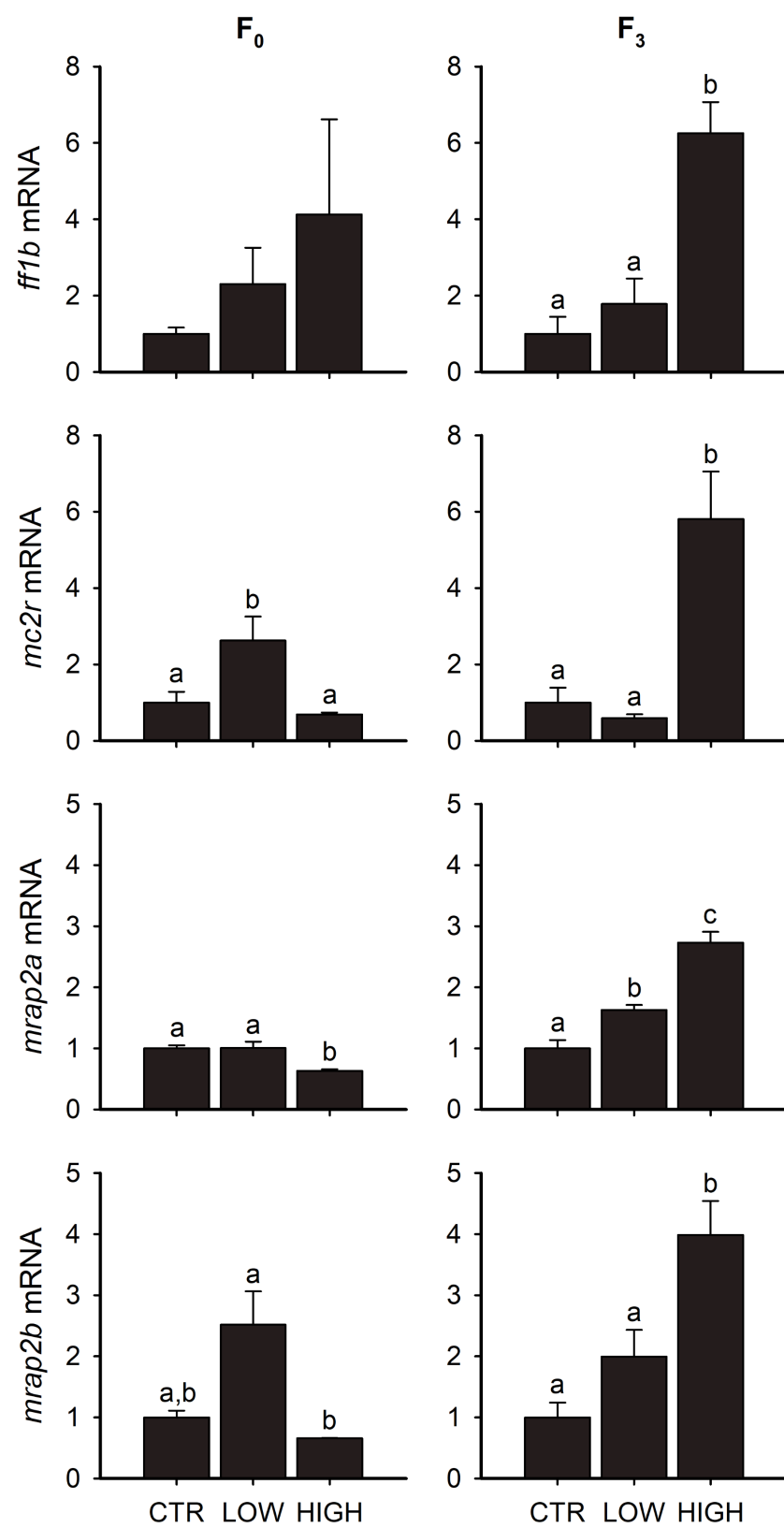


Fig. 3.4. Transcript levels of genes associated with adrenocorticotrophic hormone (ACTH) binding and interrenal development in 6 – 9 dpf ZF larvae from the CTR and FLX lineages in the generation F₀ (*Left*) and F₃ (*Right*). The FLX lineages were generated following a 6-day FLX exposure to the F₀. Transcript levels were normalized to the CTR. Data are expressed as mean ± SEM and analyzed by one-way ANOVA (on ranks for *mrap2b* mRNA from F₀), $P < 0.05$. $n = 4 - 7$ biological replicates per group in F₀ and $n = 4 - 6$ in F₃. Each replicate consists of 15 pooled larvae.

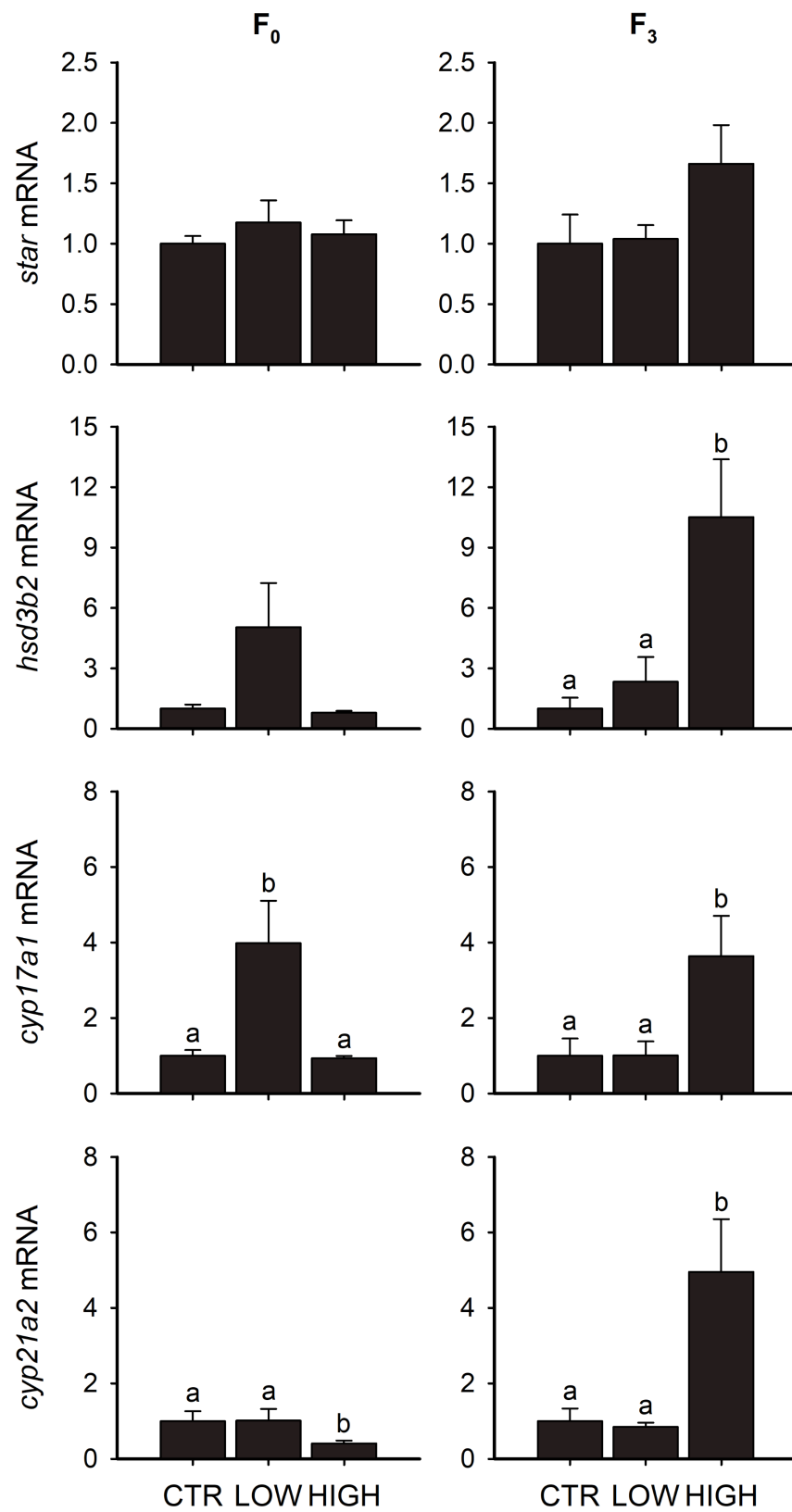


Fig. 3.5. Transcript levels of genes associated with steroidogenesis in 6 – 9 dpf ZF larvae from the CTR and FLX lineages in the generation F₀ (*Left*) and F₃ (*Right*). The FLX lineages were generated following a 6-day FLX exposure to the F₀. Transcript levels were normalized to the CTR. Data are expressed as mean ± SEM and analyzed by one-way ANOVA, $P < 0.05$. $n = 4 - 7$ biological replicates per group in F₀ and $n = 3 - 6$ in F₃. Each replicate consists of 15 pooled larvae.

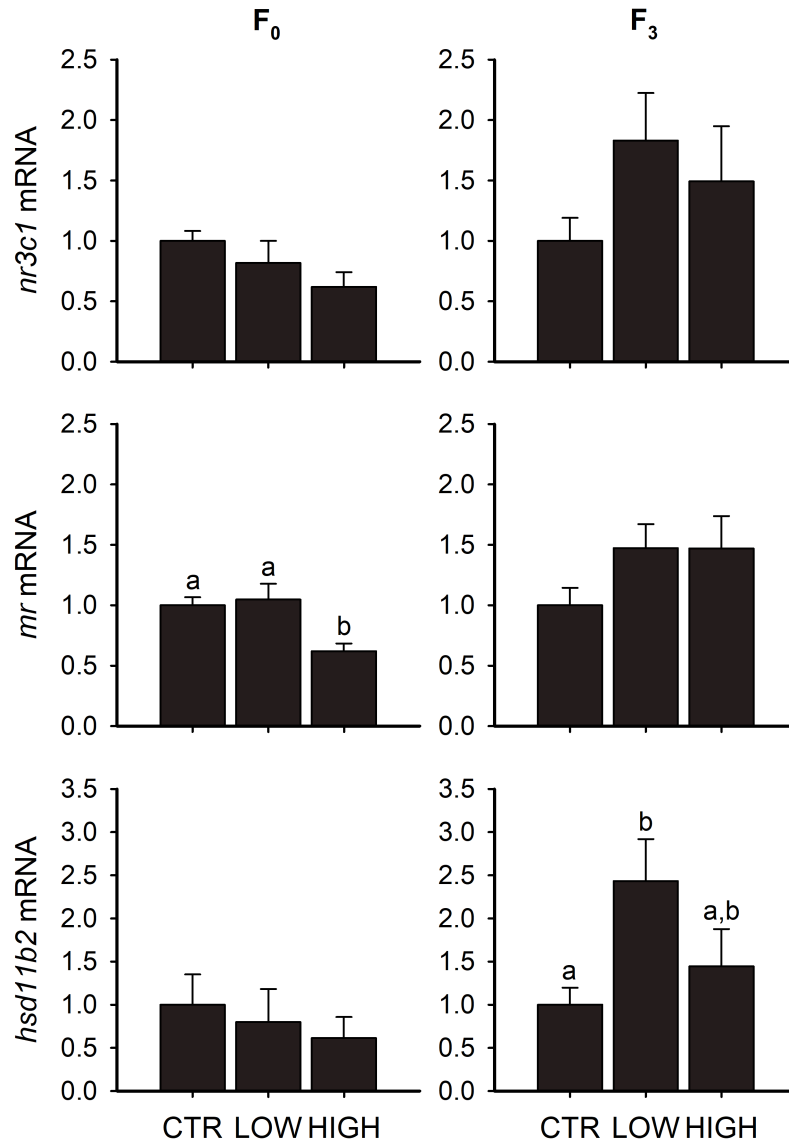


Fig. 3.6. Transcript levels of genes associated with cortisol activity regulation in 6 – 9 dpf ZF larvae from the CTR and FLX lineages in the generation F₀ (*Left*) and F₃ (*Right*). The FLX lineages were generated following a 6-day FLX exposure to the F₀. Transcript levels were normalized to the CTR. Data are expressed as mean $\pm$ SEM and analyzed by one-way ANOVA, $P < 0.05$. $n = 5 - 7$ biological replicates per group in F₀ and $n = 4 - 6$ in F₃. Each replicate consists of 15 pooled larvae.

3.5. Discussion

The timing of the molecular development of the HPI axis is well-established in ZF (Alderman and Bernier, 2009; Alsop and Vijayan, 2008; Wilson et al., 2013). By 48 hpf, the HPI axis in the larvae ZF is fully functional and able to initiate *de novo* cortisol synthesis. Since FLX increases the activity of 5-HT and its alteration is associated with the programming of the HPI/A axis, we hypothesized that FLX exposure during this critical period of the HPI axis development alters the timing of the HPI axis responsiveness to a stressor. However, in this study, we demonstrated that FLX did not accelerate or delay the ability of the HPI axis to mount a stress response following a swirling stressor. An elevation of the cortisol levels in response to the swirling stressor was only observed at 144 hpf. Prior to this time point, the HPI axis of the larvae was not responsive to the stressor, an effect that was consistent in our CTR and FLX treatments. In contrast, a study by Alsop and Vijayan (2008) showed that larvae ZF have the ability to increase cortisol levels in response to a stressor (swirling) by 97 hpf. However, other studies showed that the HPI axis is responsive to osmotic changes (Alderman and Bernier, 2009) and a stirring stressor (Nesan and Vijayan, 2016; Wilson et al., 2013) at 72 hpf. Stress-induced cortisol levels in ZF were also observed at 48 hpf following a swirling test (Eto et al., 2014). Our results are consistent with a study by Jeffrey and Gilmour (2016), where the authors observed a stress response at 144 hpf and not at 96 hpf. These discrepancies regarding the timing of the HPI axis activation in ZF are likely attributed to the intensity and duration of the applied stressor in addition to the different maternal contributions to cortisol levels in the offspring (Nesan and Vijayan, 2016). It is also important to highlight that rearing density (Ramsay et al., 2006) and the ZF strain (Gorissen et al., 2015) are known contributors to different stress responses in adult ZF, so these may also be

different between our study and those previously published. Nevertheless, the larvae in our study developed a clear stress response by 144 hpf.

Moreover, a decrease in the total cortisol levels in the larvae was observed with FLX at the end of the three exposure periods: 4 days (at 96 hpf), 5 days (at 120 hpf) and 6 days (at 144 hpf). Remarkably, this disruption of cortisol levels by FLX was not diminished in the shorter exposure period. An attenuation of 23 to 32% of cortisol levels was induced by the LOW and HIGH FLX treatments across the three exposure times. This cortisol reduction is within the range of that observed in the F₀ adult ZF exposed from 0 to 6 dpf (Chapter 2). These results support the idea that the permanent disruptive effects of FLX on the stress axis in the F₀ are induced within four days of exposure during the HPI axis development. Acute exposures to FLX during early-life have also been documented to disrupt a wide range of biological systems in larval ZF. A study by Wu et al. (2017) reported that 120 h of FLX exposure (10 µg·L⁻¹) altered the expression of key genes involved in glycolysis, gluconeogenesis and the insulin pathway in treated ZF larvae. The perturbation of some metabolites was also observed in treated ZF larvae after 94 h of FLX exposure (effects were dose dependent; 12, 70 and 700 µg·L⁻¹) in which a dysregulation of amino acids, monosaccharides, glycerophosphates, fatty acids, carboxylic acid derivatives and sugars was detected (Mishra et al., 2017). In addition to the expected alteration in the expression levels of genes associated with 5-HT signaling, FLX (effects were dose dependent; 0.52, 17, 35, 173 and 277 µg·L⁻¹) was also found to alter the transcript levels of genes involved in the transport and receptor signaling of dopamine and adrenaline following an 80-h exposure to FLX in larvae ZF (Cunha et al., 2018). Interestingly, FLX has also been shown to disrupt similar endpoints in human embryos. A protein analysis by shotgun mass spectrometry revealed that a 4-day FLX exposure (effects were dose dependent; 86.5 and 173 µg·L⁻¹) during early development disrupted the levels

of proteins involved in cell growth, survival, proliferation, and inflammatory response in whole human embryos (Kaihola et al., 2016). Apparently, exposure to FLX during the critical period of early development is detrimental to target organisms as many biological systems and functions are disrupted including the stress axis.

The transcriptional profile of the selected genes analyzed in the FLX-treated larvae differed between the F₀ and F₃ despite the persistent low cortisol phenotype displayed by both generations. Transcript levels of *crfr1* significantly decreased solely in the HIGH FLX group of the F₀. No other changes were observed related to the regulation of the neuropeptide CRF activity. The expression of the *crfr1* gene in ZF is detected at 0 and 6 hpf, and it becomes undetectable at 12 and 48 hpf (Alderman and Bernier, 2009). Therefore, the attenuated transcript levels at 6 dpf may be attributed to either a delay in the *crfr1* gene expression induced by FLX or a direct reduction in its transcriptional levels. To further investigate the effects of FLX on the HPI axis development, the expression of selected genes should be assessed over different time points.

Another important difference between the F₀ and F₃ generations concerns *fflb*, the expression of which was significantly increased only in the HIGH FLX group of the F₃. This gene is required for the development, differentiation, and function of the interrenal cells in ZF (Chai et al., 2003). This up-regulation could be associated with a coping mechanism of the F₃ treated larvae to adapt to their low cortisol levels, however, this hypothesis requires rigorous testing. Furthermore, the transcript levels of key genes associated with ACTH binding and steroidogenesis were up-regulated in the LOW treatment and down-regulated in the HIGH treatment of the F₀ treated larvae. This concentration-dependent effect of FLX was previously reported in different studies (Chiou et al., 2006; Cunha et al., 2016; Gustafsson et al., 2006; Weinberger and Klaper, 2014). Since both FLX concentrations induced the same attenuated cortisol phenotype and the key

genes directly involved in cortisol synthesis do not follow the same transcriptional patterns, we can hypothesize that the mechanism of this disruption by FLX on cortisol levels may be associated with the translational modifications at the protein level of the HPI axis regulators. Nonetheless, it is important to note that the transcript levels assessed in this study were examined in whole larvae, therefore, minor organ-specific expression differences could not be well-represented as most of the genes evaluated are expressed in a variety of tissues which may not be directly involved with the stress axis.

Surprisingly, the larvae of the F₃ generation displayed an up-regulation in the transcript levels of the genes encoding receptors and key enzymes involved in steroidogenesis. During early-life, cortisol plays an important role in a myriad of developmental processes including the circadian cycle, the serotonergic system, the growth and development of fetal organs, the development and differentiation of neurons, among others (Clayton et al., 2018; Wang and Harris, 2015). In ZF, cortisol is also involved in the hatching process of the embryo and swimming activity of larvae (Wilson et al., 2013). Therefore, the elevated transcripts levels exhibited by the F₃ larvae could be an adaptive mechanism to attempt to normalize their cortisol levels for a normal development. Interestingly and contrary to the elevated F₃ larval expression levels of the *cyp21a2* gene, our RNA-Seq analysis from our previous study in Chapter 2 performed in the whole kidney of the F₃ adult males revealed an 84% down-regulation of this transcript in the HIGH treatment. This difference in transcript levels between the larvae and adults of the F₃ generation was also observed in *star*, which was unaffected in the larvae by the HIGH treatment, whereas in the adults, its expression levels increased by 481% ($P = 0.041$). Another important gene detected in our RNA-Seq analysis of adult male kidney (Chapter 2) that was also assessed in this study was the *hsd11b2* gene which encodes for the enzyme that inactivates cortisol. In the F₃ larvae, the *hsd11b2* gene

was not altered by HIGH treatment whilst a down-regulation of 67% of its expression levels was exhibited by the adults. The mechanisms underlying the differences in these transcriptional patterns observed between the larvae and adults are unclear. Nevertheless, we hypothesized that the adults adopted different coping mechanisms to increase their cortisol levels to normal since the adaptive response elicited by the larvae failed to do so. It is also important to note that the RNA-Seq analysis was conducted in adult males while the sex of the larvae used in this study to assess the gene expression levels is unknown.

Collectively, this study demonstrated that FLX at environmentally relevant concentrations and a concentration detected in the cord blood of FLX treated mothers, disrupts the stress axis of ZF within 4 days of exposure. The transcriptional profiles of genes associated with the HPI axis in the F₀ larvae varied significantly in magnitude and direction in both treatments, despite the observation that both displayed a low cortisol phenotype. Therefore, we should be cautious when interpreting gene expression data as it may not directly reflect the disrupted physiological process. We also revealed that the larvae from the FLX lineage in the F₃ exhibited an up-regulation in the transcript levels of the majority of the selected steroidogenic-related genes, suggesting an adaptive response to the transgenerational reduction in cortisol. Cortisol is a critical regulator of adaptive and behavioral responses in animals (Egan et al., 2009; Pippal et al., 2011). In teleosts, it also plays an important role in osmoregulation, regulation of metabolism, circadian rhythms, and other developmental processes (Mommensen et al., 1999; Tsachaki et al., 2017; Wang and Harris, 2015). Considering the continuous presence of FLX in the aquatic environment and its disruptive effects on cortisol, further studies are required to examine whether FLX can alter cortisol-associated pathways. It will be also interesting to test whether adaptive responses in stress-related gene

expression levels similar to that observed in this study, are exhibited in the F₃ following FLX exposure to the same concentrations as the grand-parents.

3.6. Acknowledgments

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CHAPTER 4: Fluoxetine exposure during sexual development results in sex- and time-specific effects on the exploratory behavior of adult zebrafish

4.1. Statement of contribution

Marilyn Vera Chang, designed the study, developed the methodology, conducted the experiments and analyses and prepared the manuscript. Dr. Thomas Moon, helped with the design of the study and manuscript editing. Dr. Vance Trudeau, helped with the design of the study and manuscript editing.

4.2. Introduction

Sex steroids including androgens and estrogens are ubiquitous in all vertebrates (Munchrath and Hofmann, 2010) and through organizational and activational effects (Rosenfeld et al., 2017) they are known to play a critical role in developing sexual dimorphisms within the brain (Frye et al., 2012). The long-term organizational actions occur at the molecular and cellular levels early in development whereas activational effects are transient and occur throughout life. As a result, proper concentration and timing of exposure to steroid hormones are an important factor for normal development of the reproductive system, neural processes, brain morphology and programming of adult behaviors in vertebrates (Arnold and Breedlove, 1985; Forest, 1983; Gilmore, 2002; Morris et al., 2004; Nugent et al., 2012). Therefore, disturbances to endogenous steroid hormones during early-life may induce long-lasting alterations in juvenile/adult behaviors.

This balance in the association between the hormone levels and programming of diverse behaviors in adults across taxa may be at risk, mainly because of the growing number of pharmaceuticals and diverse contaminants in the environment. Their presence is interfering with the function of the steroid hormonal system in vertebrates including humans and fishes by acting

as endocrine disrupting chemicals (Frye et al., 2012; Guillette and Gunderson, 2001; Kinch et al., 2015; Rasier et al., 2006; Vos et al., 2000; Waring and Harris, 2005). These compounds exert their actions via antagonizing, mimicking or altering hormone synthesis and metabolism, or modulation of target receptors (Frye et al., 2012). These changes in the homeostasis of steroid hormones are impacting specific behaviors in adult aquatic organisms. For instance, embryonic zebrafish (ZF; *Danio rerio*) treated with $1.6 \mu\text{g}\cdot\text{L}^{-1}$ of the plasticizers bisphenol A (BPA; 1,000-fold lower than the accepted human daily exposure) or bisphenol S from 0 to 5 days post-fertilization (dpf) resulted in hyperactive behaviors in ZF larvae and this was associated with androgen receptor-mediated up-regulation of aromatase, an enzyme that catalyzes the conversion of androgens into estrogens (Kinch et al., 2015). It is important to note that BPA concentrations detected in the aquatic environment range from 1 to $28 \mu\text{g}\cdot\text{L}^{-1}$ (Barnes et al., 2008; Heisterkamp et al., 2004; Jin et al., 2004; Kolpin et al., 2002; Loos et al., 2010; Rudel et al., 1998). Man-made or synthetic steroid hormones are also present in the environment. A study by Zhao et al. (2018) reported that 48 h of exposure from 120 hours post-fertilization (hpf) in eleutheroembryo ZF to $153 \pm 17 \mu\text{g}\cdot\text{L}^{-1}$ progesterone significantly inhibited the locomotor activities of the larvae during the entire exposure period by 15 to 59% relative to the unexposed larvae. This is in contrast to 17α -ethinylestradiol exposure during the same period that significantly increased the locomotor behavior in the larvae ZF in a concentration and circadian stage dependent manner.

Another important endocrine disrupting chemical with emerging concerns because of its continual worldwide increase in prescription rates, even among children and pregnant women (Morkem et al., 2017; Sarginson et al., 2017), is the antidepressant fluoxetine (FLX), the active chemical in Prozac®. The continuous discharge and improper disposal into wastewater streams have led to the detection of FLX in aquatic environments at concentrations as high as $929 \text{ ng}\cdot\text{L}^{-1}$

(Bueno et al., 2007). A study by Lister et al. (2009) demonstrated that a FLX exposure of $32 \mu\text{g}\cdot\text{L}^{-1}$ in adult ZF decreased ovarian 17β -estradiol (E_2) levels in females. In adult goldfish (*Carassius auratus*), five intraperitoneal injections of $5 \mu\text{g FLX}\cdot\text{g}^{-1}$ body weight throughout 14 days also reduced E_2 levels in the plasma of the females (Mennigen et al., 2008). However, 0.54 and $54 \mu\text{g}\cdot\text{L}^{-1}$ of waterborne FLX exposure for 14 days increased plasma E_2 levels in the male goldfish (Mennigen et al., 2010a). Moreover, we previously showed that exposure to FLX from 0 to 6 dpf in ZF reduced their cortisol levels across 3 generations, consequently decreasing the locomotor and exploratory activities in adult males (Chapter 2).

The critical window of sexual development in ZF is a period of high sensitivity to sex steroids as it is suggested that the determination of the sex in addition to the differentiation and development of the gonads are mainly driven by androgens and estrogens (Luzio et al., 2015). Sexual development in ZF begins at about 10 dpf with the differentiation of the juvenile ovary in both sexes, regardless of actual sexual genotype. At approximately 21 dpf, the juvenile ovaries in males driven by the effects of androgens undergo apoptosis and are transformed into testes whereas in females, the high levels of estrogens induce the continuation of ovary development. At ~ 60 dpf both gonads are completely developed (Fig. 4.1) (Orban et al., 2009; Tong et al., 2010; Uchida et al., 2004).

Sexual development studies in ZF suggest that the window of vulnerability differs for androgens and estrogens. For instance, an early sexual developmental exposure to 17α -ethinylestradiol led to intersex ovo-testis whereas late developmental exposure from 22 to 62 dpf generated an all-female population (Andersen et al., 2003). However, exposure to 17α -methyltestosterone, a synthetic androgen, during early sex development (1 – 15 dpf) induces an all-male ZF population (Uchida et al., 2004). These studies indicate that ZF are more sensitive

to androgens during early sexual development and to estrogens at the end of the sexual developmental period.

Given that FLX has the potential to alter levels of steroids in fishes and they are known to play an important role in the programming of adult behaviors and that the vulnerability period of androgens and estrogens differ in ZF, we tested the hypothesis that exposure to FLX during early and late sexual development disrupts the behavioral responses of adult ZF in a sex- and time-dependent manner. We also examined the cortisol levels in the adult fish since there is evidence that sex steroids also modulate the stress axis in fishes (Afonso et al., 2003; McQuillan et al., 2003). These effects of sex steroids on the stress axis in mammals are well established (Kudielka and Kirschbaum, 2005; Patchev and Almeida, 1998).

4.3. Materials and methods

4.3.1. Experimental animals and exposure

Adult ZF (AB strain) were obtained from Big Al's Aquarium in Ottawa, Ontario, Canada and allowed to acclimate for 4 weeks in flow-through aquarium systems supplied with heated (28.5 ± 0.2 °C), aerated, dechloraminated City of Ottawa tap water (hereby referred to as system water) on a 14 h light:10 h dark photoperiod prior to generating the embryos for this study. Sixteen crossing cages containing system water were set up in the late afternoon with a plastic divider separating females from males in a ratio of 2F:1M and left undisturbed until the separator was removed the following morning. Fertilized eggs were collected within 1 h 45 min of spawning between 09h00 and 11h00, submerged in 0.0075% bleach for 2 min and rinsed with system water. Embryos from different spawning pairs were pooled to avoid batch effects and subsequently

divided into two groups: the first group was used for exposure period 1 and the second group for exposure period 2 (Fig. 4.1).

Embryos assigned to exposure period 1 were immediately distributed into glass Petri dishes containing either embryo medium alone (control; CTR) or supplemented with one of two concentrations of FLX (Sigma-Aldrich; Oakville, Ontario, Canada): $0.54 \mu\text{g}\cdot\text{L}^{-1}$ (LOW) or $54 \mu\text{g}\cdot\text{L}^{-1}$ (HIGH). These selected FLX concentrations were based on our previous study (Chapter 2) demonstrating a disruption of the stress axis and behavioral responses in adult ZF following a 6-day embryonic exposure. Concentrated stock solutions of FLX were prepared in the same water that was used for the embryo medium preparation, therefore, there was no need for vehicle control. The embryos from period 1 were exposed from 3 hpf to 15 dpf during the development of the juvenile ovary in ZF (Tong et al., 2010; Uchida et al., 2004), whereas the embryos from period 2 were exposed to the same FLX concentrations beginning at 15 dpf and ending at 42 dpf during a period that encompasses the period of sex differentiation in ZF (Orban et al., 2009).

Embryos from both exposure groups were reared in Petri dishes until 6 dpf at a maximum density of $1 \text{ embryo}\cdot\text{mL}^{-1}$ and maintained at 28°C without feeding. From 6 to 42 dpf, the larvae were reared in 1-L tanks containing system water at a density of $50 \text{ larvae}\cdot\text{L}^{-1}$ (Matthews et al., 2002) and maintained in a temperature-controlled ZF facility. At this point, larvae were fed three times a day with Zebrafish Management Ltd. (Winchester, England) fry food diet of the appropriate size and according to their developmental stages. The commercial feed in all stages from 16 dpf was supplemented with live *Artemia* nauplii (Artemia International LLC; Fairview, Texas, USA) once per day. The debris was cleaned and the media or exposure solutions were 90% replaced every 24 h until 42 dpf. This replacement procedure was kept consistent for both groups.

At 42 dpf, the fish were transferred into 3- and 10-L tanks (Aquatic Habitats; Apopka, Florida, USA) within a flow-through system at a density of 5 fish·L⁻¹ (Castranova et al., 2011; Matthews et al., 2002) and fed twice a day with Zeigler No.1 crumble (Aquatic Habitats; Apopka, Florida, USA). All experiments were conducted under a protocol approved by the University of Ottawa Animal Care Protocol Review Committee and undertaken in accordance with institutional animal care guidelines adhering to those of the Canadian Council on Animal Care.

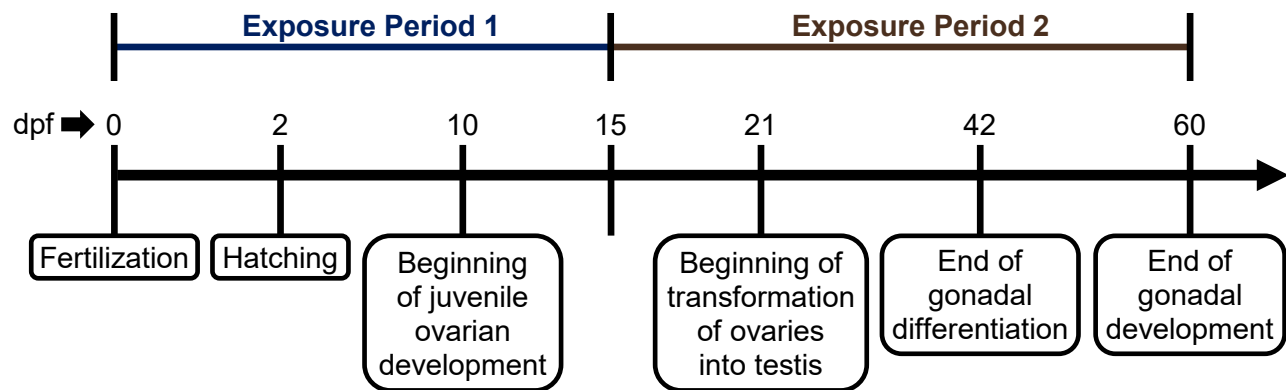


Fig. 4.1. Timeline of the sex developmental period in ZF. Exposure 1 was performed from 0 to 15 dpf during the development of juvenile ovaries while exposure 2 was conducted from 15 to 42 dpf during the sex differentiation period. The time points presented here are approximate as the sex developmental profile in ZF varies among individuals. Information was obtained from Luzio et al. (2015) and Orban et al. (2009).

4.3.2. Acute stress response

Six-month-old females and males from both exposure and CTR groups were subjected to a standardized net stressor (Ramsay et al., 2009) as described in Chapter 2 section 2.5.2. Briefly, one week prior to the stressor, the fish were transferred to the testing room and allowed to acclimate to the experimental tanks. On the day of the stress test, half of the fish from each treatment and

sex group were immediately sacrificed (Basal group), whereas the other half was subjected to the stressor (Stressed group) (Ramsay et al., 2009) between 09h15 and 10h30. The fish were sacrificed by submersion in ice-cold water and subsequently weighed, immediately snap-frozen in liquid nitrogen and stored at -80°C for whole-body cortisol analysis.

4.3.3. Cortisol extraction and quantification

Whole-body cortisol was extracted using a protocol modified from Folch et al. (1957), as described in Chapter 2 section 2.5.6. The extraction efficiency was determined by spiking homogenates with known amounts of tritiated cortisol and was calculated to be 87%. Total cortisol concentration was assessed by using a ^{125}I radioimmunoassay kit (MP Biomedicals; Solon, Ohio) according to the manufacturer's protocol followed by the measurement of the radioactive counts with a Wizard2 automatic gamma counter (PerkinElmer; Downers Grove, Illinois). The intra- and the inter-assay coefficients of variation (CV) were calculated to be 4 – 8% and 7 – 15%, respectively. Cortisol concentrations were not corrected for extraction efficiency.

4.3.4. Metirapone exposure

Adult ZF (8-months-old) of the AB strain bred in-house from a chemically clean population (naïve fish) were exposed to the 11β -hydroxylase inhibitor metirapone ($325\text{ }\mu\text{M}$; Adooq Bioscience; Burlington, Ontario, Canada) for one week as described in Chapter 2 section 2.5.4. Briefly, females and males placed in separate glass tanks at a density of $5\text{ fish}\cdot\text{L}^{-1}$, provided with adequate aeration were exposed to either system water containing metirapone or the DMSO vehicle (0.02% v/v). The water was replaced daily 1 h after feeding (including either metirapone

or DMSO). At the end of the one-week exposure, fish from both treatments were subjected to the novel tank diving test (see 4.3.5).

4.3.5. Novel tank diving test

Six-months-old females and males exposed to FLX and metyrapone-exposed adult fish were subjected to the novel tank diving test adapted from Levin et al. (2007) as described in our previous study (Chapter 2 section 2.5.7). Briefly, fish allowed to acclimatize to the testing room a week prior to the experiment were subjected to the behavioral test conducted between 09h30 and 14h30 in a trapezoid-shaped tank (Aquatic Habitats; Apopka, Florida, USA) filled with system water. The behavioral activity of each individual fish was recorded for 6 min in fresh system water. Videos were analyzed every 30 frames·s⁻¹ for a total of 10,800 frames using a validated in-house automated tracking (AT) system (Python script) (Chapter 2 section 2.6.1). Principal component analysis (PCA) was performed on the 10 calculated behavioral metrics obtained from the AT system. One PCA was conducted on the data sets across all animals (females and males). PCA yielded a single component (PC1) that strongly loads most of the behavioral metrics (Table 4.1) and explains 47% of the behavioral variance. The two variables that did not robustly contribute to PC1 were maximum speed and total distance traveled. Positive scores are associated with high exploratory and locomotor activities, whereas negative scores are linked to reduced behavioral activities.

Table 4.1. Loadings and contributions of the behavioral metrics to PC1

Behavioral metrics	Description	Loadings	Contribution (%)
Latency middle third	Delay before entering the middle third of the tank	-0.263	6.9
Latency top half	Delay before entering the top half of the tank	-0.305	9.3
Latency top third	Delay before entering the top third of the tank	-0.323	10.5
Transitions	Number of times the fish crossed into the top half of the tank	0.388	15.1
Time middle third	Total time spent in the middle third of the tank	0.383	14.7
Time top third	Total time spent in the top third of the tank	0.347	12.0
Distance middle third	Total distance spent in the middle third of the tank	0.393	15.5
Distance top third	Total distance spent in the top third of the tank	0.384	14.7
Total distance	Total distance traveled around the tank	0.099	1.0
Max speed	Maximum speed reached by the fish	-0.062	0.4

4.3.6. Statistical analyses

Cortisol data were expressed as the mean $\pm$ SEM, whereas behavioral data were presented in box plots showing the 10th and 90th percentiles. Prior to hypothesis testing, data sets were examined for normality and homogeneity of variance using the Shapiro-Wilk test and Levene median test, respectively. Statistical significance between treatments for cortisol data was determined using a two-way ANOVA followed by a Tukey's post-hoc test. Box-Cox transformations (Box and Cox, 1964) were applied when data were not normally distributed.

Alternatively, two-way ANOVA on ranks was used for data that had non-Gaussian distributions even after undergoing transformations. A Student's *t*-test or Mann-Whitney *U* Test was applied to determine significant differences within PC1 scores for the behavioral data. The level of significance for all tests was set at $\alpha < 0.05$, and all statistical analyses were performed using SigmaPlot 11.0 (Systat Software, Inc.).

4.4. Results

4.4.1. FLX reduces cortisol levels in adult ZF exposed during critical windows of sexual development

We investigated whether the two different windows of exposure to FLX during the sexual development period induced a disruption of the stress axis. A two-way ANOVA was conducted to examine the effects of the stressor and FLX treatments on cortisol levels in each of the exposure periods. Simple main effects analysis showed that the standardized net handling stressor was effective at eliciting a cortisol response in the adult females ($F_{1,34} = 110.153$, $P < 0.001$ and $F_{1,30} = 307.809$, $P < 0.001$, respectively) and males ($F_{1,37} = 119.887$, $P < 0.001$ and $F_{1,41} = 169.485$, $P < 0.001$, respectively) from the control (CTR) and FLX treatments in exposure period 1 (Fig. 4.2A) and 2 (Fig. 4.2B). However, exposure to FLX during period 1 significantly reduced the whole-body cortisol levels in adult females (40 and 42% reductions in the LOW and HIGH treatment, respectively; $F_{2,34} = 4.404$, $P = 0.020$) and males (8 and 36% reductions; $F_{2,37} = 4.596$, $P = 0.016$) from exposure period 1 (Fig. 4.2A). Treatment with FLX during exposure period 2 (Fig. 4.2B) also elicited an attenuation in cortisol levels in the adult fish but was less pronounced and only statistically significant in females (15 and 34% reductions in the LOW and HIGH treatment, respectively; $F_{2,30} = 11.348$, $P < 0.001$) and not in males ($F_{2,41} = 2.625$, $P = 0.085$). No interactions

between FLX treatment and the stressor were found in the adult females ($F_{2,34} = 2.627$, $P = 0.087$) and males ($F_{2,37} = 1.617$, $P = 0.212$) from exposure period 1 (Fig. 4.2A). In contrast, there was a statistically significant interaction between FLX treatment and the stressor in the adult females ($F_{2,30} = 4.602$, $P = 0.018$) and males ($F_{2,41} = 3.366$, $P = 0.044$) from exposure period 2.

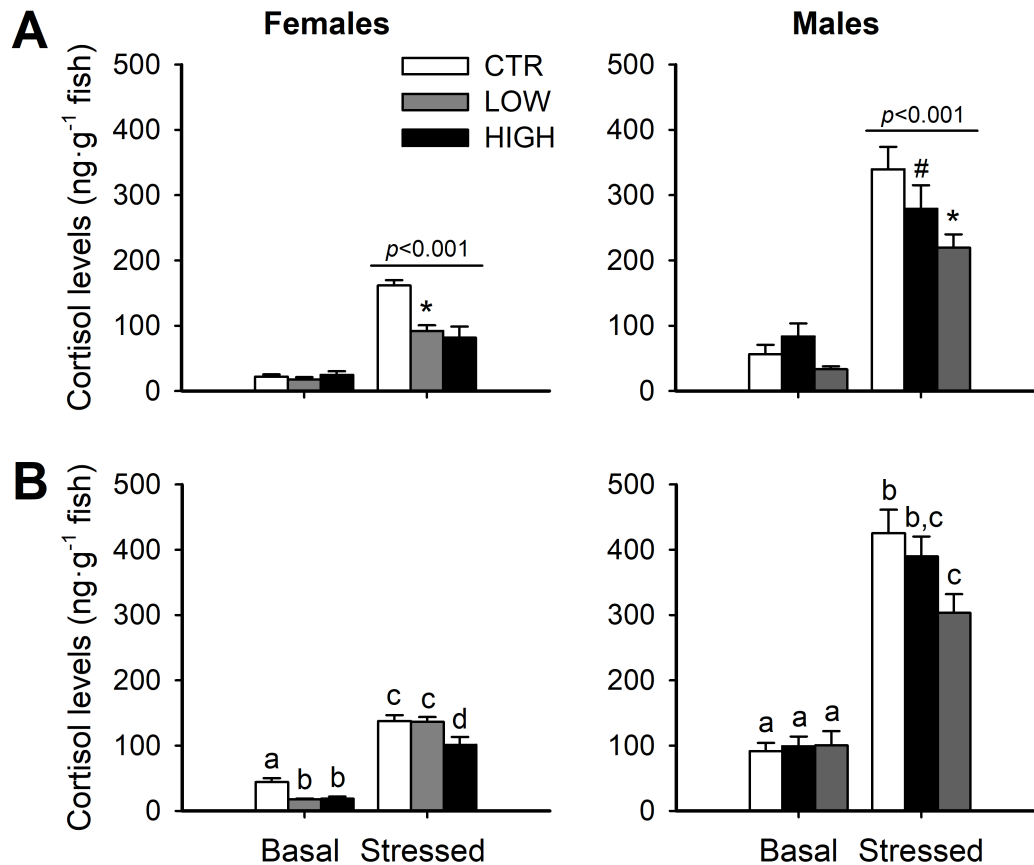


Fig. 4.2. Whole-body cortisol levels (ng·g⁻¹ fish) in adult (6 months of age) females and males following exposure to FLX (0.54 and 54 µg·L⁻¹ for LOW and HIGH, respectively) during either (A) the period of juvenile ovarian development (0 – 15 dpf) or (B) the period of sex differentiation (15 – 42 dpf) in ZF (see Fig. 4.1). The stress response was induced using a standardized net handling stress protocol. For both panels, the data are presented as mean ± SEM and analyzed by two-way ANOVA (and on ranks for data on panel A), $P < 0.05$. The letters represent statistical difference when interactions are present. P -values shown above the bars represent significant difference compared to basal. The symbols represent significant difference within the FLX

treatments: $*P < 0.05$ compared to CTR and $^{\#}P = 0.031$ compared to HIGH. $n = 4 - 8$ per group in females and $n = 6 - 10$ in males.

4.4.2. FLX exposure during sexual development results in sex- and time-specific effects on locomotor and exploratory behavior in adult ZF

We next examined whether the perturbation of the stress response by FLX also disrupted the locomotor and exploratory behaviors in 6-month-old adult ZF. Exposure to the LOW ($t(27) = 0.635$, $P = 0.531$) or HIGH ($t(27) = 1.860$, $P = 0.074$) concentrations of FLX during the development of the juvenile ovaries (period 1) did not affect these behaviors in female ZF (Fig. 4.3A). This is in marked contrast to males, where PC1 scores revealed that their exploratory and locomotor activities were significantly reduced in the HIGH FLX group ($t(27) = 2.887$, $P = 0.008$) but not in the LOW group ($t(28) = 0.843$, $P = 0.406$) compared to the CTR (Fig. 4.3A). Remarkably, in contrast to exposure period 1, FLX exposure during a period of sex differentiation (period 2) increased the locomotor and exploratory activities in the females from the LOW ($t(25) = -3.241$, $P = 0.003$) and HIGH ($t(22) = -3.758$, $P = 0.001$) groups compared to the CTR (Fig. 4.3B). No effects were observed in the behavior of the males exposed during period 2 (LOW, $t(28) = -0.741$, $P = 0.465$ and HIGH, $t(27) = -0.189$, $P = 0.852$; Fig. 4.3B).

4.4.3. Recapitulation of the behavioral responses observed in the FLX-treated fish by chemically reducing cortisol levels in naïve fish

Chemical reduction of cortisol levels by exposure to metyrapone, a steroid 11β -hydroxylase inhibitor, in naïve adult female and male ZF recapitulated the sex- and window of exposure-specific behavioral responses to the novel tank diving test observed in our FLX-treated adult fish. In the females ($U = 61$, $P = 0.021$), metyrapone exposure increased their locomotor and

exploratory activities similar to the adult females exposed to FLX during the sex differentiation period (period 2; Fig. 4.3C). In the males ($U = 56$, $P = 0.020$), metyrapone exposure decreased their behavioral responses comparable to the adult males exposed to FLX during the juvenile ovarian development in ZF (period 1; Fig. 4.3C).

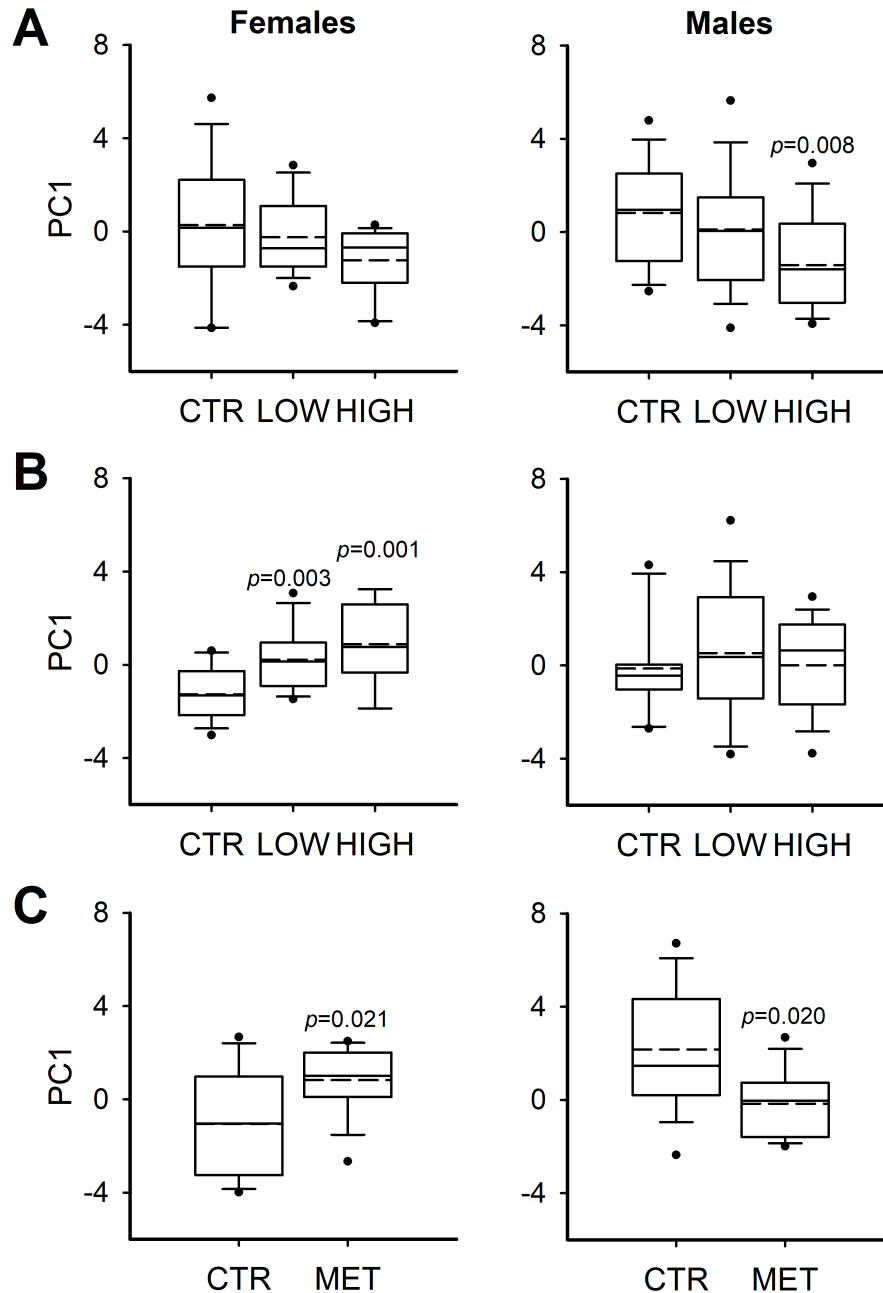


Fig. 4.3. Behavioral responses following the novel tank diving test in adult (6 months of age) ZF from (A) exposure group 1, (B) exposure group 2 and (C) the metyrapone exposure. Data were analyzed using Student's *t*-test (or Mann-Whitney *U* Test for panel C, both graphs) and are presented as box plots showing the median (solid line), the mean (dashed line), the interquartile range (box) and the whiskers embracing data within the 10th and 90th percentiles; all data outside the range of the whiskers are presented as individual data points. *P*-values shown above the bars

represent significant differences compared to CTR. $n = 14 - 15$ fish per group in panel *A*, $n = 9 - 15$ per group in panel *B* and $n = 15 - 16$ per group in panel *C*.

4.5. Discussion

Treatment with FLX during the two windows of exposure across the critical period of sexual development in ZF disrupted the stress axis in adult females and males (6 months of age). The same magnitude of disruption in cortisol levels was induced following FLX exposure during both the early sexual development, where the juvenile ovary starts to differentiate regardless of the sexual genotype of the fish, and the late sexual development during the period of gonadal differentiation. These findings are in accordance with our previous study where FLX exposure from 0 to 6 dpf reduced cortisol levels in adult (6 months of age) females and males ZF (Chapter 2). Sex differences were observed in total cortisol levels in both exposure periods regardless of the treatment. The females from the CTR exhibited a 54 to 65% lower total cortisol level compared to the CTR males. This is in contrast to our earlier study (Chapter 2) where it was shown that the females and males from the CTR across generations displayed similar total cortisol levels. Sex differences in HPA axis reactivity have been extensively studied in mammals including humans and rodents (Herman et al., 2016; Uhart et al., 2006). This sexually dimorphic stress response is partly the reflection of the organizational and activational effects of the different sex steroids present in both sexes. However, it is important to highlight that these sex-specific differences in the magnitude and duration of the HPA response to a stressor in mammals vary according to the age of the individual, estrous cycle and type and severity of the stressor (DeSantis et al., 2011; Kudielka and Kirschbaum, 2005). In ZF, evidence for sexual dimorphism of the cortisol response to a stressor is equivocal (Félix et al., 2013; Filby et al., 2010; Fuzzen et al., 2011; Oswald et al., 2012; Zhang et al., 2015). However, several studies reporting these sex differences in cortisol

levels are in accordance with our findings, where females exhibit a lower cortisol response to a stressor than males (Oswald et al., 2012; Zhang et al., 2015). Sex-dependent differences in the HPI axis reactivity have also been documented in other teleosts, such as yellow perch (*Perca flavescens*), rainbow trout (*Oncorhynchus mykiss*) and sockeye salmon (*Oncorhynchus nerka*) (Girard et al., 1998; Kubokawa et al., 2001; Øverli et al., 2006; Pottinger and Carrucj, 2000). In these teleosts, the females display higher stress-induced cortisol levels than males, however, the sex-specific cortisol differences vary according to their sexual maturity and/or their reproductive periods. Moreover, *in vitro* studies of interrenal cells (site of cortisol synthesis in teleosts) revealed that sex steroids can affect cortisol levels in teleosts, but these effects differ across species (McQuillan et al., 2003; Young et al., 1996). For instance, E₂ inhibited the ability of interrenal cells of juvenile chinook salmon (*Oncorhynchus tshawytscha*) to utilize pregnenolone as a substrate for cortisol synthesis, an effect that was absent in juvenile or mature rainbow trout (McQuillan et al., 2003). Even though our previous study (Chapter 2) and our current findings do not agree with the observations of a sexual dimorphism in the cortisol response, the duration or window of exposure to FLX did not affect the magnitude of the cortisol disruption in our adult fish. However, it remains to be determined whether the long-lasting effects of FLX manifested in the adult fish on the stress axis during exposure 1 (from 0 to 15 dpf) resulted from the same mechanism(s) of disruption as exposure 2 (from 15 to 42 dpf).

We also examined the behavioral response to novelty of the adult fish that had been exposed to FLX. The behavioral response to the novel tank diving test of the FLX-exposed adult fish varied by sex and exposure period. Our findings revealed that females are more susceptible to the effects of FLX during a period of sex differentiation (period 2), since they displayed a significant increase in their exploratory and locomotor behavior. In contrast, adult males exhibited

reduced exploratory and locomotor activities following the FLX exposure period 1, suggesting that the males are more sensitive to the long-lasting disruptive effects of FLX during their early development. We should also re-emphasize that ZF are highly sensitive to the effects of sex steroids during sexual development (Lister et al., 2009; Nagabhushana and Mishra, 2016; Pradhan and Olsson, 2016). The sensitivity of ZF to sex steroids differs by sexual developmental exposure periods. Sexual developmental studies suggest that ZF are more sensitive to androgens during early sexual development and to estrogens at the end of the sexual developmental period (Andersen et al., 2003; Uchida et al., 2004). Therefore, this sexual dimorphic effect on the behavioral response of our FLX-exposed fish across the different windows of exposure could be a reflection of the effects on sex steroid levels, which were altered by FLX in the adult ZF study by Lister et al. (2009). However, the direct involvement of sex steroids in the behavioral response of ZF following FLX treatment remains to be elucidated.

Perhaps the most striking finding of this study was the unexpected difference in the behavioral response to novelty in the females compared to the males as a result of the FLX-induced low cortisol levels. These results were supported by the behavioral response of naïve females and males following a chemical inhibition of cortisol synthesis with metyrapone. The metyrapone-exposed adult females were more active during the novel tank diving test, whereas the behavioral response of the metyrapone-treated adult males was significantly reduced relative to CTR fish. Sex differences in behaviors in ZF including boldness, cocaine withdrawal and shoaling preferences and selection have been previously reported (Engeszer et al., 2008; López Patiño et al., 2008; Oswald et al., 2012; Ruhl and McRobert, 2005). However, to our knowledge, this is the first study to report on sexual dimorphism in the locomotor and exploratory activities of adult ZF as a result

of blunted cortisol levels. These sex differences could be attributed to sex-specific coping mechanisms.

In summary, our findings revealed that FLX can permanently disrupt the stress axis by reducing the total cortisol levels in the adult fish regardless of the duration or window of exposure. However, it is unclear whether the mechanism of disruption is the same across exposure periods. We also demonstrated that the magnitude of the cortisol impairment induced by FLX does not depend on the length or timing of exposure. Furthermore, the FLX-induced attenuation in cortisol levels alters the behavioral response to a new environment in the adult fish. This study uncovered that the observed behavioral alteration depends on the window of exposure in addition to the sex of the fish; males are more sensitive to FLX during early-life (period 1) whereas females are more sensitive during a period of sex differentiation (period 2) in ZF. FLX-exposed adult females and males displayed contrasting exploratory and locomotor activities, suggesting a sexual dimorphism in the low cortisol-induced behavioral response to novelty.

This study provides evidence that FLX disrupts the stress axis and behavior of adult females and males depending on the window of exposure. However, considering the continuous discharges of FLX into the aquatic environment, fish and other aquatic life forms are constantly exposed to FLX and a myriad of other chemicals in contaminated waters. Therefore, exposure to these chemicals may pose a risk to non-target aquatic organisms such as fish, potentially affecting their fitness, survival and population dynamics (Comber et al., 2018; Yang et al., 2017b).

4.6. Acknowledgments

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CHAPTER 5: Developmental and reproductive outcomes of zebrafish following an early-life exposure to fluoxetine

5.1. Statement of contribution

Marilyn Vera Chang, designed the study, developed the methodology, conducted the experiments and analyses and prepared the manuscript. Dr. Thomas Moon, helped with the design of the study and manuscript editing. Dr. Vance Trudeau, helped with the design of the study and manuscript editing.

5.2. Introduction

Reproduction is a robust measurement of fitness which relies on complex interactions between the nervous and endocrine systems to control vertebrate reproduction through the regulation of the hypothalamic-pituitary-gonadal axis. The neuroendocrine control of reproduction is modulated by the neurotransmitter serotonin (5-HT) (Le Page et al., 2011; Prasad et al., 2015). In goldfish (*Carassius auratus*), intraperitoneal administration of 5-HT significantly elevated serum levels of gonadotropin hormone following 0.5, 1 and 2 hours post-injection in females and males (Somoza et al., 1988). In an *in vitro* study using perfused goldfish pituitaries, 5-HT also induced gonadotropin secretion in a dose-dependent manner (Somoza and Peter, 1991). In mammals and fish, 5-HT is also involved in regulating sexual behaviors, and gonadal development and maturation (Kreke and Dietrich, 2008; Li and Pelletier, 1995; Prasad et al., 2015; Tanaka et al., 1993; Vitale and Chiochio, 1993). As a consequence of the wide range of the neuroendocrine involvement of 5-HT on reproduction, any factor that disrupts the natural neurotransmitter balance may potentially disturb reproductive fitness in the affected organism.

Some pharmaceuticals are designed to alter 5-HT-mediated neurotransmission. One of these drugs is the antidepressant fluoxetine (FLX), a selective 5-HT reuptake inhibitor which is highly prescribed even among pregnant women and adolescents (Latendresse et al., 2017; Locher et al., 2017; Man et al., 2017; Morkem et al., 2017; Sarginson et al., 2017). Fluoxetine enhances 5-HT neurotransmission in the brain by selectively blocking 5-HT reuptake transporters (Frazer, 2001; Wong et al., 1995). The continuous discharge and improper disposal into wastewater streams have led to the detection of FLX in aquatic environments at concentrations as high as $929 \text{ ng}\cdot\text{L}^{-1}$ (Bueno et al., 2007). The presence of FLX in the environment concomitantly with the high degree of conservation of the neurotransmitter systems in vertebrates (Kreke and Dietrich, 2008) render aquatic vertebrates such as fish from contaminated waters susceptible to the potential effects of FLX on the reproductive system in addition to the other functions related with 5-HT neurotransmission.

Numerous studies have reported that FLX treatment in adult fish disrupt diverse reproductive endpoints. For instance, exposure to 0.54 and $54 \text{ }\mu\text{g}\cdot\text{L}^{-1}$ FLX decreased milt volume and testosterone levels in mature male goldfish whereas their 17β -estradiol (E_2) levels were significantly increased (Mennigen et al., 2010a). In contrast, in mature female goldfish, E_2 levels were reduced by FLX treatment (Mennigen et al., 2008). These results on female goldfish hormone levels were also observed in adult zebrafish (ZF; *Danio rerio*) where FLX exposure for 7 days decreased ovarian E_2 levels (Lister et al., 2009). In this study by Lister et al. (2009), the authors also reported that FLX exposure to the adult ZF reduced expression levels of genes associated with reproduction including the cytochrome P450 family 19 subfamily A polypeptide 1a (*cyp19a1a*; aromatase), follicle stimulating hormone receptor, and luteinizing hormone receptor in the adult females. Exposure to FLX ($28 \text{ ng}\cdot\text{L}^{-1}$) was also found to induce vitellogenin, an estrogen-

dependent yolk protein precursor synthesized by the liver and taken up by the growing oocytes, in male fathead minnows (*Pimephales promelas*) (Schultz et al., 2011). In a comprehensive proteomic study investigating the interactions between FLX and 17 α -ethinylestradiol, a synthetic hormone also detected in the environment, the authors demonstrated that exposure to 0.54 $\mu\text{g}\cdot\text{L}^{-1}$ FLX alone for 14 days increased the plasma vitellogenin levels in adult male goldfish. These protein levels were even higher when the male adult fish were exposed to both FLX and 17 α -ethinylestradiol (Silva de Assis et al., 2013). The number of produced eggs per copulation, and their fecundity were also significantly reduced short after a 7-day exposure to 54 $\mu\text{g}\cdot\text{L}^{-1}$ FLX in female fighting fish (*Betta splendens*) (Forsatkar et al., 2014). Mating behaviors were also altered by FLX treatment in the adult fathead minnows. A chronic 4-week exposure to 1 $\mu\text{g}\cdot\text{L}^{-1}$ FLX significantly altered nest preparation and maintenance in male fish; the FLX-treated males spent more time cleaning the nest. These fish also exhibited a decline in mating behaviors which was replaced by an increase in chasing and biting attacks towards the females (Weinberger and Klaper, 2014).

The effects of FLX on reproduction have been primarily examined following exposures in adult fish. However, we have previously shown that the disruptive effects of FLX on the locomotor and exploratory behaviors vary across different windows of exposure across sexual development (Chapter 4). We have also shown that an early developmental FLX exposure induce a transgenerational disruption of the stress axis in ZF (Chapter 2). Therefore, in this study, we investigated the effects of early-life FLX exposure on the reproductive fitness in the adult ZF across generations. We also assessed the developmental outcomes of the progeny of the exposed ZF.

5.3. Materials and methods

5.3.1. Experimental animals and FLX exposure

Larval and adult ZF from this study were generated and reared as described in Chapter 2 section 2.5.1. Briefly, at 6 months post-fertilization (mpf), 15 pairs of adult ZF (AB strain) were used to produce filial generation 0 (F_0) and each of the lineages from all subsequent generations, F_1 – F_3 . Only virgin fish were used in this experiment to avoid potential confounding effects brought about by fish with different breeding experiences on the assessment of their reproductive fitness. Pairs in F_0 to F_3 that did not spawn at trial 1 were provided a second opportunity with a different mate randomly chosen from non-spawning individuals within the same lineage to eliminate the possibility of mate preference. Mating pairs were set up in the late afternoon in crossing cages with a plastic divider that separated the female from the male and left undisturbed until the following morning. The pairs were allowed to spawn for 1 h 45 min between 09h00 and 11h00 in fresh new water.

Eggs in each generation were immediately collected and counted. At 3 hours post-fertilization (hpf), embryos from the F_0 were randomly distributed to petri dishes containing either embryo medium alone (control, CTR) or supplemented with one of two concentrations of FLX (Sigma-Aldrich; Oakville, Ontario, Canada): $0.54 \mu\text{g}\cdot\text{L}^{-1}$ (Low-FLX lineage, LFL) and $54 \mu\text{g}\cdot\text{L}^{-1}$ (High-FLX lineage, HFL) from 0 to 6 days post-fertilization (dpf). The F_1 to F_3 embryos from each lineage were distributed in plastic Petri dishes and labeled with the number assigned to their parents to monitor for any embryonic or larval developmental effects in a specific clutch. Embryos from all the generations were reared at 28°C without feeding in Petri dishes until 6 dpf. Embryo medium was renewed daily and the appropriate concentrations of FLX replaced.

Larvae and adult ZF were maintained under a 14 h light:10 h dark photoperiod in a temperature-controlled ZF facility and reared in tanks containing heated (28.5 ± 0.2 °C), aerated, dechloraminated City of Ottawa tap water. Fish from the same lineage in each generation were randomly mixed every month to avoid formation of social hierarchies and to reduce potential tank effects. All experiments were conducted following protocols approved by the University of Ottawa Animal Care Protocol Review Committee and undertaken in accordance with institutional animal care guidelines adhering to those of the Canadian Council on Animal Care.

5.3.2. Developmental parameters

The embryos/larvae from the F_0 to F_3 were monitored daily until 6 dpf and the numbers of mortalities, hatchings, and malformations were recorded. Cumulative mortality rates were calculated as the percentage of the cumulative number of embryos that died every day in each clutch (per breeding pair) relative to the number of fertilized eggs. Cumulative hatching rates were computed as the percentage of the cumulative number of embryos that hatched in each clutch relative to the number of fertilized eggs, whereas hatching success rate is the percentage of the hatched embryos per clutch that survived up to 5 dpf. Malformation rate was assessed by calculating the percentage of larvae per clutch that showed any sign of deformities relatively to the total hatched embryos (dead and alive). Percentage body phenotype was calculated as the number of larvae that displayed a white-body phenotype compared to the total number of hatched embryos (dead and alive) in each treatment (not by clutch).

5.3.3. Reproductive parameters

The assessment of the reproductive fitness of the CTR and FLX lineages from F₀ to F₃ included sex ratio, spawning rate, fertilization rate, condition factor (*K*) and gonadosomatic index (GSI). Following the two spawning trials, the 15 pairs from each lineage were euthanized and their weights and lengths were recorded. The gonads of each individual fish were removed and weighed to determine the GSI.

Spawning rate was calculated as the number of pairs that spawned in each lineage during either attempt 1 or attempt 2 divided by the total number of breeding pairs per lineage (15). Fertilization rate was determined as the percentage of the total number of fertilized eggs per breeding pair divided by the total number of eggs. *K* indices were calculated for each lineage as $K = \text{body weight (g)} \times \text{length}^{-3} \text{ (cm)} \times 100$ and GSI as $\text{gonad weight} \times \text{body weight}^{-1} \times 100$.

5.3.4. Statistical analyses

The normality of the data was assessed using the Shapiro-Wilk test, and the homogeneity of variances was checked using Levene's median test. One-way analysis of variance (ANOVA) and Tukey's post-hoc test were used to determine significant differences between CTR and FLX lineages. The data were analyzed by one-way ANOVA on ranks if data were not normally distributed. Body phenotype and sex ratio were tested with the Chi-Square test (χ^2 -test) whereas spawning rate was analyzed with Fisher's exact test. The condition factor (*K*) and GSI data were analyzed by two-way ANOVA or two-way ANOVA on ranks if data were non-parametric. All statistical analyses were performed using SigmaPlot 11.0 (Systat Software, Inc.) and statistical significance was set at $\alpha < 0.05$.

5.4. Results

5.4.1. The effects of FLX on the development of larval ZF

Different developmental parameters were assessed to test for the effects of FLX on ZF development. No significant effects on the cumulative mortality rate were found across lineages at 5 dpf from the F_0 ($F_{2,39} = 2.647$, $P = 0.084$), F_1 ($F_{2,26} = 0.151$, $P = 0.861$), F_2 ($H_2 = 1.549$, $P = 0.461$) and F_3 ($H_2 = 0.574$, $P = 0.750$) generations (Fig. 5.1). A significant effect across lineages was detected in the cumulative hatching rate of the F_0 ($F_{2,39} = 3.589$, $P = 0.037$), more specifically the larvae from the LFL displayed an increase in hatching rate compared to the CTR. In contrast, the hatching rate of the F_1 ($F_{2,26} = 0.446$, $P = 0.645$), F_2 ($H_2 = 3.032$, $P = 0.220$) and F_3 ($H_2 = 0.608$, $P = 0.738$) generations was not altered by FLX (Fig. 5.2). Moreover, FLX did not affect hatching success (Fig. 5.3) or malformation rate (Fig. 5.4) in generations F_0 ($H_2 = 3.187$, $P = 0.203$; $H_2 = 4.370$, $P = 0.112$, respectively), F_1 ($H_2 = 0.950$, $P = 0.622$; $H_2 = 0.142$, $P = 0.932$, respectively), F_2 ($H_2 = 0.307$, $P = 0.858$; $H_2 = 0.223$, $P = 0.894$, respectively) and F_3 ($H_2 = 0.157$, $P = 0.924$; $H_2 = 1.982$, $P = 0.37$, respectively).

Some of the larvae from the FLX lineages displayed a reduction in pigmentation when compared to the CTR. This white-body phenotype (White) was obvious when viewed with the naked eye (Fig. 5.5). We performed a Chi-square test to determine if there was a statistical difference between the numbers of larvae displaying the white-body phenotype compared to the larvae with normal pigmentation across lineages in each generation. No white-body phenotype was observed in the F_0 . In contrast, the larvae from the LFL and HFL in the F_1 exhibited this white-body phenotype; CTR compared to LFL ($\chi^2_1 = 447.233$, $P < 0.001$), CTR compared to HFL ($\chi^2_1 = 39.350$, $P < 0.001$) and LFL compared to HFL ($\chi^2_1 = 326.384$, $P < 0.001$). In the F_2 , only the HFL was found with this phenotype; CTR compared to HFL ($\chi^2_1 = 191.526$, $P < 0.001$) and LFL

compared to HFL ($\chi^2_1 = 236.542$, $P < 0.001$). The same patterns were observed in the F_3 , where some larvae from the HFL were the only fish affected; CTR compared to HFL ($\chi^2_1 = 100.843$, $P < 0.001$) and LFL compared to HFL ($\chi^2_1 = 127.076$, $P < 0.001$).

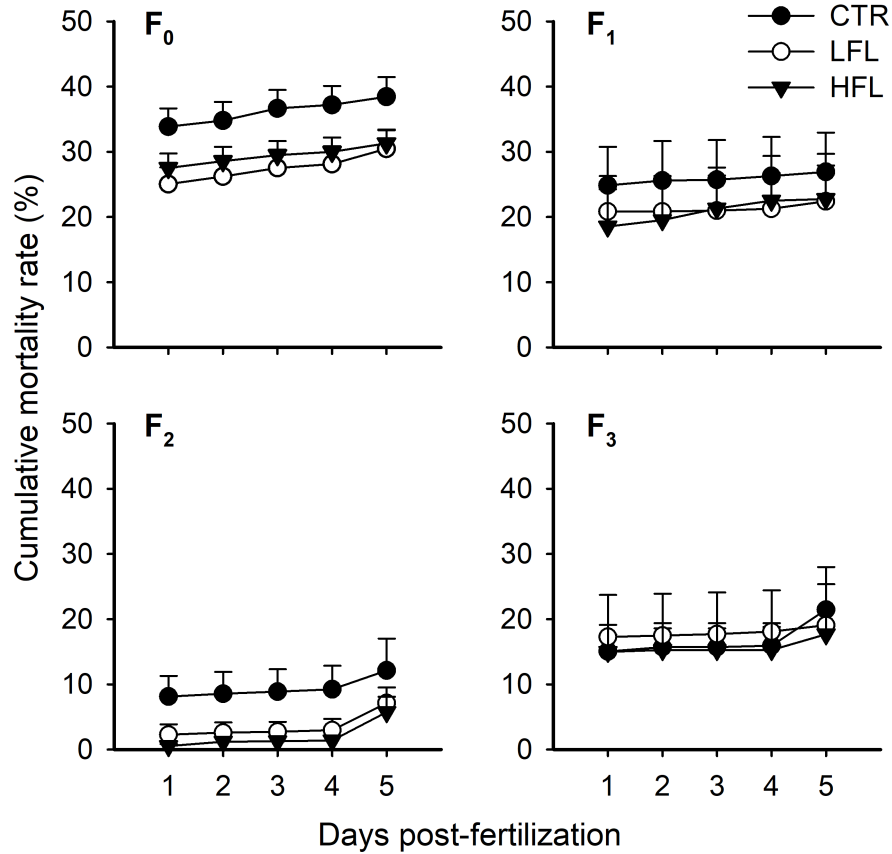


Fig. 5.1. Cumulative mortality rate of larvae from the CTR and FLX lineages. The FLX lineages were obtained from exposing the F_0 to 0.54 (LFL) and 54 (HFL) $\mu\text{g}\cdot\text{L}^{-1}$ from 0 to 6 dpf. n = individual clutches from 15 pairs per treatment after two spawning attempts. If the same pair spawned during the two attempts, only the first clutch was included in the analysis. Data presented as mean $\pm$ SEM, one-way ANOVA (on ranks: F_2 and F_3) did not show any statistical differences across treatments. Statistical tests were performed only for the last time point (5 dpf) across treatments.

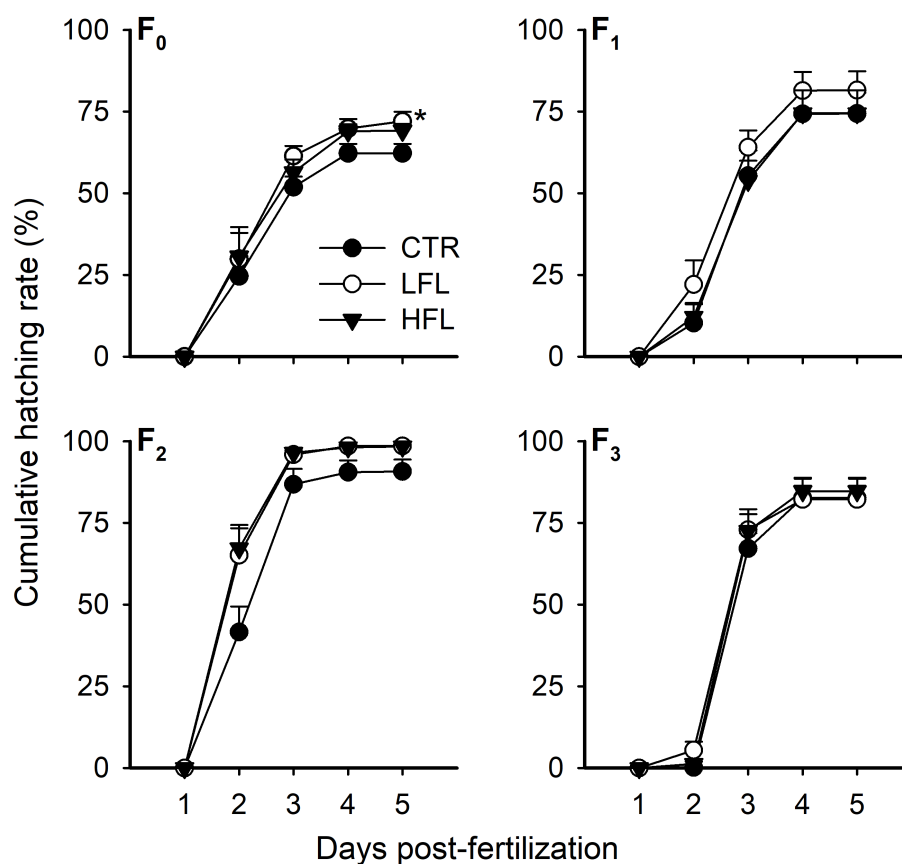


Fig. 5.2. The percentage of fertilized eggs from the CTR and FLX lineages that hatched. The FLX lineages were obtained from exposing the F_0 to 0.54 (LFL) and 54 (HFL) $\mu\text{g}\cdot\text{L}^{-1}$ from 0 to 6 dpf. n = individual clutches from 15 pairs per treatment after two spawning attempts. If the same pair spawned during the two attempts, only the first clutch was included in the analysis. Data presented as mean $\pm$ SEM, one-way ANOVA (on ranks: F_2 and F_3), $P < 0.05$. Statistical analysis was performed only for the last time point (5 dpf) across treatments. * $P = 0.026$ for LFL compared to the CTR.

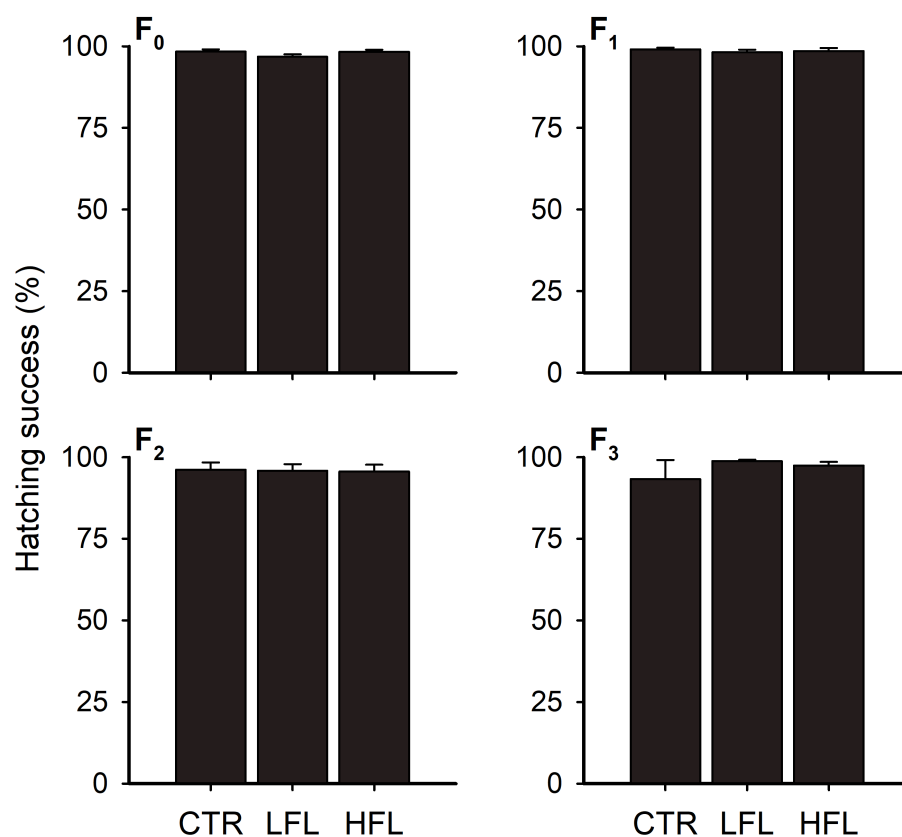


Fig. 5.3. The percentage of hatched embryos from the CTR and FLX lineage that survived up to 5 dpf. The FLX lineages were obtained from exposing the F₀ to 0.54 (LFL) and 54 (HFL) $\mu\text{g}\cdot\text{L}^{-1}$ from 0 to 6 dpf. n = individual clutches from 15 pairs per treatment after two spawning attempts. If the same pair spawned during the two attempts, only the first clutch was included in the analysis. Data expressed as mean $\pm$ SEM; one-way ANOVA on ranks did not reveal any statistical differences across treatments.

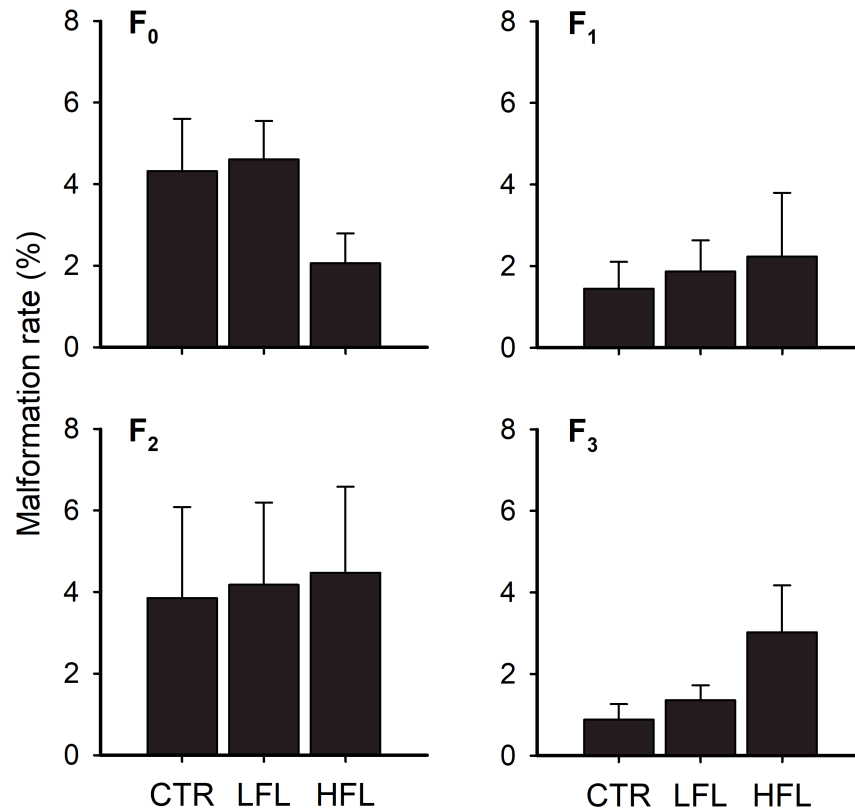


Fig. 5.4. The percentage of hatched embryos from the CTR and FLX lineages that showed any indication of deformities. All hatched larvae were included in this analysis even if the larva did not survive. The FLX lineages were obtained from exposing the F₀ to 0.54 (LFL) and 54 (HFL) $\mu\text{g}\cdot\text{L}^{-1}$ from 0 to 6 dpf. n = individual clutches from 15 pairs per treatment after two spawning attempts. If the same pair spawned during the two attempts, only the first clutch was included in the analysis. Data expressed as mean $\pm$ SEM; one-way ANOVA on ranks did not find any statistical differences across treatments.

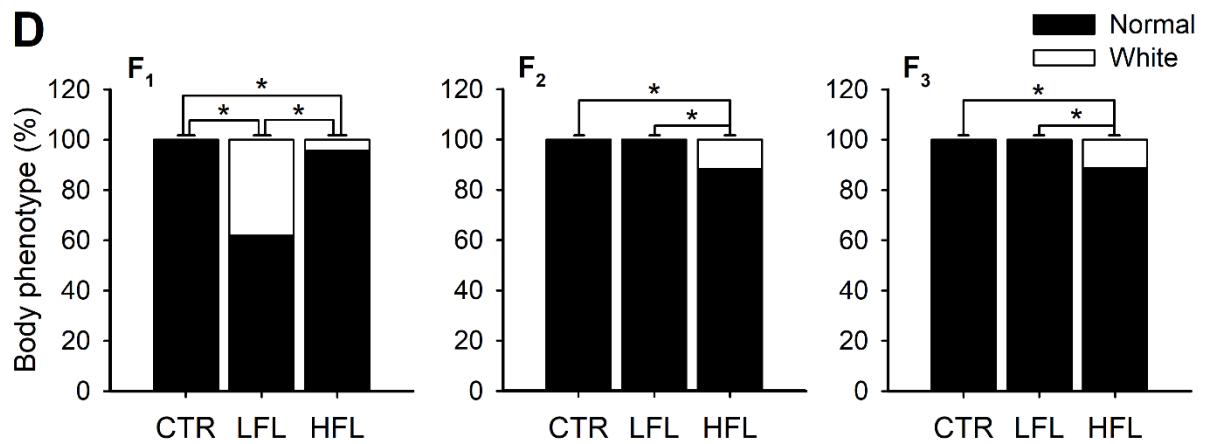
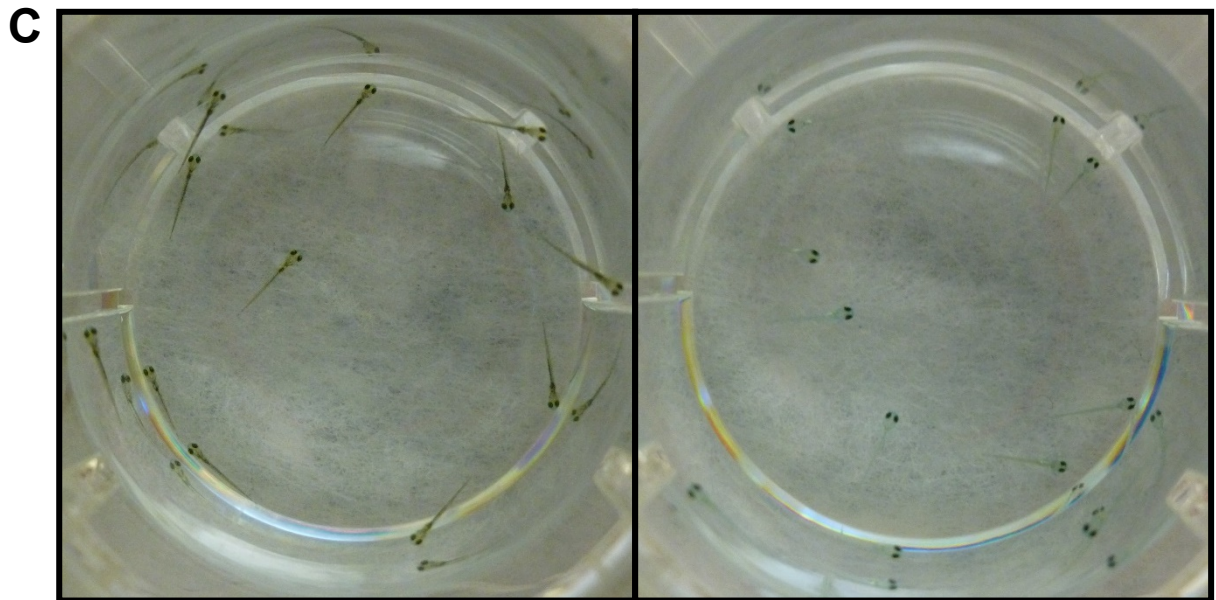
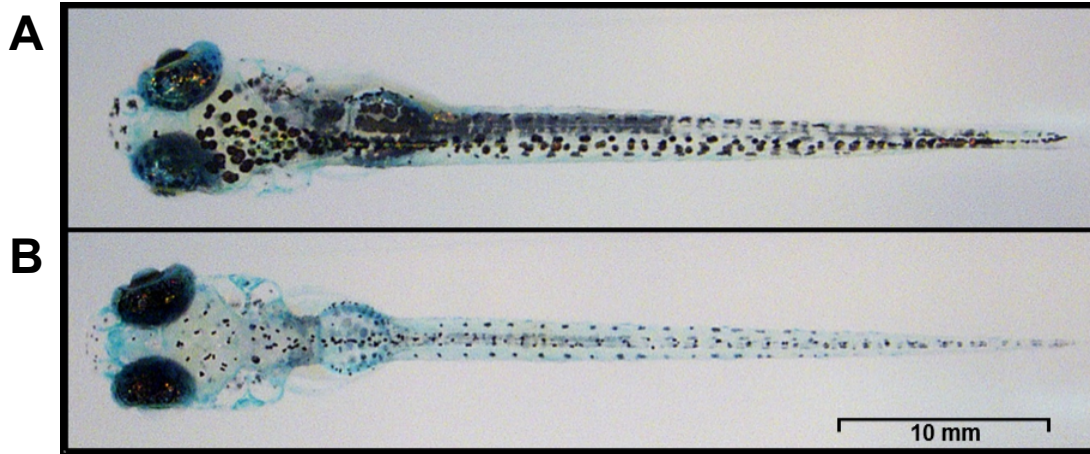


Fig. 5.5. Body phenotype of the larvae from the CTR and FLX lineages. The FLX lineages were generated from exposing the F₀ to 0.54 (LFL) and 54 (HFL) $\mu\text{g}\cdot\text{L}^{-1}$ from 0 to 6 dpf. (A) Normal pigmentation of ZF larvae observed under the microscope. (B) Reduced pigmentation observed in some of the larvae from the FLX lineage observed under the microscope. This type of body phenotype is described as “White” in the figure. (C) Group of 6 dpf larvae displaying the normal pigmentation patterns (*Left*) and the white-body phenotype (*Right*). (D) Percentage of the normal pigmentation and white-body phenotype observed during the developmental stage of the larva. The percentage describes the hatched larvae from 15 pairs per treatment that displayed each of the two phenotypes (total number of larvae: 914 to 1,181 for F₁; 1,560 to 1,937 for F₂; 737 to 1,114 for F₃). Data expressed as a percentage of the body phenotype, * $P < 0.001$, Chi-square test.

5.4.2. Embryonic/larval exposure to FLX does not affect reproductive fitness of adult ZF

We assessed the sex ratio of adult ZF to determine whether FLX induced either masculinization or feminization of the population. We performed a Chi-square test with the number of females and males across lineages (Fig. 5.6). No significant differences were found in the F₀; CTR compared to LFL ($\chi^2_1 = 0.102$, $P = 0.749$), CTR compared to HFL ($\chi^2_1 = 0.299$, $P = 0.585$) and LFL compared to HFL ($\chi^2_1 = 0.008$, $P = 0.928$). In the F₁, there was a significant 11% increase in the number of males from the LFL compared to the HFL ($\chi^2_1 = 4.997$, $P = 0.025$). No other differences in the sex ratio from the F₁ were observed; CTR compared to LFL ($\chi^2_1 = 0.724$, $P = 0.395$) and CTR compared to HFL ($\chi^2_1 = 1.361$, $P = 0.243$). A 17% increase in the number of males from the generation F₂ was also observed in the HFL compared to the CTR ($\chi^2_1 = 6.466$, $P = 0.011$). No other sex ratio differences were observed; CTR compared to LFL ($\chi^2_1 = 0.129$, $P = 0.719$) and LFL compared to HFL ($\chi^2_1 = 3.778$, $P = 0.052$). In the F₃, the LFL group produced 13% more males relative to the CTR ($\chi^2_1 = 9.367$, $P = 0.002$) and 19% more than the HFL ($\chi^2_1 = 17.930$, $P < 0.001$). The sex ratio did not vary in the CTR compared to HFL from the F₃ ($\chi^2_1 = 1.395$, $P = 0.238$).

We also examined spawning rates in the CTR and FLX lineages (Fig. 5.7). However, no significant differences were found in the F_0 , CTR compared to LFL ($P = 1.000$), CTR compared to HFL ($P = 1.000$) and LFL compared to HFL ($P = 1.000$); F_1 , CTR compared to LFL ($P = 1.000$), CTR compared to HFL ($P = 0.651$) and LFL compared to HFL ($P = 1.000$); F_2 , CTR compared to LFL ($P = 0.682$), CTR compared to HFL ($P = 1.000$) and LFL compared to HFL ($P = 1.000$); and F_3 , CTR compared to LFL ($P = 1.000$), CTR compared to HFL ($P = 0.109$) and LFL compared to HFL ($P = 0.245$).

The fertilization rate of the eggs produced by the 15 pairs in each lineage from F_0 to F_3 was also evaluated (Fig. 5.8). No significant effects of FLX exposure were found in the F_0 ($H_2 = 4.972$, $P = 0.083$), F_2 ($H_2 = 4.209$, $P = 0.122$) and F_3 ($H_2 = 1.994$, $P = 0.369$). In contrast, the fertilization rate of the F_1 pairs ($H_2 = 14.310$, $P < 0.001$) was significantly affected by FLX. A Tukey's post-hoc test revealed that the fertilization rate was reduced in the adults from the HFL compared to the adults from the CTR group in the F_1 .

A two-way ANOVA (on ranks for F_0) was conducted to examine the effects of sex and lineages (CTR and FLX) on the K -factor of the adult (6 months of age) females and males (Fig. 5.9). The K -factor was significantly lower in males compared to females (simple main effect of sex) in the F_0 ($F_{1,80} = 114.372$, $P < 0.001$), F_1 ($F_{1,82} = 54.580$, $P < 0.001$), F_2 ($F_{1,81} = 48.712$, $P < 0.001$) and F_3 ($F_{1,82} = 69.921$, $P < 0.001$). In addition, FLX did affect the K -factor within the generations: F_0 ($F_{2,80} = 0.199$, $P = 0.820$), F_1 ($F_{2,82} = 0.807$, $P = 0.450$), F_2 ($F_{2,81} = 1.104$, $P = 0.337$) and F_3 ($F_{2,82} = 0.620$, $P = 0.541$). There was no statistically significant interaction between the effects of sex and lineages on K -factor in F_0 ($F_{2,80} = 0.053$, $P = 0.949$), F_1 ($F_{2,82} = 0.010$, $P = 0.905$), F_2 ($F_{2,81} = 0.074$, $P = 0.929$) and F_3 ($F_{2,82} = 0.244$, $P = 0.784$).

Furthermore, we also conducted a two-way ANOVA on ranks to determine the effects of sex and lineages on the GSI (Fig. 5.10). As expected, the GSI was significantly lower in males compared to females in the F_0 ($F_{1,82} = 256.692$, $P < 0.001$), F_1 ($F_{1,81} = 273.786$, $P < 0.001$), F_2 ($F_{1,81} = 246.994$, $P < 0.001$) and F_3 ($F_{1,84} = 266.777$, $P < 0.001$). No significant effects of FLX on GSI was evident in the F_0 ($F_{2,82} = 0.931$, $P = 0.398$), F_1 ($F_{2,81} = 1.812$, $P = 0.170$), F_2 ($F_{2,81} = 0.362$, $P = 0.698$) and F_3 ($F_{2,84} = 1.753$, $P = 0.179$). A significant interaction was found between sex and lineages on the GSI in F_1 ($F_{2,81} = 3.478$, $P = 0.036$), in which the males from the LFL group exhibited a 23% lower GSI than the males from the HFL. In contrast, no interactions were observed in F_0 ($F_{2,82} = 1.135$, $P = 0.327$), F_2 ($F_{2,81} = 0.749$, $P = 0.476$) and F_3 ($F_{2,84} = 0.688$, $P = 0.506$).

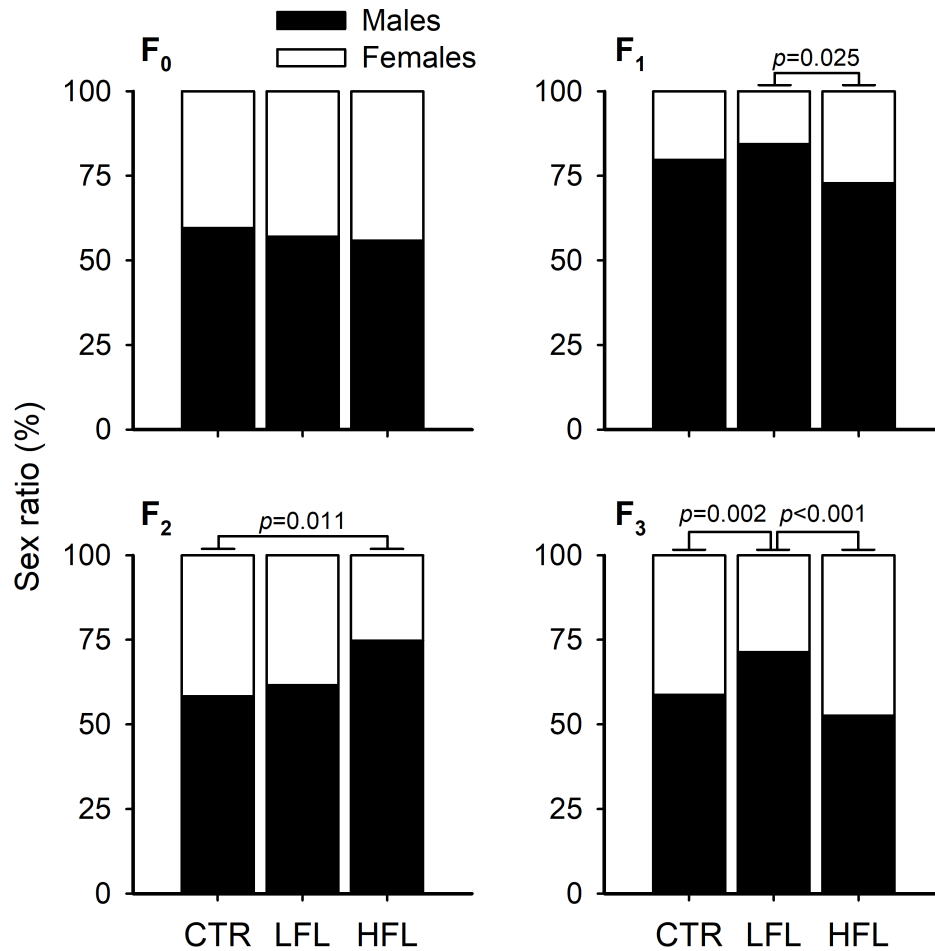


Fig. 5.6. The female to male ratio of adult ZF from the CTR and FLX lineages (0.54 (LFL) and 54 (HFL) $\mu\text{g}\cdot\text{L}^{-1}$) in each generation. Data were collected from the entire population of CTR and FLX lineages at 6 mpf. Analysis following Chi-square test, $P < 0.05$. $n = 163$ ZF for CTR, 142 (LFL), 154 (HFL) in F₀; 128 (CTR), 147 (LFL), 140 (HFL) in F₁; 115 (CTR), 99 (LFL), 119 (HFL) in F₂; 240 (CTR), 332 (LFL), 192 (HFL) in F₃.

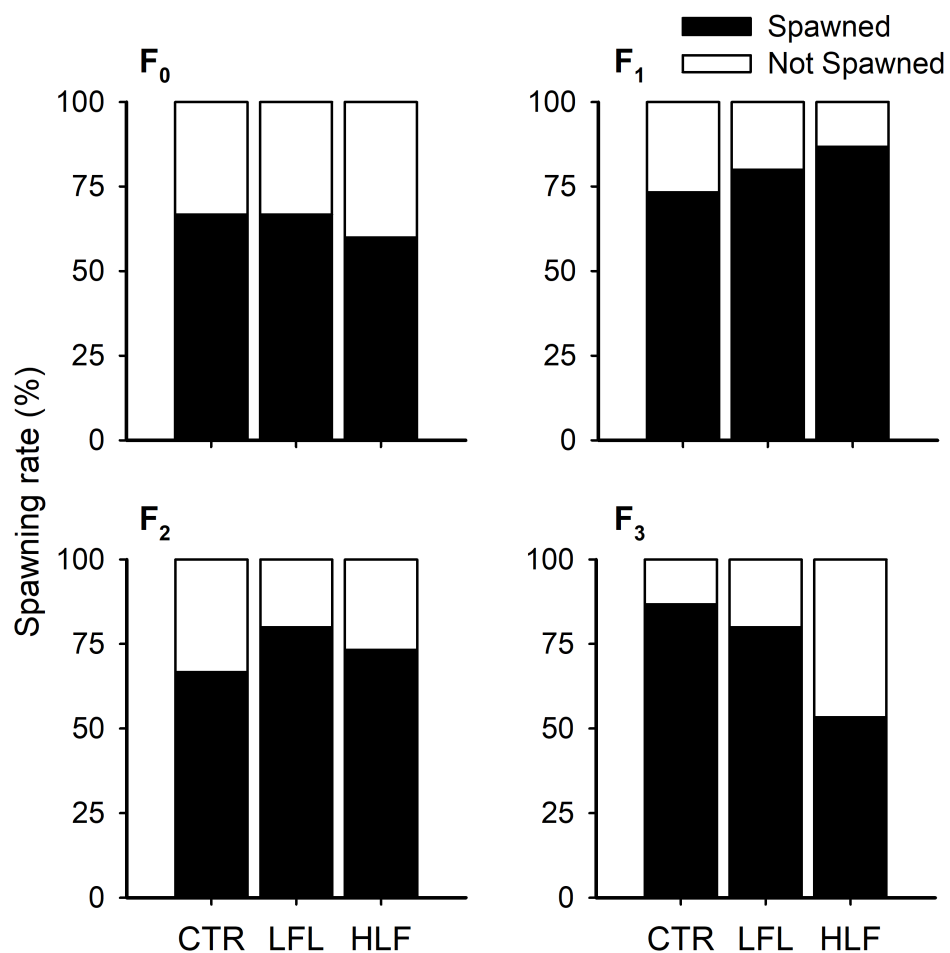


Fig. 5.7. Spawning rate of adult ZF from the CTR and FLX lineages (0.54 (LFL) and 54 (HFL) $\mu\text{g}\cdot\text{L}^{-1}$). Data were collected from 15 adult (6 months) pairs per treatment after two spawning attempts. The Fisher's exact test found no significant differences in any of the generations displayed.

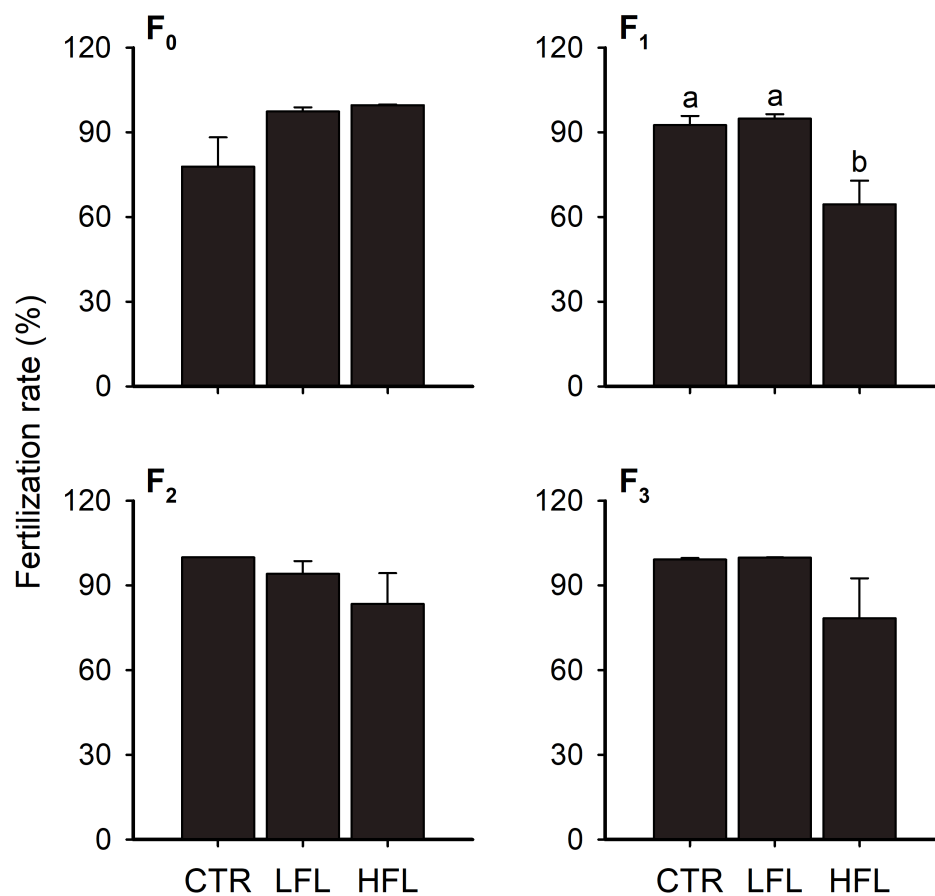


Fig. 5.8. The percentage of fertilized eggs from the mating of adult (at 6 months of age) ZF of the CTR and FLX lineages (0.54 (LFL) and 54 (HFL) $\mu\text{g}\cdot\text{L}^{-1}$). Data were collected from 15 pairs per treatment after two spawning attempts. Data presented as mean $\pm$ SEM, one-way ANOVA on ranks, $P < 0.05$.

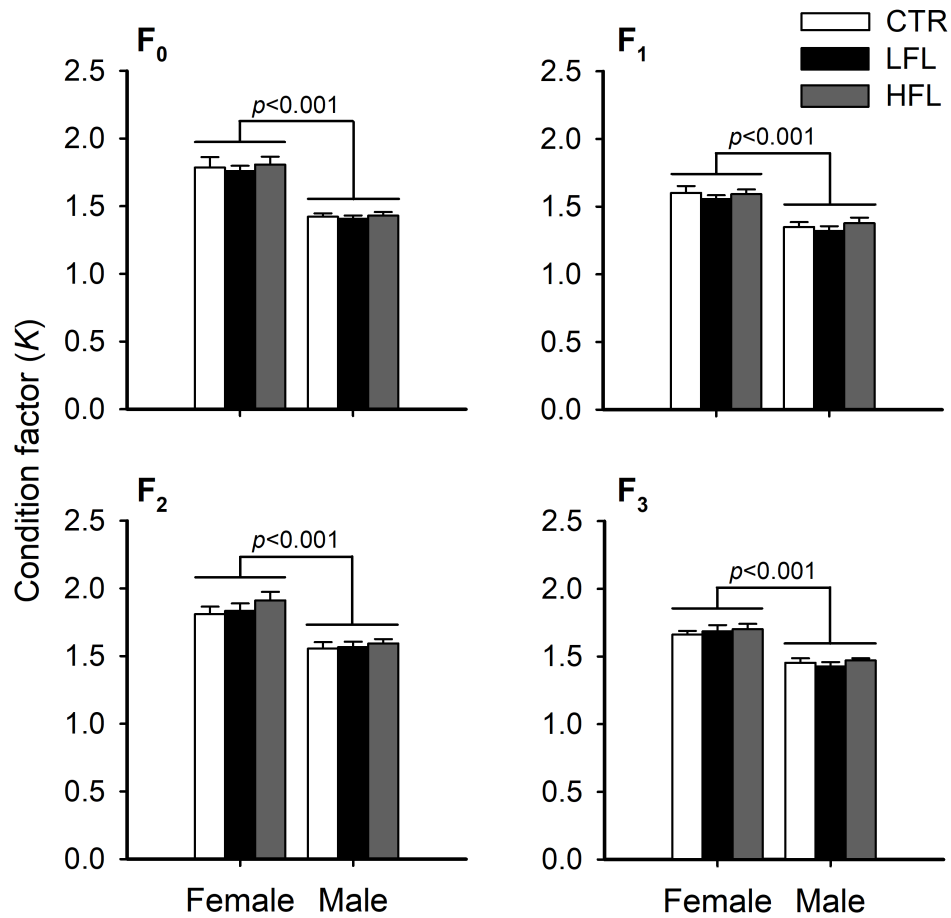


Fig. 5.9. Condition factor (K) of adult (at 6 months of age) ZF from the CTR and FLX lineages (0.54 (LFL) and 54 (HFL) $\mu\text{g}\cdot\text{L}^{-1}$). Data are displayed as mean $\pm$ SEM, $n = 15$ biological replicates per group, two-way ANOVA (on ranks: F₀), $P < 0.05$.

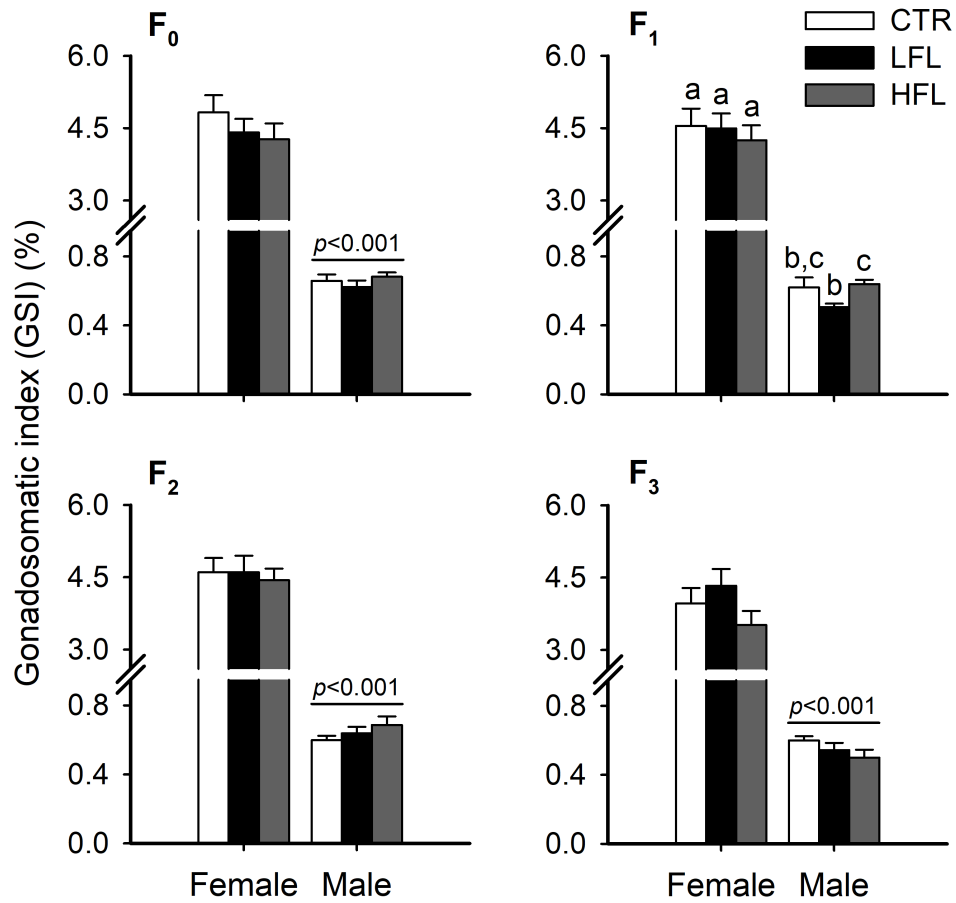


Fig. 5.10. Gonadosomatic index (GSI) of adult (at 6 months of age) ZF across 3 generations of the CTR and FLX lineages (0.54 (LFL) and 54 (HFL) $\mu\text{g}\cdot\text{L}^{-1}$). Data are displayed as mean $\pm$ SEM, $n = 15$ biological replicates per group, two-way ANOVA on ranks, $P < 0.05$. The letters represent statistical difference when interactions are present. P -values displayed above the bars represent significant differences compared within sex.

5.5. Discussion

Our findings revealed that human therapeutic and environmentally relevant concentrations of FLX did not affect the development of larvae ZF. Exposure to FLX from 0 to 6 dpf did not induce any alterations in the total mortality rate, hatching rate and the extent of malformations in the F₀ or any subsequent generations (F₁ to F₃). These results on the effects of FLX on the developmental endpoints are consistent with other studies that exposed larvae ZF to similar FLX concentrations (Mishra et al., 2017; Park et al., 2012). Although these studies showed no effect by FLX on their F₀ larvae, to our knowledge, we are the first study to report that FLX did not affect mortality, hatching and malformation rates in the descendants (F₁ to F₃ generations) of the FLX-treated fish.

In contrast to the lack of effects on mortality and hatching, a hypopigmentation pattern was observed in the F₁ to F₃ larvae from specific FLX lineages. This phenotype was found in 38% of the LFL larvae in the F₁, whereas only 4% of the HFL was affected. In contrast, the pigmentation pattern in LFL larvae from the F₂ and F₃ generations was not altered. However, 12% and 11% of the HFL larvae from the F₂ and F₃, respectively, showed a reduction in their pigmentation. This abnormality in the pigmentation induced by FLX was also observed in a study by Cunha et al. (2016), where exposure to 277 µg·L⁻¹ FLX for 80 h affected 35% of the larvae ZF. An *in vitro* study on isolated scale melanophores (pigmented cells) from tilapia (*Oreochromis mossambicus*) showed that exposure to 5-HT and FLX induced melanosomes (pigments) dispersion (Salim et al., 2013). The aggregation or translocation of melanosomes towards the center is associated with light color, whereas dispersion or translocation towards the periphery gives a darker tint of the dorsal surface (Sugimoto et al., 2005). Treatment with FLX has been shown to increase plasma 5-HT levels in adult Gulf toadfish (*Opsanus beta*) (McDonald et al., 2011; Morando et al., 2009).

However, in our study, we did not measure 5-HT levels in either the F₀ or in any of the subsequent generations. Therefore, it is uncertain whether 5-HT levels differed in the exposed larvae compared to the larvae from the FLX lineages in the F₁ to F₃.

Other important modulators of the pigmentation patterns or the translocation of melanosomes are adrenaline and noradrenaline. High expression of the adrenoceptor beta 2 surface a (*adrb2a*) in larval ZF skin, morpholino-mediated inhibition of *adrb2a* and resultant hypopigmentation (Wang et al., 2009) indicate the involvement of adrenaline and noradrenaline signaling in pigmentation. A study by Xu and Xie (2011) demonstrated that other adrenoreceptors including adrenoceptor alpha 1 and alpha 2 are also involved in the dispersion and aggregation of the melanosomes in ZF skin. Interestingly, the transcriptional profile of the adult male kidney ZF from our previous study (Chapter 2) revealed that adrenergic signaling in the F₀ was significantly up-regulated in the LFL compared to the CTR, whereas the HFL from the F₃ exhibited a down-regulation of this signaling. Therefore, our previous results support that disruption of the adrenergic signaling by FLX in the F₃ may play a role in the hypopigmentation pattern exhibited by the F₃ larvae from the FLX lineage. In teleosts, the kidney is composed of many cell types including epithelial cells (Flik et al., 2006), hence it is not surprising if melanocytes are also found in this tissue. Nevertheless, it remains to be established whether the alteration of the adrenergic signaling by FLX in the skin is associated with the reduction in pigmentation observed in our F₁ to F₃ larvae. A study by Cunha et al. (2018) reported that exposure to high concentrations of FLX (173 and 276.8 $\mu\text{g}\cdot\text{L}^{-1}$) induced a down-regulation of the adrenergic receptors transcripts in ZF embryos. The difference observed in the adrenergic related pathways between our FLX-exposed fish (F₀) and the FLX-exposed embryos from Cunha et al. (2016) may be attributed to the different concentrations of FLX that our fish were exposed to. However, since whole-embryos were

assessed in the study by Cunha et al. (2018), it is impossible to associate the alteration of the adrenergic receptor gene expression specifically to melanophores.

We also investigated the reproductive fitness of the adult ZF from the FLX lineages in generations F₀ to F₃. A significant increase in the proportion of males was found in the HFL from the F₂, however, this effect disappeared in the F₃. We also observed an increase in males in the LFL only from the F₃. These effects on the sex ratio were not very pronounced nor consistent across generations. Furthermore, no differences were found in spawning, fertilization rate, *K*-factor and GSI of these adult fish from the FLX lineages. Therefore, these findings demonstrated that a 6-day early developmental exposure to FLX has a minor impact on the reproductive system in ZF. To our knowledge, our study is the first to examine the long-term effects of short embryonic FLX exposure on reproduction in the adult fish. However, our results are supported by studies where exposures to FLX in fish was conducted during adulthood. For instance, in a study using adult Japanese medaka (*Oryzias latipes*), the authors reported that a 14-day FLX exposure (at concentrations of 0.1, 0.5, 1 and 5 µg·L⁻¹) did not alter egg production, fertilization or spawning rate in adult fish (Foran et al., 2004). Moreover, a 4-week exposure to FLX at concentrations of 0.1, 1 and 10 µg·L⁻¹ did not affect the number of spawned eggs in fathead minnows, whereas at 100 µg·L⁻¹ FLX a significant reduction in egg numbers was observed (Weinberger and Klaper, 2014). Our results also agree with a study in rats, where developmental exposure to FLX was not detrimental for the sexual reproduction in adult females but instead it facilitated proceptive and receptive behaviors including an increase in the lordosis quotient, and a reduction in rejection behavior (Rayen et al., 2014). In contrast, Lister et al. (2009) showed that adult ZF exposed to 32 µg·L⁻¹ FLX decreased the average egg numbers spawned by the females. However, the lack of

effects of FLX on GSI was consistent between our study and that of Lister et al. (2009) who observed no change in FLX-exposed adult ZF.

In summary, we have demonstrated that exposure to human physiological (HFL) and environmentally relevant (LFL) concentrations of FLX does not affect the development of ZF, yet it has the potential to alter the pigmentation patterns of subsequent generations. Moreover, we found that FLX does not disrupt the reproductive capability in either adult ZF or in their progeny, suggesting that exposure to FLX during early development does not affect the reproductive fitness of the fish. However, FLX affects other components of fitness in fish as it was demonstrated in our previous study (Chapter 2) where a 6-day embryonic exposure to FLX impaired the stress response and decreased the locomotor and exploratory activities in adult male ZF across generations. The findings in this current study on the lack of effects of early-life FLX exposure on survival and reproduction is evidence that the pronounced effect of FLX on the stress axis and behavior is not attributed to selection. Normal reproduction allows for efficient transfer and inheritance of the reduced cortisol response and behavioral effects observed in our FLX-treated fish from one generation to the other, increasing the likelihood of an epigenetic mode of transgenerational inheritance.

5.6. Acknowledgments

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CHAPTER 6: Venlafaxine exposure in F4 embryos intensifies the reduced cortisol phenotype in the adult females and altered sex steroid levels in adult zebrafish

6.1. Statement of contribution

Marilyn Vera Chang, designed the study, developed the methodology, conducted the experiments and analyses and prepared the manuscript. Dr. Thomas Moon, helped with the design of the study and manuscript editing. Dr. Vance Trudeau, helped with the design of the study and manuscript editing.

6.2. Introduction

Pregnancy and the postpartum period are accompanied by an increase in vulnerability to psychiatric disorders particularly depression and anxiety (Meltzer-Brody et al., 2018). Psychiatric disorders during pregnancy are associated with poor maternal health and with numerous adverse outcomes for the offspring including impairments in cognitive and physical abilities and increased risk to develop neuropsychiatric disorders (Huizink et al., 2004; 2003; Susser et al., 2016; Van den Bergh et al., 2008). A variety of pharmacological agents exist today which are used as treatment for these mood disorders. The selective serotonin reuptake inhibitor (SSRI) family of antidepressants especially Fluoxetine (FLX, Prozac®), are the first-line of treatment for pregnant women with these affective disorders (Latendresse et al., 2017; Locher et al., 2017; Man et al., 2017; Morkem et al., 2017).

Even though SSRIs are highly tolerable and efficient at treating mood disorders (Mace and Taylor, 2000), in cases of treatment resistance or low remission rates, serotonin-norepinephrine reuptake inhibitors (SNRI) may be used as an alternative (Furu et al., 2015). The therapeutic mechanism of action of the SNRIs involves blocking presynaptic reuptake transporters of

serotonin, norepinephrine, and to a lesser extent, dopamine, resulting in increased extracellular monoamine concentrations within the central nervous system and thereby an increase in neurotransmission (Holliday and Benfield, 1995; Safeekh and Pinto, 2009). Venlafaxine (VEN; Effexor XR®, Fig. 1.2) is the most frequently prescribed drug from the SNRI family (Gribbin et al., 2011).

Venlafaxine, similar to FLX, has been detected in the cord blood and amniotic fluid of pregnant women that are therapeutically treated with this drug, thereby increasing the monoamines levels target by VEN in the fetus (Bellantuono et al., 2015). Concentrations of VEN in the cord blood of treated pregnant women ranges from 9 to 232 $\mu\text{g}\cdot\text{L}^{-1}$ (Ewing et al., 2015; Loughhead et al., 2006; Rampono et al., 2004; Rampono et al., 2009). Venlafaxine treatment during pregnancy may disturb sensitive developmental processes in the brain of the fetus since serotonin, norepinephrine, and dopamine play important roles during fetal development (Bogi et al., 2018). This may lead to neurobehavioral consequences later in life as was observed in our previous study using FLX (Chapter 2). Exposure to human physiologically relevant concentrations of FLX during zebrafish (ZF; *Danio rerio*) embryogenesis permanently disrupted the stress axis in the adult fish, an effect that persisted in their progeny at least to the F₃ generation without diminution. We also found that this hypocortisol phenotype in the FLX lineages was associated with decreased locomotor and exploratory activities in the adult males for 2 generations.

Since genetic predisposition plays an important factor in clinical depression (Albert et al., 2012; Krishnan and Nestler, 2008; Shelton, 2007) and depressed individuals sometimes fail to properly respond to SSRI medications, it is therefore expected that many generations of the same family can be treated with a different family of antidepressants. Moreover, this is also true in the

environment where aquatic organisms are exposed to a variety of chemicals including many families of antidepressants.

Given that in pregnant women FLX is the first-line of treatment for depression and VEN is the alternative, we recreated this situation using ZF to encompass both human and environmental concerns. We tested the hypothesis that FLX exposure during embryogenesis alters the response of the descendants to VEN treatment during early-life. We specifically focused on the stress axis and behavioral responses to new environments since we previously demonstrated that these were disrupted by FLX (Chapter 2). Moreover, since there is evidence of VEN ($10 \mu\text{g}\cdot\text{L}^{-1}$) disrupting embryo production following a 2-week exposure in adult ZF (Galus et al., 2013), we examined the development and reproduction of fish following VEN treatment. For this study, we used our control (CTR) and FLX lineages that we previously generated (see Chapter 2).

6.3. Materials and methods

6.3.1. Experimental animals and exposure

The fish used in this study were obtained as described in Chapter 2 section 2.5.1. Briefly, at 6 months post-fertilization (mpf), 15 adult ZF pairs were bred to generate filial generation 0 (F_0). At 3 hours post-fertilization (hpf), the F_0 embryos were randomly assigned to glass petri dishes containing either embryo medium alone (CTR; $0.94 \mu\text{M}$ methylene blue, 4.9 mM NaCl, 0.17 mM KCl, 0.33 mM $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$, 0.33 mM $\text{CaCl}_2\cdot 2\text{H}_2\text{O}$) or supplemented with one of two concentrations of FLX (Sigma-Aldrich; Oakville, Ontario, Canada): $0.54 \mu\text{g}\cdot\text{L}^{-1}$ (LOW FLX lineage, LFL) and $54 \mu\text{g}\cdot\text{L}^{-1}$ (HIGH FLX lineage, HFL) from 0 to 6 days post-fertilization (dpf). No other FLX exposures were carried out on the subsequent generations. Fifteen pairs of virgin adult ZF (6 mpf) from each lineage were used to produce generations F_1 to F_4 .

Immediately after 1 h 45 min of spawning of the F₃ pairs, F₄ embryos from each lineage (CTR, LFL and HFL) were arbitrarily divided into two groups (see Fig. 6.1) and placed in glass petri dishes containing either embryo medium alone (0 µg·L⁻¹ VEN) or supplemented with a nominal concentration of 5 µg·L⁻¹ VEN (Sigma-Aldrich; Oakville, Ontario, Canada). Concentrated stock solutions of VEN were prepared in the same water that was used for the embryo medium preparation, therefore, there was no need for vehicle control. Exposure to VEN was conducted from 3 hpf to 6 dpf. The nominal VEN concentration used in this study was environmentally relevant (Arnnok et al., 2017; Couperus et al., 2016; Metcalfe et al., 2010). Embryos throughout the study were reared in Petri dishes until 6 dpf at a maximum density of 1 embryo·mL⁻¹ and maintained at 28 °C without feeding. The embryo media or exposure solutions were renewed daily concomitantly with the removal of dead embryos. Larvae were fed three times a day with Zebrafish Management Ltd. (Winchester, England) fry food diet of the appropriate size and according to their developmental stage and from 60 dpf until adulthood, fish were fed twice daily with Zeigler No.1 crumble (Aquatic Habitats; Apopka, Florida, USA). The commercial feed in all stages from 16 dpf was supplemented with live *Artemia* nauplii (Artemia International LLC; Fairview, Texas, USA) once per day.

Larvae and adult ZF were maintained under a 14 h light:10 h dark photoperiod in a temperature-controlled ZF facility and reared in tanks containing heated (28.5 ± 0.2 °C), aerated, dechloraminated City of Ottawa tap water (hereby referred as system water). Fish from the same lineage in each generation were randomly mixed every month to avoid formation of social hierarchies and to reduce potential tank effects. All experiments were conducted following protocols approved by the University of Ottawa Animal Care Protocol Review Committee and

undertaken in accordance with institutional animal care guidelines adhering to those of the Canadian Council on Animal Care.

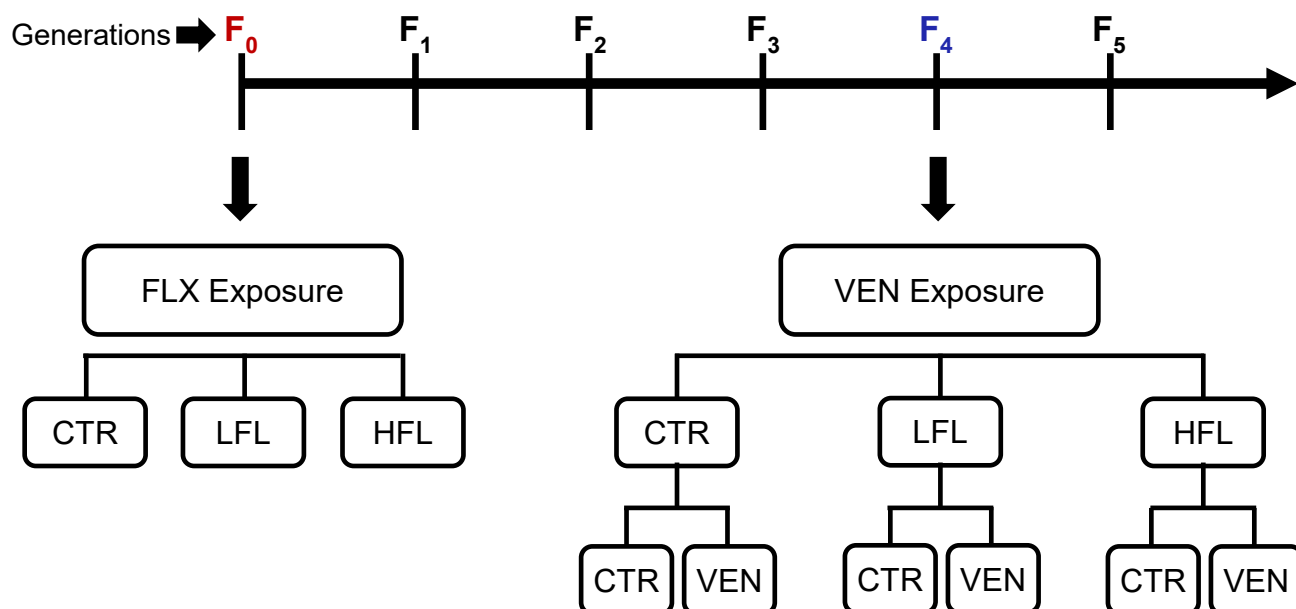


Fig. 6.1. Schematic representation of the experimental design following the introduction of venlafaxine (VEN; $5 \mu\text{g}\cdot\text{L}^{-1}$), a member of the serotonin-norepinephrine reuptake inhibitor (SNRI) family. The exposure to VEN was performed from 0 to 6 dpf in the F₄ embryo ZF from the CTR and FLX lineages. The FLX lineages was generated from exposing the F₀ embryos to $0.54 \mu\text{g}\cdot\text{L}^{-1}$ (LFL) and $54 \mu\text{g}\cdot\text{L}^{-1}$ (HFL) from 0 to 6 dpf.

6.3.2. Developmental parameters

The F₄ and F₅ embryos/larvae were monitored daily until 6 dpf and the number of mortalities, hatchings, and malformations was recorded. Cumulative mortality rate was calculated as the percentage of the cumulative number of embryos that died every day in each clutch (per breeding pair) relative to the number of fertilized eggs. Cumulative hatching rate was calculated as the percentage of the cumulative number of embryos that hatched in each clutch relative to the

number of fertilized eggs, and hatching success rate is the percentage of the hatched embryos per clutch that survived up to 6 dpf. Malformation rate was assessed by calculating the percentage of larvae per clutch that showed any sign of deformities relatively to the total hatched embryos (dead and alive).

6.3.3. Acute stress response

Six-month-old females and males from each lineage and VEN-exposed groups were subjected to the standardized net stressor as described in Chapter 2 section 2.5.2. Briefly, a week prior to the stressor, the adult fish were transferred and allowed to acclimate to the experimental room. During this period, fish were fed normally with Zeigler No.1 crumble (Aquatic Habitats; Apopka, Florida, USA) at 10h00 and 15h00. On the day of the stress test, half of the fish from each treatment were immediately sacrificed (Basal group), whereas the other half was subjected to a standardized net stressor (Stressed group) (Ramsay et al., 2009) between 09h15 and 10h30. The fish were sacrificed by submersion in ice-cold water and subsequently weighed, immediately snap-frozen in liquid nitrogen and stored at -80°C for whole-body hormonal analysis.

6.3.4. Lipids extraction and quantification

Whole-body lipids were extracted using a protocol modified from Folch et al. (1957), as described in Chapter 2 section 2.5.6. Whole-body extraction efficiencies were determined by spiking homogenates with known amounts of the appropriate radioactive isotope (^{14}C or ^3H) and were 87% for cortisol, 85% for 17β -estradiol (E_2) and 89% for testosterone, 91% for cholesterol and 71% for triglycerides; values were not corrected for individual extraction efficiency.

Total cortisol concentration was assessed by using a ^{125}I radioimmunoassay kit (MP Biomedicals; Solon, Ohio) according to the manufacturer's protocol followed by the measurement of the radioactive counts with a Wizard2 automatic gamma counter (PerkinElmer; Downers Grove, Illinois). The intra- and the inter-assay coefficients of variation (CV) were calculated to be 4-8% and 7-15%, respectively. Total 11-ketotestosterone (11-KT) levels were estimated using a commercial enzyme-linked immunosorbent assay (ELISA) (Cayman Chemical; Burlington, Ontario, Canada) as per the manufacturer's protocol. The intra- and the inter-assay CV were 4-12% and 5-18%, respectively. Total E_2 and testosterone levels were determined by using commercially available ELISA kits (TECO Diagnostic; Anaheim, California, USA) according to the manufacturer's instructions. The intra- and the inter-assay CV for these ELISA assays were calculated to be, for E_2 5-8% and 7-13%, respectively and for testosterone 7-9% and 9-19%, respectively. Total cholesterol and triglycerides concentrations were measured using colorimetric enzyme assay kits (TECO Diagnostic). The intra- and the inter-assay CV for these assays were calculated to be, for cholesterol 1-7% and 9-11%, respectively and for triglycerides 1-8% and 8-10%, respectively. For all ELISA experiments, absorbance values were estimated using a microplate spectrophotometer (SpectraMAX Plus 384; Molecular Devices; USA).

6.3.5. Novel tank diving test

Six-month-old females and males from each lineage and VEN-exposed groups were subjected to the novel tank diving test adapted from Levin et al. (2007) as described in our previous study (Chapter 2 section 2.5.7). Briefly, fish were allowed to acclimatize to the testing room in 3-L tanks ($16 \text{ fish} \cdot \text{tank}^{-1}$) one week prior to the experiment. The behavioral test was conducted between 9h30 and 14h30 over a 5-day period in a trapezoid-shaped tank (24 cm along the bottom

× 29 cm at the top × 15 cm high × 7 cm width; Aquatic Habitats; Apopka, Florida, USA) filled with system water. The behavioral activity of each individual fish was recorded for 6 min in fresh system water. Videos were analyzed every 30 frames·s⁻¹ for a total of 10,800 frames using a validated in-house automated tracking (AT) system (Python script) (Chapter 2 section 2.6.1). Principal component analysis (PCA) was performed separately for females and males on the 10 calculated behavioral metrics obtained from the AT. PCA yielded a single component (PC1) that strongly loaded most of the behavioral metrics (Table 6.1) and explained 59% and 63% of the behavioral variance for males and females, respectively. The two variables that did not robustly contribute to PC1 were maximum speed and total distance traveled. Positive scores are associated with high exploratory and locomotor activities, whereas negative scores are linked to reduced behavioral activities.

Table 6.1. Loadings and contributions of the behavioral metrics to PC1

Behavioral metrics	Description	Females		Males	
		Loadings	Contribution (%)	Loadings	Contribution (%)
Latency middle third	Delay before entering the middle third of the tank	-0.273	7.5	-0.185	3.4
Latency top half	Delay before entering the top half of the tank	-0.348	12.1	-0.288	8.3
Latency top third	Delay before entering the top third of the tank	-0.342	11.7	-0.321	10.3
Transitions	Number of times the fish crossed into the top half of the tank	0.371	13.8	0.399	15.9
Time middle third	Total time spent in the middle third of the tank	0.379	14.4	0.396	15.7
Time top third	Total time spent in the top third of the tank	0.354	12.5	0.384	14.8
Distance middle third	Total distance spent in the middle third of the tank	0.377	14.2	0.394	15.5
Distance top third	Total distance spent in the top third of the tank	0.355	12.6	0.383	14.6
Total distance	Total distance traveled around the tank	0.109	1.2	0.107	1.2
Max speed	Maximum speed reached by the fish	0.004	0.0	-0.059	0.4

6.3.6. Assessment of reproductive endpoints in F₄ adults

Fifteen adult pairs (6 mpf) from each of the 6 groups were used for the assessment of reproduction. Pairs that did not spawn at trial 1 were provided a second opportunity with a different mate randomly chosen from non-spawning individuals within the same group to eliminate the possibility of mate preference. The assessment of the reproductive fitness included sex ratio, spawning rate, fertilization rate and condition factor (*K*). For the assessment of the *K*-factor, between 15 and 37 adult fish from each group were euthanized and their weights and lengths were recorded. Spawning rate was calculated as the number of pairs that spawned in each lineage during either attempt 1 or attempt 2 divided by the total number of breeding pairs (15) per group. Fertilization rate was determined as the percentage of the total number of fertilized eggs per breeding pair divided by the total number of eggs. *K* indices were calculated for each group as $K = \text{body weight (g)} \times \text{length}^{-3} \text{ (cm)} \times 100$.

6.3.7. Statistical analyses

Statistical analyses were performed using SigmaPlot 11.0 (Systat Software, Inc.). Prior to hypothesis testing by analysis of variance (ANOVA), the normality of the data was assessed using the Shapiro-Wilk test, and the homogeneity of variances was checked using the Levene median test. The post-hoc test used to examine significant differences within groups was the Tukey's post-hoc test. The data for the developmental parameters including mortality rate, hatching rate, hatching success and malformation rate in addition to levels of steroids (sex steroids and cortisol) and lipids (cholesterol and triglycerides) were expressed as the mean $\pm$ SEM. The developmental parameters, steroids and lipids were assessed using either a two-way ANOVA or the non-parametric two-way ANOVA on ranks test (see captions for more information). Box-Cox (Box

and Cox, 1964) transformation was applied when steroids and lipids data were not normally distributed. Since levels of sex steroids and lipids were obtained from adult fish subjected to the standardized net stress, we first performed a three-way ANOVA with the variables: FLX treatment, VEN treatment and cortisol conditions (basal or stress-induced). No significant effect of cortisol conditions was observed, therefore we excluded cortisol conditions from all subsequent analyses related to steroids and lipids.

The PC1 scores representing the exploratory and locomotor behaviors of the adult fish were presented in box plots showing the 10th and 90th percentiles and the statistical significance was determined using a two-way ANOVA on ranks. Sex ratio was tested with the Chi-Square test (χ^2 -test) whereas spawning rate was analyzed with the Fisher's exact test. The condition factor (K) was analyzed by two-way ANOVA for females and two-way ANOVA on ranks for males. The level of significance for all tests was set at $\alpha < 0.05$.

6.4. Results

6.4.1. VEN exposure to the F₄ embryos/larvae from the CTR and FLX lineages does not affect development of the F₄ or F₅ larvae

The effects of VEN and the FLX lineages on different developmental parameters of the F₄ larvae were determined. A significant difference was found in the mortality rate of the larvae from the FLX lineages not exposed to VEN ($F_{2,56} = 5.728$, $P = 0.005$). A Tukey's post-hoc test revealed that the mortality rate of the larvae from the LFL was reduced compared to the larvae from the CTR. Exposure to VEN did not affect the mortality rate of the F₄ larvae ($F_{1,56} = 0.039$, $P = 0.844$). No interactions were found between VEN exposure and the FLX lineages on the mortality rate of the F₄ larvae ($F_{2,56} = 0.997$, $P = 0.375$; Fig. 6.2A).

The hatching rate of the larvae from the FLX lineages was statistically different than that of the CTR ($F_{2,57} = 5.506$, $P = 0.007$), more specifically the LFL larvae had a higher hatching rate than the CTR larvae. However, no effect was observed on the hatching rate following VEN treatment ($F_{1,57} = 0.237$, $P = 0.628$). No interactions were found between VEN exposure and the FLX lineages on the hatching rate of the F₄ larvae ($F_{2,57} = 0.459$, $P = 0.634$; Fig. 6.2B).

The hatching success of the F₄ larvae was significantly affected by FLX exposure to the F₀ ($F_{2,54} = 10.496$, $P < 0.001$). The hatching success of the LFL larvae was increased compared to that of the CTR. No effects were found on the hatching success following VEN treatment ($F_{1,54} = 0.168$, $P = 0.683$). No interactions were found between VEN exposure and the FLX lineages on the hatching success of the F₄ larvae ($F_{2,54} = 2.629$, $P = 0.081$; Fig. 6.2C).

Malformation rate was significantly altered in the larvae from the FLX lineages ($F_{2,54} = 10.778$, $P < 0.001$). The LFL larvae had a reduction in the malformation rate relative to the CTR. VEN treatment did not induce any effect on malformation rate of the F₄ larvae ($F_{1,54} = 3.882$, $P = 0.054$). No interactions were found between VEN exposure and the FLX lineages on the malformation rate of the F₄ larvae ($F_{2,54} = 0.696$, $P = 0.503$; Fig. 6.2D).

We also assessed the same developmental parameters in the F₅ larvae, the first generation of the fish that were exposed to VEN. However, no significant differences on the mortality ($F_{2,48} = 3.045$, $P = 0.057$; Fig. 6.2A) and hatching rate ($F_{2,48} = 2.817$, $P = 0.070$; Fig. 6.2B) were found across FLX lineages in the F₅ larvae. Conversely, hatching success ($F_{2,47} = 3.566$, $P = 0.036$; Fig. 6.2C) and malformation rate ($F_{2,47} = 3.566$, $P = 0.036$; Fig. 6.2D) in the F₅ larvae were significantly affected by FLX treatment to the F₀. A post-hoc test revealed that the LFL displayed higher hatching success and lower malformation rate compared to the HFL larvae.

VEN exposure to the F₄ did not affect any of the developmental parameters of the F₅ larvae including mortality rate ($F_{1,48} = 0.085$, $P = 0.772$; Fig. 6.2A), hatching rate ($F_{1,48} = 0.040$, $P = 0.843$; Fig. 6.2B), hatching success ($F_{1,47} = 0.858$, $P = 0.359$; Fig. 6.2C) and malformation rate ($F_{1,47} = 0.858$, $P = 0.359$; Fig. 6.2D).

Moreover, there was no statistically significant interaction between VEN exposure and the FLX lineages on the mortality rate ($F_{2,48} = 1.816$, $P = 0.174$; Fig. 6.2A), hatching rate ($F_{2,48} = 2.053$, $P = 0.139$; Fig. 6.2B), hatching success ($F_{1,47} = 2.226$, $P = 0.119$; Fig. 6.2C) and malformation rate ($F_{1,47} = 2.226$, $P = 0.119$; Fig. 6.2D) of the F₅ larvae.

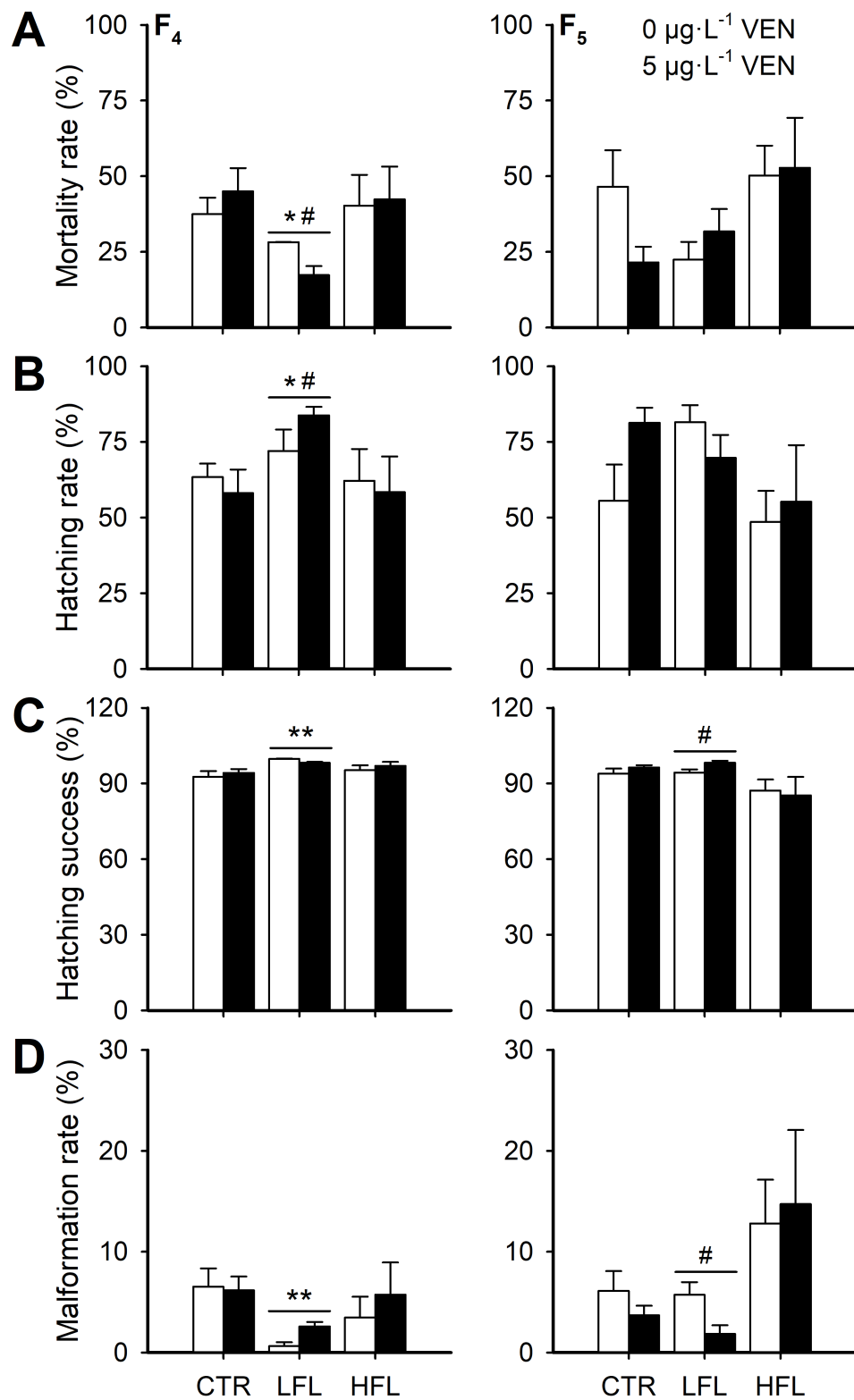


Fig. 6.2. Developmental parameters of the F₄ and F₅ larvae ZF from the CTR and FLX lineages following VEN exposure to the F₄. (A) Cumulative mortality rate of the larvae at 6 dpf. (B) Percentage of fertilized eggs that hatched. (C) Percentage of hatched embryos that survived up to 6 dpf. (D) Percentage of hatched embryos (dead and alive) that showed any sign of deformities. The FLX lineages were obtained from exposing the F₀ to 0.54 (LFL) and 54 (HFL) $\mu\text{g}\cdot\text{L}^{-1}$ from 0 to 6 dpf (see Fig. 6.1). Exposure to 5 $\mu\text{g}\cdot\text{L}^{-1}$ VEN was conducted from 0 to 6 dpf in the F₄ larvae. n = individual clutches from 15 pairs per group after two spawning attempts. If the same pair spawned during the two attempts, only the first clutch was included in the analysis. Data presented as mean $\pm$ SEM, two-way ANOVA (on ranks: B, C and D). The asterisks represent statistical difference within the FLX lineages; * P < 0.01 compared to CTR; ** P < 0.001 compared to CTR; # P < 0.05 compared to HFL.

6.4.2. Exposure of F₄ embryos/larvae to VEN intensifies the reduced cortisol phenotype in the adult females from the HFL lineage

Basal cortisol levels in the F₄ adult females were significantly affected by early FLX treatment to the F₀ ($F_{2,68} = 9.419$, $P < 0.001$). This effect was observed in the HFL adults displaying lower basal cortisol levels compared to the CTR fish. The VEN treatment intensified this reduction of the basal cortisol levels ($F_{1,68} = 7.479$, $P = 0.008$). However, no interactions were found between VEN exposure and the FLX lineages on the basal cortisol levels of the F₄ adult females ($F_{2,68} = 1.581$, $P = 0.213$; Fig. 6.3A).

The cortisol levels in the F₄ adult females following the standardized net stressor were also affected by FLX treatment to the F₀ ($F_{2,74} = 15.645$, $P < 0.001$). A Tukey's post-hoc test revealed that the stress-induced cortisol levels in the females from the HFL were reduced relative to the CTR and these blunted levels were further reduced by VEN treatment ($F_{1,74} = 6.125$, $P = 0.016$). Moreover, a significant interaction was found between VEN treatment and the FLX lineages on the stress-induced cortisol levels of the F₄ adult females ($F_{2,74} = 3.217$, $P = 0.046$; Fig. 6.3A). The HFL group exposed to VEN had the lowest cortisol levels.

In the F₄ adult males, basal cortisol levels did not change across FLX lineages ($F_{2,94} = 2.841$, $P = 0.063$) and they were not affected by VEN exposure ($F_{1,94} = 0.470$, $P = 0.495$). However, there was a statistically significant interaction between the effects of VEN exposure and the FLX treatment to the F₀ on the basal cortisol levels of the F₄ males ($F_{2,94} = 8.581$, $P < 0.001$; Fig. 6.3B). The HFL group exposed to VEN exhibited the lowest cortisol levels across all groups and the LFL group exposed to VEN had the highest cortisol levels across the groups that were not exposed to VEN.

The stress-induced cortisol levels in the F₄ adult males was significantly altered by FLX treatment to the F₀ ($F_{2,105} = 32.889$, $P < 0.001$). The adult males from the LFL and HFL displayed a reduction in their cortisol levels relative to the CTR following the net stressor. However, VEN exposure did not induce any changes in the stress-induced cortisol levels ($F_{1,105} = 3.595$, $P = 0.061$). No interactions were found between VEN exposure and the FLX lineages on the stress-induced cortisol levels of the F₄ adult males ($F_{2,105} = 0.471$, $P = 0.626$; Fig. 6.3B).

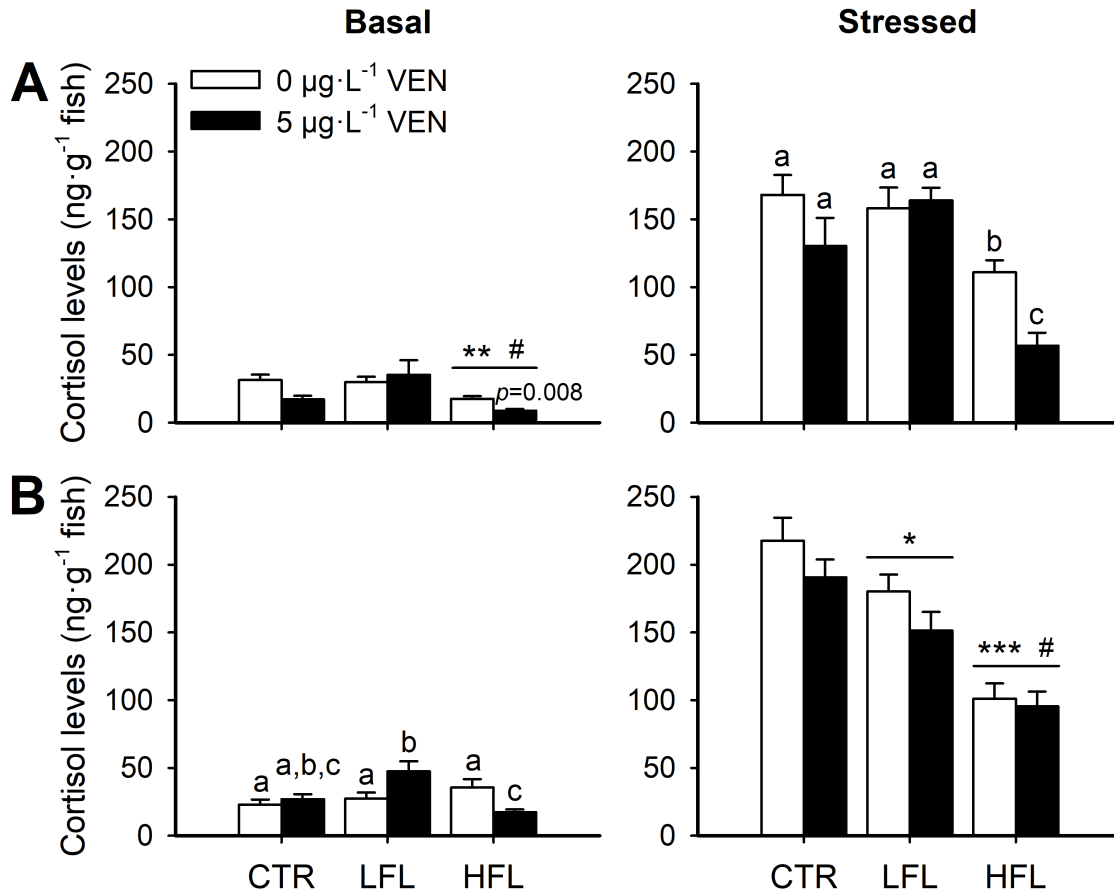


Fig. 6.3. Whole-body cortisol levels ($\text{ng}\cdot\text{g}^{-1}$ fish) in the F_4 adult (A) females and (B) males from the CTR and FLX lineages following exposure to $5\ \mu\text{g}\cdot\text{L}^{-1}$ VEN during early development (see Fig. 6.1). The stress response was induced using a net handling stress protocol. The FLX lineages were obtained from exposing the F_0 to 0.54 (LFL) and 54 (HFL) $\mu\text{g}\cdot\text{L}^{-1}$ from 0 to 6 dpf. Exposure to $5\ \mu\text{g}\cdot\text{L}^{-1}$ VEN was conducted from 0 to 6 dpf in the F_4 larvae. For panel A and B, the data are presented as mean $\pm$ SEM and analyzed by two-way ANOVA, $P < 0.05$. The letters represent statistical difference when interactions are present. P -values shown above the bars denote significant difference compared to the $0\ \mu\text{g}\cdot\text{L}^{-1}$ VEN group. The symbols represent significant difference within the FLX lineages: $*P = 0.013$ compared to CTR; $**P = 0.006$ compared to CTR; $***P < 0.001$ compared to CTR and $\#P < 0.001$ compared to LFL. $n = 7 - 19$ per group in females and $n = 16 - 19$ in males.

6.4.3. Exposure of F₄ larvae to VEN alters whole-body sex steroid levels in the F₄ adult ZF independent of FLX lineage

The whole-body testosterone levels in the F₄ adult females (6 mpf) did not vary across FLX lineages ($F_{2,145} = 2.854$, $P = 0.061$) nor were they affected by VEN exposure ($F_{1,145} = 0.003$, $P = 0.956$). However, there was a statistically significant interaction between VEN exposure and the FLX lineages on the whole-body testosterone levels of the F₄ adult females ($F_{2,145} = 4.951$, $P = 0.008$; Fig. 6.4A), where the HFL group that was not exposed to VEN had higher total testosterone levels compared to the HFL group exposed to VEN. In the adult males (6 mpf), total testosterone levels did not vary across FLX lineages ($F_{2,202} = 0.521$, $P = 0.595$), but they were significantly reduced by VEN exposure ($F_{1,202} = 84.215$, $P < 0.001$). No interactions were found between VEN exposure and the FLX lineages on the total testosterone levels of the F₄ adult males ($F_{2,202} = 1.034$, $P = 0.357$; Fig. 6.4A).

The total 11-KT levels in the adult females (6 mpf) were not affected by FLX treatment to the F₀ ($F_{2,148} = 3.032$, $P = 0.051$), however, VEN exposure significantly increased their 11-KT levels ($F_{1,148} = 10.778$, $P = 0.001$). No interactions were found between VEN exposure and the FLX lineages on the total 11-KT levels of the F₄ adult females ($F_{2,148} = 1.970$, $P = 0.143$; Fig. 6.4B). In the adult males (6 mpf), total 11-KT levels were not affected by FLX treatment to the F₀ ($F_{2,206} = 2.500$, $P = 0.085$) and in contrast to females, VEN exposure significantly reduced 11-KT levels ($F_{1,206} = 5.620$, $P = 0.019$). No interactions were found between VEN exposure and the FLX lineages on the total 11-KT levels of the F₄ adult males ($F_{2,206} = 1.651$, $P = 0.194$; Fig. 6.4B).

The whole-body E₂ levels in the adult females (6 mpf) did not change across FLX lineages ($F_{2,144} = 0.923$, $P = 0.399$), but VEN exposure significantly increased their E₂ levels ($F_{1,144} = 7.289$,

$P = 0.008$). Interestingly, a significant interaction was found between VEN exposure and the FLX lineages on the total E_2 levels of the F₄ adult females ($F_{2,144} = 8.792$, $P < 0.001$; Fig. 6.4C), where all groups exposed to VEN (CTR, LFL and HFL) and the HFL group that was not exposed to VEN had the highest E_2 levels. In contrast to females, total E_2 levels in the adult males (6 mpf) were statistically altered across FLX lineages ($F_{2,205} = 3.911$, $P = 0.022$). A Tukey's post-hoc test revealed that the total E_2 levels from the HFL group were higher compared to the CTR group. Exposure to VEN did not affect the total E_2 levels in the adult males ($F_{1,205} = 0.012$, $P = 0.914$). Moreover, no interactions were found between VEN exposure and the FLX lineages on the total E_2 levels of the F₄ adult males ($F_{2,205} = 0.296$, $P = 0.744$; Fig. 6.4C).

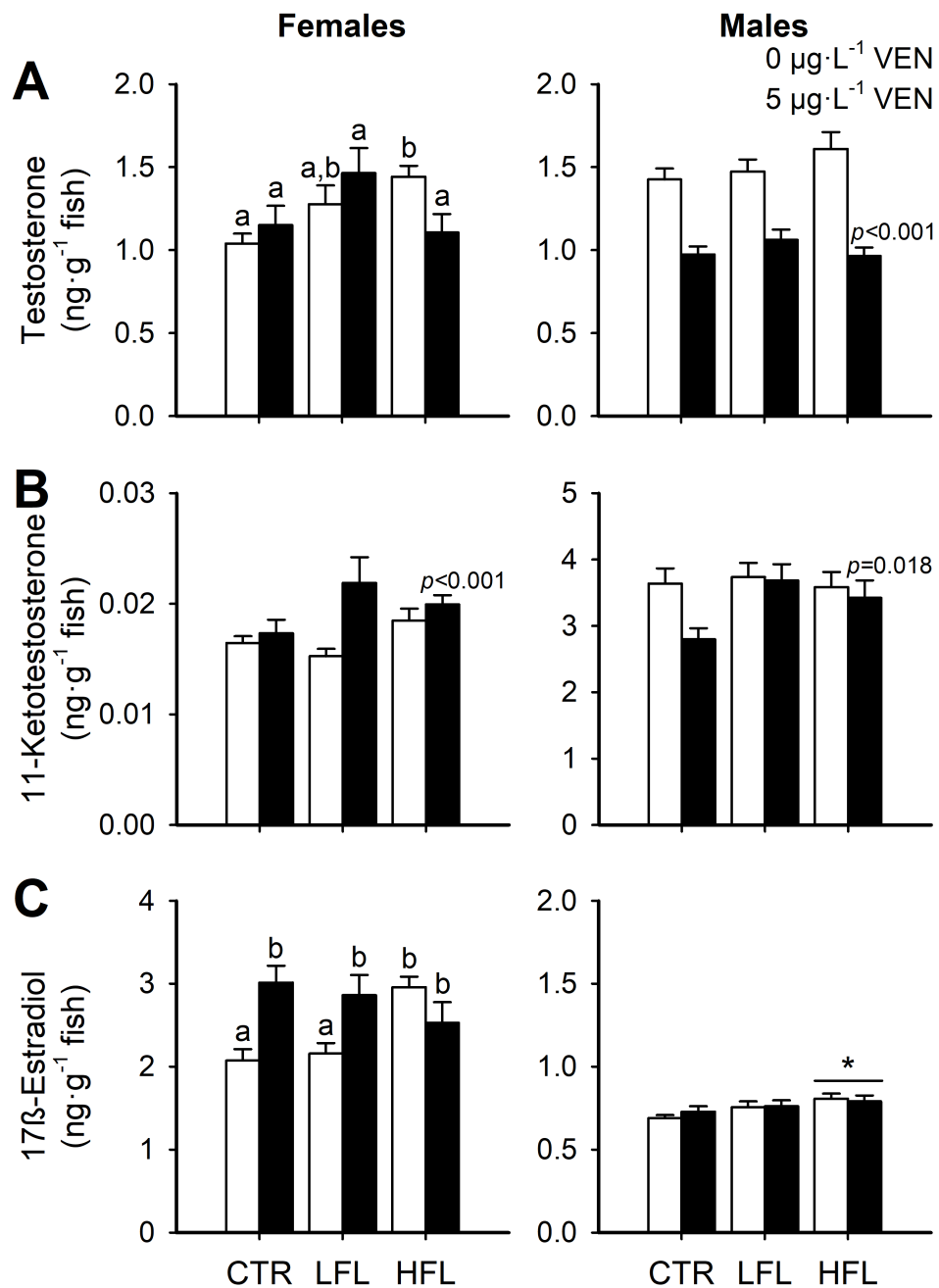


Fig. 6.4. Whole-body levels of sex steroids ($\text{ng}\cdot\text{g}^{-1}$ fish) in the F₄ adult females (*Left*) and males (*Right*) from the CTR and FLX lineages following exposure to $5\ \mu\text{g}\cdot\text{L}^{-1}$ VEN during early development (see Fig. 6.1). The FLX lineages were obtained from exposing the F₀ to 0.54 (LFL) and 54 (HFL) $\mu\text{g}\cdot\text{L}^{-1}$ from 0 to 6 dpf. Exposure to $5\ \mu\text{g}\cdot\text{L}^{-1}$ VEN was conducted from 0 to 6 dpf in the F₄ larvae. (A) Total testosterone levels; $n = 14 - 37$ females and $n = 34 - 35$ males per group. (B) Total 11-ketotestosterone (11-KT) levels; $n = 15 - 36$ females and $n = 34 - 36$ males in each group. (C) Total 17 β -estradiol (E₂) levels; $n = 14 - 36$ females and $n = 34 - 36$ males per group. Data in all panels are expressed as mean $\pm$ SEM and analyzed by two-way ANOVA (on ranks: 11-KT in females; E₂ in females and males), $P < 0.05$. The letters represent statistical differences when interactions are present. P -values shown above the bars denote significant difference compared to the $0\ \mu\text{g}\cdot\text{L}^{-1}$ VEN group. The asterisk represents significant difference within the FLX lineages: * $P = 0.015$ compared to CTR.

6.4.4. The effects of VEN exposure on the whole-body cholesterol and triglycerides levels in the F₄ adult ZF

Whole-body cholesterol levels in the adult females (6 mpf) were significantly affected by FLX exposure to the F₀ ($F_{2,143} = 3.971$, $P = 0.021$). A Tukey's post-hoc test revealed that total cholesterol levels were significantly reduced in the HFL group compared to the CTR group. However, VEN exposure did not change the total cholesterol levels in the adult females ($F_{1,143} = 0.472$, $P = 0.493$). Moreover, no interactions between VEN exposure and the FLX lineages were detected on the total cholesterol levels of the F₄ adult females ($F_{2,205} = 0.264$, $P = 0.769$; Fig. 6.5A). Similar to females, total cholesterol levels in the adult males (6 mpf) were significantly altered by FLX exposure to the F₀ ($F_{2,196} = 7.597$, $P < 0.001$). However, a Tukey's post-hoc test revealed that total cholesterol levels were significantly higher in the LFL group compared to the CTR and HFL group. No effects were found following VEN exposure on the total cholesterol levels ($F_{1,196} = 0.032$, $P = 0.857$). No interactions between VEN exposure and the FLX

lineages were detected on the total cholesterol levels of the F₄ adult males ($F_{2,196} = 0.183$, $P = 0.833$; Fig. 6.5A).

Whole-body triglycerides levels in the adult females (6 mpf) significantly varied across FLX lineages ($F_{2,146} = 10.376$, $P < 0.001$). A Tukey's post-hoc test revealed that the total triglycerides levels were significantly lowered in the LFL and HFL groups compared to the CTR group. Moreover, VEN exposure also significantly reduced total triglycerides levels in the adult females ($F_{1,146} = 6.871$, $P = 0.010$). However, no interactions between VEN exposure and FLX lineages were detected on the total triglycerides levels of the F₄ adult females ($F_{2,146} = 2.520$, $P = 0.084$; Fig. 6.5B). In contrast, total triglycerides levels in the adult males (6 mpf) did not vary across FLX lineages ($F_{2,204} = 0.164$, $P = 0.849$) nor were they affected by VEN exposure ($F_{1,204} = 0.303$, $P = 0.583$). No interactions between VEN exposure and the FLX lineages were found on the total triglycerides levels of the F₄ adult males ($F_{2,204} = 0.276$, $P = 0.759$; Fig. 6.5B).

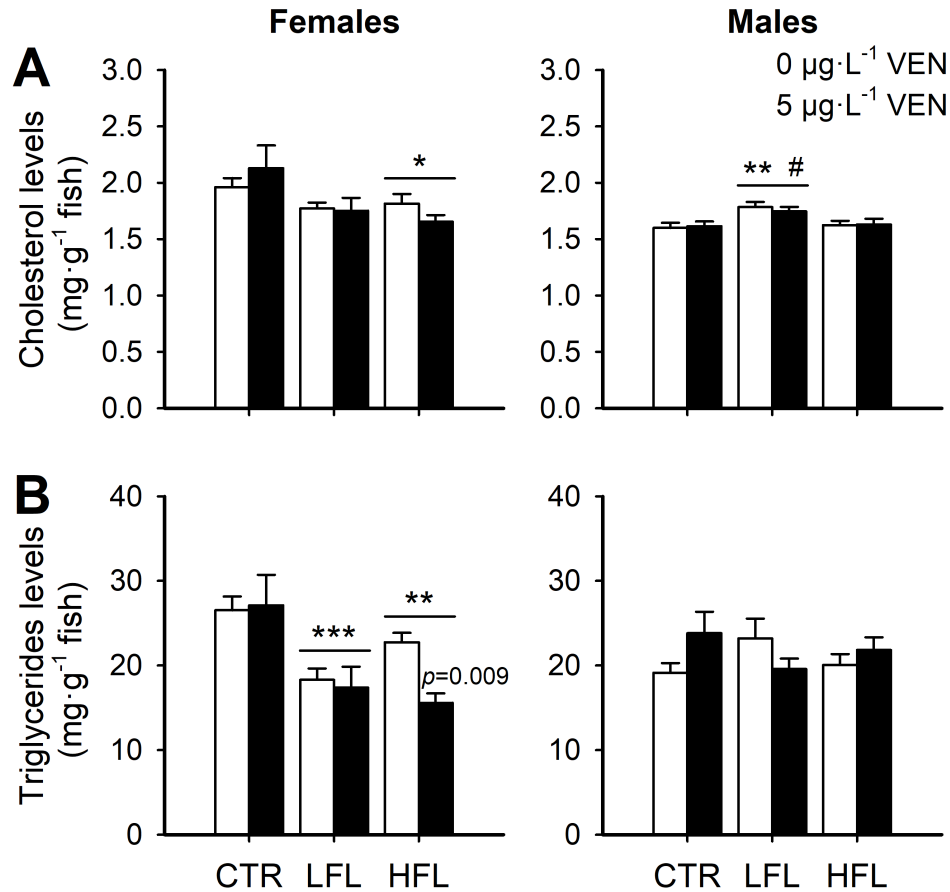


Fig. 6.5. Whole-body lipid levels ($\text{mg}\cdot\text{g}^{-1}$ fish) in the F_4 adult females (*Left*) and males (*Right*) from the CTR and FLX lineages following exposure to $5\ \mu\text{g}\cdot\text{L}^{-1}$ VEN during early development (see Fig. 6.1). The FLX lineages were obtained from exposing the F_0 to 0.54 (LFL) and 54 (HFL) $\mu\text{g}\cdot\text{L}^{-1}$ from 0 to 6 dpf. Exposure to $5\ \mu\text{g}\cdot\text{L}^{-1}$ VEN was conducted from 0 to 6 dpf in the F_4 larvae. (A) Total cholesterol levels; $n = 13 - 37$ females and $32 - 35$ males in each group. (B) Total triglycerides levels; $n = 15 - 36$ females and $33 - 36$ males per group. Data in both panels are expressed as mean $\pm$ SEM and analyzed by two-way ANOVA (on ranks: cholesterol in females; triglycerides in females and males), $P < 0.05$. P -values shown above the bars denote significant difference compared to the $0\ \mu\text{g}\cdot\text{L}^{-1}$ VEN group. The symbols represent significant differences within the FLX lineages: * $P = 0.019$ compared to CTR; ** $P < 0.005$ compared to CTR; *** $P < 0.001$ compared to CTR; # $P = 0.004$ compared to HFL.

6.4.5. Exposure of F₄ embryos/larvae to VEN does not alter locomotor and exploratory behaviors of adult ZF

The locomotor and exploratory behaviors of the adult females at 6 mpf following the novel tank diving test were significantly altered across FLX lineages ($F_{2,86} = 4.158$, $P = 0.019$). A Tukey's post-hoc test revealed that both activities in the HFL adult females were significantly reduced compared to the CTR fish. However, VEN exposure did not affect the behavior of the adult females subjected to the novel tank diving test ($F_{1,86} = 0.222$, $P = 0.639$). Moreover, no interactions between VEN exposure and the FLX lineages were found on the locomotor and exploratory activities in the F₄ adult females ($F_{2,86} = 0.617$, $P = 0.542$; Fig. 6.6A).

In contrast to females, no alterations of locomotor and exploratory activities in the adult males at 6 mpf across FLX lineages ($F_{2,89} = 1.321$, $P = 0.272$) were evident. Exposure to VEN did not affect the behaviors of the adult males following the novel tank diving test ($F_{2,89} = 1.491$, $P = 0.225$). There was a statistically significant interaction between VEN exposure and the FLX lineages on the locomotor and exploratory activities following the novel tank diving test in the F₄ adult males ($F_{2,89} = 4.729$, $P = 0.011$; Fig. 6.6B). The fish from the HFL group treated with VEN displayed lower locomotor and exploratory activities compared to the fish from the CTR and LFL group that were also exposed to VEN.

6.4.6. Exposure of F4 embryos/larvae to VEN does not disrupt reproduction in adult ZF

We first examined the differences in sex ratios across groups that were not exposed to VEN (CTR, LFL and HFL) (Fig. 6.7A). There was a significant 11% increase in the number of males from the LFL compared to the CTR ($\chi_1 = 7.263$, $P = 0.007$). No differences in the sex ratio were found between the HFL and the CTR ($\chi_1 = 0.146$, $P = 0.703$) nor between the HFL and the LFL ($\chi_1 = 2.627$, $P = 0.105$). Among the groups that were exposed to VEN (Fig. 6.7A), no differences in the sex ratio were found between the CTR exposed to VEN and the LFL ($\chi_1 = 3.722$, $P = 0.054$) or the HFL ($\chi_1 = 1.007$, $P = 0.316$). However, a significant 18% increase was found in the number of males from the LFL compared to the HFL ($\chi_1 = 11.323$, $P < 0.001$). Additionally, sex ratios between the groups that were not exposed to VEN and the groups exposed to VEN revealed a statistical 15% increase in the males from the CTR exposed to VEN compared to the CTR not exposed to VEN ($\chi_1 = 6.188$, $P = 0.013$). There was also a significant 25% increase in males from the LFL exposed to VEN compared to the CTR not exposed to VEN ($\chi_1 = 31.275$, $P < 0.001$). However, no significant difference in the sex ratio was found between the CTR not exposed to VEN compared to HFL exposed to VEN ($\chi_1 = 1.451$, $P = 0.228$). No differences in the sex ratio between LFL not exposed to VEN and CTR exposed to VEN ($\chi_1 = 0.358$, $P = 0.550$) and between LFL not exposed to VEN and HFL exposed to VEN ($\chi_1 = 0.314$, $P = 0.575$) were observed. Contrarily, a statistical 14% increase in males was found in the LFL exposed to VEN compared to the LFL not exposed to VEN ($\chi_1 = 11.438$, $P < 0.001$). The sex ratio between HFL not exposed to VEN and CTR exposed to VEN ($\chi_1 = 3.300$, $P = 0.069$) and between HFL not exposed to VEN and HFL exposed to VEN ($\chi_1 = 0.398$, $P = 0.528$) did not vary. However, the LFL exposed to VEN group had a significant 22% increase in males compared to the HFL not exposed to VEN group ($\chi_1 = 19.756$, $P < 0.001$).

Furthermore, the K -factor in the adult females did not vary across FLX lineages ($F_{2,147} = 1.002$, $P = 0.369$) but it was significantly increased following VEN treatment ($F_{1,147} = 27.326$, $P < 0.001$). We also found a significant interaction between the effects of VEN exposure and the FLX lineages on the K -factor in the F₄ adult females ($F_{2,147} = 6.472$, $P = 0.002$; Fig. 6.7B). In contrast to females, the K -factor in the adult males was significantly affected by FLX exposure to the F₀ ($F_{2,206} = 4.098$, $P = 0.018$); the LFL group had a reduced K -factor compared to the CTR. However, VEN exposure did not alter the K -factor of the adult males ($F_{1,206} = 0.282$, $P = 0.596$). Also, there was a statistically significant interaction between the effects of VEN exposure and FLX lineages on K -factor in the F₄ adult males ($F_{2,206} = 4.909$, $P = 0.008$; Fig. 6.7B).

We also examined spawning rates in the CTR and FLX lineages from the non-exposed and VEN exposed groups (Fig. 6.7C). In the groups from the CTR and FLX lineages that were not exposed to VEN, we did not find any significant differences in spawning rates; CTR compared to LFL ($P = 0.450$), CTR compared to HFL ($P = 0.710$) and LFL compared to HFL ($P = 1.000$). However, in the groups from the CTR and FLX lineages that were exposed to VEN, we found a 53% decrease in spawning rates from the HFL compared to the LFL ($P = 0.009$). No other significant changes in spawning rates were found among the groups that were exposed to VEN; CTR compared to LFL ($P = 0.450$) and CTR compared to HFL ($P = 0.128$). The spawning rates between the groups that were not exposed to VEN and those that were exposed to VEN did not change (CTR not exposed to VEN compared to CTR exposed to VEN ($P = 1.000$); CTR not exposed to VEN compared to LFL exposed to VEN ($P = 0.450$); CTR not exposed to VEN compared to HFL exposed to VEN ($P = 0.128$); LFL not exposed to VEN compared to CTR exposed to VEN ($P = 0.450$); LFL not exposed to VEN compared to LFL exposed to VEN ($P = 1.000$); HFL not exposed to VEN compared to CTR exposed to VEN ($P = 0.710$), and HFL

not exposed to VEN compared to LFL exposed to VEN ($P = 1.000$), except for the HFL exposed to VEN, where spawning rates were significantly reduced by 53.3% compared to the LFL not exposed to VEN group ($P = 0.009$) and 47% compared to the HFL not exposed to VEN group ($P = 0.025$).

The fertilization rate of the eggs produced by the 15 pairs in each of the groups were also evaluated (Fig. 6.7D). However, the fertilization rate did not vary across FLX lineages ($F_{2,47} = 2.775$, $P = 0.073$) nor were these rates affected by VEN exposure ($F_{1,47} = 0.357$, $P = 0.553$). Moreover, no interactions between VEN exposure and the FLX lineages were found on the fertilization rate in the F₄ adult fish ($F_{2,47} = 1.807$, $P = 0.175$; Fig. 6.7D).

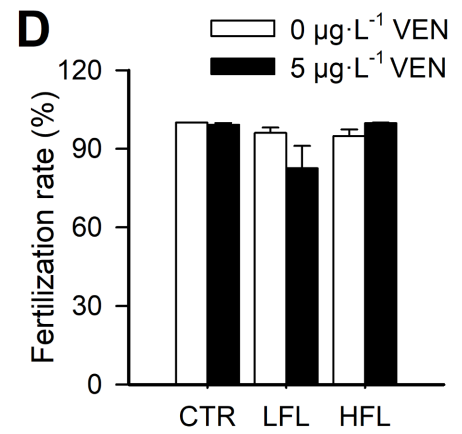
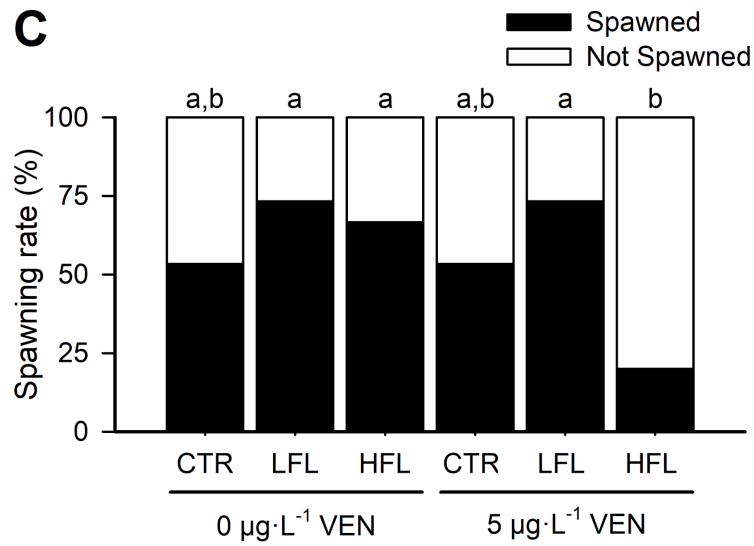
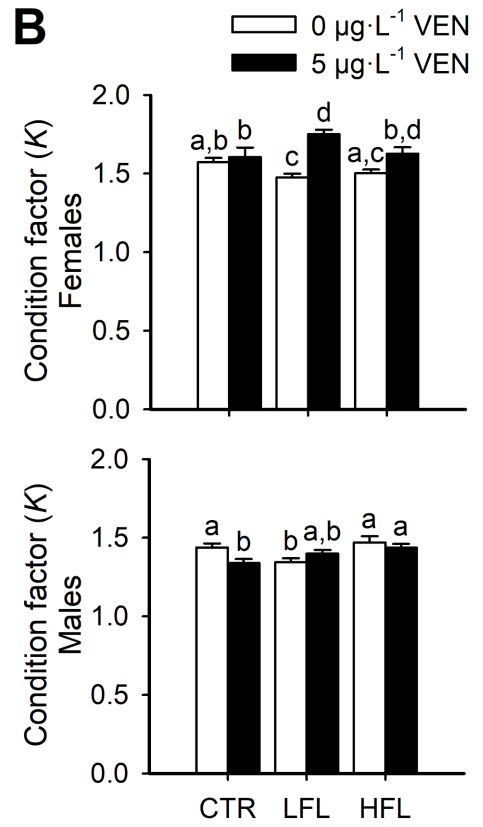
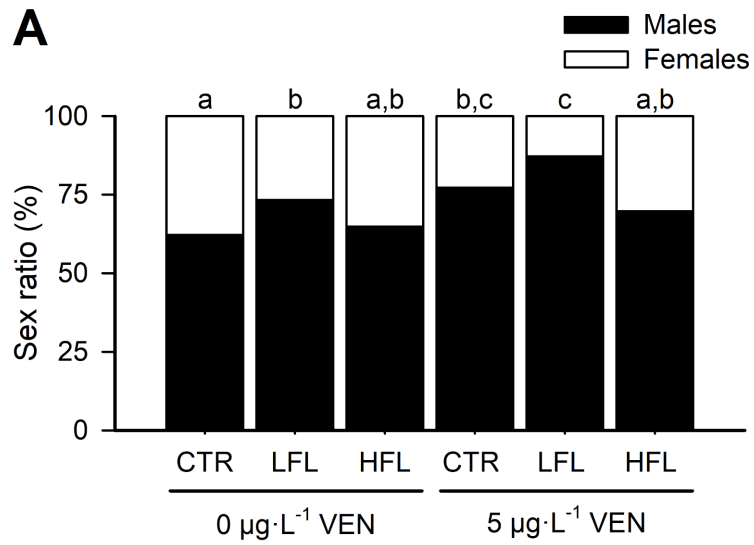


Fig. 6.7. Reproductive endpoints of the F₄ adult ZF from the CTR and FLX lineages exposed to VEN from 0 to 6 dpf. (A) The female to male ratio of adult ZF at 6 mpf. Analysis using the Chi-square test, $P < 0.05$; $n = 270$ ZF for CTR, 281 (LFL), 125 (HFL) in the $0 \mu\text{g}\cdot\text{L}^{-1}$ VEN; 92 (CTR), 172 (LFL), 99 (HFL) in the $5 \mu\text{g}\cdot\text{L}^{-1}$ VEN. (B) Condition factor (K) of adult (6 mpf) ZF. Data are displayed as mean $\pm$ SEM, $n = 15 - 37$ females per group and $n = 34 - 36$ males per group, two-way ANOVA (on ranks: for males), $P < 0.05$. (C) Spawning rate of 15 adult (6 mpf) pairs per group after two spawning attempts. Data was analyzed using Fisher's exact test, $P < 0.05$. (D) The percentage of fertilized eggs from the mating of the 15 pairs per group after two spawning attempts. Data presented as mean $\pm$ SEM, two-way ANOVA on ranks did not find any significant difference on the percentage of fertilized eggs at $P < 0.05$.

6.5. Discussion

Our findings illustrate a “two-hit” model of antidepressants where the “hits” span generations. In this instance the first hit (FLX treatment during embryogenesis—recreating prenatal exposure to the first-line of treatment in pregnant women with affective disorders) predisposed a future generation to respond to a second hit (VEN treatment during embryogenesis—recreating prenatal exposure to an alternative treatment in case of FLX response failure), which further altered the adult phenotype (discuss below). The first hit of this model corresponds to the exposure of ZF embryos from 0 to 6 dpf to FLX, an event that occurred four generations earlier. Exposure of the F₀ to $0.54 \mu\text{g}\cdot\text{L}^{-1}$ FLX (LFL group) affected the development of the F₄ larvae independent of the second hit, VEN exposure. An increase in survival, hatching rate and hatching success were recorded for these F₄ larvae. Malformation rate in these larvae was also significantly reduced. These effects were not observed in the larvae from the FLX lineages in the F₀ to F₃ generations (Chapter 5). Furthermore, these developmental effects on the LFL larvae were not observed in the F₅ larvae, suggesting that the developmental effects experienced in the F₄ are minor and not persistent.

Exposing the F₄ generation to VEN (the second hit) from 0 to 6 dpf did not affect any developmental parameters in the F₄ or F₅ larvae regardless if they were from the CTR or either FLX lineage. A study by Galus et al. (2013) reported that exposure to 0.5 µg·L⁻¹ VEN (~1.5 to 72 hpf) increased mortality rate in larvae ZF, however, these effects were not observed using 10 µg·L⁻¹ VEN. Our findings on the lack of effects on mortality rate in our F₄ larvae following VEN exposure agree with a study by Parrott and Metcalfe (2017) that found that 167 to 168 days of exposure (from fertilization) to 0.88, 8.8, and 88 µg·L⁻¹ VEN did not affect mortality rates in fathead minnows (*Pimephales promelas*). These authors also reported that the hatching and malformation rates in the F₁ larvae were unaffected by these same exposure conditions (Parrott and Metcalfe, 2017), supporting our results with the F₅ larvae.

In our previous study (Chapter 2), we demonstrated that early developmental exposure to FLX disrupts the stress axis by reducing total cortisol levels in adult ZF across 3 generations. Strikingly, this disruption in the stress axis is still observed in the F₄. Similar effects to our F₀ to F₃ generations (Chapter 2) on the sex-specific cortisol disruption were observed in the F₄. FLX exposure to the F₀ induced a 6 to 34% decrease in stress-induced cortisol levels whereas in males a 17 to 54% reduction in the stress-induced cortisol levels were observed in our adults from the FLX lineage. The stress response of these F₄ adult ZF was also affected by FLX exposure to our F₀ generation. The females from the LFL and HFL groups responded to only 94 and 68% of the CTR fish to the net stressor, respectively, while males had an even lower ability to respond to the stressor; 74 and 34% of the ability of the CTR in the LFL and HFL, respectively.

Interestingly, VEN exposure to the F₄ exacerbated the reduction in the stress-induced cortisol levels solely in the adult females from the HFL group. Since this impairment was only observed in the HFL group, it indicates that the human physiological concentration of FLX

(54 $\mu\text{g}\cdot\text{L}^{-1}$) can potentially permanently alter the way that the body reacts or responds to another antidepressant. However, it will be interesting to determine if the 54 $\mu\text{g}\cdot\text{L}^{-1}$ FLX exposure alters the response of the body to other chemicals as well. Moreover, this effect of VEN on the cortisol levels was absent in the adult males regardless of FLX exposure, suggesting a sex-specific VEN effect on the cortisol levels. The lack of effect of VEN on stress-induced cortisol levels was also observed following a 7-day treatment to 0.2 and 1.0 $\mu\text{g}\cdot\text{L}^{-1}$ in juvenile rainbow trout (Best et al., 2014).

The sex steroid levels of the F₄ adult ZF were not affected by FLX exposure to the F₀ except for E₂ in males from the HFL in which we observed a 17% increase in their total E₂ levels compared to the CTR. However, this increase in E₂ levels does not seem to be biologically significant as no effects were found in their reproductive capability. Moreover, the levels of sex steroids assessed in this study were whole-body. We feel that these results on total sex steroid levels should be interpreted with caution as sex steroids are synthesized in many tissues (Balthazart and Ball, 2006; Li et al., 2015) as a result, the total levels may not be indicative of the concentration of hormone to which specific target tissues are exposed.

Exposure of the F₄ larvae to VEN for 6 days decreased total testosterone and 11-KT levels by 26 to 36% and 8 to 23%, respectively, relative to the CTR in the adult males and regardless of FLX treatment. In females, VEN alone increased their total E₂ levels by 17 to 45% compared to the CTR. It also increased their 11-KT levels by 5 to 33%. A 7-day exposure to 0.2 and 1.0 $\mu\text{g}\cdot\text{L}^{-1}$ VEN in juvenile rainbow trout altered the handling stressor-mediated expression levels of key steroidogenic enzymes including steroidogenic acute regulatory protein (levels increased) and cytochrome P450 side chain cleavage (levels decreased) in the head kidney of rainbow trout (Best et al., 2014). These results on the alteration of the expression levels of key steroidogenic enzymes

by VEN exposure (Best et al., 2014) support our findings on VEN-induced impairment of sex steroid and cortisol levels observed in our study. However, the assessment of the expression levels of genes associated with steroidogenesis is required to better elucidate the underlying mechanism(s) for the observed alteration in both, sex steroid and cortisol levels.

Another important component of steroidogenesis was also affected in the F₄ adult ZF by FLX treatment to the F₀. Total cholesterol levels, the precursor for steroid synthesis including cortisol, were 7% lower in the adult females from the HFL compared to the CTR fish, whereas the males from the LFL group experienced a 12% increase. However, VEN exposure did not affect total cholesterol levels in either females or males. Furthermore, whole-body triglycerides levels were reduced by 14 to 31% in the adult females from the LFL and HFL groups. Exposure to VEN also induced a reduction in total triglycerides levels in the adult females regardless of the FLX lineage. Male levels, however were not affected by FLX. It is unclear from this study the significance of the reduction in triglycerides levels in the adult females but it will be important to determine its potential effects since triglycerides are also a major source of energy reserve, especially in winter for certain fish species (Driedger et al., 2009).

Reduced cortisol levels following FLX exposure to the F₀ decreased locomotor and exploratory activities in the F₀ to F₂ adult males following the novel tank diving test (Chapter 2). However, this behavioral phenotype in the adult fish from the FLX lineage disappeared in the F₃ generation (Chapter 2). In this study, we demonstrated that the F₄ adult females from the HFL group exhibited reduced activities compared to the CTR, an effect that was absent in the males. Exposure to VEN did not alter the behavioral responses of the adult fish to the novel tank diving test in either females or males. Parrott and Metcalfe (2018) also reported that VEN exposure at 0.88 and 8.8 µg·L⁻¹ from fertilization for 162 – 163 days post-hatch in fathead minnows did not

alter the nest-defense behaviors in these fish. However, other studies have reported that VEN can alter a variety of behaviors in fish. For instance, a 6-day exposure during adulthood to 50, 250 and 500 $\mu\text{g}\cdot\text{L}^{-1}$ VEN in hybrid striped bass (*Morone saxatilis* x *Morone chrysops*) increased their time to capture prey (Bisesi et al., 2014). Moreover, a microinjection of 1 and 10 ng VEN in ZF embryos (1–4 cell stage) reduced the activity and distance travelled in the larvae following light and dark behavioral tests (Thompson et al., 2017). In adult male mosquitofish (*Gambusia holbrooki*), 100 $\mu\text{g}\cdot\text{L}^{-1}$ VEN for one week disrupted their normal circadian rhythmicity (Melvin, 2017).

Reproductive capability is also an important measure of fitness in fish. In our previous study (Chapter 5), we did not find any major effects in the sex ratio, spawning rate, fertilization rate, *K*-factor and gonadosomatic index in our fish from the FLX lineage in generation F_0 to F_3 . Here, we report that three of our groups, LFL exposed and not exposed to VEN and CTR exposed to VEN, had an 11 to 25% higher number of males compared to the CTR. However, the spawning rate of these adults were > 50%. Interestingly, in our HFL group that was exposed to VEN during embryogenesis, only 20% of the pairs spawned (3 of the 15 pairs) even after the two spawning attempts with different partners, however, the fertilization rate of their eggs was ~ 100%. We also found that the *K*-factor of our adult females and males from the FLX lineage were significantly affected by VEN exposure, but this effect was <3% in females and <7% in males. Overall VEN exposure by itself did not affect reproduction in our adult ZF. Our findings of little effect of VEN on ZF reproduction was also observed in fathead minnows (Parrott and Metcalfe, 2017). Exposure to 0.88 and 8.8 $\mu\text{g}\cdot\text{L}^{-1}$ VEN for 167 to 168 days from fertilization in fathead minnows did not affect the *K*-factor or egg production in the adult fish. Moreover, the egg quality or fertilization rate of the F_1 were also unaffected by exposure of the parent fish to VEN (Parrott and Metcalfe, 2017). These authors observed an increase in egg production per female solely in the fish that were

exposed for 167 to 168 days to $88 \mu\text{g}\cdot\text{L}^{-1}$ VEN (Parrott and Metcalfe, 2017). In contrast, adult ZF exposed for 6 weeks during adulthood to $10 \mu\text{g}\cdot\text{L}^{-1}$ VEN demonstrated a reduction in viable egg production per female (Galus et al., 2013).

We conclude that exposure to environmentally ($0.54 \mu\text{g}\cdot\text{L}^{-1}$; LFL) and human physiologically ($54 \mu\text{g}\cdot\text{L}^{-1}$; HFL) relevant concentrations of FLX from 0 to 6 dpf disrupted cortisol levels especially under stressful conditions in adult ZF across at least 4 generations. These disruptive effects on cortisol levels were sex-dependent, with males being more sensitive to FLX during early embryogenesis than females. Perhaps the most striking finding of this study was the unexpected exacerbation of the blunted cortisol levels following exposure of the F_4 generation to $5 \mu\text{g}\cdot\text{L}^{-1}$ VEN (from 0 to 6 dpf) observed in our adult females from the HFL group. These findings support our hypothesis and suggest that FLX exposure of the great-great-grandparents (F_0) permanently and most likely epigenetically shaped the response of future generations and especially females, to antidepressants. We also found that VEN exposure has the potential to alter steroidogenesis and specifically sex steroid levels regardless of the FLX lineage. Our research emphasizes the need to conduct further studies in humans to determine the potential implications in the approach of using different antidepressants as treatments for depression in different generations of a given family or even in the same individual.

Our findings also highlight potential risks to aquatic wildlife given that in the environment we can detect a myriad of chemicals which may be synergistically affecting the aquatic population. Due to improper disposal and human excretion, VEN and its metabolites have been detected in the aquatic environment at concentrations as high as $\sim 4 \mu\text{g}\cdot\text{L}^{-1}$ (Arnnok et al., 2017; Couperus et al., 2016; Metcalfe et al., 2010). The pseudopersistence of VEN and FLX in the environment, concomitantly with the high degree of conservation of neurotransmitter systems in vertebrates

(Kreke and Dietrich, 2008), render aquatic organisms such as fish susceptible to the effects of antidepressants in contaminated aquatic ecosystems.

6.6. Acknowledgments

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CHAPTER 7: General Discussion and future directions

7.1. Overview of the problematic

The findings reported in this doctoral thesis contribute to our understanding of human and aquatic toxicology of fluoxetine (FLX; Prozac®), a member of the selective serotonin (5-HT) reuptake inhibitor family. Given that FLX readily cross the placenta, a fetus from a FLX-treated pregnant woman is at risk from the disruptive effects of extracellular 5-HT over-stimulation induced by FLX exposure (Ewing et al., 2015; Kaihola et al., 2016; Loughhead et al., 2006; Rampono et al., 2009). One of the prominent roles of 5-HT that we explored in this study is its involvement in the development and programming of the stress axis during this highly plastic stage of development. The mechanism(s) by which 5-HT exerts its programming actions is through epigenetic modifications (Weaver et al., 2014), and due to their stability, epigenetic modifications have the potential to be transmitted to subsequent generations (Le Dantec et al., 2015; Nestler, 2016; Skinner, 2014). Moreover, the ever-increasing rates of prescriptions have unfortunately led to an escalation in environmental discharges of FLX with aquatic concentrations detected as high as 929 ng·L⁻¹ (Arnnok et al., 2017; Bueno et al., 2007; Comber et al., 2018). Therefore, its continuous release into the environment, concomitantly with the conservation of FLX targets across vertebrates and of the biochemical pathways and functions of 5-HT (Mennigen et al., 2011), render aquatic organisms such as fish, another target for the possible unintended effects of this drug.

In light of all this evidence, we tested the hypothesis that FLX disrupts the stress axis and that the resultant phenotype is transmitted to the descendants. The FLX concentrations studied were environmentally relevant (0.54 µg·L⁻¹) or comparable to concentrations detected in the cord

blood of FLX-treated pregnant women ($54 \mu\text{g}\cdot\text{L}^{-1}$). All the findings collected throughout my doctoral studies are summarized in Fig. 7.1.

7.2. Thesis summary and discussion

This is the first study to demonstrate that exposure to FLX during the first 6 days of life leads to an impairment of cortisol synthesis for at least 4 consecutive generations in adult zebrafish (ZF; *Danio rerio*) without diminution, even though the descendants were not directly exposed to FLX. (Chapter 2 and 6). This effect was more pronounced and persistent in males than females (Chapter 2). Since cortisol is primarily synthesized in the interrenal cells in fish or the adrenocortical cells in mammals (Dores and Garcia, 2015; Kilianova et al., 2006), we examined the responsiveness of the interrenal cells to adrenocorticotrophic hormone stimulation in the FLX-treated adult males from the exposed generation 0 (F_0) and the unexposed F_3 . Our findings revealed that FLX induced a transgenerational disruption of the ability of the interrenal cells to synthesize cortisol. Transcriptomic and pathway analyses of the kidney containing interrenal cells of adult males from the F_0 and F_3 generations uncovered many biological pathways and functions associated with steroidogenesis that were altered by early-life FLX exposure of the F_0 . These findings provide further insights into the underlying mechanism(s) of the transgenerational disruption induced by FLX.

An unexpected yet interesting finding was the reduction in the locomotor and exploratory activities following the novel tank diving test observed in our males from the FLX-treated groups for 2 consecutive generations, specifically at the human-relevant concentration. We subsequently linked these behavioral alterations to the low cortisol phenotype exhibited by the males (Chapter 2). Even though our FLX-treated females also displayed a reduction in cortisol levels, we

did not observe any alteration in their behaviors in our transgenerational model. We later (Chapter 4) demonstrated that females are susceptible to the FLX-induced low cortisol effects during a different window of development, the sex differentiation period (15 to 42 days post-fertilization, dpf). Another novel finding from this study was the sexual dimorphic behavioral response observed in the exploratory and locomotor activities induced by the low-cortisol levels of our FLX-treated fish (Chapter 4). In the females, their behavioral activity increased as a result of the hypocortisolism compared to the control. In contrast, in males we observed a reduction in their behavioral response to the novel tank diving test as previously discussed. It is worth noting that the novel tank diving paradigm (Levin et al., 2007) used in this study is also considered a measurement of anxiety-like responses similar to the open field test conducted in rodents (Bencan et al., 2009; Cachat et al., 2011; Mocelin et al., 2015). Contrary to our findings, studies in ZF following an exposure during adulthood showed that FLX increases their behavioral response (or attenuates their anxiety-like behavior as described in these studies) regardless of the sex of the fish (Cachat et al., 2010; 2011; Egan et al., 2009).

We also demonstrated that FLX induced the disruption of the cortisol levels in the larvae within 4 days of exposure (Chapter 3). To better understand the molecular disruption of the stress axis and to investigate the coping mechanism(s) of the larvae immediately following the 6-day FLX exposure, we assessed key genes associated with the stress axis in the whole-larvae of generation F₀ (Chapter 3). These findings revealed that the transcriptional profile of the genes assessed in the F₀ varied in magnitude and direction in both treatments, despite the same low cortisol phenotype induced by both the environmental- and human-relevant concentrations. We also showed an up-regulation in the transcript levels of steroidogenic-related genes and a down-regulation of a gene involved in the inactivation of cortisol in the F₃ larvae ancestrally exposed to

the human-relevant concentration of FLX. These findings on the transcript levels of the selected genes in the larvae from F₀ and F₃ suggest that the larvae adopted specific coping mechanism(s) to the disruptive effects of FLX depending on the exposure concentration and the filial generation. These specific effects of FLX to the filial generation were further supported by the reduction in the pigmentation patterns observed solely in the descendants of the exposed fish (F₁ to F₃) while it was absent in the F₀ (Chapter 5). It is also noteworthy that the reproductive fitness of our FLX-treated fish or their descendants was not affected (Chapter 5). Normal reproduction in our transgenerational model allowed for an efficient transfer and inheritance of all the pronounced effects of FLX on the stress axis, behavior and pigmentation from one generation to the next, increasing the likelihood of an epigenetic mode of transgenerational inheritance.

We also constructed a “two-hit” model of antidepressant exposure. The aim of this model was to recreate and examine the effects of predisposing a future generation by exposing the F₀ to FLX (first hit) to respond to a second hit by exposing the fish to venlafaxine (VEN, as an alternative treatment in case of FLX response failure) (Chapter 6). The first hit corresponded to the previously discussed exposure of ZF embryos from 0 to 6 dpf to FLX, whereas the second hit corresponded to exposure of ZF embryos from 0 to 6 dpf to VEN, an event that occurred four generations later (in the F₄). Perhaps the most striking finding of this two-hit model was the unexpected exacerbation in the attenuation of cortisol levels observed in our adult females exposed to VEN that were ancestrally exposed (in the F₀) to the human-relevant concentration of FLX (54 µg·L⁻¹). We also found that VEN exposure has the potential to alter steroidogenesis and specifically sex steroid levels regardless of the FLX treatment. These findings support our hypothesis and suggest that FLX exposure of the great-great-grandparents (F₀) permanently and most likely epigenetically shaped the response of future generations to other antidepressants.

7.3. Significance of these findings

As emphasized in the overview above (section 7.1), these findings are of a human and aquatic animal concern. The cortisol response to stressors is a critical regulator of adaptive and behavioral responses in vertebrates (Egan et al., 2009; Pippal et al., 2011). In teleosts, cortisol also plays an important role in osmoregulation, regulation of metabolism, circadian rhythms, and other developmental processes (Mommensen et al., 1999; Tsachaki et al., 2017; Wang and Harris, 2015). In this study, we uncovered sex-specific locomotor and behavioral alterations resulting from the FLX-induced disruption in cortisol levels. However, there are a myriad of other physiological processes modulated by cortisol, hence the potential implications of this cortisol disruption are of high concern. For instance, chronic hypocortisolism has been associated with long-term detrimental effects to human health including burnouts, chronic fatigue, fibromyalgia, immune disorders, and post-traumatic stress disorder, among others (Bakusic et al., 2017; Demitrack and Crofford, 1998; Fries et al., 2005; Nijhof et al., 2014; Wichmann et al., 2017). More strikingly, evidence on hyporeactivity of the HPA axis in children prenatally exposed to SSRIs through maternal treatment already exists (Davidson et al., 2009; Oberlander et al., 2008).

Given this evidence of the transgenerational effects of developmental FLX exposure on the stress axis and behavioral responses, an investigation is required to determine whether these effects occur in humans as FLX is generally the first-line of pharmacological treatment in pregnant women suffering from affective disorders (Latendresse et al., 2017; Locher et al., 2017; Man et al., 2017; Morkem et al., 2017; Sarginson et al., 2017). Furthermore, although highly speculative, if these effects could be replicated in humans, would we expect great-great-grandchildren (~80 years from when FLX was first introduced into the market) being affected from an exposed generation to psychological disorders associated with low cortisol levels (see Fig. 7.1)? This will

eventually lead to an increase in other pharmacological treatments to reduce or treat these other disorders. Finally, given that levels of FLX detected in the aquatic environment (Mennigen et al., 2010a) also reduced the stress response over at least 4 generations, our findings highlight potential risks to natural populations of fish. It is important to highlight that aquatic organisms and even humans are exposed to many different chemicals in their environments (Comber et al., 2018; Yang et al., 2017a). Therefore, exposure to these chemicals may be synergistically acting and affecting the fitness of all organisms as it was observed with our two-hit model.

7.4. Future directions

The extensive new data presented in this thesis provide numerous possible directions for future research. In addition to what has been outlined in the individual chapters, we identified four main lines of investigation that should be considered:

1. Epigenetic mechanisms are generally transmitted through the germline (Nilsson and Skinner, 2015; Skinner, 2014; Xin et al., 2015). However, whether the transgenerational transmission of the FLX effects is either paternal or maternal remains to be investigated.
2. Assuming that this transgenerational inheritance occurs by epigenetic mechanisms, it will be essential to identify which of the myriad of epigenetic mechanisms are involved.
3. The transcriptomic profiling and pathway analysis revealed many other genes and processes transgenerationally disrupted by early-life FLX exposure. Many of these were related to kidney functions, immune response, hematopoietic and epithelial cells processes, among others. Therefore, future studies should explore the implications of these disrupted biological pathways by FLX.

4. Similar studies to this one should be conducted using other pharmaceuticals that are highly prescribed. For instance, a 2017 report from the U.S.A. stated that hypertension drugs accounted now for the highest prescription rates (30% of all prescriptions) (Aitken, 2018).

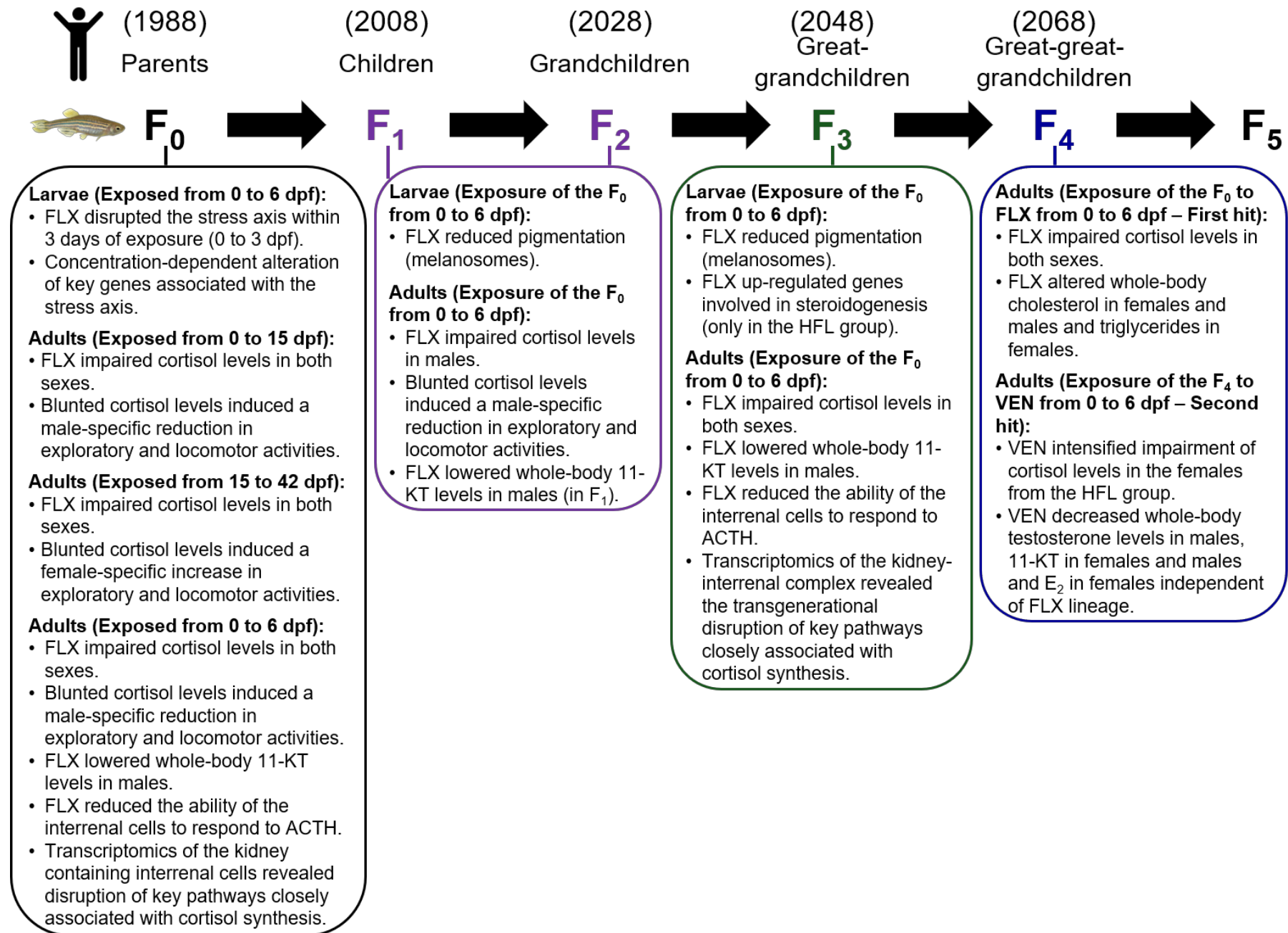


Fig. 7.1. Transgenerational disruption resulting from early-life exposure to FLX in ZF using human ($54 \mu\text{g}\cdot\text{L}^{-1}$) and environmentally ($0.54 \mu\text{g}\cdot\text{L}^{-1}$) relevant drug concentrations. The first year (i.e. 1988) in the human timeline corresponds to the year that FLX was introduced into the U.S.A. market (Wong et al., 1995). In the timeline, each generation of humans is separated by 20 years as it is the average age that women give birth according to Tietze (1957).

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