

**Corticosteroidogenesis as a Target of Endocrine Disruption for the Antidepressant
Fluoxetine in the Head Kidney of Rainbow Trout (*Oncorhynchus mykiss*)**

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Abstract

Fluoxetine (FLX), the active ingredient of Prozac™, is a member of the selective serotonin reuptake inhibitor (SSRI) class of anti-depressant drugs and is present in aquatic environments worldwide. Previous studies reported that FLX is an endocrine disruptor in fish, bioconcentrating in tissues including the brain. Evidence implicates that serotonin influences the activity of the hypothalamo-pituitary-interrenal (HPI) stress axis, thus exposure to FLX may disrupt the teleost stress response. This study examined *in vitro* cortisol production in rainbow trout (*Oncorhynchus mykiss*) head kidney/interrenal cells exposed to FLX and ¹⁴C-pregnenolone metabolism in head kidney microsome preparations of FLX-exposed trout. Results indicated that cells exposed *in vitro* to increasing concentrations of FLX had lower cortisol production and cell viability (versus control) and microsomes isolated from trout exposed to 54 µg/L FLX had higher pregnenolone metabolism versus those of control and low FLX-exposed (0.54 µg/L) trout.

Résumé

La Fluoxétine (FLX), produit actif de Prozac™, fait partie de la classe des antidépresseurs nommée Inhibiteurs sélectifs de la Recapture de la Sérotonine (IRS). La FLX est un médicament retrouvé dans l'environnement aquatique du monde entier. Des études précédentes indiquent que la FLX est un perturbateur endocrinien et qu'elle a le pouvoir de se concentrer dans les tissus des poissons, et notamment dans le cerveau. Ceci implique donc que la sérotonine influence l'axe hypothalamo-pituitaire-interrenal (HPI), axe régulant le stress. Ainsi l'exposition à FLX peut perturber la réponse du stress chez les téléostéens. Le but de cette étude a été d'une part, d'étudier la production *in vitro* de cortisol dans les cellules interrenales de truite arc-en-ciel (*Oncorhynchus mykiss*) exposées au FLX et d'autre part, d'étudier le métabolisme du ¹⁴C-prégnénolone dans des préparations de microsomes du pronéphros chez des truites préalablement exposées au FLX. Les résultats indiquent que les cellules exposées *in vitro* à des concentrations de FLX modérées à hautes ont une production de cortisol inférieures et une baisse de viabilité contre des cellules non-exposées; les microsomes des truites exposées à 54 µg/L FLX ont un métabolisme plus élevé de prégnénolone contre ceux des truites du groupe contrôle et de l'exposition à faible concentration (0.54 µg/L) de FLX.

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List of Abbreviations

ACBD – acyl-CoA binding domain	NADH – nicotinamide adenine dinucleotide hydride
ACh – acetylcholine	NADPH – nicotinamide adenine dinucleotide phosphate
ACTH – adrenocorticotrophic hormone	NAD ⁺ – nicotinamide adenine dinucleotide
ANT – adenine nucleotide translocator	NEpi – norepinephrine
AVT – arginine vasotocin	NIMH – National Institute of Mental Health
BCA – bicinchoninic acid	norFLX – norfluoxetine hydrochloride
BSA – bovine serum albumin	NPO – nucleus preopticus
CART1 – cocaine and amphetamine regulated transcript	NPY – neuropeptide Y
CCAC – Canadian Council on Animal Care	NSAID – non-steroidal anti-inflammatory drug
CNS – central nervous system	OCD – obsessive-compulsive disorder
CRF/H – corticotropin releasing factor/hormone	OMM – outer mitochondrial membrane
CSM – Committee on Safety of Medicines	P – statistical probability
CYP450 – cytochrome P450 enzyme	P4 – progesterone
DA – dopamine	P450 _{sc} – cytochrome P450 side chain cleavage enzyme
DOI – 2,5-dimethoxy-4-iodoamphetamine	P5 – pregnenolone
Dpm – disintegrations per minute	PBR – peripheral-type benzodiazepine receptor
eMEM – enriched minimal essential media	PCV – posterior cardinal vein
Epi – epinephrine	PGF _{2α} – prostaglandin F _{2α}
ER – estrogen receptor	PKA – protein kinase A
E2 – 17β-estradiol	POMC – pro-opio melanocortin
FDA – U.S. Food and Drug Administration	PCP – personal care product
FDx – ferredoxin	PPCP – pharmaceutical and personal care product
FDxR – ferredoxin reductase	PVN – paraventricular nucleus
FFA – free fatty acids	qRT-PCR – quantitative real-time polymerase chain reaction
FLX – fluoxetine hydrochloride	SE – standard error
FST – forced swimming test	SERT – serotonin transporter
GEM – gemfibrozil	SSNRI – Selective Serotonin Reuptake Inhibitor
GR – glucocorticoid receptor	SSRI – Selective Serotonin Reuptake Inhibitor
GRE – glucocorticoid response element	StAR – steroidogenic acute regulatory protein
HPA/I – hypothalamo-pituitary-adrenal/interrenal	T – testosterone
IMM – inner mitochondrial membrane	TCA – tricyclic antidepressant
IMMS – inter-mitochondrial membrane space	TLC – thin layer chromatography
IP – intraperitoneal	VDAC – voltage dependent anion channel
IU – international unit	WHO – World Health Organization
LDH – lactate dehydrogenase	11β-OHase – 11β-hydroxylase
LOEC – lowest observed effect concentration	17,20P – 17,20β-dihydroxy-4-pregnene-3-one
MAO – monoamine oxidase	
MC2R – melanocortin type 2 receptor	
MEM – minimal essential media	
MR – mineralocorticoid receptor	
N – sample size	

17-OHase – 17 α -hydroxylase
17-P4 – 17 α -hydroxyprogesterone
17-P5 - 17 α -hydroxypregnenolone
21-OHase – 21-hydroxylase
3 β -HSD – 3 β -hydroxysteroid
dehydrogenase
5-HIAA – 5-hydroxyindoleacetic acid
5-HT – serotonin
8-OH-DPAT – 8-hydroxy-2
(di-n-propylamino) tetralin

Chapter 1. Introduction

1.0 The Problem

Depressive disorders affect more than 120 million people worldwide (WHO, 2011) making these disorders one of today's most pressing public health issues (Hinkelmann et al., 2009). Depression is a common but serious illness (NIMH, 2008; WHO 2011) affecting both the social and professional activities of its sufferers (Lajeunesse et al., 2008). The estimated costs of treatment, medication, loss of productivity and premature death associated with depression in 2006 were 14.4 billion dollars per year in Canada alone (Lajeunesse et al., 2008). Today, depression is one of the leading causes of debilitating (WHO, 2011) and chronic diseases (Health Canada, 2009) and the majority of its sufferers require treatment in order to recover (NIMH, 2008).

A common treatment option for alleviating depressive disorders is anti-depressant medication from the class of pharmaceuticals known as Selective Serotonin Reuptake Inhibitors (SSRIs). Since their introduction in the 1980s (Moret et al., 2009), the use of SSRIs in depression and human therapeutics has increased steadily (Kreke and Dietrich, 2008), largely due to the mild side-effect profiles and effectiveness of these drugs in patients (Peretti et al., 2000). As a result of their growing use and limited biodegradation (Redshaw et al., 2008), SSRIs are now found in aquatic environments worldwide (Christensen et al., 2009; Metcalfe et al., 2003; Kolpin et al., 2002). Although environmental concentrations of these pharmaceuticals are in the range of ng/L to low µg/L (Kreke and Dietrich, 2008), aquatic organisms, like fish, may be affected by exposures at these sublethal levels because of the highly specific mode of action of these drugs (Kreke and Dietrich, 2008). Fish benefit both natural ecosystems and human societies in numerous and invaluable ways (Holmlund and

Hammer, 1999). Food production, medicine, water purification and even recreational activities (Holmlund and Hammer, 1999) such as tourism and fishing, are just a few examples of the services fish provide to human society. Global aquaculture production is reported to have increased on average by 10% per year since the 1990s (Food and Agriculture Organization, 2000), demonstrating that the need for fish and the services they provide to human populations is growing. In addition, many vital ecosystem processes such as food web structures, nutrient recycling and productivity (McIntyre et al., 2007) are highly influenced and maintained by fish activities.

The numbers of dispensed SSRI prescriptions continue to increase each year making it likely that fish species will be continuously exposed to these pharmaceuticals and any negative impact they may have including, endocrine disruption. As fish provide necessary services to both human societies and natural ecosystems, it is important to understand how environmental contaminants, such as SSRIs, may impact the physiology and overall health of these indispensable aquatic organisms.

This research focuses on how the hormonal and enzymatic activity of the teleost endocrine stress axis is impacted by SSRI exposure. Evaluating the effects of SSRI exposure on cortisol production and head kidney steroidogenesis in rainbow trout (*Oncorhynchus mykiss*) will allow me to assess whether environmentally relevant levels of these anti-depressants are capable of altering the physiological stress response and potentially jeopardizing fish survival. This introductory chapter will examine the concept of stress, explain how vertebrate organisms, specifically fish, respond and adapt to stressors and show how the stress response can be influenced by SSRI exposure. The uses, activity and environmental interaction of the SSRIs will also be discussed, and a more detailed review of

the properties and endocrine disrupting abilities of fluoxetine hydrochloride (FLX, ProzacTM) – the SSRI upon which my research is based – will be provided.

1.1 The Stress Response in Fish

1.1.1 Homeostasis, Stress and Stressors

Homeostasis is a regulated state of constancy by which an animal's internal processes and conditions are maintained despite changing or perturbing conditions in the external environment (Moyes and Schulte, 2008). This complex and dynamic equilibrium (Chrousos, 1997; Wendelaar Bonga, 1997) is what permits an animal to survive the demands placed upon it (Selye, 1973). More specifically, these demands are related to intrinsic or extrinsic stimuli commonly called *stressors* (Wendelaar Bonga, 1997). Stressors are also considered to be destabilizing stimuli (Pottinger, 2003) as they can produce effects that destabilize an animal's homeostasis (Wendelaar Bonga, 1997). In an environmental context, Pottinger (2003) categorized stressors into four classes: i) physical (abiotic and biotic), e.g. temperature, flow regimes, predator and parasite damage; ii) chemical, e.g. alterations in dissolved oxygen, exposure to pollutants; iii) physiological, e.g. starvation, disease; and, iv) psychological, e.g. territoriality, threat of predation, etc. When the homeostatic norm is destabilized or threatened by the action of a stressor, the animal is in a condition known as *stress* (Wendelaar Bonga, 1997; Norris, 2000; Barton, 2002). Physical, chemical and any perceived stressors (Barton, 2002) can trigger stress in an animal and elicit a coordinated set of compensatory and/or adaptive behavioral and physiological responses, collectively known as the *stress response* (Selye, 1973; Wendelaar Bonga, 1997). A usual misconception is that stress in itself is detrimental to an animal (Barton, 2002), however stress actually plays a key role in the ability of vertebrates to perform necessary life functions and allows them to

survive and/or adjust to threats or changes they encounter in the environment (Schreck, 2010). However, if the stressor(s) an animal encounters are particularly severe and/or chronic, the stress response can become maladaptive and harmful to the organism and could ultimately result in death (McEwen, 2000; Barton, 2002; Hinkelmann, 2009).

1.1.2 The Vertebrate Stress Response

The physiological cascade known as the vertebrate stress response is initiated when a stressor is sensed or perceived by a vertebrate organism (Schreck, 2010). The perception of a stressor signals the central nervous system (CNS), specifically the hypothalamus, to stimulate preganglionic sympathetic neurons that synapse and release acetylcholine (ACh) onto cells (modified neurons) of the adrenal medulla that release catecholamines into the bloodstream (Moyes and Schulte, 2006). Catecholamines are amine neurotransmitters including epinephrine (Epi) and norepinephrine (NEpi) that induce physiological changes in peripheral cells and tissues in response to stress (Hart et al., 1989). Examples of such changes include: increased blood glucose, decreased gut motility, increased oxygen transport to tissues and energy mobilization (Hart et al., 1989; McCarty and Gold, 1996). The changes induced by the release of these neurotransmitters prepare the animal to physically react to a stressor if needed, making the catecholamines an integral part of the physiological stress response in all vertebrates (Hart et al., 1989; McCarty and Gold, 1996; Wendelaar Bonga, 1997).

Stressors also stimulate the activity of the hypothalamo-pituitary-adrenal (HPA) axis (Fig. 1.1). This axis not only permits an organism to adapt to stressful conditions but also supports normal physiological functioning (Kudielka et al., 2006). Any perceived threat or change triggers an animal's hypothalamus to produce and secrete corticotropin-releasing hormone (CRH). CRH is a major hypothalamic regulating neuropeptide that signals the

anterior pituitary to secrete adrenocorticotrophic hormone (ACTH) (Norris, 2000; Dedovic et al., 2009) into the blood circulation, where it binds to melanocortin type 2 receptors (MC2R) (Flik et al., 2006) on the adrenal cortex (Dedovic et al., 2009). In response to this binding, cortisol and/or corticosterone are synthesized in the adrenal cortex and released as end products of the stimulation of the HPA axis (Chrousos and Gold, 1992; Norris, 2000).

The stress axis in fish is fundamentally similar to the HPA axis except that corticosteroid production in fish occurs in diffuse cells that make up what is called the interrenal tissue located around the walls of the post-cardinal vein; thus the fish stress axis is called the HPI or hypothalamic-pituitary-interrenal axis (Wendelaar Bonga, 1997). In teleosts, stress sensory information is received by the hypothalamic nucleus preopticus (NPO) (Flik, 2006), which releases CRH (Stolte et al., 2008). The axons of the NPO CRH cells project directly to the pituitary gland (Flik, 2006), in particular the cells of the rostral pars distalis, which produce ACTH. ACTH is released into the circulation where it binds to its receptor (MC2R) on the interrenal cells of the head kidney and stimulates the production and secretion of cortisol (Stolte et al., 2008). Teleost fish also release catecholamines, generally before cortisol, that are stored in chromaffin cells (adrenal medulla homologue) dispersed throughout the head kidney and specifically within the posterior cardinal vein (PCV) (Wendelaar Bonga, 1997; Barton, 2002). When a stressor is initially encountered, the CNS (hypothalamus) signals sympathetic nerve fibers to stimulate the release of Epi and/or NEpi via the cholinergic receptors of innervated chromaffin cells (Barton, 2002). Catecholamines exert both stimulatory and inhibitory actions on fish physiology, including increasing blood flow to the gills and altering plasma free fatty acid (FFA) content in order to maintain homeostasis (Wendelaar Bonga, 1997) (Fig. 1.2).

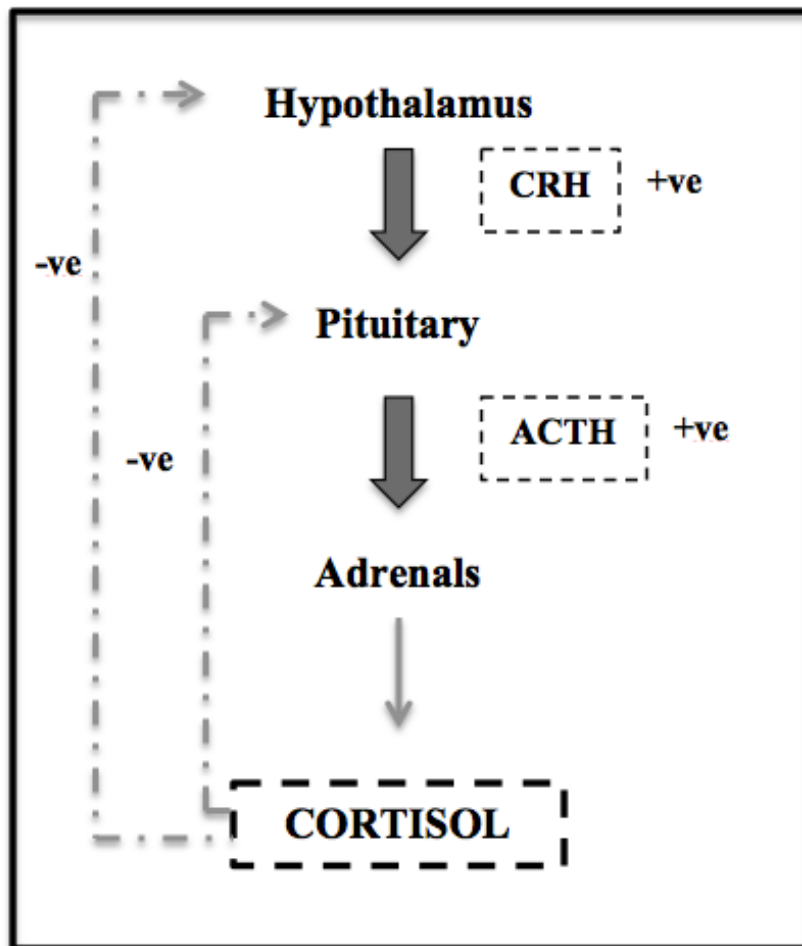


Figure 1.1. The Hypothalamo-Pituitary-Adrenal (HPA) Axis. CRH - corticotropin-releasing hormone; ACTH – adrenocorticotrophic hormone; -ve = negative feedback; +ve = positive feedback. Modified from Young, 2004

1.1.3 Importance of Cortisol

Catecholamines are an integral part of the vertebrate stress response (Hart et al., 1989; McCarty and Gold, 1996) but glucocorticoids such as cortisol, are essential for the long term management of physiological stress in vertebrates including humans and teleost fishes. Cortisol is not stored in steroidogenic cells but its production and release are stimulated in response to stressors or corticotropic agents like ACTH (Geslin et al., 2004). Cortisol synthesis begins with cholesterol stored in steroidogenic cells and involves the activity of several steroidogenic enzymes. The first and rate-limiting step of cortisol synthesis is the transfer of cholesterol across the inner mitochondrial membrane (Gravel and Vijayan, 2006; Papadopoulos, 2004). The movement of cholesterol across the inner mitochondrial membrane is stimulated by the cAMP intracellular signaling cascade – through protein kinase A (PKA) –, which is stimulated by the binding of ACTH to MC2Rs (Midzak et al., 2011). An ensemble of both cytoplasmic and mitochondrial proteins mediate the complex transportation of cholesterol across the aqueous inter mitochondrial membrane space (Fig. 1.3) (Midzak et al., 2011). When steroidogenesis is stimulated, the inner mitochondrial proteins CYP11A1, ferredoxin reductase (FdxR) and ferredoxin (Fdx) associate together, and with the adenine nucleotide translocator (ANT) protein (Midzak et al., 2011). ANT, along with the outer mitochondrial membrane proteins including, the voltage dependent anion channel (VDAC) and peripheral-type benzodiazepine receptor (PBR) is believed to be involved in forming a contact site between the outer and inner mitochondrial membranes, thereby allowing hydrophobic cholesterol to cross the aqueous inter-mitochondrial membrane space (Midzak et al., 2011). The steroidogenic acute regulatory (StAR) protein functionally promotes the translocation of cholesterol at the outer mitochondrial membrane,

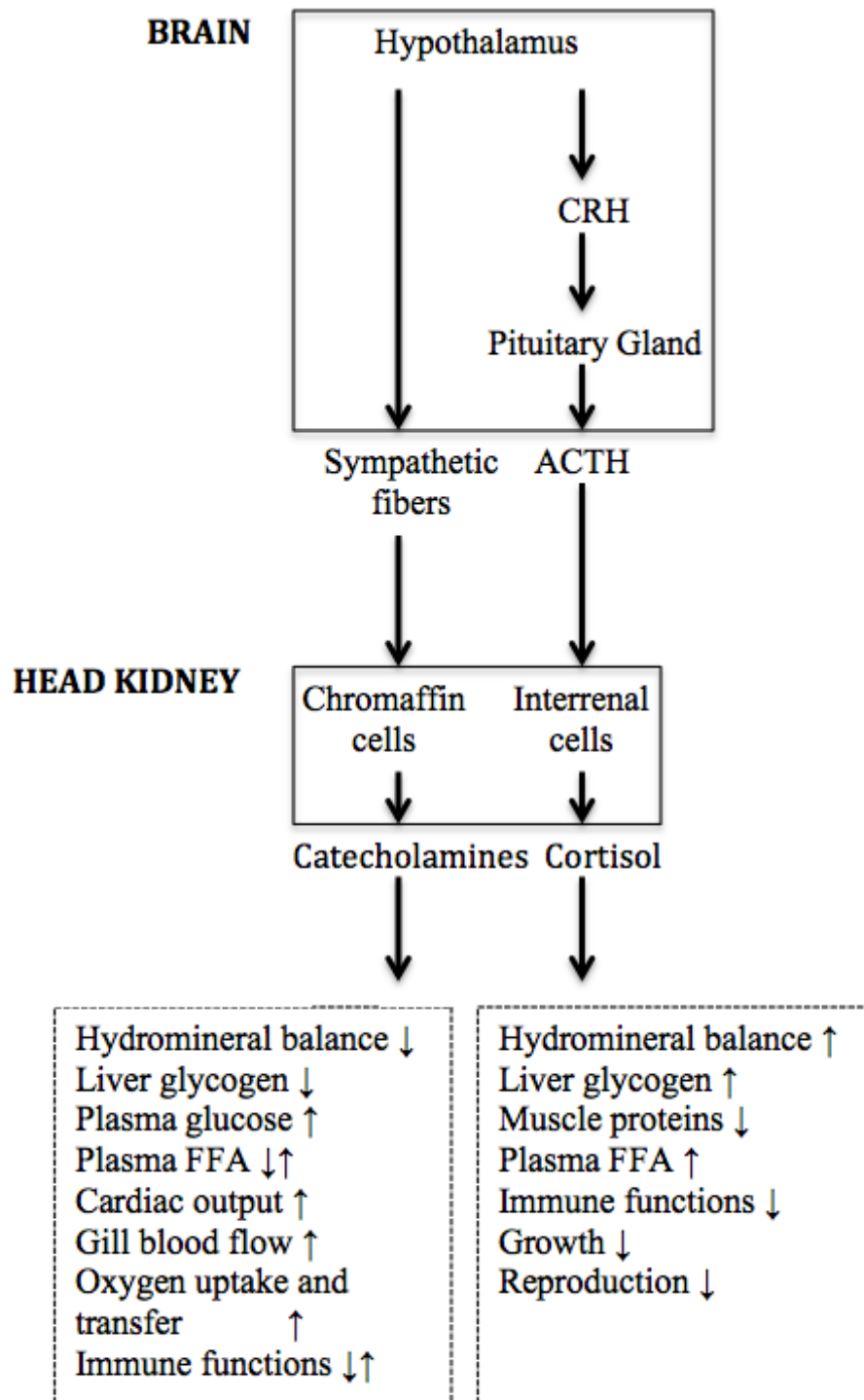


Figure 2.2. The neuroendocrine elements of the teleost stress response. ↑ = stimulatory; ↓ = inhibitory. Adapted from Wendelaar Bonga, 1997.

while PKA, a cytoplasmic protein, is involved in the clustering of the PBR protein pair, an event that is required for initiating the steroidogenic pathway (Midzak et al., 2011). ACBD3, an acyl-CoA binding domain (ACBD) family protein, is a PKA associated protein that scaffolds PKA's subunits to the PBR protein pair (Midzak et al., 2011) and is therefore, necessary in allowing the two PBR subunits to associate. Another ACBD protein, ACBD1, also acts on PBR to stimulate steroidogenesis (Midzak et al., 2011).

Once across the inner mitochondrial membrane, cholesterol is converted to pregnenolone by side chain cleavage mediated by the P450_{scc} (side chain cleavage) enzyme complex, specifically CYP11A1 (Geslin et al., 2004, Midzak et al., 2011). Pregnenolone leaves the mitochondrion and enters the endoplasmic reticulum, where it undergoes a series of isomerizations and hydroxylations by 17 α -hydroxylase (17-OHase), 3 β -hydroxysteroid dehydrogenase (3 β -HSD), 21-hydroxylase (21-OHase), and 11 β -hydroxylase (11 β -OHase), ultimately producing cortisol (Fig. 1.4) (Geslin et al., 2004; Papadopoulos, 2004). The synthesis and release of cortisol permits an animal to deal with encountered stressors by redistributing the body's energy, acting on neurotransmitter systems, and ultimately changing the animal's behavior (Wendelaar Bonga, 1997).

Cortisol exerts its cellular effects by crossing the cell plasma membrane and binding to intracellular proteins called glucocorticoid receptors (GRs) (Bamberger et al., 1996; Mommsen et al., 1999), which are expressed in nearly all vertebrate cell types (Kudielka et al., 2006). When cortisol binds to the cytosolic GR, this GR-cortisol complex moves to the nucleus where it binds to specific gene promoter sequences known as GREs (glucocorticoid response elements) thereby acting as a transcription factor (Bamberger, 1996; Mommsen et al., 1999). The increased release and binding of cortisol results in physiological changes at

the level of organ systems and includes changes in development, immune capacity and energy biosynthesis (Shreck, 2010). Cortisol also auto-regulates HPA activity by binding to GRs in the central and pituitary components of the stress axis, thereby exerting negative feedback on CRH and ACTH release from the hypothalamus and anterior pituitary, respectively (Young, 2004). Even at a resting state, cortisol is released in a circadian fashion to maintain regular physiological functions including normoglycemia and arterial tension (Bamberger et al., 1996). In fact, hypoactivity of cortisol production and release is associated with autoimmune disorders including lupus erythematosus, fibromyalgia, chronic fatigue, neurodermatitis and increased immune mediated inflammation (Kudielka et al., 2006). Over-production of cortisol (or hyperactivity of the HPA axis) results in increased adiposity and insulin resistance (Van Cauter et al., 1998). In addition, human patients with Cushing's syndrome (i.e. high cortisol) exhibit disturbances in their mood, cognition and overall behavior (Stokes, 1995). Perhaps the most important finding for the purpose of this research is that a number of studies have linked over-activity of the HPA axis to serious diseases including depression (Hinkelmann et al., 2009; Mitani et al., 2006; Maes et al., 1991; McEwen, 2000).

The ability to maintain homeostasis is essential for the survival of any organism, but cortisol is particularly important in fish as it plays a role in water and mineral regulation (Gilmour, 2005), and therefore serves as a ligand for both the glucocorticoid (GR) and mineralocorticoid (MR) receptors (Stolte et al., 2008). As with the catecholamines, cortisol has both stimulatory and inhibitory effects on physiological functioning in fish (Wendelaar Bonga, 1997). When released, cortisol induces changes in gill chloride cell morphology (Laurent and Perry, 1990), alters intermediary metabolism (e.g. inhibiting protein synthesis)

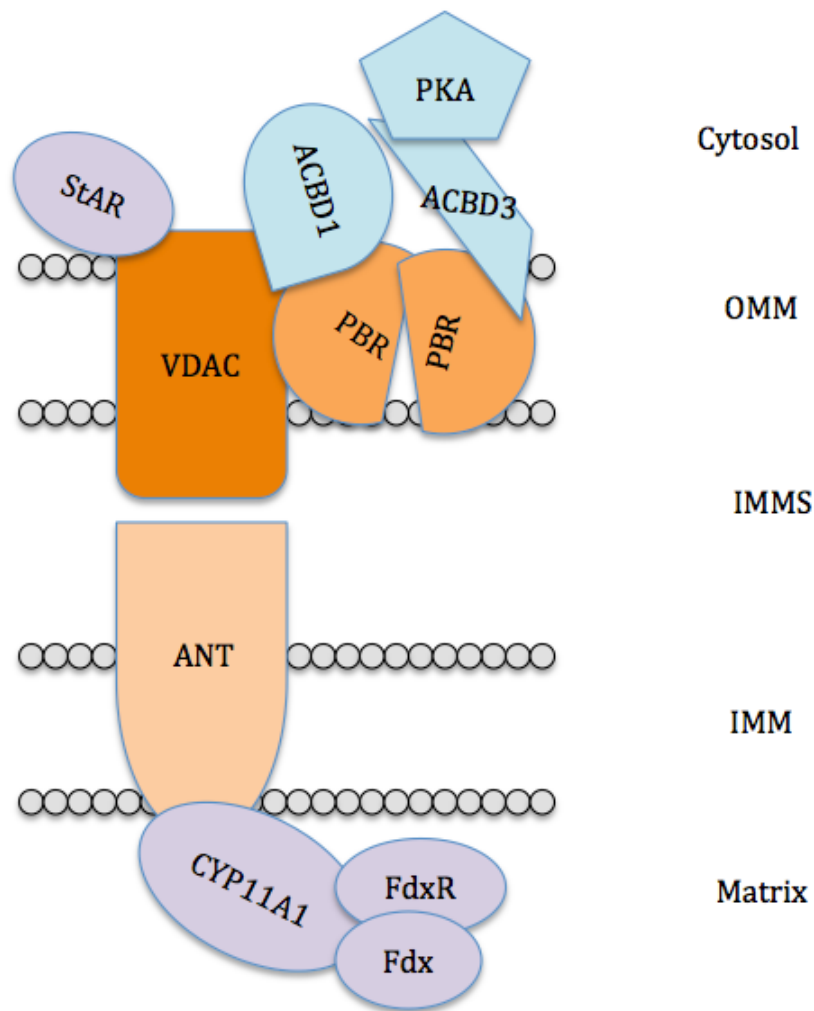


Figure 1.3. The protein ensemble involved in the translocation of cholesterol for steroidogenesis. The cytosolic proteins: Protein Kinase A (PKA), acyl-CoA binding domain 3 (ACBD3) and acyl-CoA binding domain 1 (ACBD1) are shown in blue; mitochondrial matrix proteins: steroidogenic acute regulatory protein (StAR), CYP11A1, ferrodoxin (Fdx) and ferrodoxin reductase (FdxR) are shown in purple; Outer mitochondrial membrane (OMM) proteins: voltage dependent anion channel (VDAC) and peripheral-type benzodiazepine receptor (PBR) and inner mitochondrial membrane (IMM) protein: adenine nucleotide translocator (ANT) are shown in orange. IMMS= inter-mitochondrial membrane space. Adapted from Midzak et al., 2011.

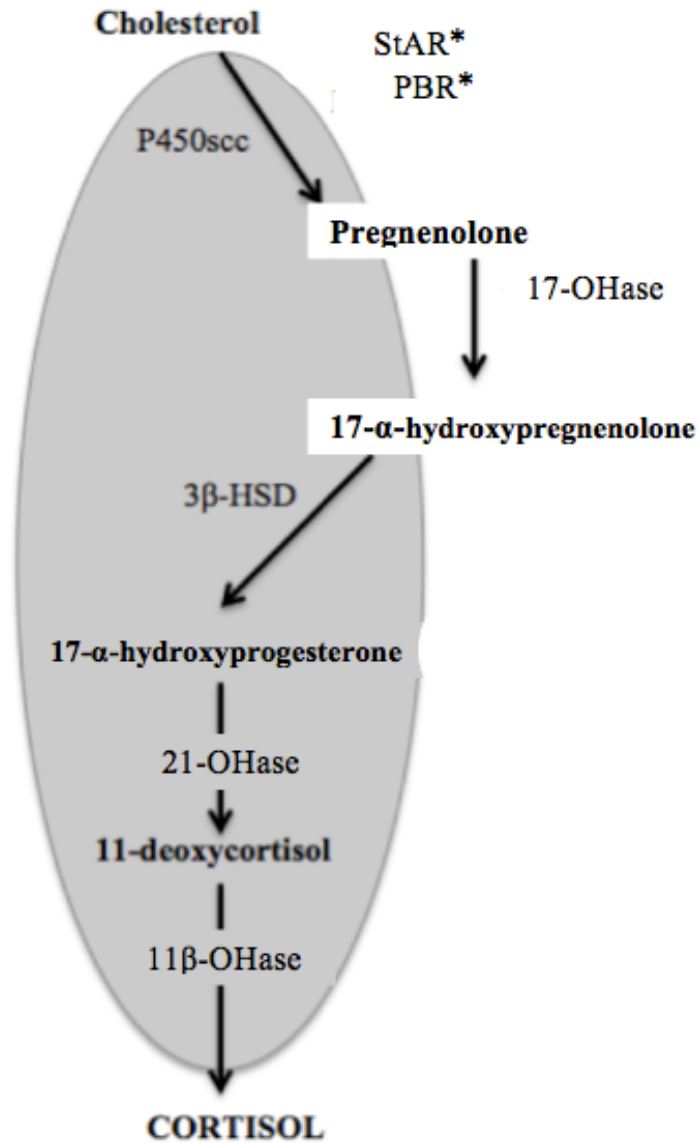


Figure 1.4. Steroidogenic Pathway of Cortisol Biosynthesis. The mitochondrial compartment is represented by the grey circle; other enzymatic reactions occur within the cytosol. StAR = steroidogenic acute regulatory protein; PBR = peripheral-type benzodiazepine receptor; P450scc = Cytochrome P450 side chain cleavage; 17 α -H = 17 α -hydroxylase ; 3 β -HSD = 3 β -hydroxysteroid dehydrogenase; 21-H = 21-hydroxylase; 11-B-H = 11 β -hydroxylase; * = involved in cholesterol transport to the inner mitochondrial membrane. Modified from Mommsen et al., 1999.

(Van der Boon et al., 1991), reduces gut motility and increases liver glycogen concentration (Wendelaar Bonga, 1997; Mommsen et al. 1999) (Fig. 1.2).

Prolonged elevation of glucocorticoids in fish may result in suppression of reproduction, decreased body weight and immunosuppression (Wendelaar Bonga, 1997; Weyts et al., 1999; Norris, 2000). Prolonged administration of glucocorticoids also results in deficits in learning and memory (Lupien and McEwen, 1997) as well as atrophy of neurons in the hippocampi of a number of animal models (Young, 2004).

Cortisol can exert its effects on virtually every physiological system in the majority of vertebrates (Kudielka et al., 2006), thus its biological effects will greatly influence the well-being of individual organisms and ultimately impact the reproductive status, growth and general health and fitness of entire animal populations (Norris, 2000).

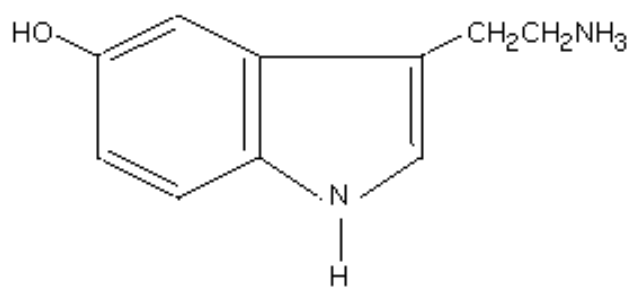
1.1.4 Role of Serotonin

Serotonin (Fig. 1.5) is a neurotransmitter that is involved in the modulation of mood, emotion, sleep and appetite (Schloss and Williams, 1998). Accordingly, the serotonergic system is implicated in the regulation of many behavioral and physiological functions (Schloss and Williams, 1998). Thirty years of research on stress and serotonin supports that numerous components of serotonergic systems are sensitive to stressors and that serotonin itself is an important component of the physiological network that allows adaptation to stress (Chaouloff et al., 1999).

Many studies demonstrate that stressors induce hyperactivity of brain serotonin metabolism in mammals, fish (Khan and Deschaux, 1997) and other species. Serotonin itself has been shown to mediate the release of the catecholamines E and NEpi from rainbow trout chromaffin cells (Fritsche et al., 1993). Male broiler chicks injected with concentrations of

corticotropin releasing factor (CRF) and serotonin had a serotonin-mediated decrease in the excitatory behaviors (i.e. vocalizations, frequency of spontaneous activity) induced by CRF injections (Zhang et al., 2004). Serotonin injections also stimulated corticosterone release (Zhang et al., 2004). Winberg et al. (1997) injected rainbow trout (*Oncorhynchus mykiss*) with different concentrations of 8- hydroxy-2(di-n-propylamino) tetralin (8-OH-DPAT), a known agonist of the serotonin receptor subtype 5-HT_{1A} , and reported a dose-dependent increase in trout plasma cortisol levels. Lepage et al. (2000) reported that exposing wild and domesticated sea trout (*Salmo trutta*) to a novel environment and/or a predator resulted in higher brain levels of serotonin (5-HT) and its major metabolite 5-hydroxyindoleacetic acid (5 HIAA). Heisler et al. (2007) determined that serotonergic activity was necessary for stress/HPA axis activation in mice. They also reported that approximately half of the CRH-containing neurons located in the paraventricular nucleus (PVN) of the hypothalamus, co-expressed serotonergic receptor mRNA (Heisler et al., 2007). Human studies reported that abnormal serotonergic activity resulted in abnormalities in the endocrine stress response leading to diseases such as depression (Jorgensen et al., 1998; Hinkelmann et al., 2009; Mitani et al., 2006; Maes et al., 1991; McEwen, 2000).

These findings support a role for the serotonergic system in the stress response; thus exposure to an SSRI that alters serotonin (5-HT) content in the synapse could potentially affect the stress axis and alter the ability of non-target organisms (i.e. fish) to respond to stressors.



Serotonin, 5-HT

Figure 1.5. Chemical structure of serotonin or 5-hydroxytryptophan (5-HT). Source: <https://www.msu.edu/user/espino11/chemical.htm>

1.2 Selective Serotonin Reuptake Inhibitors

1.2.1 *Physical and Chemical Properties*

The Selective Serotonin Reuptake Inhibitors (SSRIs) are a structurally heterogeneous group of drugs (Schreiber and Pick, 2006) designed to be selective for the brain serotonergic system so that deficits in serotonin or 5-HT concentrations, which are associated with the symptoms of depressive disorders, are remediated (CSM, 2004). Fluvoxamine (LuvoxTM), fluoxetine (ProzacTM), paroxetine (PaxilTM), sertraline (ZoloftTM), citalopram (CelexaTM) and escitalopram (LexaproTM) are the six compounds comprising the SSRI class (Schreiber and Pick, 2006; Hiemke and Härtter, 2000). The SSRIs differ in structure (Fig. 1.6), but are also differentiated by pharmacokinetic properties (Table 1.1) including: stereochemistry, protein binding, metabolism, inhibition of metabolic enzymes and drug-drug interactions (DeVane, 1999; Hiemke and Härtter, 2000). For example, fluoxetine (FLX) and citalopram are enantiomeric and promoted as racemic mixtures (DeVane, 1999), whereas fluvoxamine has no chiral centers and therefore, no optical isomers (DeVane, 1999; Perucca et al., 1994). The half-lives of the SSRIs are all different, ranging from 20 h (paroxetine) to over 4 days (FLX) and many have active metabolites with even longer half lives (CSM, 2004). Sertraline, citalopram (Hiemke and Härtter, 2000) and escitalopram (Søgaard et al., 2005) show linear pharmacokinetics meaning that the concentration of the drug present inside the body is proportional to the dose consumed and that the rate of elimination is proportional to the concentration (Makoid and Vuchetich, 1999). Fluoxetine, fluvoxamine, and paroxetine demonstrate non-linear pharmacokinetics (Hiemke and Härtter, 2000) meaning that the concentrations and rates of elimination of these drugs are not proportional to the dose consumed; the plasma drug concentrations at steady state are disproportionately higher than

those that would be predicted by single doses (DeVane, 1999) and the half lives of these compounds increase with multiple dosing (Bergstrom et al., 1988). The only shared neurobiological property among the SSRI class of antidepressants is the capacity to potentially block the 5-HT reuptake process (Vaswani et al., 2003; Blier and de Montigny, 1999). Despite their rational design (Hiemke and Härtter, 2000), it was demonstrated that some of the SSRIs are not as selective for serotonergic proteins as initially thought (Schreiber and Pick, 2006). Several SSRIs are found to have binding affinities for other monoamine transporters and proteins (CSM, 2004), which influence neurogenic processes like dopamine reuptake inhibition, muscarinic cholinergic antagonism, noradrenaline reuptake inhibition, nitric oxide synthase inhibition, etc. (Schreiber and Pick, 2006). Along with their physical and chemical differences, the binding affinities of the SSRIs for other, non-serotonergic transporter and receptor proteins, contribute to why some of these drugs have efficacy in treating anxiety disorders other than depression (Schreiber and Pick, 2006; DeVane, 1999) and why not all the SSRIs are approved for treating all the same disorders (Clineaero, 2011).

1.2.2 Mechanism of Action

SSRIs block the serotonin transporter (SERT) from taking-up serotonin released by pre-synaptic serotonergic neurons from synaptic junctions within the brain (Fig. 1.7) (Hemeryck and Belpaire, 2002). This action results in increased serotonin concentrations within the synapse and at the postsynaptic neuron receptor sites (Kwon and Armbrust, 2006). However, research indicates that the pharmacology of SSRIs is more complex, as the effects of these drugs are not limited simply to inhibiting the reuptake of serotonin (Schreiber and Pick, 2006; CSM, 2004). Changes in both the number of receptors and receptor function at the synapse can also result from SSRI treatment (Hiemke and Härtter, 2000).

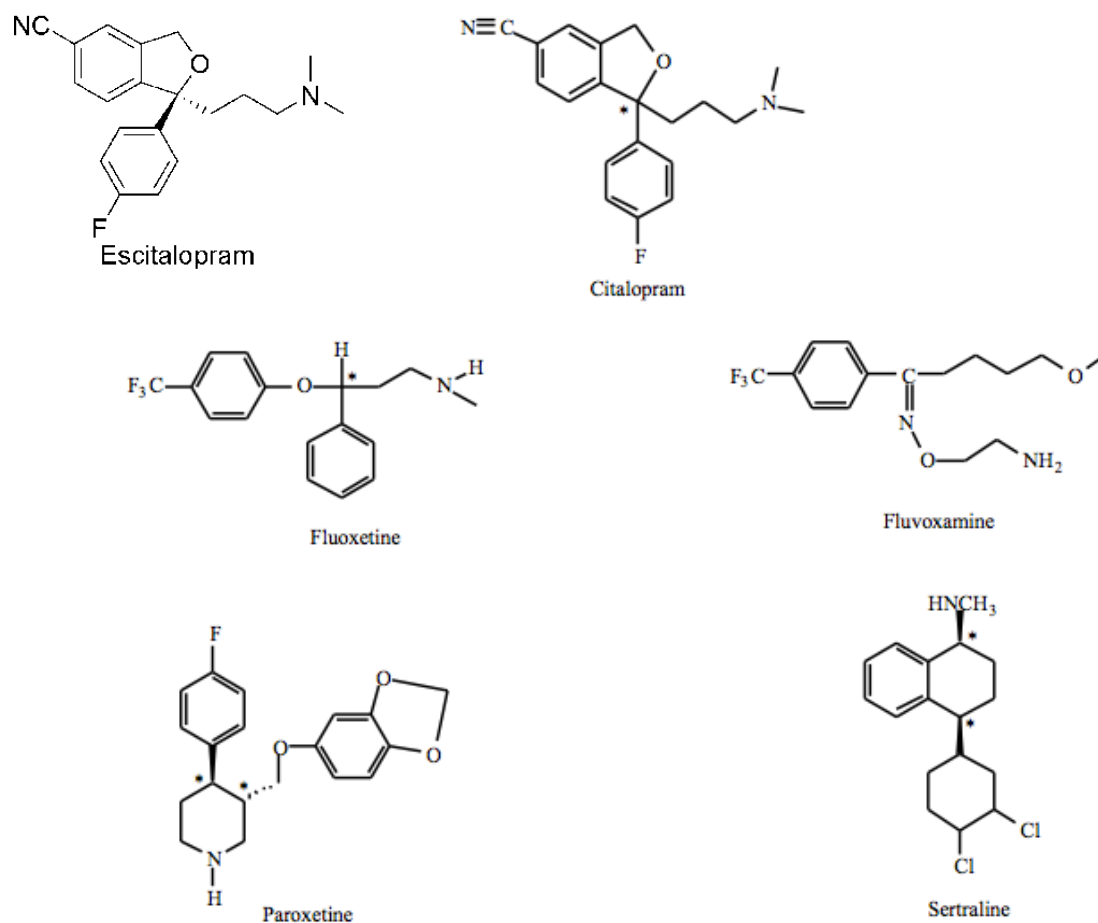


Figure 1.6. Chemical structures of the SSRIs. Structures of escitalopram (LexaproTM), citalopram (CelexaTM), fluoxetine (ProzacTM), fluvoxamine (LuvoxTM), paroxetine (PaxilTM) and sertraline (ZoloftTM) are shown. An asterisk (*) indicates a chiral center. Sources: Hemeryck and Belpaire, 2002 and Eildal et al., 2008.

Table 1.1. Pharmacological characteristics of five SSRIs and venlafaxine, an SSNRI. S=S enantiomer; R=R enantiomer; NF= Norfluoxetine; NA= Noradrenaline; 5HT= 5-hydroxytryptamine; COMT= Catechol-o-methyl transferase. Modified from CSM, 2004.

Drug	Fluoxetine	Fluvoxamine	Paroxetine	Sertraline	Citalopram	Venlafaxine
Affinity	S>R 1.5x; NF S>R 2.0x	Selective	Most potent Lower selectivity	Second most potent	Highly selective	NA and 5HT re-uptake blocker
Half-life	1-4 days	15-28 h	20 h	26 h	36 h	5 h
Active metabolites	Yes	No	No	Yes (5-10%)	Yes	Yes
Steady State	>4 weeks	10 days	7-14 days	5-7 days	6-10 days	3 days
Non-linear kinetics	Yes	Yes	Yes	No	No	No
Metabolizing enzymes	2D6, 2C9, 2C19, 3A4	2D6, 1A2	COMT, 2D6	3A4, 2D6	2D6, 2C19, 3A4	2D6, 3A3/4
Enzyme inhibition	2D6	1A2, 2C19	2D6	Minimal	Not relevant	Weak inhibitor 2D6
Receptor effects	5HT _{2A} , M, D ₂ , β	-	M	D, α_1	A ₁ , H ₁	-

Furthermore, it can take up to 3 weeks or more for the therapeutic effects of the SSRIs to have their full therapeutic effect in humans (Taylor et al., 2006), suggesting that the mechanism of action of SSRIs goes beyond simply inhibition of the SERT transporter (CSM, 2004). It is reported that at the start of treatment SSRIs induce a reduction in the firing activity of 5-HT neurons, due to an initial increase in the activity of the somatodendritic 5-HT_{1A} auto-receptors, which control the firing frequency of 5-HT neurons (Blier and de Montigny, 1999). This reduction in firing reduces the frequency of action potentials in the pre-synaptic neuron and thus 5-HT release and binding to postsynaptic sites, thereby preventing any augmentation of 5-HT transmission (Wilkinson et al. 1991). Two to three weeks later, the normal firing activity of the 5-HT neurons returns because the 5-HT_{1A} auto-receptors are desensitized by the continuous SSRI treatment and the antidepressant effects are initiated (Blier and de Montigny, 1999). The symptoms of depression are linked to numerous brain regions including the hippocampus, suprachiasmatic nucleus of the hypothalamus, amygdala, lateral geniculate body and the facial motor nucleus (Blier and de Montigny, 1999). Additionally, the SSRIs themselves are also effective in treating disorders where postsynaptic 5-HT_{1A} subtype receptors are not involved, such as panic disorder (Blier and de Montigny, 1999). To complicate matters even further is the suggestion that the therapeutic response to SSRIs may depend on human allelic variants in the SERT gene (Kim et al., 2000). These findings demonstrate that the SSRIs can have clinically significant secondary properties (and treatment effects) that can distinguish one drug in this class from another (Stahl, 1998). The multiple binding affinities of the SSRIs for multiple receptor proteins further emphasizes the complex pharmacology of these antidepressants and that their precise mode of action remains unclear (Schreiber and Pick, 2006; CSM, 2004).

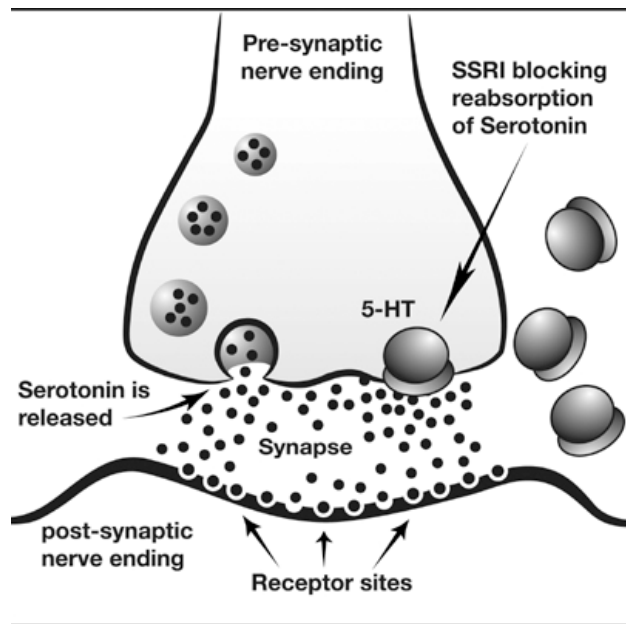


Figure 1.7. Mode of action of SSRIs at a serotonergic neuron synapse. Source: <http://withfriendship.com/images/g/34484/SSRI-picture.jpg>

1.2.3 Other Antidepressants Selective for Serotonin

A class of antidepressants known as the Selective Serotonin Norepinephrine Reuptake Inhibitors (SSNRIs) includes the compounds venlafaxine, duloxetine hydrochloride and milnacipran (Caccia, 1998) and is designed to inhibit the reuptake of serotonin as well as norepinephrine (Khetan and Collins, 2007). Along with the SSRIs, these SSNRI compounds are highly successful and popular in the treatment of depressive disorders. Venlafaxine (EffexorTM), the first SSNRI released onto the drug market, was the 10th largest selling pharmaceutical worldwide in both 2005 – generating \$3.8 billion dollars in revenue – and 2006 (Alonso et al., 2010; Khetan and Collins, 2007). In 2007, 17.2 million prescriptions of venlafaxine were dispensed in the U.S. making this SSNRI the sixth most commonly prescribed antidepressant on the U.S. retail market (Verispan VONA, 2007). In one study examining the concentrations of antidepressants in wastewater and a stream water system in the U.S., venlafaxine was the most commonly observed antidepressant compound (Schultz and Furlong, 2008). Although this SSNRI is found in a number of water systems, its presence was only recently reported and thus the amount of ecotoxicological data available on venlafaxine and the SSNRIs in general is relatively limited (Alonso et al., 2010). The rising number of venlafaxine prescriptions is likely due to the observation that venlafaxine has demonstrated a more robust and earlier onset of action in the treatment of depression compared to the SSRIs (Feighner, 1999); however, it has also been shown to have a 30-fold greater affinity for the 5-HT transporter versus the NEpi transporter, and therefore inhibits serotonin reuptake more than norepinephrine uptake (Gilmor et al., 2002; Bymaster et al., 2001; Caccia, 1998) further emphasizing serotonin's role in both the onset and successful treatment of depression and anxiety disorders.

1.2.4 Metabolism of the SSRIs

The SSRIs are lipid soluble, high clearance drugs subject to degradation by multiple metabolic pathways (Gardiner and Begg, 2006), but principally metabolized in the liver by a group of heme-containing proteins known as the cytochrome P450 enzymes (CYP450) (Gardiner and Begg, 2006; CSM, 2004; Hemeryck and Belpaire, 2002; Hiemke and Härtter, 2000; DeVane, 1999). These enzymes are mainly present in hepatocytes but also the small intestine, lungs, kidneys, and brain (Gardiner and Begg, 2006), and are largely responsible for the oxidation of many endogenous substrates including drugs and contaminants (Hemeryck and Belpaire, 2002). If two or more P450 enzymes share an amino acid sequence identity greater than 40%, they are classified into the same CYP family (e.g. CYP1, CYP2, etc.) and further classified into the same subfamily if their sequence identity is greater than 55% (e.g. CYP2D) (Hemeryck and Belpaire, 2002). Some of the CYP isoforms involved in the breakdown of SSRIs and related antidepressants include CYP1A2, 2C9, 2C19, 2D6 and 3A4 (Table 1.1) (CSM, 2004). Although each CYP isoform can metabolize many different drug substrates and multiple P450 enzymes can metabolize the same drug, most substrates have a high affinity for one particular P450 isoform (Hemeryck and Belpaire, 2002). The enzyme affinities of the SSRIs are important to consider because when SSRI compounds are metabolized, they often produce metabolites that retain the capacity to block serotonin reuptake and/or are capable of inhibiting the metabolic functions of P450 enzymes (DeVane, 1999). SSRI substrates will compete with other drug substrates for catalytic sites on the same CYP isoform, which can result in the inhibition of the metabolism of these other drugs (Hemeryck and Belpaire, 2002). The level of this inhibition is dependent on both drug dose and the pre-existing metabolic status of the patient (Lam et al., 2002), but competing drug

substrates may still influence the degree of drug-drug interactions (DeVane, 1999).

Following hepatic metabolism, most SSRIs are excreted to some extent in the urine, however certain SSRIs such as sertraline are significantly eliminated (i.e. ~50%) in the feces (Hiemke and Härtter, 2000). All SSRIs are extensively metabolized prior to excretion from the body as less than 12% of any SSRI is found to be excreted in urine as the parent compound (DeVane, 1999).

1.2.5 Uses and Popularity of SSRIs

SSRIs first arrived on the drug market in the late 1980s with the launch of ProzacTM and since then, they have become one of the most frequently prescribed classes of drugs worldwide (Currie, 2005). From 1990-1998, the number of antidepressant prescriptions dispensed from community pharmacies in Australia rose from 5.1 million to 8.2 million (McManus et al., 2000). This increase consisted of a rapid market uptake of the SSRIs and a 25% decrease in prescriptions of the tricyclic antidepressants (TCAs) (McManus et al., 2000). The estimated number of SSRI prescriptions dispensed in Canada increased by 80% to >15.6 M prescriptions between 1999 and 2003 (Fig. 1.8) (IMS Health, 2003). Up until 2006, there was a 320% increase in the usage of SSRIs in Norway and it is estimated that approximately 4% of the Norwegian population is currently being treated with at least one SSRI (Vasskog et al., 2006). In 2007, the SSRIs escitalopram, paroxetine and sertraline were all among the 200 most widely dispensed pharmaceuticals in the United States (Schultz et al., 2010). Increases in the prescribing of SSRIs ranging from 50% to 400% have also been reported in the United Kingdom, Italy and other European countries since the 1990s (Ministry of Health, 2007). Compared with other types of antidepressants (i.e. TCAs, monoamine oxidase inhibitors, etc.), the SSRIs are found to be of comparative efficiency

(Anderson, 2000) but better tolerated by suffers of anxiety and depression (Anderson, 2000; Vasskog et al., 2006). This has resulted in significantly fewer SSRI-treatment discontinuations due to adverse side effects (Anderson, 2000), as observed with the other antidepressant classes (Gumnick and Nemeroff, 2000). SSRIs have gradually squeezed out older-type antidepressants, like the TCAs, so that they now comprise 81% of the depression drug market (IMS Health, 2003).

The use, popularity and effects of these drugs have influenced people's language, culture and even some views on health and disease (Currie, 2005). The influence of SSRIs has become so wide spread in fact that some human populations are described as living in the "Age of Prozac" (Currie, 2005). The ease of their use (Hotopf et al., 1996), advantageous safety profile and success in treating major depression has resulted in SSRIs being the first-line of therapy in the treatment of minor cases of depression and other psychiatric disorders thought to be associated with abnormalities of the serotonin system (Hiemke and Härtter, 2000; Schreiber and Pick, 2006). Furthermore, SSRIs are emerging as possible treatments for other ailments ranging from mood disorders to menopause (Pflumm, 2011). For example, escitalopram could one day be an alternative to hormone replacement therapy in menopausal women (Pflumm, 2011). During a 2-month trial, 55% of the women taking escitalopram experienced half the number of hot flashes as they did before treatment. When administered to mice with Huntington's disease, paroxetine suppressed the neurodegenerative effects of the disease and increased overall survival of the affected mice (Duan et al., 2004). SSRIs are also showing promise in treating neuro-developmental disorders that cause repetitive behaviors, from autism to intellectual disability (Pflumm, 2011). With the scope of their treatment expanding the number of SSRI prescriptions will likely increase, leading to the

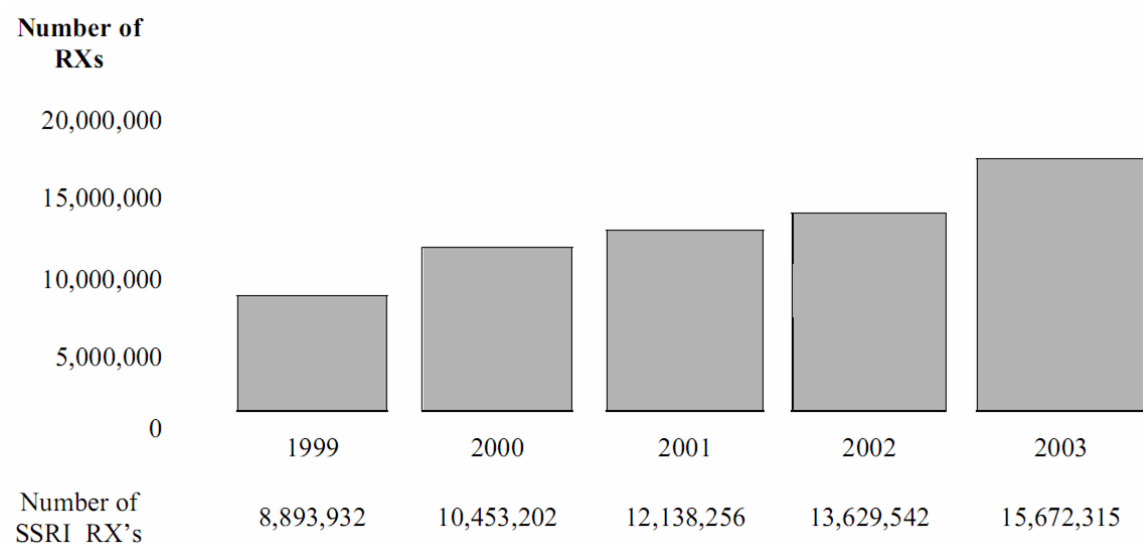


Figure 1.8. Estimated number of SSRI prescriptions dispensed in Canada (1999-2003). Modified from IMS Health, 2003.

potential for increased accumulation of these compounds and their metabolites in aquatic environments.

This further accumulation will potentially lead to greater accumulation and endocrine disruption in aquatic organisms, which will eventually lead to harmful and possibly fatal toxicity in these organisms.

1.2.6 Sources and Pathways in the Environment

Human pharmaceuticals including the SSRIs enter aquatic environments from sewage treatment plants (Kwon and Armbrust, 2006). SSRIs enter these treatment plants primarily through human excretion and from flushing unconsumed pills down the toilet (Schultz et al., 2010; Ruhoy and Daughton, 2008). Other sources include waste from veterinary therapeutics and effluent discharges from industrial plants, hospitals, etc. (Ruhoy and Daughton, 2008; Brooks et al., 2005). Current sewage treatment technologies are not equipped to remove SSRI-type pharmaceuticals from wastewaters (Alonso et al., 2010). Consequently, these compounds can remain in sewage-treatment plant effluents and enter into aquatic systems including surface and ground waters (Demeestere et al., 2010; Kreke and Dietrich, 2008). Total SSRI concentrations in effluent watersheds are reported to range from 840 ng/L to 3.2 µg/L (Metcalf et al., 2010). Over time, SSRIs degrade by processes such as photolysis and bacterial degradation (Kwon and Armbrust, 2006; Redshaw et al., 2008). However, the stability of these compounds and their relative resistance to breakdown (Kwon and Armbrust, 2006) results in these compounds persisting in the environment for extended periods of time. It was reported that SSRIs bioconcentrate in the tissues of fish (Smith et al., 2010; Brooks et al., 2005; Mennigen et al., 2010; Schultz et al., 2010) and are rapidly adsorbed by and persist in sediments (Kreke and Dietrich, 2008; Kwon and Armbrust, 2006). Furthermore, their

growing use in human therapeutics means that substances like SSRIs are continuously being discharged into receiving waters and aquatic species are being chronically exposed to them even at low concentrations (Kreke and Dietrich, 2008).

Christensen et al. (2007) reported on experiments using a combination of five SSRIs in varying amounts. Results indicated that SSRIs showed a “concentration addition” meaning that the degree of toxicity exhibited by one SSRI at a particular concentration is additive to the toxicities generated by the other SSRIs. Therefore, the effects of SSRIs on aquatic organisms will be more detrimental if the concentration of total SSRIs, and possibly other pharmaceutical compounds, present in the environment is high or increases over time (Christensen et al., 2007).

1.3 Fluoxetine Hydrochloride

1.3.1 Physical and Chemical Properties

FLX has a phenoxyphenylpropanamine chemical structure (see Fig. 1.6) that contributes to its inhibition of monoamine uptake. However, it is the trifluoromethyl substituent that confers the majority of FLX’s potency and selectivity as an SSRI (Fuller et al., 1991). FLX is a racemic mixture of two enantiomers (Hiemke and Härtter, 2000) with the S-enantiomer being ~1.5-times more potent in inhibiting serotonin reuptake than the R-enantiomer (Gram, 1994). FLX is a lipophilic drug with the highest volume of distribution (14-100 L/kg) of any SSRI (Catterson and Preskorn, 1996) indicating its ability to accumulate extensively in tissues (Hiemke and Härtter, 2000). It is also reported to bind highly (>94%) to serum proteins including albumin and alpha-1-acid glycoprotein (Holladay et al., 1997).

Following oral administration, FLX is nearly completely absorbed across the intestine and is carried through the portal circulation to the liver where it is metabolized extensively by CYP450 enzymes (Gardiner and Begg, 2006) (Fig. 1.9). FLX's main metabolite is demethylated norfluoxetine (norFLX), which has comparable pharmacological activity to FLX, but a longer half-life (Mandrioli et al., 2006). The CYP2D6 enzyme produces primarily S-norFLX, with other P450 isoforms (i.e. CYP3A4) contributing to S-FLX metabolism when CYP2D6 becomes saturated (Gardiner and Begg, 2006). R-FLX is metabolized by both CYP2D6 and CYP2C9 (Margolis et al., 2000), however inhibition of CYP2D6 metabolism has been shown to occur when FLX use is chronic (Amchin et al., 2001; Gardiner and Begg, 2006). The half-life of FLX in humans ranges from 1-4 days (Benfield et al., 1986; Gram, 1994; Kreke and Dietrich, 2008) while that of norFLX ranges from 7-15 days (Benfield et al., 1986; Gram, 1994; Hiemke and Härtter, 2000). This would imply that 6 to 8 weeks of FLX treatment would be needed in order for FLX to reach steady-state levels (Hemeryck and Belpaire, 2002). However, when administered in high doses or with multiple dosing, FLX demonstrates non-linear pharmacokinetics (Hiemke and Härtter, 2000) and under steady-state conditions, the concentrations of racemic norFLX normally exceed those of racemic FLX (Hemeryck and Belpaire, 2002). The half-lives of these compounds further predict that chronic dosing of FLX will result in plasma accumulation of FLX and norFLX that will persist for more than 1 month (DeVane, 1999). Along with their extended half-lives, the ability of these SSRIs to inhibit more than one P450 isoform makes it more likely for drug-drug interactions to occur even after FLX-treatment has ended (CSM, 2004). Nevertheless, the FLX dose and metabolic status of the individual will ultimately determine both the accumulation of FLX and norFLX and the extent of drug-drug interaction (Lam et

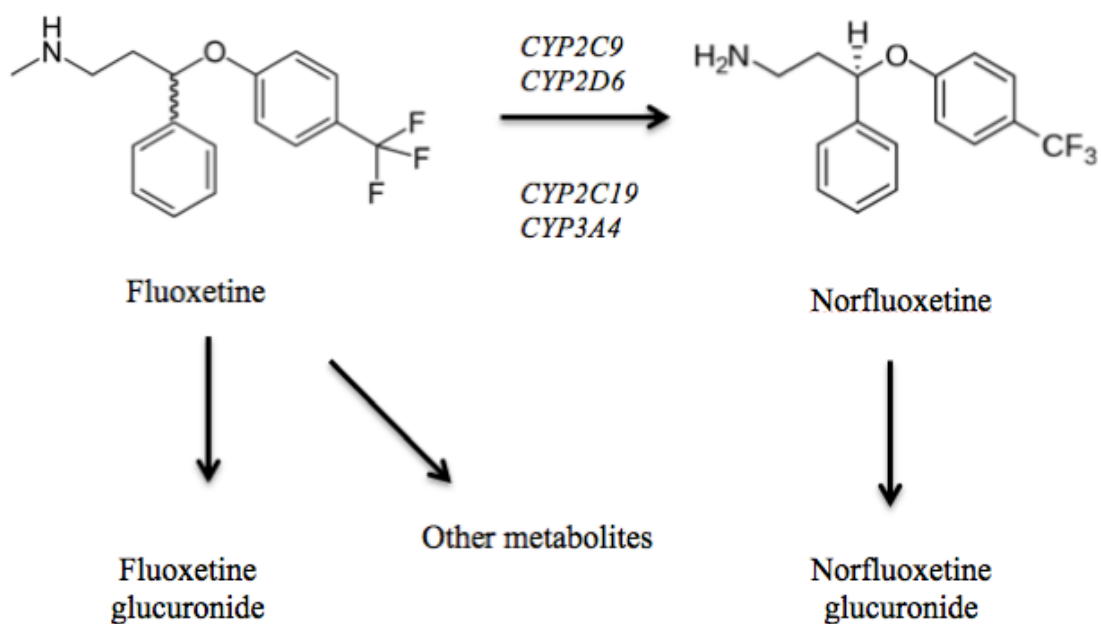


Figure 1.9. Phase I metabolism of fluoxetine hydrochloride. Modified from Hiemke and Härtter, 2000.

al., 2002). Despite its lipophilicity and high volume of distribution, FLX is still partially excreted from the human body as the parent compound, FLX hydrochloride (~11%), its active metabolite norFLX (7%) and other metabolites (7-8%) (Benfield et al., 1986; DeVane, 1999).

1.3.2 Uses and Environmental Presence

Fluoxetine hydrochloride (FLX, ProzacTM) was first approved for use in 1986 making it the first SSRI available for prescription (Henry and Black, 2008). Since then, ProzacTM has been prescribed to more than 54 million people worldwide (Eli Lilly, 2005), making it the most frequently prescribed antidepressant in history (Ecks and Basu, 2009). The U.S. Food and Drug Administration (FDA) has approved the use of FLX in the treatment of major depressive disorder, obsessive compulsive disorder (OCD), panic disorder, anxiety and other mood disorders (FDA, 2010; Eli Lilly, 2011). FLX is also the only drug of its class currently approved for treating depression in children and adolescents (Hetrick et al., 2010). Studies examining the use of FLX in the treatment of non-anxiety disorders have shown promising results, including treatment of patients suffering from bulimia and binge eating disorders (Pflumm, 2011) and today, FLX is FDA approved for the treatment of bulimia nervosa (Eli Lilly, 2011). More recently, a group of researchers tested the effectiveness of FLX in treating recovering stroke patients. The double-blind placebo-controlled trial involved 118 ischaemic stroke sufferers, half of which were given 20 mg FLX per day, in addition to regular stroke recovery therapy (Chollett et al., 2011). The researchers reported that the patients treated with FLX (starting 5-10 days after stroke onset) for three months had greater recovery of motor skills than the patients who had received a placebo (Chollett et al., 2011). FLX is partially excreted from the human body and often, as with its SSRI counterparts, improperly

disposed of, thus its presence is reported in aquatic systems and environments worldwide with concentrations ranging from 12-540 ng/L (Brooks et al., 2003; Kreke and Dietrich, 2008). FLX is subjected to bacterial degradation over time (Redshaw et al., 2008), however one study that looked at the stability of FLX in aquatic environments found that FLX is relatively resistant to hydrolysis, photolysis and microbial degradation and also quickly adsorbed by sediments where it shows persistence (Kwon and Armbrust, 2006). Of greater concern is the finding that FLX accumulates in the tissues of aquatic organisms. A study by Brooks et al. (2005) sampled populations of bluegill (*Lepomis macrochirus*), channel catfish (*Ictalurus punctatus*), common carp (*Cyprinus carpio*), and black crappie (*Pomoxis nigromaculatus*) from both a pharmaceutical and personal care product (PPCP)-dominated and a control stream in north Texas. They examined brain, liver and muscle tissues of these fish and found that concentrations of FLX and norFLX in the tissues from fish originating from the PPCP-dominated stream exceeded 0.1 ng/g (Brooks et al., 2005). Schultz et al. (2010) sampled SSRI concentrations in two different effluent-impacted streams in the U.S. They also took tissue samples from populations of white suckers (*Catostomus commersoni*) in the stream and found concentrations (ng/g) of FLX and its metabolites in both the streambed sediments and fish brain tissues (Schultz et al., 2010).

1.3.3 Toxicity in Vertebrates

The majority of published studies examining the toxicological effects of SSRIs in vertebrates have studied FLX hydrochloride (Prozac™). Environmental concentrations of FLX are reported to be low (ng/L), however its high potency, selectivity (Fuller et al., 1991) and lipophilicity (Catterson and Preskorn, 1996) makes it likely that non-target organisms will experience exposure effects even at sublethal levels. As such, the scientific literature

reports numerous studies that examined the effects of FLX exposures on non-human vertebrates. In recent years, these studies have shown that FLX is capable of altering the physical, behavioral, endocrine and physiological processes of both aquatic and terrestrial vertebrates.

1.3.3.1 Physical/Behavioral Effects

Physical parameters and/or behaviors that can be affected by FLX exposure include feeding, mobility, growth and the overall activity levels of an animal. Page et al. (1999) reported that male Sprague-Dawley rats given FLX (10 mg/kg) had increased mobility and swimming behaviors (versus non-dosed rats) during a forced swimming test (FST). Lynn et al. (2007) exposed male betta fish (*Betta splendens*) to 3 µg/mL FLX for 3 h and then placed a mirror in each experimental tank in order to project a reflection and thereby induce an aggressive response from the betta fish. They compared the intensity and frequency of aggressive behaviors (i.e. opercula flaring, broadside displays, etc.) in bettas exposed and unexposed to FLX (Lynn et al., 2007). Aggressive behaviors significantly declined in the FLX-exposed fish (versus the amount observed in fish under control conditions). Stanley et al. (2007) also investigated the behavioral responses of fathead minnows (*Pimephales promelas*) exposed to sublethal levels of S-FLX and R-FLX. The S-FLX enantiomer had a lowest observed effect concentration (LOEC) of 101 µg/L for minnow survival, while the R-FLX enantiomer had an LOEC of 170 µg/L. S-FLX significantly reduced growth at 51 µg/L and feeding rate was reduced in a dose-dependent manner for all FLX enantiomer treatments (Stanley et al., 2007).

Beulig and Fowler (2008) examined the effects of FLX on active avoidance learning

in goldfish (*Carassius auratus*). A comparison of the escape and avoidance to shock response was made between fish receiving either chronic administration of FLX, a 5 HT_{1A} receptor antagonist or control treatments. The FLX-exposed group performed the lowest number of “avoids”, while the receptor blocked group performed the most (Beulig and Fowler, 2008). Furthermore, the FLX-treated fish were observed to be the least active in their holding tank, which suggested that active avoidance learning may be stimulated by the 5 HT_{1A} receptor antagonist and consequently blunted by FLX exposure. One additional study exposed hybrid striped bass (*Morone saxatilis* x *M. chrysops*) to FLX for 6 days, allowed a 6-day recovery period and then assessed the ability of the bass to capture prey as an index of performance (Gaworecki and Klaine, 2008). The exposed bass demonstrated a FLX concentration- and duration-dependent decrease in their ability to capture prey. Furthermore, an increase in the time for the bass to capture prey correlated with decreased brain serotonin activity suggesting FLX in the water modified brain serotonin content.

These studies demonstrate the wide range of physical and/or behavioral parameters that can be modified by FLX exposure in both mammalian and aquatic vertebrates. FLX exposure results in reduced growth, feeding and aggressive or predatory behaviors. It can also impact survival, learning capacity, mobility and overall activity level, potentially through its endocrine effects on synaptic 5-HT concentrations.

1.3.3.2 Endocrine and Physiological Effects

FLX acts by inhibiting the serotonergic neuronal uptake pump, SERT (Vaswani, 2003), and potentially has binding affinities for other 5-HT receptor proteins within the central nervous system (Schreiber and Pick, 2006). As such, physiological (and endocrine)

processes including hormone production and gene expression could be impacted by FLX exposure, as demonstrated in a number of studies.

Damjanoska et al. (2003) used rats to examine the effects of FLX exposure on the hypothalamic serotonergic receptor 5-HT_{2A}. Rats were injected with FLX daily (10 mg/kg/day IP.) for 2, 3, 7, 21 or 42 days. FLX treatments of 21 or 42 days reduced the ACTH response to injections of a 5-HT_{2A} agonist, 2,5-dimethoxy-4-iodoamphetamine (DOI) (Damjanoska et al., 2003). After 21 days of FLX treatment, ACTH and corticosterone responses were found to potentiate at 30 min post-DOI injection. In addition, chronic FLX treatments were found to potentiate 5-HT_{2A} receptor signaling in the frontal cortex of rats, but differentially altered the signaling of 5-HT_{2A} receptors found in CRF neurons of the paraventricular nucleus (Damjanoska et al., 2003). Semsar et al. (2004) experimentally enhanced serotonergic neurotransmission using FLX in the bluehead wrasse (*Thalassoma bifasciatum*). The mRNA expression of arginine vasotocin (AVT) – a hormone with corticotropic actions (Wendelaar Bonga, 1997) – was measured and found to be 2-fold lower in the FLX-treated males compared to saline-treated controls in all regions of the preoptic area of the hypothalamus (Semsar et al., 2004).

Mennigen et al. (2008) examined the effects of FLX on neuroendocrine function and the reproductive axis in female goldfish. Goldfish received intraperitoneal (IP) injections of FLX (5 µg FLX/g body weight) twice weekly for 14 days. FLX-treated goldfish significantly reduced plasma estradiol levels by approximately 3-fold (Mennigen et al., 2008). Expression of estrogen receptor (ER) β₁ mRNA was also significantly reduced by 4-fold and 1.7-fold in the hypothalamus and telencephalon, respectively. In addition, qRT-PCR confirmed that isotocin – a neuropeptide that stimulates reproductive behavior in fish – mRNA was down

regulated 6- and 5-fold in the hypothalamus and telencephalon, respectively (Mennigen et al., 2008). These results demonstrated that FLX can potentially affect sex hormones and alter the genes involved in the reproductive processes of female goldfish. A further study by Mennigen et al. (2009) examined food intake and weight gain in sexually regressed female goldfish IP-injected with FLX. After 13 days of FLX injections, a significant decrease in both food intake and weight gain resulted. In the hypothalamus, both CRF1 and NPY mRNAs were up regulated 2.3-fold while CART1 mRNA was up-regulated 3.2-fold and NPY mRNA down-regulated 2.3-fold in the telencephalon (Mennigen et al., 2009). These results indicated that altering serotonergic tone by FLX treatment reduced food intake and weight gain and increased the expression of potent inhibitory feeding neuropeptides (Mennigen et al., 2009). Mennigen et al. (2010) also examined the effects of waterborne FLX on the reproductive axis of sexually mature male goldfish. Male goldfish were exposed to FLX at 0.54 µg/L and 54 µg/L for 14 days prior to receiving a vehicle or sex pheromone stimulus consisting of either 4.3 nM 17,20β-dihydroxy-4-pregnene-3-one (17,20P) or 3 nM prostaglandin F_{2α} (PGF_{2α}) (Mennigen et al., 2010). Basal milt volume significantly decreased in fish exposed to 54 µg/L FLX while pheromone-stimulated milt volume decreased at the 0.54 µg/L and 54 µg/L FLX exposures (Mennigen et al., 2010). Fluoxetine (54 µg/L) was also found to inhibit both basal and pheromone-stimulated testosterone levels (Mennigen et al., 2010).

Morando et al. (2009) inserted FLX implants (50 µg/g) in gulf toadfish (*Opsanus beta*) and found cortisol levels increased 4-fold after 24 h. Lister et al. (2009) examined the impact of nominal concentrations of FLX (0.32, 3.2, 32 µg/L) on reproduction in sexually mature female zebrafish (*Danio rerio*) over a 7-day period. Compared with the control

groups, FLX at 32 µg/L significantly reduced the average number of eggs spawned by approximately 4.5-fold. Quantitative real time PCR (qRT-PCR) analysis showed that ovarian aromatase, follicle stimulating hormone receptor and luteinizing hormone receptor gene expression were significantly reduced by FLX (Lister et al., 2009). These findings suggest that disruptions to the synthesis of ovarian steroids and the actions of gonadotropins may underlie the negative influence of FLX on ovarian estradiol and spawning levels (Lister et al., 2009).

The results of these studies demonstrate that FLX has the potential to modify gene expression in the brains of non-target organisms. These modifications can impact the production of hormones involved in reproduction, feeding and other endocrine systems, thereby impacting whole animal physiology.

1.4 Hypothesis and Research Objectives

The endocrine stress axis is a fundamental regulatory system that allows vertebrates to adjust to dynamic environments and effectively handle stress (Stolte et al., 2008). Many previous studies have demonstrated the ability of pharmaceuticals, including SSRIs, to disrupt endocrine processes in non-target aquatic organisms (Daughton and Ternes, 1999; Fent et al., 2006). However, few of these studies have focused on how the endocrine stress response of these organisms is affected by SSRI exposure. Therefore, the overall purpose of my research is to establish whether exposure to an SSRI, i.e. FLX, affects the endocrine stress response in a model teleost fish species, the rainbow trout *Oncorhynchus mykiss*. Rainbow trout are a practical model for toxicology research because they demonstrate sensitivity to a variety of chemical substances, and more is known about their physiology and biology than any other fish species (Thorgaard et al., 2002). Furthermore, their relatively

large size allows for large amounts of specific tissues and cell types to be isolated and analyzed (Thorgaard et al., 2002), which will largely benefit my research objectives.

1.4.1 Hypothesis

Based on the finding that serotonin concentration influences the stress axis in vertebrates, I hypothesize that exposure to FLX, an SSRI, will increase the activity of the HPI axis in teleost fish. I further predict that: 1) *in vitro* cortisol production will be higher in cells exposed to FLX and norFLX; and, 2) the steroidogenic pathway will be up-regulated in rainbow trout exposed to waterborne FLX.

1.4.2 Research Objectives

My first research objective is to determine the effects of FLX exposure on *in vitro* cortisol production using the interrenal cells of stressed and non-stressed rainbow trout. This will be accomplished by isolating and exposing trout head kidney cells to different concentrations of FLX and its active metabolite, norFLX, and examining ACTH-induced cortisol production and cell viability *in vitro*. A second research objective will determine how FLX alters the synthesis and intermediates involved in the steroidogenic pathway for cortisol production in rainbow trout. The effects of FLX exposure on this pathway will be assessed by exposing trout to in-tank water concentrations of FLX, evaluating plasma cortisol concentrations of FLX-exposed fish, isolating trout head kidney microsomes and evaluating the formation of pathway intermediates using a radioactive tracer.

The outcomes of this research will help elucidate how an SSRI-type pharmaceutical can impact the endocrine stress axis of aquatic vertebrates and contribute to expanding the literature available on this topic, ultimately establishing whether this particular drug may impact the fitness of a fish species in a SSRI-contaminated river.

Chapter 2

***In vitro* Cortisol Production of Rainbow Trout Head Kidney Cells**

Exposed to Fluoxetine and Norfluoxetine

2.0 Introduction

Cortisol is the main corticosteroid in teleost fish (Ings et al., 2011) and is produced on-demand from interrenal cells located in the head kidney (anterior to the kidney) (Fig. 2.1) (Hontela, 1998). Physical, chemical or psychological stressors (Dedovic *et al.*, 2009), trigger the activation of the hypothalamo-pituitary-interrenal (HPI) axis, resulting in the increased production and release of cortisol (Brodeur et al., 1997; Bender *et al.*, 2008). When released, cortisol targets the gills, intestine and liver (Gregory and Wood, 1999) exerting effects on intermediary metabolism (Van Der Boon et al., 1991; Laflamme et al., 2000), the immune system (Laflamme et al., 2000) and overall energy distribution (Wendelaar Bonga, 1997; Flik et al., 2006), assisting the fish to overcome the applied disturbance (Flik et al., 2006).

Cortisol is also involved in other physiological processes including reproduction (Laflamme et al., 2000; Bender et al., 2008), feeding (Bernier and Peter, 2001; Pankhurst et al., 2008) and osmoregulation (Wendelaar Bonga, 1997; Gregory and Wood, 1999; Gilmour, 2005), thus functioning as both a glucocorticoid and mineralocorticoid hormone in most teleosts (Norris, 2000; Stolte et al., 2008). The mineralocorticoid aldosterone is found in fish but at such low concentrations that it is considered to be physiologically insignificant (McCormick and Bradshaw, 2006; Wendelaar Bonga, 1997). Consequently, if cortisol secretion is compromised in teleosts, multiple physiological processes may be impacted (Gregory and Wood, 1999; Weyts et al., 1997; Weyts et al., 1999; Gilmour, 2005; Bender et al., 2008; Laflamme et al., 2000).

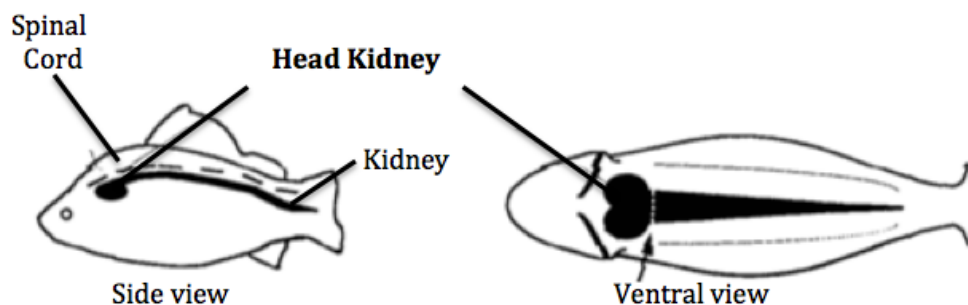


Figure 2.1. Anatomical position of the teleost head kidney. Modified from Brodeur et al., 1997.

Fish encounter numerous stressors in their aquatic environment on a daily basis. These stressors can range from predator avoidance to water pollution resulting from human activities (Barton, 2002). Human pharmaceuticals, personal care products (PCPs) and other anthropogenic chemicals have been detected in water supplies and wastewater effluents around the world (Westerhoff et al., 2005), and some of these compounds have demonstrated a potential to disrupt cortisol production in freshwater teleosts (Leblond et al., 2001; Ings et al., 2011). Exposure to heavy metals, pesticides and other organic contaminants at environmentally relevant concentrations has resulted in decreased ACTH-stimulated cortisol production (*in vitro*) and reduced cell viability in teleost head kidney tissues (Leblond et al., 2001; Brodeur et al., 1997). Studies have also shown that the stressor-mediated plasma cortisol response in teleosts is impaired by pharmaceuticals such as non-steroidal anti-inflammatory drugs (NSAIDs) (Gravel and Vijayan, 2007) and medications like mitotane (o, p'-DDD) (Ilan and Yaron, 1983).

Fluoxetine hydrochloride (FLX), the active ingredient of ProzacTM, is a selective serotonin reuptake inhibitor (SSRI) type antidepressant that targets the central nervous system (CSM, 2004), selectively blocking the reuptake of serotonin into presynaptic neurons (Peretti

et al., 2000). After oral consumption and CYP-mediated metabolism, FLX is partially excreted from the human body as both FLX (~10%) and metabolites that include the active norfluoxetine norFLX (Benfield *et al.*, 1986). When excreted or improperly disposed of, FLX moves into aquatic environments via sewage treatment effluents (Mennigen *et al.*, 2010; Kwon and Armbrust, 2006). Both FLX and norFLX have been shown to deposit in fish tissues (Paterson and Metcalfe, 2008; Brooks *et al.*, 2005) and research concludes that FLX is an endocrine disrupter in fish. Several studies have demonstrated that FLX – at environmentally relevant concentrations – modifies the gene expression and production of estrogens and other reproductive hormones, thereby impacting reproductive processes in several teleost species (Schultz *et al.*, 2011; Mennigen *et al.*, 2010; Mennigen *et al.*, 2008; Foran *et al.*, 2004). Other studies demonstrate that FLX alters feeding and induces anxiolytic effects in fish (Mennigen *et al.*, 2009; Egan *et al.*, 2009). While the number of studies reporting on FLX's endocrine-disrupting abilities is large, little research has focused on whether direct exposure to FLX or another SSRIs can alter cortisol production in fish. In the zebrafish (*Danio rerio*) study by Egan *et al.* (2009), whole-body cortisol concentrations decreased in response to FLX exposure, but this observation was not investigated further.

The primary objective of this study was to determine the effects of FLX exposure on *in vitro* cortisol production in rainbow trout head kidney cells to test the hypothesis that FLX and norFLX exposure alters cortisol production, thereby potentially altering the stress response and impacting other physiological processes in teleost fish. A second objective was to assess head kidney cell viability following *in vitro* exposure to FLX and norFLX to test the hypothesis that initially exposing rainbow trout to waterborne FLX will affect *in vitro* cortisol production and cell viability.

2.1 Materials and Methods

2.1.1 Chemicals

Minimum essential media (MEM), adrenocorticotrophic hormone (ACTH) and NADH were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fluoxetine hydrochloride (FLX) was purchased from Sigma-Aldrich (St. Louis, MO, USA) and norfluoxetine hydrochloride (norFLX) from TLC PharmaChem Inc. (Vaughan, ON). Collagenase-dispase was purchased from Roche Canada (Mississauga, ON), imidazole from BDH Laboratory Supplies (VWR, Mississauga, ON) and pyruvate from Boehringer (Ingelheim, Germany). Cortisol assay kits were purchased from MP Biomedicals, LLC (Solon, OH, USA).

2.1.2 Fish and Tank Maintenance

Juvenile female rainbow trout (*Oncorhynchus mykiss*) purchased from Linwood Acres Trout Farm (Campbellcroft, ON) were acclimated for a minimum of 2 weeks in 1200 L fiberglass tanks supplied with oxygenated, dechloraminated City of Ottawa tap water at 13°C. Trout were fed at 0.5% body weight daily with a commercial diet (Regular Trout Floating Food, Martin Mills, Tavistock, ON) and kept at a constant 12 hr light:dark photoperiod (7 a.m. – 7 p.m.). All experiments were performed following a protocol approved by the University of Ottawa Animal Care Protocol Review Committee and adhered to published guidelines of the Canadian Council on Animal Care (CCAC) regarding the use of animals in teaching and research.

2.1.3 Exposure to FLX

Eight trout (83.3 ± 1.1 g) were moved to each of three 70 L fiberglass tanks and allowed to acclimate for one week; feeding was suspended at this point. A stock FLX concentration (3 mg/mL) was made in 95% ethanol and the tanks received FLX to give

nominal concentrations of 0, 0.54 and 54 µg/L for a period of 12 days. Tank FLX concentrations were maintained by a static renewal procedure. The entire volume of water in each tank, along with the corresponding FLX treatment dose, was renewed every 48 h. This was done by draining and replacing one half of the original tank water in each exposure tank with new oxygenated, dechloraminated City of Ottawa tap water at 13°C, 3 times per tank during each renewal. The control tank received renewed treatments of 95% ethanol only. This renewal scheme was used in order to minimize waste build up and ensure that FLX concentrations remained relatively constant in the tanks, but also to minimize stress to the trout.

Tank water temperature and chemical parameters including pH, nitrate, nitrite and ammonia were monitored throughout the entire experiment and maintained within normal ranges. Tanks were covered to minimize light exposure and reduce external stressors.

On day 12, half (4 fish) of the trout in each tank were immediately netted and anaesthetized with benzocaine (35 mg/L), a caudal blood sample immediately removed (1 mL EDTA-coated syringe; 23 gauge needle) and the trout subsequently euthanized by spinal transection. The remaining 4 trout were netted and exposed to air for 30 sec. Following air exposure, the trout were returned to their tanks for 30 min to allow cortisol levels to reach those induced by the air-stress (Ilan and Yaron, 1983; Lima et al., 2006). Fish were then anaesthetized, blood sampled, and euthanized as above.

2.1.4 Head Kidney Cell Preparation

Head kidney cells were isolated using a modification of the method described by Mimeault et al. (2007). Fish were anaesthetized with benzocaine (30-35 mg/L), a blood sample removed for subsequent cortisol determination, and euthanized as above. The

vasculature was perfused through the post-cardinal vein with 2 x 20 mL Cortland saline (124 mM NaCl, 2.0 mM KCl, 4.1 mM MgSO₄, 5.0 mM NaHCO₃, 0.8 mM glucose, 1.2 mM KH₂PO₄ and 2.0 mM CaCl₂).

The entire head kidney was removed from the trout and transferred to a 15 mL plastic tube containing 2 mL eMEM (MEM supplemented with 75 µM bovine serum albumin (MP Biomedicals, Canada, cat no. 105033) and 26 mM NaHCO₃ adjusted to pH 7.4) with 2 mg/mL collagenase-dispase. The tissue was initially disrupted by pressing the tissue against the inner side of the culture tube using a small spatula followed by a 60 min enzymatic digestion at room temperature with constant agitation on a low speed shaker; after 30 min, cells were resuspended every 10 min using a glass pipette. The cell suspension was filtered sequentially through 200 and 70 µm nylon membranes, and the filtrate centrifuged at 260 xg (Hermle Z360K, Mandel Scientific Company Ltd.) for 5 min at 12°C. The supernatant was discarded and the cell pellet was resuspended in 1 mL of fresh eMEM. Cell numbers were estimated using a hemocytometer and density was adjusted to 50 million cells/mL. A 150 µL volume of this cell resuspension was added to the wells of a 96 well plate and incubated at 12.5°C with gentle agitation for 2 h to establish basal cortisol production in the cells. Optimal cell densities and incubation times were determined in previous experiments (Mimeault et al., 2007; Leblond et al., 2001).

2.1.5 In vitro Exposure to FLX and norFLX

Following the 2 h pre-incubation, the plate was centrifuged at 140 x g (Hermle Z360K) for 3 min at 12°C (Leblond et al., 2001) and the supernatant recovered for cortisol determinations. Cells were resuspended by gentle aspiration using a pipet in eMEM containing 0, 0.004, 0.04, 0.4, 4, 40 or 400 µM FLX or norFLX generated by serial dilution

of a stock drug concentration dissolved in DMSO (99%; final DMSO concentration 0.3% in each well). The plate was subsequently incubated at 12.5°C with gentle agitation for 1 h.

2.1.6 Stimulation of Head Kidney Cells

Following the 1 h FLX/norFLX exposure, 1 IU/mL ACTH (dissolved in filtered, autoclaved MilliPore® water) was added to the cells to stimulate cortisol production; this concentration of ACTH was reported to give maximal cortisol production in previous experiments (Mimeault et al., 2007; Leblond et al., 2001). The cells were carefully resuspended and incubated for an additional 1 h at 12.5°C with gentle agitation. After this 1 h incubation, the cell suspensions were pipetted into 1.5 mL tubes. Fifty µL of each cell suspension was transferred to a separate 1.5 mL tube and used for the lactate dehydrogenase (LDH) assay. The additional cell suspension was centrifuged for 2 min at 20 000 x g (Beckman Coulter Microfuge R centrifuge). The resulting supernatant was placed in a 0.6 mL tube and frozen in liquid nitrogen for later assessment of cortisol production.

2.1.7 Radioimmunoassays

2.1.7.1 Cortisol

Cortisol concentrations were assessed using an ¹²⁵I RIA kit (MP Biomedicals, Irvine, CA, USA) as per the manufacturer's instructions and read using a Packard® Cobra™ Auto-Gamma Counter.

2.1.7.2 Adrenocorticotrophic hormone (ACTH)

Plasma ACTH concentrations were assessed using a double antibody (hACTH) ¹²⁵I RIA kit (MP Biomedicals, Irvine, CA, USA) as per the manufacturer's instructions and read using a Packard® Cobra™ Auto-Gamma Counter.

2.1.8 Cell Viability Assay

Head kidney cell viability was assessed using lactate dehydrogenase (LDH) activities. The 50 μ L of cell suspension was centrifuged at 200 x g for 2 min at 4°C and LDH activity in the resulting supernatant was assessed. Total LDH activity was determined by adding deionized water to the cell pellet, vortexing and then centrifuging the cells at 20 000 x g for 2 min. LDH activity was assessed immediately following the oxidation of 0.35 mM NADH in the presence of 50 mM imidazole and 4.5 mM pyruvate with 10 μ L supernatant in a 96 well plate. Cell viability is presented as the percentage of LDH detected with live cells (supernatant) versus the total LDH activity (pellet and supernatant).

2.1.9 Statistical Analysis

Two-way ANOVAs using the Holm Sidak method were performed, where possible, using SigmaPlot™ 11 (Systat Software, Inc., San Jose, CA) as indicated in figure captions. Values of $p < 0.05$ were accepted as significant. Results are graphed using SigmaPlot™ 11 (Systat Software, Inc., San Jose, CA).

2.2 Results

2.2.1 *In vitro* Cortisol Production and Cell Viability

Cortisol production and cell viability in isolated head kidney cells exposed to increasing concentrations of FLX and norFLX are shown in Figures 2.2-2.5. Cortisol production and cell viability was evaluated in head kidney cells isolated from rainbow trout previously exposed to 0, 0.54 or 54 µg/L FLX. Conditions in the 3 holding tanks and experimental manipulations used for the waterborne FLX exposure experiments were identical with the exception of the FLX treatment administered to the FLX tanks. The head kidneys from 4 trout were combined in order to achieve a cell concentration of 50 million cells/mL for the *in vitro* drug exposures.

2.2.1.1. *Unstressed Fish*

In vitro cortisol production in cells isolated from unstressed control (0 µg/L) trout was higher than production in cells isolated from either the 0.54 or 54 µg/L FLX-exposed trout (Figs. 2.2 and 2.3), at all *in vitro* concentrations of FLX and norFLX, except 400 µM. Cortisol production between the 0.54 or 54 µg/L FLX-exposed cells showed no observable differences or consistent trends. Unfortunately, statistical analysis could not be used to detect statistical differences within and between treatments at each of the *in vitro* FLX and norFLX exposures. The amount of head kidney tissue isolated from each group of FLX-treated trout, yielded a cell concentration large enough to expose only one cell sample per *in vitro* drug concentration. In unstressed control head kidney cells, cortisol production was relatively equal from 0 to 40 µM FLX (Fig. 2.2) but notably decreased at 400 µM FLX. Cortisol production in 0.54 and 54 µg/L FLX-treated, unstressed trout head kidney cells was low across all FLX treatments, not exceeding 4 ng/ml.

Control-treated cells exposed to norFLX had cortisol production similar to FLX at the 0.004 and 400 μ M concentrations (Fig. 2.3). Between concentrations of 0.04 and 4 μ M norFLX, cortisol production was ~2 fold greater than production in cells exposed to identical concentrations of FLX. Interestingly, at 40 μ M norFLX, cortisol production was lower than in head kidney cells exposed to 40 μ M FLX.

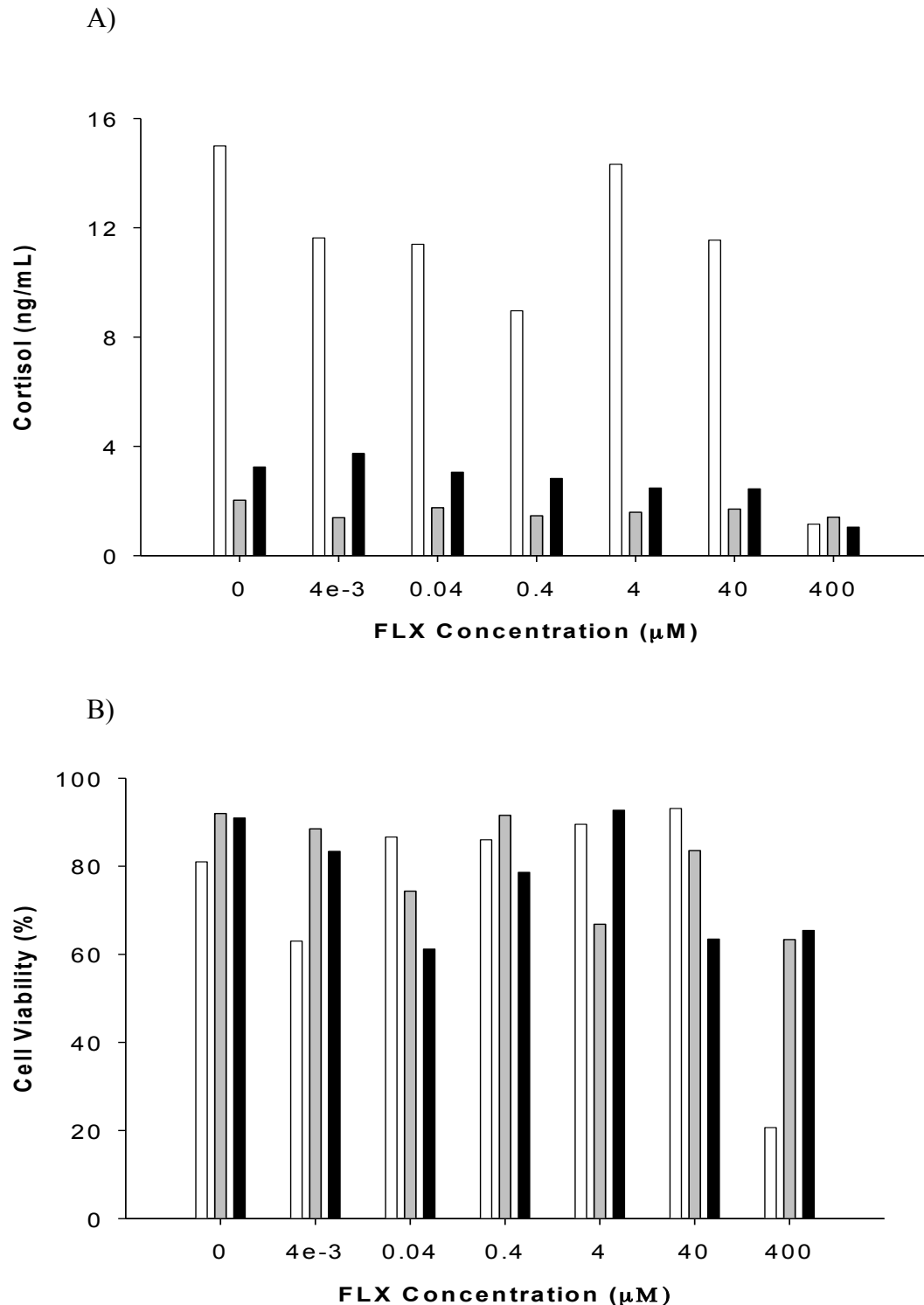
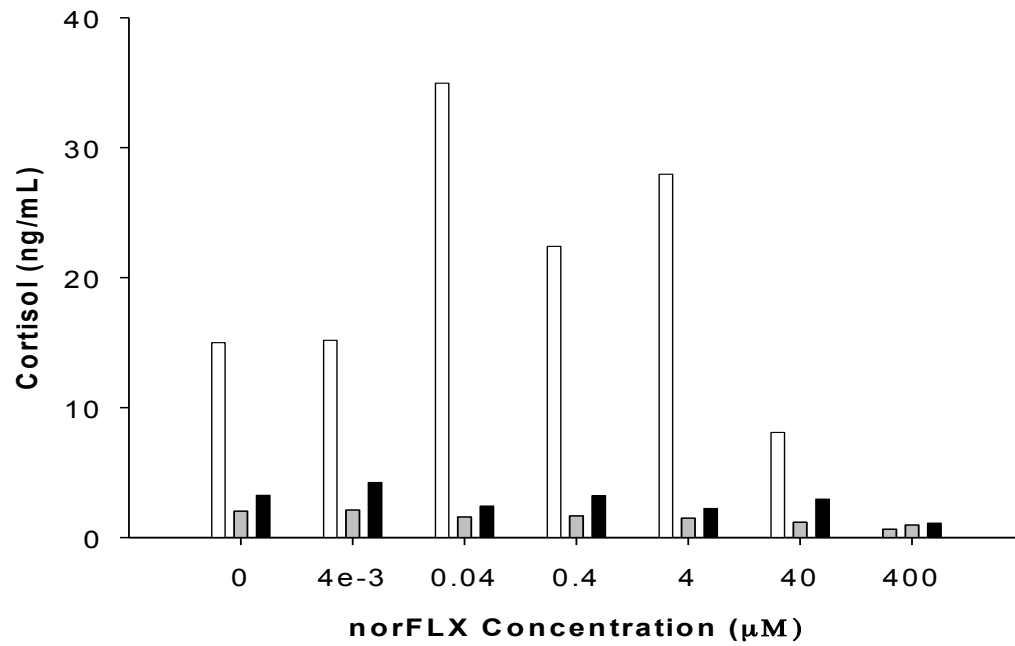


Figure 2.2. ACTH-stimulated cortisol production (*in vitro*) A) and cell viability B) in dispersed head kidney cells exposed to increasing concentrations of FLX. Cells isolated from unstressed control (0 μg/L FLX) (white bars), 0.54 μg/L (grey bars) and 54 μg/L (black bars) FLX-treated rainbow trout. Cells (50 million cells/mL) were incubated in the presence of FLX concentrations for 60 min and subsequently stimulated with ACTH (1 IU/mL eMEM) for one additional hour. Cell viability is presented as the percentage of LDH detected with live cells (supernatant) versus the total LDH activity (pellet and supernatant) x 100%.

A)



B)

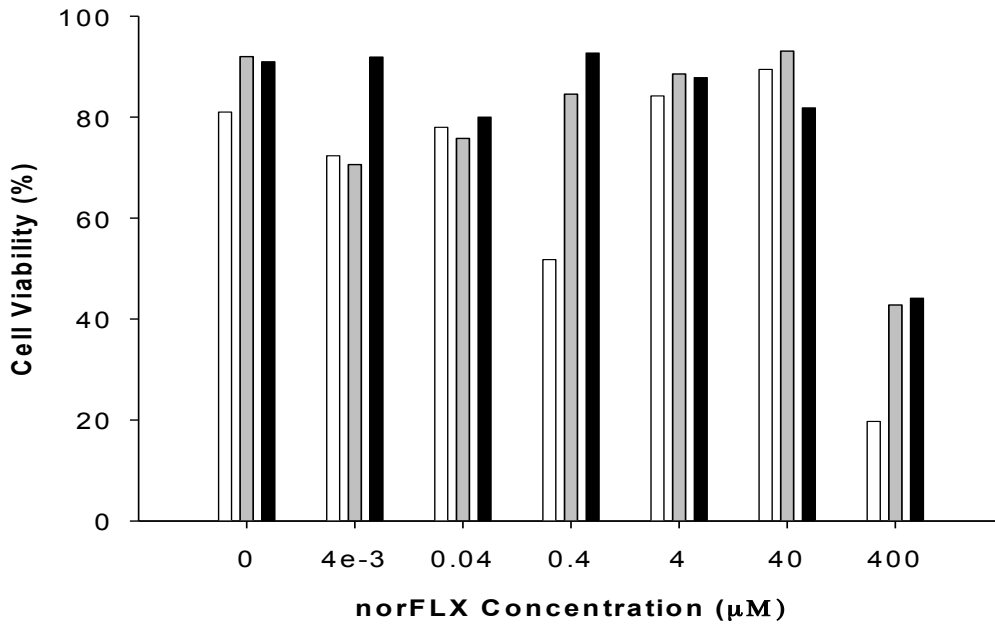


Figure 2.3. ACTH-stimulated cortisol production (*in vitro*) A) and cell viability B) in dispersed head kidney cells exposed to increasing concentrations of norFLX. Cells isolated from unstressed control (0 μg/L FLX) (white bars), 0.54 μg/L (grey bars) and 54 μg/L (black bars) FLX-treated rainbow trout. Cells (50 million cells/mL) were incubated in the presence of norFLX concentrations for 60 min and subsequently stimulated with ACTH (1 IU/mL eMEM) for one additional hour. Cell viability is presented as the percentage of LDH detected with live cells (supernatant) versus the total LDH activity (pellet and supernatant) x 100%.

2.2.1.2. *Air-Exposed (Stressed) Fish*

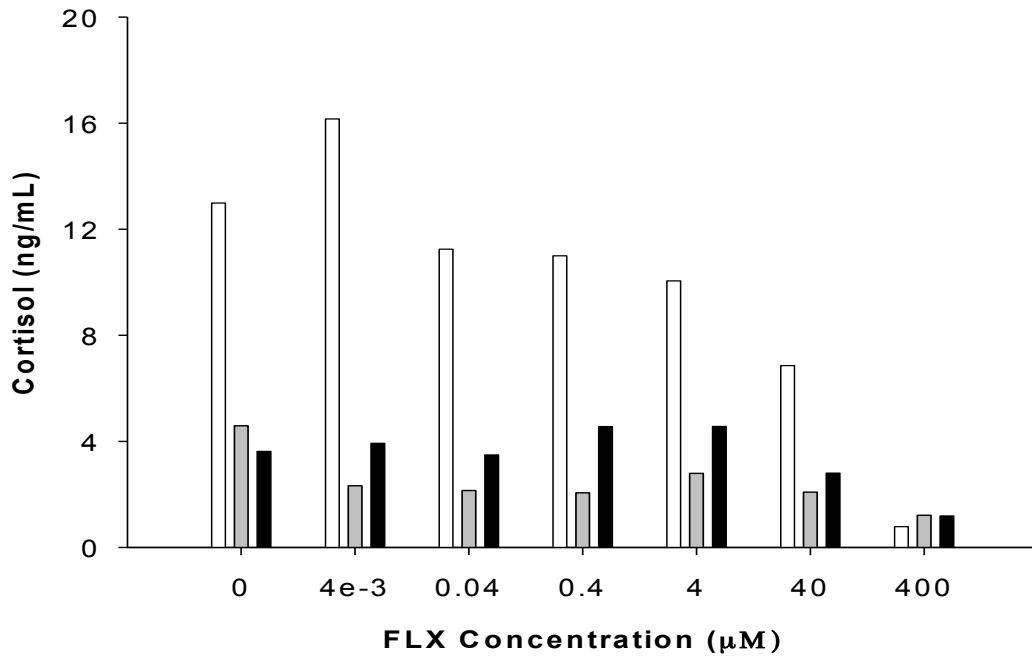
As observed in the isolated unstressed head kidney cells, *in vitro* cortisol production in cells isolated from air-stressed control (0 µg/L) trout was higher than production in cells isolated from either the 0.54 or 54 µg/L FLX-exposed trout (Figs. 2.4 and 2.5), at all *in vitro* concentrations of FLX and norFLX, except 400 µM. Cortisol production between the 0.54 or 54 µg/L FLX-exposed cells showed no observable differences or consistent trends. Statistical analysis could not be used to detect statistical differences within and between treatments at each of the *in vitro* FLX and norFLX exposures, as the total isolated cell concentration was large enough to provide only one cell sample per *in vitro* drug concentration. In the air-stressed fish cells, a slight but consistent decrease was observed with increasing concentrations of FLX from 0.04 to 400 µM FLX. As with the unstressed fish cells, cortisol production was lowest at the 400 µM FLX concentration. No trends were observed in cortisol production in the 0.54 and 54 µg/L FLX-exposed fish cells, but production was observed to be ~4 times lower (versus control) across all FLX concentrations, except 400 µM.

Air-stressed cells exposed to increasing concentrations of norFLX demonstrated similar cortisol production to the FLX-exposed cells (Fig. 2.5). No trends in cortisol production were observed across the norFLX exposures. However, as with FLX, cortisol production decreased at 40 µM norFLX and was lowest in cells exposed to 400 µM norFLX.

2.2.1.3. *Cell Viability*

Cell viability was relatively constant between the control, 0.54 and 54 µg/L FLX-treated (unstressed and air-stressed) head kidney cells. However, viability was notably lower across all three treatments in cells exposed to the 400 µM FLX and norFLX concentrations (*in vitro*).

A)



B)

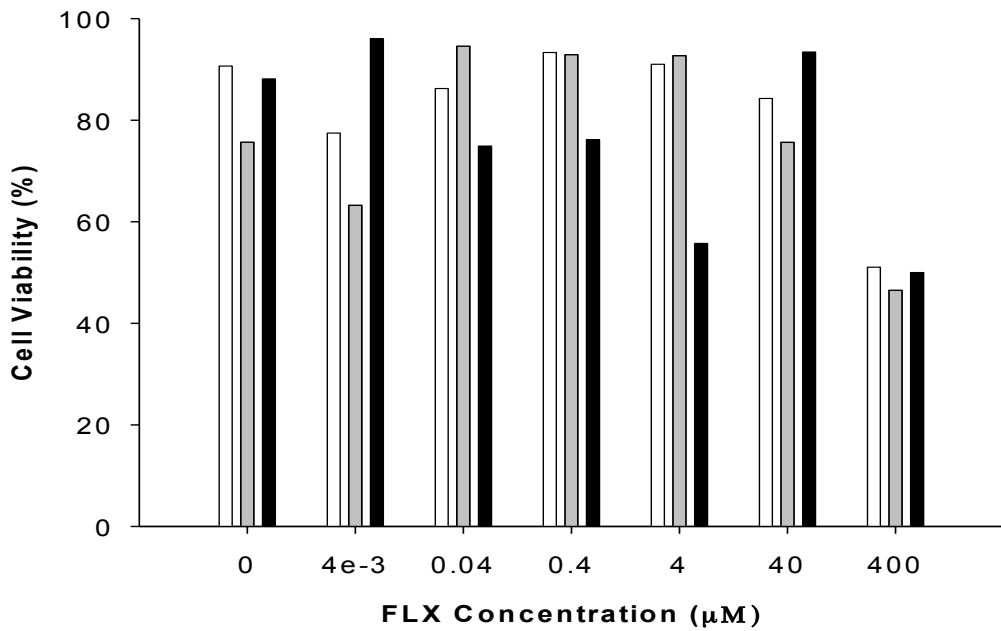


Figure 2.4. ACTH-stimulated cortisol production (*in vitro*) A) and cell viability B) in dispersed head kidney cells exposed to increasing concentrations of FLX. Cells isolated from air-stressed control (0 μg/L FLX) (white bars), 0.54 μg/L (grey bars) and 54 μg/L (black bars) FLX-treated rainbow trout. Cells (50 million cells/mL) were incubated in the presence of FLX concentrations for 60 min and subsequently stimulated with ACTH (1 IU/mL eMEM) for one additional hour. Cell viability is presented as the percentage of LDH detected with live cells (supernatant) versus the total LDH activity (pellet and supernatant) x 100%.

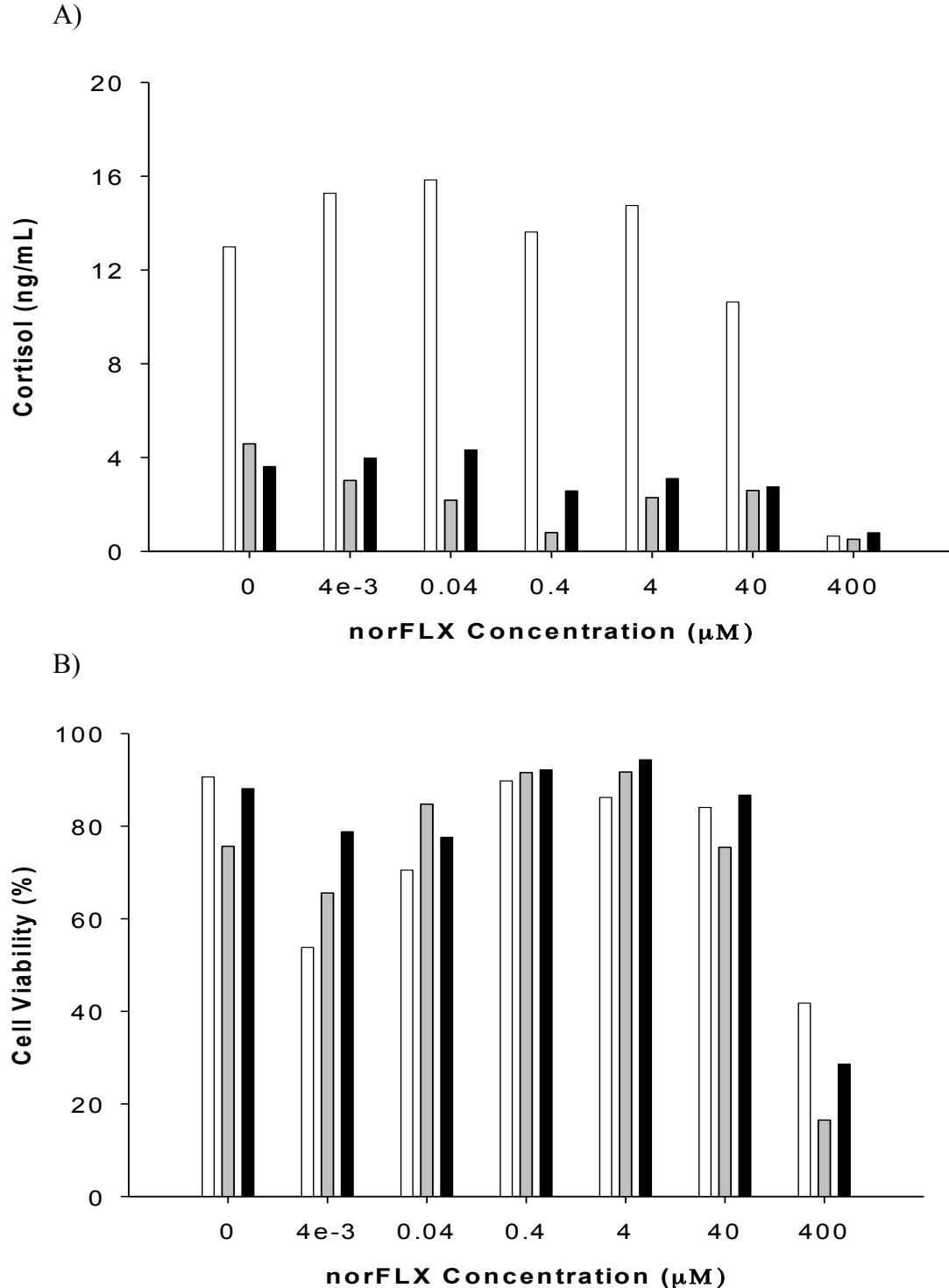


Figure 2.5. ACTH-stimulated cortisol production (*in vitro*) A) and cell viability B) in dispersed head kidney cells exposed to increasing concentrations of norFLX. Cells isolated from air-stressed control (0 μg/L FLX) (white bars), 0.54 μg/L (grey bars) and 54 μg/L (black bars) FLX-treated rainbow trout. Cells (50 million cells/mL) were incubated in the presence of norFLX concentrations for 60 min and subsequently stimulated with ACTH (1 IU/mL eMEM) for one additional hour. Cell viability is presented as the percentage of LDH detected with live cells (supernatant) versus the total LDH activity (pellet and supernatant) x 100%.

2.2.2 Plasma Cortisol

Plasma cortisol concentrations were assessed in control and FLX-treated and unstressed and air-stressed trout (Fig. 2.6). Comparison using two-way ANOVA (Holm-Sidak method) showed that plasma cortisol concentrations in air-stressed trout were significantly higher than unstressed ($P < 0.001$) trout within all treatments. In unstressed trout exposed to the highest FLX dose (54 $\mu\text{g/L}$), cortisol concentrations were $>310\%$ higher than those from trout in the control treatment ($P < 0.021$) and $>470\%$ higher than trout exposed to the low FLX dose (0.54 $\mu\text{g/L}$) ($P < 0.001$). In air-stressed trout, cortisol concentrations were significantly higher ($>160\%$) in high-dosed FLX trout compared to low-dosed FLX trout ($P < 0.016$). Plasma cortisol concentrations in control, air-stressed trout were not significantly different from those in low and high FLX-treated, air-stressed trout.

2.2.3 Plasma ACTH

Plasma ACTH concentrations were assessed in FLX-treated and unstressed and air-stressed trout are presented in Figure 2.7. Comparison using two-way ANOVA (Holm-Sidak method) showed that plasma ACTH concentrations in air-stressed trout were significantly higher than those of unstressed trout within the control treatment ($P < 0.001$) but differences were not significant within the low- or high-dose FLX treatments. ACTH concentrations in unstressed trout exposed to the high FLX dose (54 $\mu\text{g/L}$) were significantly lower (~ 3 -fold) than those in both the control ($P = 0.01$) and low (0.54 $\mu\text{g/L}$) ($P = 0.013$) FLX-treated trout.

ACTH concentrations were significantly higher ($P < 0.001$) in the control trout versus both the 0.54 $\mu\text{g/L}$ (36%) and 54 $\mu\text{g/L}$ (44%) FLX-treated trout. Plasma ACTH concentrations in the 0.54 and 54 $\mu\text{g/L}$ FLX-treated, air-stressed trout were not significantly different from one another.

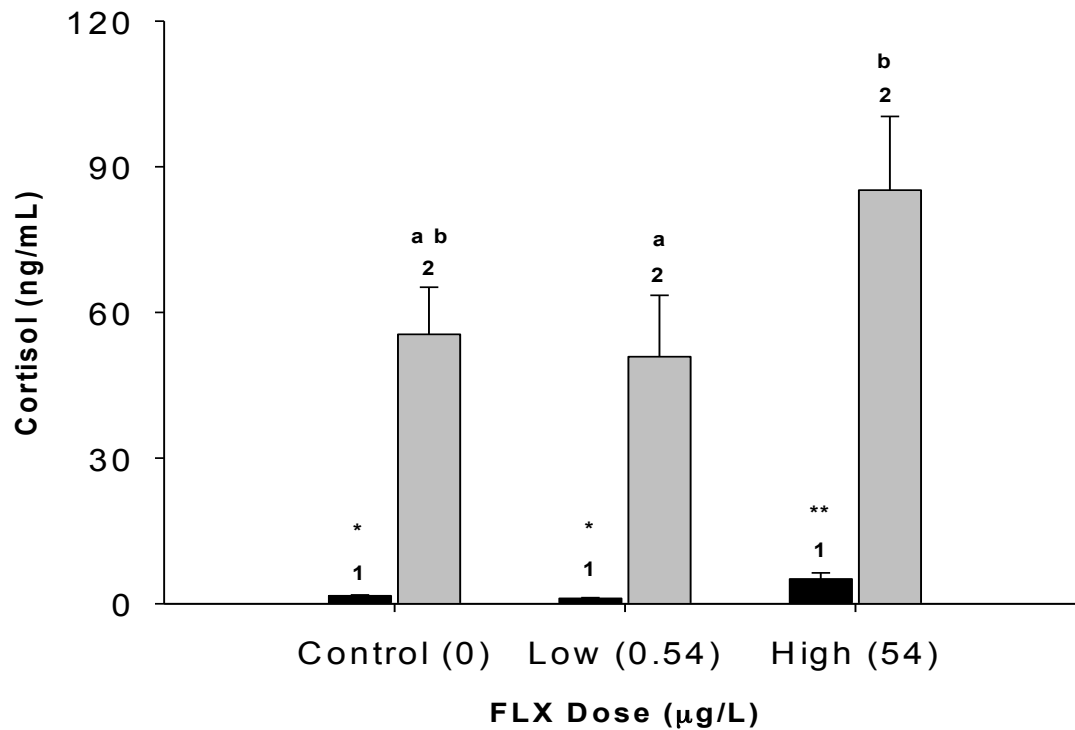


Figure 2.6. Plasma cortisol concentrations (ng/mL) in rainbow trout exposed to 0, 0.54 and 54 µg/L waterborne FLX. Cortisol concentrations were determined from blood samples taken from the caudal vasculature of unstressed (black columns) and air-stressed (grey columns) trout. Means (+SE) of control (0 µg/L; n=11), control + air-stress (n=10), low (0.54 µg/L; n=11), low + air-stress (n=11), high (54 µg/L; n=11) and high + air-stress (n=11) FLX treatments are presented. Statistical differences between FLX treatments and the unstressed and air-stressed conditions were determined using two-way ANOVA (Holm-Sidak method). 1, 2 = denotes significant differences between cortisol concentrations in unstressed and air-stressed trout within each FLX treatment; *, ** = denotes significant differences in cortisol concentrations in unstressed trout exposed to different FLX treatments; a, b = denotes significant differences in cortisol concentrations in air-stressed trout exposed to different FLX treatments.

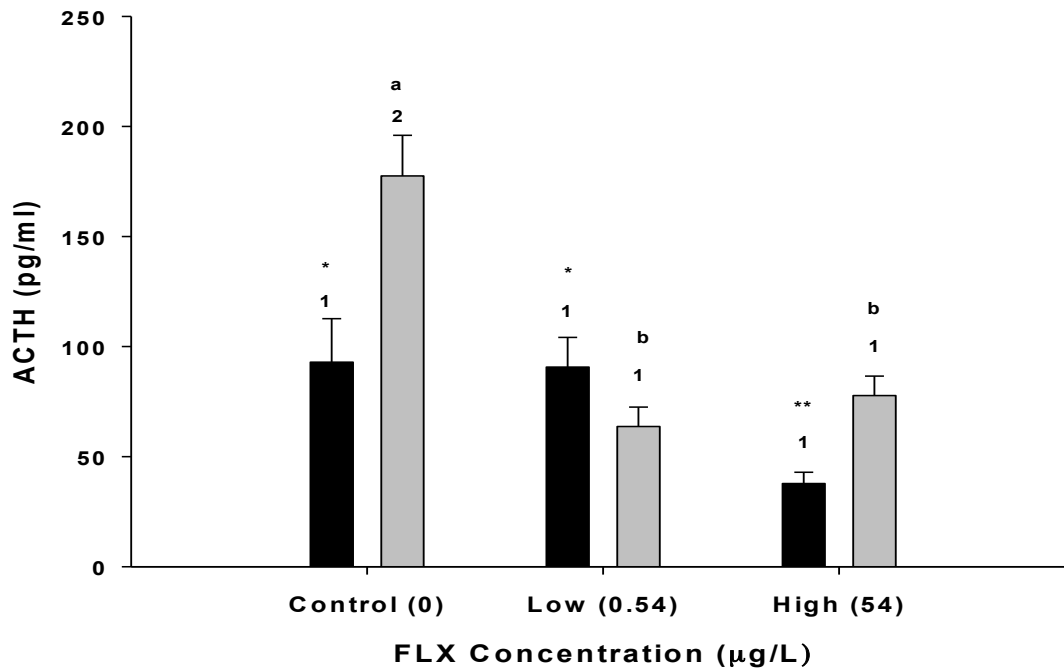


Figure 2.7. Plasma ACTH concentrations (pg/mL) in rainbow trout exposed to 0, 0.54 and 54 µg/L waterborne FLX. ACTH concentrations were determined from blood samples taken from the caudal vasculature of unstressed (black columns) and air-stressed (grey columns) trout. Means (+SE) of control (0 µg/L; n=4), control + air-stress (n=4), low (0.54 µg/L; n=4), low + air-stress (n=4), high (54 µg/L; n=4) and high + air-stress (n=4) FLX treatments are presented. Statistical differences between FLX treatments and the unstressed and air-stressed conditions were determined using two-way ANOVA (Holm-Sidak method). 1, 2 = denotes significant differences between ACTH concentrations in unstressed and air-stressed trout within each FLX treatment; *, ** = denotes significant differences in ACTH concentrations in unstressed trout exposed to different FLX treatments; a, b = denotes significant differences in ACTH concentrations in air-stressed trout exposed to different FLX treatments.

2.3 Discussion

2.3.1 *In vivo Stress Hormones*

This experiment demonstrated that cortisol production in rainbow trout can be altered by exposure to FLX. Cortisol synthesis and release begins when the hypothalamus releases CRF and subsequently stimulates the anterior pituitary to release the corticotropic agent, ACTH (Ings et al., 2011; Wendelaar Bonga, 1997). ACTH is released into the circulation and binds to melanocortin 2 receptors (MC2R) on the steroidogenic interrenal cells of the teleost head kidney, stimulating cortisol biosynthesis (Ings et al., 2011; Flik et al., 2006). In control (unstressed and non-FLX-treated) rainbow trout, mean plasma ACTH concentration was 92.9 ± 19.8 pg/mL (Fig. 2.11). Pottinger et al. (1996) found similar levels (99 ± 11 pg/mL) in resting rainbow trout and Swift (1982) reported mean plasma ACTH concentrations in free-swimming rainbow trout (*Salmo gairdneri*) to be between 97 ± 7 and 120 ± 17 pg/mL. Mean plasma cortisol concentration in the control (unstressed) trout was 1.3 ± 0.2 ng/mL. Although this is a very low concentration, basal or resting levels of cortisol in salmonids have been reported to be <5 ng/mL (Wendelaar Bonga, 1997).

Exposure to one or more stressors triggers an increase in the activity of the HPI axis (Yao and Denver, 2007), which includes a rise in both plasma ACTH and cortisol levels (Metz et al., 2004; Wendelaar Bonga, 1997; Balm and Pottinger, 1995; Sumpter et al., 1986). Such an occurrence was observed in the control trout of the present experiment. Plasma ACTH concentrations in trout subjected to the acute air-stress had a mean value of 177.5 ± 18.4 pg/mL, while cortisol concentrations had a mean value of 62.3 ± 9.2 ng/mL. Again, these values were comparable to those observed by Pottinger et al. (1996), though variations in stress-induced plasma ACTH and cortisol found between different studies are generally

attributed to the type and severity of the stressor used to induce the teleost stress response (Arends et al., 1999). Fish subjected to the air-stress were placed back in their tanks and allowed to recover for 30 min a time period necessary to achieve peak cortisol concentrations following exposure to a stressor and then begin to drop back towards resting levels (Lima et al., 2006; Ilan and Yaron, 1983).

There is evidence that serotonin is involved in the regulation of HPI axis activity in teleosts, including the presence of a 5-HT receptor subtype (5-HT_{1A}) in the salmonid brain and that administering a 5-HT_{1A} agonist (8-OH-DPAT) results in a 10-fold increase in plasma cortisol levels in rainbow trout (Lawrence and Hemingway, 2003; Winberg et al., 1997). Furthermore, exposure to stressors, such as confinement, social subordination and predation, are shown to increase serotonergic activity in the brains of both mammalian and teleost fish species (Schjolden et al., 2006; Øverli et al., 2005; Winberg et al., 1997; Winberg and Nilsson, 1993). SSRI pharmaceuticals have been chemically designed to inhibit the reuptake of serotonin into presynaptic neurons, thereby increasing serotonin availability within neuronal synaptic clefts, allowing 5-HT molecules to continually interact with postsynaptic receptors (Kwon and Armbrust, 2006).

The higher plasma cortisol concentrations (5.6 ± 1.6 ng/mL) observed in unstressed trout exposed to 54 µg/L FLX could result from an increase in serotonin availability. As mentioned previously, administering a 5-HT receptor agonist did result in higher plasma cortisol concentrations in rainbow trout (Winberg et al., 1997) and enhanced brain serotonergic activity is shown to correlate with high plasma ACTH and cortisol concentrations in teleosts (Bernier and Peter, 2001; Winberg et al., 1993). The plasma ACTH concentrations were significantly lower in high-dosed (54 µg/L) FLX trout (versus control

and low-dosed FLX), which may indicate that the higher cortisol concentration was acting to negatively feedback on the production and secretion of ACTH, as has been demonstrated previously (Young, 2004; Wendelaar Bonga, 1997). However, this result does not agree with the finding of Winberg et al. (1993) who reported that high serotonin concentrations correlated with high plasma ACTH levels. Some studies on rats have argued that serotonin actually down-regulates ACTH output (Van Loon et al., 1981; Telegdy et al., 1976), yet others have demonstrated the opposite (Spinedi and Negro-Vilar, 1983; Holmes et al., 1982; Buckingham et al., 1979). Along with being an SSRI, FLX is also a moderately potent inhibitor of dopamine (DA) reuptake (Stanford, 1996) and there is evidence that DA can inhibit basal ACTH secretion in fish (Randall and Conte, 2009; Metz et al., 2004; Olivereau et al., 1988), and stress-induced changes in brain serotonergic activity (Höglund et al., 2001). If DA is acting on the pituitary counteracting the serotonin-mediated increase in ACTH, this may mean that FLX is also exerting an influence at the level of the hypothalamus.

Intraperitoneal injections of FLX increased CRF mRNA levels in the hypothalamus of goldfish (Mennigen et al., 2009) and serotonin itself does modulate CRF expression *in vitro* and *in vivo* (Itoi et al., 1998). Certain SSRIs are reported to reduce the activity of CRF neurons (Nemeroff and Owens, 2004); therefore, the down-regulation of ACTH in the high-dosed FLX trout could be occurring indirectly through reduced CRF release (Telegdy et al., 1975). Unfortunately, any modification to CRF resulting from FLX exposure cannot be confirmed in this study, as CRF concentrations and expression levels were not measured. If CRF expression and/or production were being modulated through FLX exposure – or serotonin concentrations – one would predict that any effects exerted on CRF would be carried down the HPI axis (i.e. low CRF leads to low ACTH which leads to low cortisol).

However, basal cortisol levels were observed to be higher in high-dosed FLX trout and not significantly different than those of control in low-dosed FLX trout. In teleosts, atrial natriuretic factor, angiotensin II, growth hormone, thyroxin, arginine vasotocin and the catecholamines all have corticotropic actions (Wendelaar Bonga, 1997), which can impact the production and release of cortisol. For example, Fritsche et al. (1993) demonstrated that serotonin mediates catecholamine release in rainbow trout; therefore perhaps the high concentration of plasma cortisol in the high FLX-treated trout is a result of increased production and stimulation of the CAs – resulting from increased 5-HT concentrations (Fritsche et al., 1993) – or the actions of another corticotropic factor. Again, this statement cannot be confirmed, as ACTH was the only corticotropic factor measured.

While mean basal levels of cortisol were significantly higher than those observed in the control and low (0.54 µg/L) FLX-exposed trout, it was not much higher than some of the basal plasma cortisol concentration ranges previously reported in salmonids (Høgåsen, 1998; Wendelaar Bonga, 1997). Basal levels of cortisol in salmonids are reported to be variable (Høgåsen, 1998), however cortisol concentrations in teleosts do cycle both on a daily and seasonal bases, which could impact physiological processes like reproduction (Laflamme et al., 2000; Høgåsen, 1998). The cortisol measurements in this study were taken from juvenile rainbow trout at approximately the same time of day. Therefore, though basal cortisol concentrations in 54 µg/L FLX-exposed trout did not exceed some of the previously reported basal/resting cortisol levels in teleosts (Norris, 2007; Høgåsen, 1998), continued exposure to FLX may potentially alter the daily and/or seasonal fluctuations of cortisol, which may compromise physiological cycles. FLX has already been shown to alter sex hormones in male and female goldfish (Mennigen et al., 2008; Mennigen et al., 2010) and affect feeding

(Mennigen et al., 2009). Alterations in feeding, reproduction and other physiology could eventually lead to altered cortisol production, as these drug-induced changes will ultimately disturb homeostasis, which stimulates the production and release of cortisol (Schreck, 2010; Barton, 2002). If possible, plasma ACTH and cortisol concentrations should be determined in sexually mature teleosts exposed to FLX at different times of year to determine how daily, and potentially seasonal cortisol fluctuations are influenced by SSRI exposure.

Plasma ACTH concentrations in high-dosed FLX trout subjected to 30 sec of air-stress were not significantly higher compared to the levels observed in their unstressed counterparts. Plasma cortisol concentrations however, were highest in these trout (Fig. 2.6). It is widely accepted that cortisol exerts negative (inhibitory) feedback on the HPI axis, so it is likely that the minimal increase in mean ACTH concentration in these fish, is the result of feedback inhibition on ACTH secretion at the anterior pituitary (Norris, 2000). Cortisol levels in the unstressed, high-dosed FLX trout were already higher – possibly due to a higher availability of serotonin – and ACTH levels were significantly lower than the other treatment groups suggesting that negative feedback by cortisol were already occurring in the resting state. Winberg and Lepage (1998) demonstrated that socially subordinate rainbow trout had elevated telencephalon and brain stem 5-HIAA/5-HT ratios, plasma cortisol levels and pituitary POMC mRNA concentrations after just 1 day of subordination. Therefore, as stressors are reported to induce serotonergic activity (Lawrence and Hemingway, 2003; Winberg and Lepage, 1998), and FLX is a drug designed to increase serotonergic activity, the high cortisol concentrations may be attributed to the increased serotonin concentrations resulting from FLX exposure. The low plasma ACTH and cortisol levels observed in air-stressed rainbow trout exposed to the low dose (0.54 µg/L) of FLX could be attributed to

several factors. It must also be noted that unstressed, low dose FLX-exposed rainbow trout did not vary significantly from the plasma ACTH and cortisol concentrations observed in the control trout. As such, only the rise in ACTH and cortisol concentrations that resulted from stressor exposure impacted blood levels at this FLX concentration. A logical explanation for this difference, especially compared to air-stressed, 54 µg/L FLX-exposed trout, would be the large difference in the administered FLX doses. A larger dose would likely have a greater or more widespread impact on serotonergic activity in a vertebrate organism. The prescribed dose of FLX to human patients varies based on the anxiety disorder being treated (Eli Lilly, 2011). For example, a patient suffering from panic disorder is prescribed 10 mg FLX per day, while one suffering from bulimia nervosa requires a treatment dose of 60 mg FLX per day (Eli Lilly, 2011). These differences in doses are based on research that show that different anxiety disorders demonstrate abnormalities in different serotonergic receptors, which do not all respond identically to one specified FLX treatment or dose (Blier and de Montigny, 1999; Lesch et al., 1991). Consequently, different FLX doses and even different treatment periods with FLX are required (Eli Lilly, 2011) in order for a disorder to be successfully relieved. In regards to the rainbow trout, a 5-HT receptor subtype (5-HT_{1A}) has been shown to be involved in the teleost stress response (Winberg et al., 1997), however, how this receptor would respond to an SSRI cannot presently be confirmed. SSRIs have been shown to initially prevent the augmentation of 5-HT transmission in humans, due to an SSRI induced reduction of 5-HT neuron firing (Blier and de Montigny, 1999; Wilkinson et al. 1991). Perhaps the 12-day FLX exposure, at 0.54 µg/L, prevented increased 5-HT transmission, which resulted in the reduced activity of the teleost stress axis. Additionally, monoamine oxidase (MAO) activity in the hypothalamus of juvenile rainbow trout does increase during confinement

stress (Schjolden et al., 2006). MAOs catalyze the oxidative deamination of neurotransmitters and biogenic amines (Edmondson et al., 2004), which would contribute to decreasing serotonin concentrations in the synaptic cleft (El-Ganzouri et al., 1985). The high dose FLX-exposed trout were treated for the same length of time (12 days) as the low dose FLX-exposed trout, but demonstrated different results. This suggests that drug dose plays a role in determining how 5-HT receptors and transmission in teleosts will be affected by SSRI exposure. If the 54 µg/L FLX dose resulted in increased serotonergic activity within the 12 day exposure period, it is possible that both the inhibitory feedback by cortisol on the teleost stress axis and the increased MAO activity resulting from stress, were overridden by the FLX-induced increase in serotonin. The involvement and/or modification of other 5-HT (and non-5-HT) receptors resulting from FLX is also a possibility.

2.3.2 *In vitro* Cortisol Production

The optimal cell density, incubation time and ACTH concentration for *in vitro* cortisol production in dispersed head kidney (interrenal) cells were determined in previous studies (Mimeault et al., 2007). FLX and norFLX were dissolved in DMSO, where the final DMSO concentration in each cell well was 0.3%. At this concentration, cortisol production and cell viability were not affected or compromised by the presence of DMSO (Mimeault et al., 2007). Therefore, any observed changes in cortisol production (*in vitro*) would be a result of FLX or norFLX exposure.

Cortisol production in head-kidney cells isolated from non-FLX exposed, unstressed rainbow trout demonstrate that *in vitro* exposure to FLX and norFLX resulted in impaired cortisol production only at extremely high concentrations (i.e. 400 µM). Seven previously conducted experiments (data not shown), following the same experimental protocol as the

present study but using only FLX, demonstrated similar results to those found in the present (control) trial. This observed reduction in cortisol can at least partially, be attributed to the reduced cell viability that occurred when the head kidney/interrenal cells were exposed to the 400 μ M concentrations. ACTH-induced cortisol production in head-kidney cells isolated from unstressed trout exposed to 0.54 and 54 μ g/L FLX was lower than that observed in cells isolated from control fish (Figs. 2.2 and 2.3). As observed with cells from the control treatment, *in vitro* cortisol production and cell viability were lowest at the 400 μ M FLX and norFLX exposures. Most important however, is the observation that cortisol production was low in the 0.54 and 54 μ g/L FLX-exposed cell groups even in cells exposed to an *in vitro* drug concentration of 0 μ M. This indicates that the waterborne FLX exposure impacted the responsiveness of head kidney/interrenal cells to ACTH stimulation. Interestingly, plasma cortisol concentrations indicate that cortisol production was higher in 54 μ g/L FLX-exposed trout – unstressed and stressed – than control and 0.54 μ g/L FLX-treated trout, yet *in vitro* ACTH-stimulated cortisol production was extremely low (versus control cells). Such data suggest that more upstream components of the teleost stress axis are responsible for alterations in cortisol production. Removing head kidney cells from rainbow trout eliminates all upstream and external influences (Wendelaar Bonga, 1997) on cortisol production, including that of the serotonergic system (Schjolden et al., 2006; Lawrence and Hemingway, 2003). The removal of these influences would reduce, if not eliminate stimulation of cortisol production. As control cells produced more cortisol upon ACTH stimulation, it is likely that the differences in production are attributed, at least in part, to the level of serotonergic activity amongst the different waterborne FLX treatments. In addition, without external influences or an intact stress axis, cortisol could be freely exerting autocrine or paracrine

feedback inhibition on interrenal cells, as glucocorticoid receptors are expressed in the adrenal cortex of mammals and head kidney/interrenal cells of at least some fish (Vazzana et al., 2010; Loose et al., 1980). In addition, differences in cortisol production could be the result of different interrenal cell yields in the cell wells. All trout used in this experiment were approximately the same size and head kidney cells were counted using a hemocytometer, and resuspended in order to ensure that a similar cell concentration was pipetted into each well. However, isolated head kidney tissue does not only consist of the steroidogenic interrenal cells (Hontela et al., 2008) and resuspension of the cell solution does not guarantee that the interrenal cells will be evenly distributed amongst each well. In order to determine the relative concentrations of steroidogenic cells in the isolated head kidney tissues, a method such as differential cell staining for steroidogenic enzyme, 3 β -HSD, would need to be used in order to identify the interrenals from other head kidney cell types (Hontela et al., 2008). Furthermore, it has been shown that an enlargement in the nuclear diameters of the interrenal cells indicates increased steroidogenic activity (Rotllant et al., 2003). Therefore, cortisol production will also be impaired/lower if the enlargement of interrenal cells is impeded by FLX exposure. Steroidogenic cell concentration and activity are both important, particularly because cortisol is not stored, but synthesized ‘on demand’ (Geslin and Auperin, 2004).

The effects of norFLX – the active metabolite of FLX – on *in vitro* cortisol production were evaluated because, like FLX, norFLX is present in the aquatic environment (Brooks et al., 2005) and is reported to have comparable pharmacological activity, but a longer half-life than FLX (Mandrioli et al., 2006). norFLX is also reported to be a more potent inhibitor of serotonin reuptake than FLX (Sigma-Aldrich, 2011). Unstressed head

kidney cells exposed to norFLX had cortisol production similar to FLX at the 0.004 and 400 μM concentrations. However, between concentrations of 0.04 and 4 μM norFLX, cortisol production was ~ 2 fold greater than production in cells exposed to identical concentrations of FLX. Some research has implied that head kidney tissues express serotonergic receptors (Fritsche et al., 1993), so this could be a result of a unique norFLX stimulation of these serotonergic receptors, at these concentrations. At 40 μM norFLX, cortisol production was lower than in head kidney cells exposed to 40 μM FLX. As norFLX has been reported to have a greater potency than FLX (Sigma-Aldrich, 2011), it is possible that this metabolite has a lower LOEC than its parent compound and/or begins to exert harmful physiological effects at lower concentrations than FLX.

Overall, no trends or differences compared to the FLX concentrations could be established across the norFLX exposures. No concrete conclusions from these *in vitro* observations can be made because, as previously mentioned, statistical analyses could not be performed on this *in vitro* data, as only one experimental trial (with one sample for each FLX and norFLX exposure) was conducted. Accepting the data presented, however it is clear that both in-water exposure and *in vitro* head kidney cell exposures to FLX will modify cortisol release.

The highest environmental concentration reported for FLX is 0.54 $\mu\text{g/L}$ (Brooks et al., 2003), a concentration that is much lower to the *in vitro* FLX and norFLX concentrations used here (e.g. 400 μM = 138 mg/L). Therefore, environmentally relevant concentrations of FLX (and norFLX) do not appear to compromise head kidney cell integrity or viability. However, changes in cortisol and ACTH plasma concentrations (*in vivo*) were observed at the 0.54 $\mu\text{g/L}$ FLX concentration (see Section 2.3.1). The low production of cortisol

observed in cells isolated from low and high FLX-dosed trout could be due to a low yield of interrenal cells in these cell samples. The proportion of steroidogenic cells to other cells in the head kidney of rainbow trout (*O. mykiss*), has been reported to only be 1:8000 (Hontela et al., 2008), which would result in very low cortisol production even in the no drug-exposed fish. Furthermore, FLX could potentially be altering the yield of these interrenal cells via effects on head kidney tissues overall. Combined SSRI concentrations in the environment are reported to range from 840 ng/L to 3.2 µg/L (Metcalfé et al., 2010). However, their continuous input into the environment and slow degradation (Redshaw et al., 2008) are likely to result in increased environmental concentrations that could impact steroidogenesis in teleost head kidneys – as seen in the plasma concentrations of rainbow trout – and impact the overall health of aquatic life (Christensen et al., 2007).

2.4 Conclusions

Alterations in plasma cortisol concentrations and fluctuations can compromise the stress response (this thesis), osmoregulation (McCormick, 2001), immune function (Laflamme et al., 2000) and reproductive capacity in teleosts (Schreck et al., 2001). These physiological changes would result in changes to overall fish behaviors and could lead to reduced fish population sizes and a general decline in the adaptability and survival capacity of fish (McCormick, 2001; Barton et al., 1987). This study demonstrated that high (54 µg/L) waterborne FLX exposure of juvenile rainbow trout results in higher plasma cortisol concentrations, but lower plasma ACTH concentrations (versus control and low dose FLX-exposed trout). At an environmentally relevant FLX concentration (0.54 µg/L), both plasma cortisol and ACTH concentrations are lower than concentrations in control and high FLX-exposed trout subjected to an acute air-stress, which suggests that FLX has dose-dependent

effects on teleosts. These results lend support to my original hypothesis, stating that exposure to FLX, an SSRI, will alter the activity of the HPI axis in teleost fish. In addition, the *in vitro* results suggest that interrenal integrity is compromised at higher exposure concentrations (i.e. 40 and 400 μ M) of FLX and norFLX and that potential differences in interrenal cortisol production exist between FLX and its active metabolite, norFLX. However, these conclusions are preliminary, as additional experiments are required to validate these statements. This research specifically contributes to elucidating the endocrine disrupting abilities of FLX on the teleost stress response, a topic that has received minimal focus.

Chapter 3

Cortisol Steroidogenesis in Head Kidney Microsomes of Rainbow Trout (*Oncorhynchus mykiss*)

3.0 Introduction

Pharmaceuticals and personal care products (PPCPs) have been detected in water supplies and wastewater effluents worldwide (Westerhoff et al., 2005). Wastewater effluents are a primary route into aquatic environments for pharmaceuticals and PPCPs, as these substances are partially excreted from the human body or disposed of through sewage (Schultz and Furlong, 2008). Active pharmaceutical ingredients show a wide range of persistence in aquatic environments, potentially leading to chronic exposure of aquatic species (Kreke and Dietrich, 2008; Khetan and Collins, 2007). Most pharmaceuticals have a high therapeutic index and their effects are often elicited at very low concentrations with little or no toxicity (Hontela, 2006). As such, the presence of pharmaceutical ingredients in water systems has raised concerns about the potential effects of these chemicals on non-target, aquatic species (Hontela, 2006).

Certain xenobiotics, including the analgesic ibuprofen and the lipid regulator, gemfibrozil (GEM), have been found to impair steroidogenesis of the reproductive hormones, 17 β -estradiol (E2) and testosterone (T), by targeting the enzymes involved in the steroidogenic pathway (Han et al., 2010; Mimeault et al., 2005). Teleost exposure to ibuprofen and the anti-inflammatory, salicylate has resulted in a significant drop in gene expression of steroid acute regulatory (StAR) protein and peripheral-type benzodiazepine receptor (PBR), two proteins involved the transport of cholesterol into the inner mitochondrial membrane for conversion to pregnenolone by the P450_{scc} (side chain cleavage

enzyme) (Gravel and Vijayan, 2006; Mimeault et al., 2005).

Cortisol is the principal corticosteroid in teleost fishes and the rise in its circulating concentrations after stressor exposure has been shown to play a key role in enabling fish to regain homeostasis (Gravel and Vijayan, 2006; Barton, 2002; Mommsen et al., 1999). Stressors trigger the hypothalamus to release corticotropin-releasing factor (CRF), which subsequently stimulates the anterior pituitary to release ACTH, the primary corticotropic agent (Ings et al., 2011; Wendelaar Bonga, 1997). ACTH is released into the circulation and then binds to melanocortin 2 receptors (MC2R) on the steroidogenic interrenal cells of the teleost head kidney, stimulating cortisol biosynthesis (Ings et al., 2011; Flik et al., 2006). Cholesterol is translocated to the inner mitochondrial membrane by the StAR protein and PBR (Gravel and Vijayan, 2006; Papadopoulos, 2004) and then converted to pregnenolone (P5) by the P450_{scc} (side chain cleavage) enzyme (Aluru et al., 2005). P5 undergoes a series of isomerization and hydroxylation reactions that are catalyzed by 17 α -hydroxylase (17-OHase), 3 β -hydroxysteroid dehydrogenase (3 β -HSD), 21-hydroxylase (21-OHase), and 11 β -hydroxylase (11 β -OHase), resulting in the production of cortisol (Fig. 3.1) (Geslin and Auperin, 2004).

Selective serotonin reuptake inhibitor (SSRIs) antidepressants are among the most commonly detected pharmaceuticals in both surface water and wastewater effluents (Corcoran et al., 2010). These antidepressants are prescribed to patients suffering from major depression or other anxiety disorders, and alleviate depressive symptoms by increasing serotonergic neurotransmission via inhibition of the serotonin reuptake transporter enzyme, SERT (Daughton and Ternes, 1999). The majority of studies examining the exposure effects of SSRIs on aquatic organisms have used fluoxetine hydrochloride (FLX, ProzacTM). This

research has shown that FLX exposure can increase the expression of potent inhibitory feeding neuropeptides, modulate the expression of genes involved in reproductive function and disrupt reproductive behavior, reduce growth, feeding and predatory behaviors in numerous fish species (Mennigen et al., 2010; Mennigen et al., 2009; Gaworecki and Klaine, 2008; Mennigen et al., 2008; Stanley et al., 2007). Very few of these studies have focused on whether FLX (or other SSRIs) can disrupt cortisol production in teleost fish, specifically the cortisol response to stressors.

This present study examined cortisol steroidogenesis in head-kidney microsomes isolated from rainbow trout exposed to waterborne concentrations of FLX. The isolated microsomes were incubated with ^{14}C -pregnenolone substrate in order to test the hypothesis that FLX exposure alters cortisol biosynthesis in a teleost fish. Research examining the impact of SSRI exposure on corticosteroid production is lacking; therefore, the results of this study will determine if an antidepressant pharmaceutical can alter steroidogenesis in teleost fish and potentially alter the teleost stress response, thereby expanding this area of research.

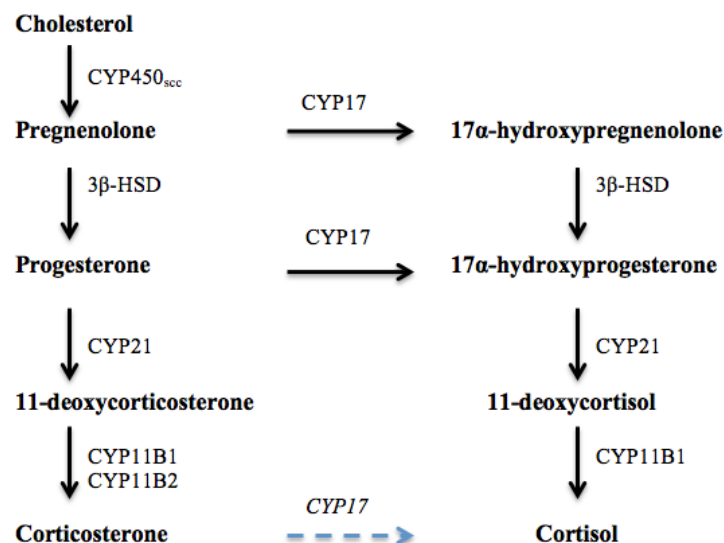


Figure 3.1. Steroidogenesis of Cortisol Following Translocation of Cholesterol Across the Inner Mitochondrial Membrane. The enzyme(s) involved in each metabolic conversion are listed beside the appropriate arrow(s). The dashed arrow represents an enzymatic conversion shown to occur in fish.

3.1 Materials and Methods

3.1.1 Chemicals

Fluoxetine hydrochloride (FLX), bicinchoninic acid (BCA) and copper (II) sulphate were purchased from Sigma-Aldrich (St. Louis, MO, USA). Albumin bovine (BSA) was purchased from MP Biomedicals, LLC (Irvine, CA, USA). [4-¹⁴C]-pregnenolone substrate (50 mCi/mmol, Amersham Radiochemicals), steroid carriers (Steraloids), solvents, nicotinamide cofactors (Sigma Chemicals) and other chemicals were provided by Dr. Gerard M. Cooke (Health Canada).

3.1.2 Fish and Maintenance

Juvenile female rainbow trout (*Oncorhynchus mykiss*) purchased from Linwood Acres Trout Farm (Campbellcroft, ON) were acclimated for more than 2 weeks in 1200 L fiberglass tanks. As a preemptive treatment for potential disease, all trout were treated with a 1 hr bath of 125 ppm formalin every second day during the first week after delivery.

Tank systems were supplied with oxygenated, dechloraminated City of Ottawa water at a temperature of 13°C. Trout were fed at 0.5% body weight daily with a commercially available pellet diet (Regular Trout Floating Food, Martin Mills, Tavistock, ON). Each holding tank initially contained 300 trout of relatively equal size and at a constant 12 h light: dark photoperiod (7 a.m. – 7 p.m.). All experimentation was performed following a protocol approved by the University of Ottawa Animal Care Protocol Review Committee and adhered to the principles established by the Canadian Council on Animal Care (CCAC) regarding the use of animals in research.

3.1.3 Exposure to FLX

Three groups of rainbow trout were exposed to 0, 0.54 and 54 µg/L of waterborne FLX for a period of 12 days. Each of six 70 L fiberglass tanks contained eight trout (87.9 ± 1.4 g), which were acclimated for over one week and fasted for the complete duration of the acclimation and FLX exposure periods. Prior to its addition to the aquaria, FLX was dissolved in 95% ethanol (3 mg/ml).

FLX concentrations in the exposure tanks were maintained by a static renewal procedure. The entire volume of water in each tank, along with the corresponding FLX treatment dose, was renewed every 48 h. The control tank received renewed treatments of 95% ethanol only.

Tank water temperature was monitored, maintained and renewed at 13°C. Chemical parameters including pH, nitrate, nitrite and ammonia were also monitored throughout the entire experiment. Garbage bags were placed over top each tank, in order to minimize light exposure to the trout and reduce the impact of external stressors.

At the end of the FLX exposure period, half of the trout in each tank were immediately anaesthetized with benzocaine (35 mg/L) and were subsequently euthanized by severing the spine. The remaining trout were netted and exposed to air for 30 sec. Following this induced stressor; the trout were returned to their tanks for 30 min allowing cortisol levels to reach those induced by the air-stress (Ilan and Yaron, 1983; Lima *et al.*, 2006). Fish were then anaesthetized with benzocaine (35 mg/L) and were euthanized by severing the spine.

3.1.4 Head Kidney Tissue Isolation

Following euthanasia, a lengthwise dissection cut was made in the ventral surface of each fish and the entire digestive tract was removed. Complete head kidneys were removed

from the trout and quickly transferred to 1.5 ml tubes. Tissue samples were immediately flash frozen in liquid nitrogen and stored at -80°C.

3.1.5 Preparation of Head Kidney Microsomes

Head kidney samples were removed from -80°C, weighed and placed on ice in corresponding Sorvall centrifuge tubes. Identically treated samples were combined together, so that the sample content weight of each tube was 1 g. A 2 ml aliquot of cold Tris homogenizing buffer (50 mM Tris, 20% glycerol, 142 mM EDTA, 1 M NaOH; pH 7.4) was added to each centrifuge tube. Head kidneys were homogenized for 5 sec using a Polytron homogenizer (setting #4), set on ice for 30 sec and then homogenized for an additional 5 sec. Following homogenization, additional metabolism buffer was added to each centrifuge tube, resulting in a buffer volume equal to four times the weight of the tissue sample. Sample tubes were placed in a Sorvall RC 50 Plus centrifuge and spun at 10 000 x g for 10 min at 4°C. The resulting supernatant was poured into a second set of Sorvall centrifuge tubes and spun again at 10 000 x g for 10 min at 4°C. Supernatant was then decanted into corresponding ultracentrifuge tubes and spun at 39 000 rpm (176,000 $g_{\max}$) for 1 hr at 4°C in a Beckmann L7 Ultracentrifuge. The resultant supernatant was discarded and the microsomal pellet was resuspended in a metabolism buffer volume equivalent to half the weight of the original tissue sample. The pellets were detached from the ultracentrifuge tubes using a tiny spatula and then decanted into glass tissue grinder tubes. Microsomal pellets were ground 5-6 times with a motorized tissue grinder, transferred to corresponding glass tubes and vortexed. A 50 μ l volume of microsomes from each sample, was added to a corresponding round-bottomed glass tube containing 3 mL of ^{14}C -pregnenolone substrate solution (pregnenolone 1 μM

(75,000 dpm) NADPH (500 μ M), NAD⁺ (500 μ M) in assay buffer pH 7.4). The remaining volume of microsomes was kept at -20°C for subsequent protein quantification.

Substrate tubes were incubated in a 37°C water bath with gentle agitation. At 30, 60 and 90 min of incubation time, each tube was vortexed and 1 ml aliquot of substrate solution was withdrawn and transferred to corresponding round bottomed glass test tubes containing 5 ml ethyl acetate and steroid carriers (30 μ g each of: pregnenolone, progesterone, 17 α -hydroxypregnenolone, 17 α -hydroxyprogesterone, deoxycorticosterone, corticosterone, 11-deoxycortisol, cortisol). Immediately after the addition of the solution aliquots, the test tubes were vortexed in order to stop the metabolic reaction and extract the steroids. Phase separation was achieved by centrifugation at 1000 rpm for 10 min in an IEC Centra-7 centrifuge. Following centrifugation, test tubes were placed in an acetone-dry ice bath, freezing the lower aqueous layer while allowing the organic layer to be decanted into corresponding conical glass centrifuge tubes. The centrifuge tubes were placed in a Savant Speed Vac Plus SC210A for 1-2 hrs, allowing the liquid contained in each sample to evaporate to dryness. Steroid residues were then resuspended in 50 μ l of a 1:1 chloroform: methanol solution and vortexed. The sample tubes were rotated during resuspension and vortexing, in order to ensure that any steroids dried on the sides of the tubes would be resuspended in solution. Sample tubes were tightly capped and the resuspended steroid solutions were left at room temperature overnight.

3.1.6 Steroid Separation by Thin Layer Chromatography

The following morning, tubes were uncapped and any dried steroid residues were resuspended in 50 μ l of a 1:1 chloroform: methanol solution. Each steroid extract was applied to a corresponding Thin Layer Chromatography (TLC) silica gel G plate using a

capillary tube. Steroid samples were applied at points of origin located in the bottom left-hand corner of the TLC plates, with no application spot exceeding 1 cm in diameter.

The plates were developed in chloroform: acetone (17:3 v/v) in the first dimension. The plates were removed from the solvent and allowed to air dry in the fume hood. The plates were then developed in the second dimension in hexane: ethyl acetate (10:7 v/v) run twice. When completely dry, each TLC plate was wrapped smoothly and tightly in SaranTM original saran wrap and placed face up in a light-tight exposure cassette. A storage phosphor screen was laid directly over each plate. Prior to placement over the plates, phosphor screens were exposed to a light source (Image Eraser) to ensure removal of any latent radioactivity from previous usage. The exposure cassettes were sealed shut and left overnight, permitting the transfer of radioactive images from the TLC plates to the phosphor screens.

The following day, phosphor screens were removed from the exposure cassettes and the transferred radioactive steroid images were detected and scanned by the Storm 820 optical scanner (Amersham Biosciences Corp., Piscataway, NJ, USA). The Storm was set in phosphore mode, pixilation at 100 microns, excitation at 635 nm (red) and emission at 390 BP. After scanning, all phosphor screens were exposed to electric light (Image Eraser) to allow for later use. TLC plates were boxed and stored for additional and future analysis if needed.

3.1.7 Steroid Analysis

Steroid metabolites concentrations were determined using ImageQuant TL Analysis software (Amersham Biosciences Corp., Piscataway, NJ, USA). Scanned radioactive images were imported from the Storm 820 (Amersham Biosciences Corp.) into ImageQuant and analyzed using the 'Analysis Toolbox' software tool. Carrier steroids were located by

exposure to UV light and iodine vapor and used to identify the migration position of each steroid and determine which steroids were present in each radioactive TLC steroid separation.

The percentage of radioactivity contained in each steroid compound was determined by measuring the degree of color contrast between the selected compound and the blank background. Steroid concentration was determined by converting the percentage of radioactivity to pmol/mg protein using the protein concentration determined in each microsome sample.

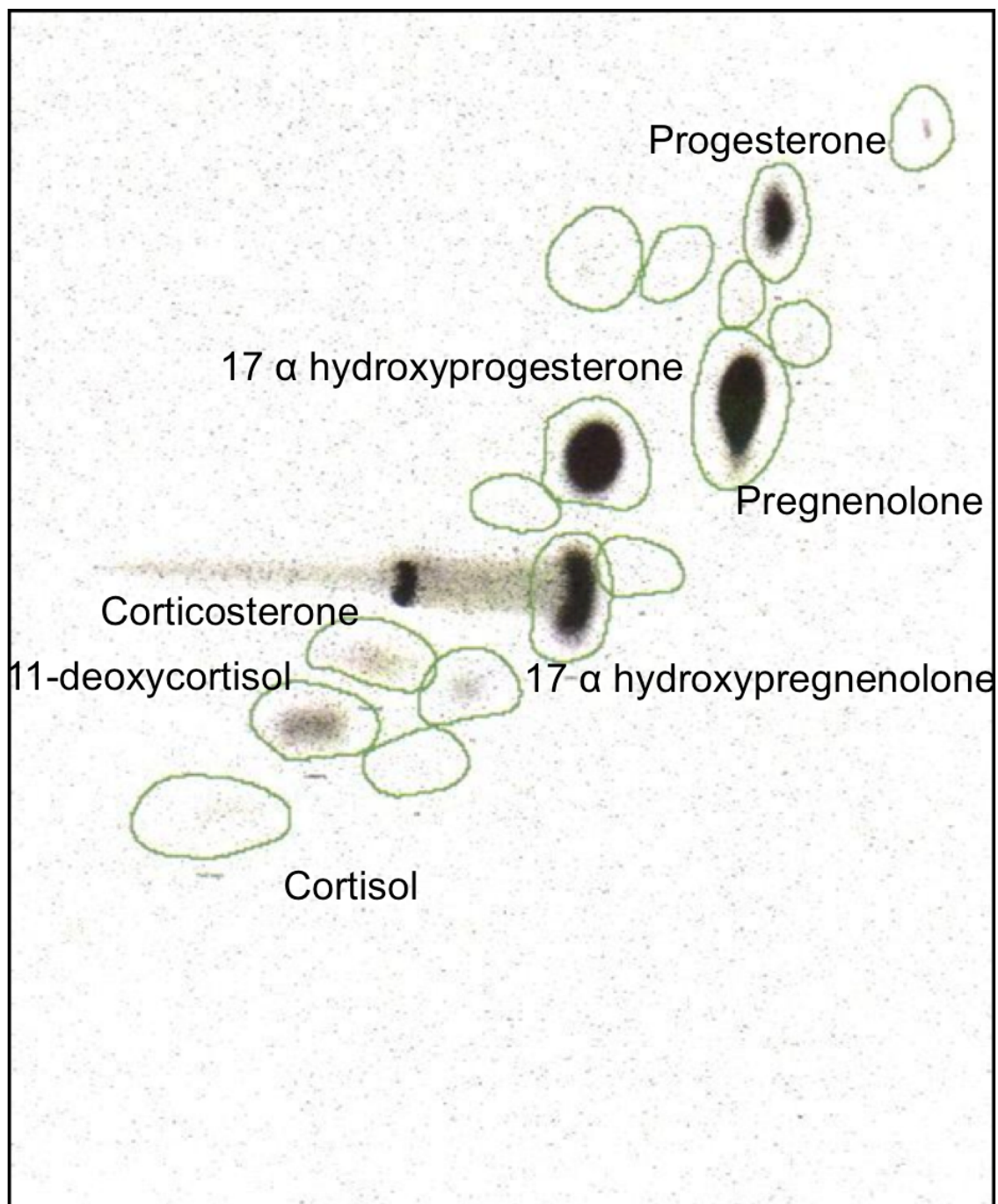


Figure 3.2. Image of TLC chromatography plate showing ^{14}C -pregnenolone metabolism in control head kidney microsomes of rainbow trout

3.1.8 Protein Quantification

The protein concentration of each microsome sample was determined using the Bicinchoninic Acid (BCA) Protein assay. A 10 mg/ml albumin bovine (BSA) solution was prepared in Tris-HCl homogenizing buffer (100 mM Tris, 250 mM sucrose, 25 mM KCl, 5 mM MgCl₂, 7 mM mercaptoethanol, 100 mM HCl, 500 µM NADPH; pH 7.4) and a serial dilution was done in order to get BSA concentrations of 10, 8, 6, 5, 4, 2, 1, 0.5 and 0 mg/ml and establish a standard curve. Microsome samples were removed from -20°C, diluted 20x in Tris-HCl homogenizing buffer and kept on ice.

A solution of 20 ml BCA and 400 µl copper (II) sulphate was made in a Pyrex® glass beaker and kept at room temperature. Ten µl aliquots of the diluted samples and standard solutions were added (in triplicate) to wells of a 96-well microplate. A 200-µl aliquot of the BCA-copper (II) sulphate solution was added to each well and the microplate was incubated for 18 min at 37°C in a microplate heat block. Absorbance was read at 560 nm using a spectrophotometer and the Softmax® Pro software program (Molecular Devices Corp.).

3.1.9 Statistical Analysis

One-way ANOVA with Bonferroni's Multiple Comparison Test was performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA, USA; www.graphpad.com). Values of $p < 0.05$ were accepted as significant. Results were graphed using GraphPad Prism (GraphPad Software) with steroid metabolism over time presented by nonlinear regression curves, calculated using polynomial (second order) equations.

3.2 Results

Cortisol biosynthesis in rainbow trout head kidney microsomes was determined by incubating the isolated microsomes with ^{14}C -pregnenolone (1 μM , 75,000 dpm). Steroid metabolite production in each microsomal sample was quantified and analyzed at 30, 60 and 90 min of incubation time, as described in Section 3.1. Microsomal samples were isolated from head-kidney tissue samples each weighing 1 g. Metabolite concentrations (pmol/mg protein) were determined using the protein concentration of each microsomal sample.

In all microsomal samples isolated from control (0 $\mu\text{g/L}$), 0.54 $\mu\text{g/L}$ FLX- and 54 $\mu\text{g/L}$ FLX-treated trout, the radioactively labeled metabolites: pregnenolone (P5), progesterone (P4), corticosterone, 17 α -hydroxypregnenolone (17P5), 17 α -hydroxyprogesterone (17P4) and 11-deoxycortisol (see Figure 3.2) were visible and quantified at all incubation time points. A number of unknown metabolites were also produced in the microsomal samples, but the concentrations of these were very low and did not differ significantly between the control, air-stressed and drug exposed samples. 11-deoxycorticosterone and cortisol were not traceable in any of the microsomal samples. Corticosterone production was not significantly different between any of the microsomal samples. Table 3.1 summarizes the significant differences found in metabolite production between all microsomal samples.

3.2.1 Steroidogenesis in Control Head Kidney Microsomes

In the control (no FLX or air-stress exposure) microsomal samples, ^{14}C -P5 conversion to P4 and 17P5 was relatively equal. At 90 min of incubation time, $\sim 5.48 \times 10^4$ pmol/mg protein of the ^{14}C -labeled P5 substrate had been metabolized to downstream

metabolites (Fig. 3.2). Concentrations of the initial metabolites of ^{14}C -P5 metabolism were 1.88×10^3 pmol/mg protein for P4 (Fig. 3.3) and 1.78×10^3 pmol/mg protein for 17P5 (Fig. 3.4). Therefore, neither ^{14}C -P5 conversion to P4 (via 3β -HSD) nor 17P5 (via 17-OHase) was shown to be a predominant pathway in ^{14}C -P5 metabolism in head-kidney microsomes isolated from control (i.e. non-stressed and non-FLX-treated) rainbow trout. Concentrations of the more downstream metabolites were 1.24×10^3 , 2.26×10^3 and 5.09×10^2 pmol/mg protein for corticosterone (Fig. 3.5), 17P4 (Fig. 3.6) and 11-deoxycortisol (Fig. 3.7), respectively.

3.2.2 Effects of Air-Stress on Steroidogenesis in Control Head Kidney Microsomes

Microsomes isolated from non-FLX-treated (control) trout subjected to a 30 sec air-stress had metabolized $\sim 6.83 \times 10^4$ pmol/mg protein of P5 at 90 min of incubation. P4 concentration was 4.0×10^3 pmol/mg protein at 90 min, and production was significantly higher ($P < 0.05$) than P4 production in the control (no air-stress) microsomal sample. Corticosterone was at a concentration of 1.14×10^3 pmol/mg protein, while 17P5 concentration was 1.99×10^3 pmol/mg protein at the end of the incubation period. 17P4 concentration was 1.03×10^4 pmol/mg protein and its production was higher, but not significantly higher, than 17P4 production in the control (no air-stress) microsomes. 11-deoxycortisol was at a concentration of 9.38×10^2 pmol/mg protein. Compared with the metabolite production and concentrations formed in the control (no air-stress) microsomal samples, exposure to an acute stress shifts the metabolic pathway of ^{14}C -P5 towards cortisol being the final metabolic end product (versus corticosterone).

3.2.3 Effects of FLX Exposure on Steroidogenesis in Head Kidney Microsomes

Microsomes exposed to a low (0.54 µg/L) FLX concentration exhibited conversion of ^{14}C -P5 that was significantly greater than that in the control (no air-stress) microsomes ($P < 0.05$). At 90 min of incubation, 7.45×10^4 pmol/mg protein of ^{14}C -P5 had been converted to downstream metabolites. Production of 17P5, 17P4 and 11-deoxycortisol was significantly higher ($P < 0.05$) in this low-dosed FLX microsomal sample (versus the control sample).

A concentration of 7.24×10^4 pmol/mg protein of ^{14}C -P5 had been converted to downstream metabolites (at 90 min of incubation) in microsomes isolated from rainbow trout exposed to the high (54 µg/L) FLX concentration. Conversion of ^{14}C -P5 and production of 17P4 were both significantly higher than in the control microsomal sample ($P < 0.05$).

3.2.4 Effects of Air-Stress Combined with FLX Exposure on Steroidogenesis in Head Kidney Microsomes

The combination of a low FLX concentration and 30 sec air-stress resulted in $\sim 6.90 \times 10^4$ pmol/mg protein of ^{14}C -P5 having been converted at 90 min of incubation. P4 and corticosterone were at concentrations of 3.00×10^3 and 1.18×10^3 pmol/mg protein, respectively. 17P5 was at a concentration of 1.90×10^3 pmol/mg protein and its production was significantly lower than in microsomes exposed to 0.54 µg/L FLX only ($P < 0.05$).

The microsomal sample isolated from rainbow trout exposed to 54 µg/L FLX and a 30 sec air-stress demonstrated the greatest metabolism of ^{14}C -P5 with a concentration of only 2.46×10^4 pmol/mg protein of unconverted ^{14}C -P5 remaining at the end of the 90 min incubation period. The conversion of ^{14}C -P5, as well as production of P4, 17P5, 17P4 and 11-deoxycortisol was significantly higher than that in control microsomes. The production of

17P5, 17P4 and 11-deoxycortisol were all significantly higher in the 54 $\mu\text{g/L}$ FLX plus air-stress sample versus the 0.54 $\mu\text{g/L}$ FLX plus air-stress microsomal sample ($P < 0.05$).

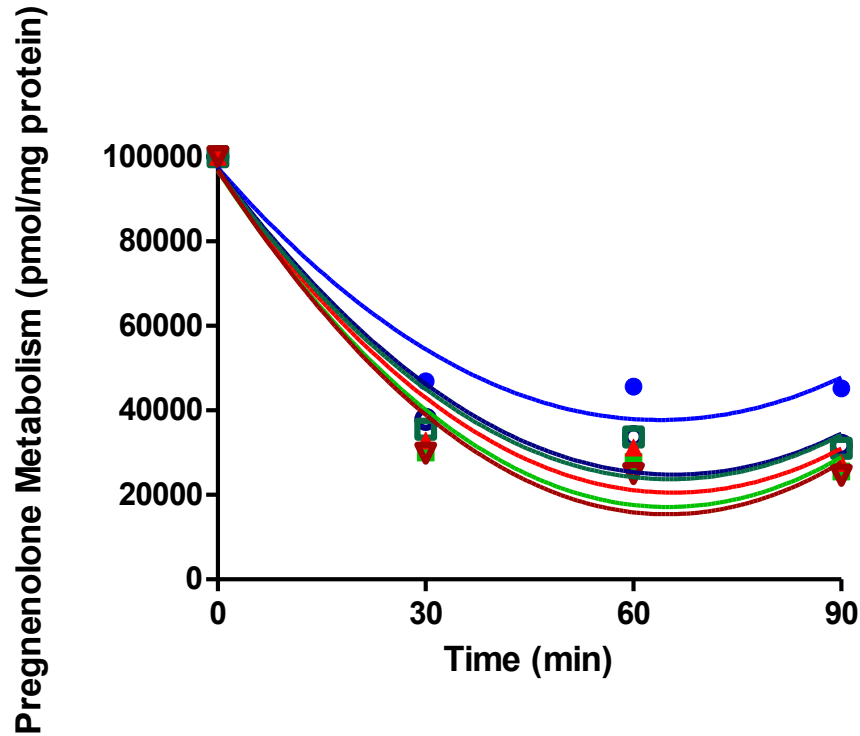


Figure 3.3. Time course of ^{14}C -pregnenolone (P5) metabolism by microsomes isolated from rainbow trout head kidneys. Metabolism in microsomes isolated from trout exposed to FLX treatments of 0 $\mu\text{g/L}$ (Control) (●), 0 $\mu\text{g/L}$ plus air-stress (○), 0.54 $\mu\text{g/L}$ (■), 0.54 $\mu\text{g/L}$ plus air-stress (□), 54 $\mu\text{g/L}$ (▲) and 54 $\mu\text{g/L}$ plus air-stress (▼) are presented. Nonlinear regression curves were drawn and calculated – using polynomial, second order equations – by GraphPad Prism (GraphPad Software, San Diego, CA, USA).

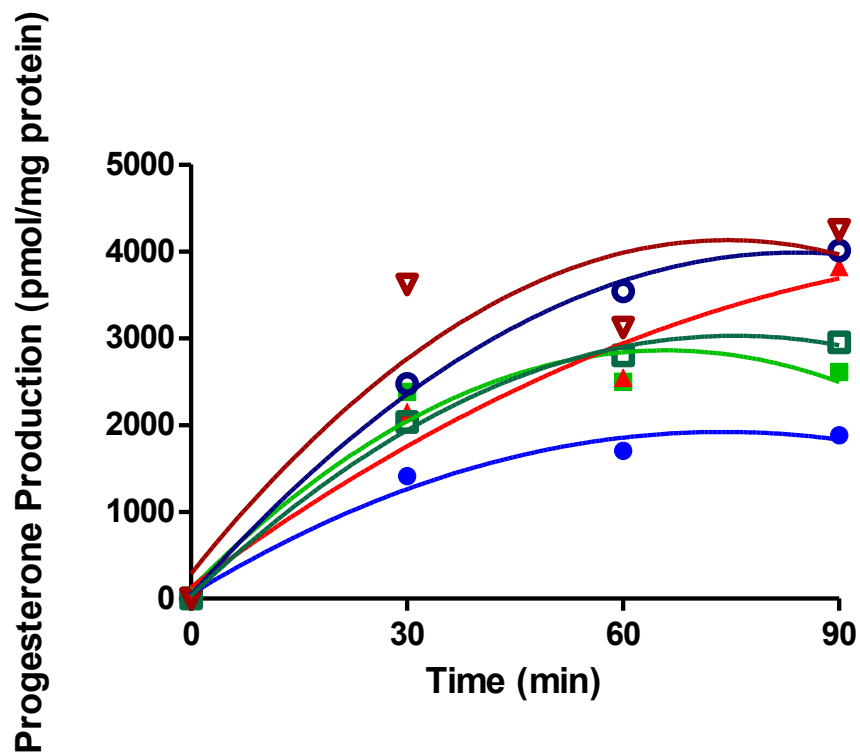


Figure 3.4. Time course of progesterone (P4) production by microsomes isolated from rainbow trout head kidneys. P4 production in microsomes isolated from trout exposed to FLX treatments of 0 µg/L (Control) (●), 0 µg/L plus air-stress (○), 0.54 µg/L (■), 0.54 µg/L plus air-stress (□), 54 µg/L (▲) and 54 µg/L plus air-stress (▽) are presented. Nonlinear regression curves were drawn and calculated – using polynomial, second order equations – by GraphPad Prism (GraphPad Software, San Diego, CA, USA).

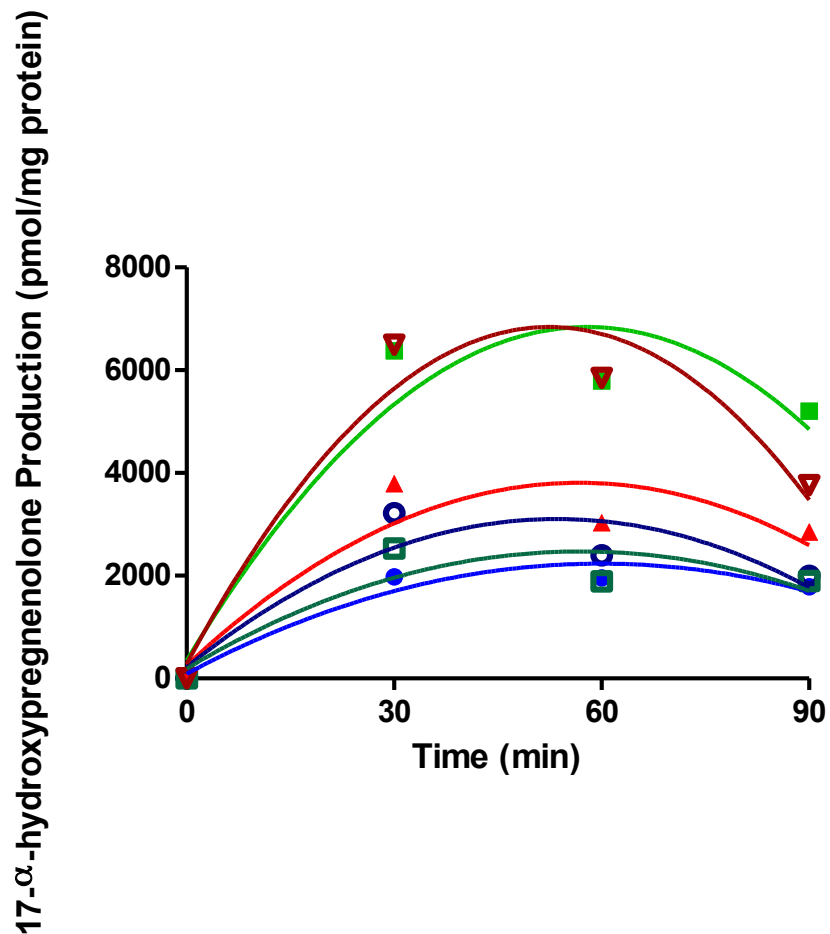


Figure 3.5. Time course of 17 α -hydroxypregnenolone (17P5) production by microsomes isolated from rainbow trout head kidneys. 17P5 production in microsomes isolated from trout exposed to FLX treatments of 0 μ g/L (Control) (●), 0 μ g/L plus air-stress (○), 0.54 μ g/L (■), 0.54 μ g/L plus air-stress (□), 54 μ g/L (▲) and 54 μ g/L plus air-stress (▼) are presented. Nonlinear regression curves were drawn and calculated – using polynomial, second order equations – by GraphPad Prism (GraphPad Software, San Diego, CA, USA).

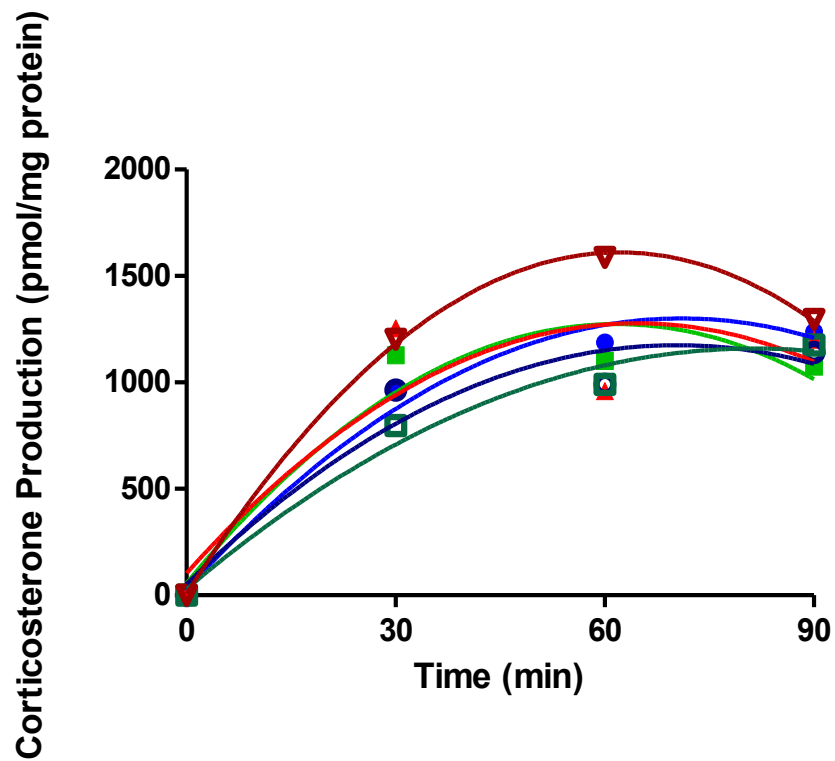


Figure 3.6. Time course of corticosterone production by microsomes isolated from rainbow trout head kidneys. Corticosterone production in microsomes isolated from trout exposed to FLX treatments of 0 µg/L (Control) (●), 0 µg/L plus air-stress (○), 0.54 µg/L (■), 0.54 µg/L plus air-stress (□), 54 µg/L (▲) and 54 µg/L plus air-stress (▼) are presented. Nonlinear regression curves were drawn and calculated – using polynomial, second order equations – by GraphPad Prism (GraphPad Software, San Diego, CA, USA).

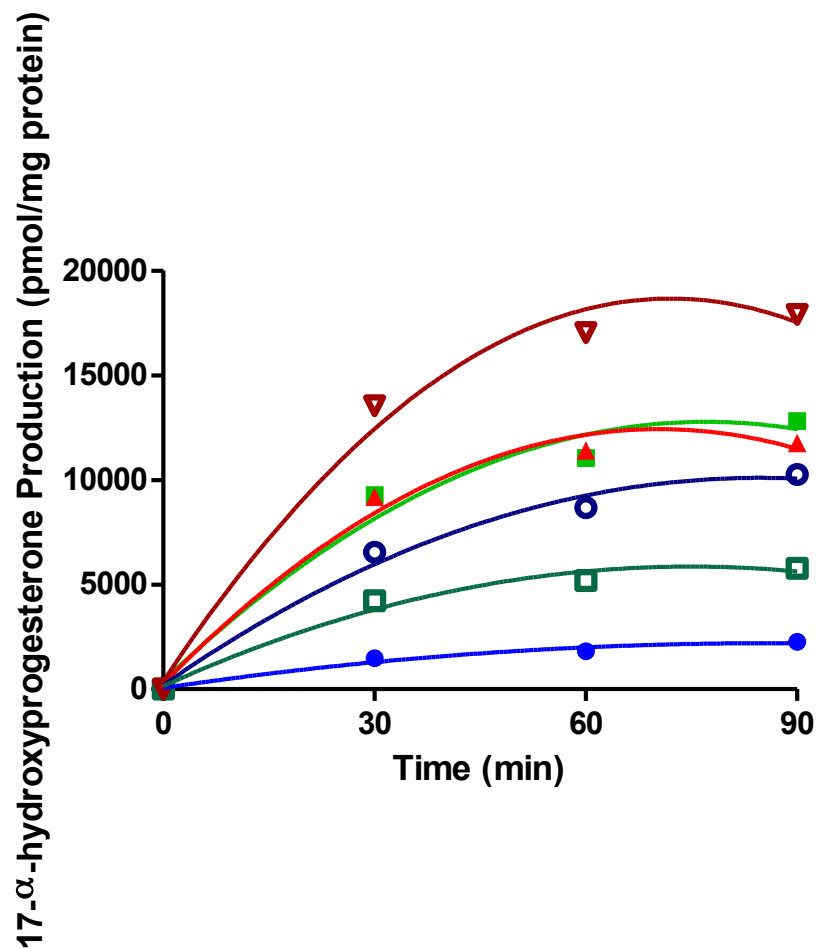


Figure 3.7. Time course of 17α -hydroxyprogesterone (17P4) production by microsomes isolated from rainbow trout head kidneys. 17P4 production in microsomes isolated from trout exposed to FLX treatments of 0 $\mu\text{g/L}$ (Control) (●), 0 $\mu\text{g/L}$ plus air-stress (○), 0.54 $\mu\text{g/L}$ (■), 0.54 $\mu\text{g/L}$ plus air-stress (□), 54 $\mu\text{g/L}$ (▲) and 54 $\mu\text{g/L}$ plus air-stress (▼) are presented. Nonlinear regression curves were drawn and calculated – using polynomial, second order equations – by GraphPad Prism (GraphPad Software, San Diego, CA, USA).

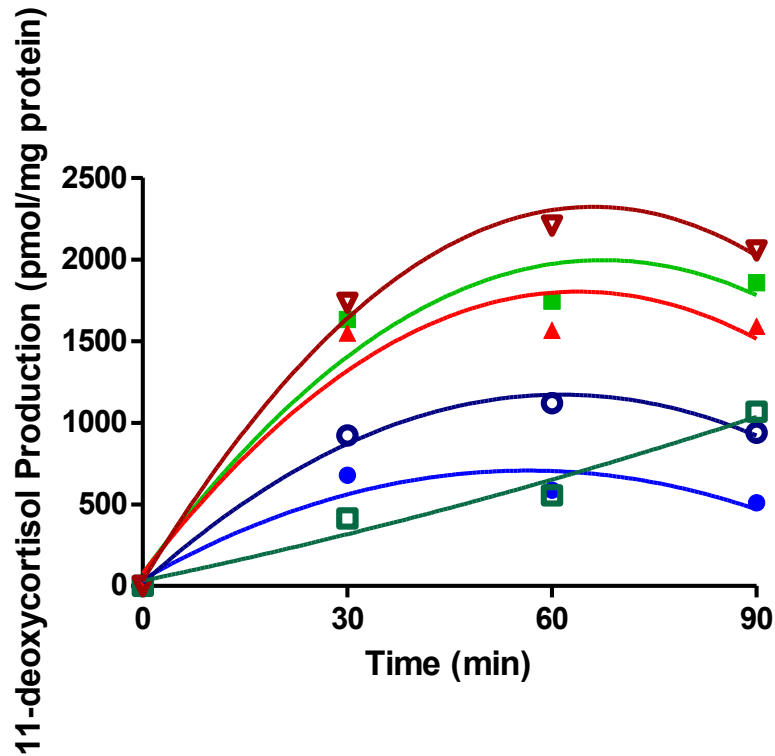


Figure 3.8. Time course of 11-deoxycortisol production by microsomes isolated from rainbow trout head kidneys. 11-deoxycortisol production in microsomes isolated from trout exposed to FLX treatments of 0 µg/L (Control) (●), 0 µg/L plus air-stress (○), 0.54 µg/L (■), 0.54 µg/L plus air-stress (□), 54 µg/L (▲) and 54 µg/L plus air-stress (▽) are presented. Nonlinear regression curves were drawn and calculated – using polynomial, second order equations – by GraphPad Prism (GraphPad Software, San Diego, CA, USA).

Table 3.1. Comparison of ^{14}C -pregnenolone metabolism and steroid metabolite production in isolated head kidney microsomes. Metabolite production in microsomal samples was measured at 30, 60 and 90 min of incubation with ^{14}C -P5 substrate at 37°C. C = control; L = low dose FLX; H = high dose FLX; S = air-stressed; ↑ = significantly higher metabolism or metabolite production (vs. comparison treatment); ↓ = significantly lower metabolism or metabolite production (vs. comparison treatment).

FLX Treatment	Metabolite						Comparison Treatment
	P5*	P4	Cortico.	17P5	17P4	11-deoxy	
C (0 µg/L)		↓					C+S
	↓						L+S
	↓	↓		↓	↓	↓	H+S
	↓			↓	↓	↓	L
	↓				↓		H
L (0.54 µg/L)				↑			C+S
				↑			L+S
							H+S
H (54 µg/L)							C+S
							L+S
C+S							H+S
L+S				↓	↓	↓	

* = indicates that the metabolism (not production) of the metabolite is being compared between treatments

3.3 Discussion

This study investigated the effects of FLX exposure on corticosteroidogenesis in isolated head-kidney microsomes of rainbow trout (*O. mykiss*). Microsomes are vesicle-like artifacts, formed from the endoplasmic reticulum when cells are lysed (Voet and Voet, 2004), that have been successfully used in previous *in vitro* studies investigating: steroid metabolism, enzyme inhibition and drug-drug interactions (Lacarelle et al., 1991; Kominami et al., 1992). In this present study, microsomes were used in order to assess ^{14}C -pregnenolone metabolism and ultimately, cortisol production in head-kidneys isolated from trout exposed to control (0 $\mu\text{g/L}$), low- (0.54 $\mu\text{g/L}$) and high- (54 $\mu\text{g/L}$) dose FLX treatments. My original hypothesis stated that FLX exposure alters cortisol biosynthesis in teleost fish and I further predicted that corticosteroidogenesis would be up-regulated in rainbow trout exposed to waterborne FLX. The results obtained from this experimental study support both my hypothesis and my prediction regarding the activity of the steroidogenic pathway. In control microsomes ^{14}C -P5 was converted to P4 and 17P5 relatively equally, suggesting that at a resting state, the steroidogenic pathway produces corticosterone and cortisol in relatively equal amounts under the *in vitro* conditions used in this study. Microsomes isolated from trout exposed to an acute stressor (i.e. 30 sec of air-stress) had greater concentrations of the steroid metabolites leading to the formation of cortisol (versus corticosterone). This suggests that stressors stimulate the steroidogenic pathway to shift towards the production of more cortisol, emphasizing its role as the primary stress hormone in teleost fish (Arends et al., 1999). Exposure to 0.54 $\mu\text{g/L}$ FLX also resulted in greater conversion of ^{14}C -P5 and increased production of 17P5, 17P4 and 11-deoxycortisol than observed in control microsomes. Similar results were observed with the 54 $\mu\text{g/L}$ FLX, which suggests that FLX

disrupts homeostasis in teleosts and/or is perceived as a stressor by the HPI axis. The greatest shift towards cortisol production was observed in microsomes isolated from trout exposed to 54 µg/L FLX and exposed to an air-stress. These results implicate a stressor- and/or FLX-mediated up regulation of 3β-HSD and 17-OHase expression and/or their enzymatic activity, especially because production of the 17P4 metabolite demonstrated the greatest variation amongst the different microsomal samples, according to stressor and/or FLX exposure. Unfortunately, this implication of altered enzymatic activity cannot be confirmed, as RNA expression of steroidogenic enzymes was not measured in this study. P4 production (via 3β-HSD) was only significantly higher in the control plus air-stress and 54 µg/L plus air-stress microsomes (versus control), while 17P5 concentrations differed much more significantly across the microsomal samples (Table 3.1). This suggests that FLX, and even stress, does not impact P4 and 17P5 production and conversion equally. If these two intermediates were impacted equally, the concentration differences of P4 and 17P5 between samples would be nearly identical and the concentrations within each sample would be relatively equal. Furthermore, microsomes from 0.54 µg/L FLX trout demonstrated higher production of 17P5 than air-stressed control and 0.54 µg/L FLX microsomes. This observation could be indicative of an FLX-mediated modification in a more upstream component of the HPI axis, such as modulation in CRF expression (Itoi et al., 1998) or in the head kidney cell response to ACTH (Vazzana et al., 2010; Norris, 2000), that occurs at this particular drug concentration and exposure time. However, because substrate conversion and steroid metabolite production is occurring along a pathway, lower concentrations in the more upstream metabolites (i.e. P4 and 17P5) could be the result of a higher conversion rate to the more downstream metabolites (i.e. 17P4, 11-deoxycortisol) resulting from stress or FLX. The

54 µg/L FLX plus air-stress microsomes produced the highest concentration of 11-deoxycortisol, which further supports that FLX stimulates the same response from interrenal microsomal enzymes as an external, physical stressor (Barton, 2002). Significant differences in steroid metabolism and metabolite production did not demonstrate consistency or additive effects amongst the stressed and FLX-treated microsomes, possibly suggesting that there are dose-dependent differences in enzymatic conversion rates between the FLX-treated microsomes. 11-deoxycorticosterone was not traceable in any of the microsomal samples, likely due to the rapid conversion of this metabolite to corticosterone. Still, no significant differences in corticosterone production were observed between any of the control, FLX- and/or stress-treated microsomal samples. CYP21 (21-OHase) is the enzyme responsible for the conversion of P4 to 11-deoxycorticosterone, while CYP11B1 (11β-OHase) and CYP11B2 (18-OHase) convert 11-deoxycorticosterone to corticosterone (Margioris et al., 2006). However, 21-OHase also converts 17P4 to 11-deoxycortisol and 11β-OHase converts 11-deoxycortisol to cortisol (Mommensen et al., 1999). As significant differences in 11-deoxycortisol production (via 21-OHase) were observed among the different microsomal samples, it's likely that enzymatic activity is affected by stress and FLX, but also influenced by substrate availability. An up regulation in the activities of 3β-HSD and 17-OHase – resulting in greater 17P4 production – would reduce the amount of substrate available for enzymatic conversions downstream of P4. Corticosterone is present in some fish species, where it has glucocorticoid and mineralocorticoid properties and functions similar to those of cortisol (Høgåsen, 1998). However, fish also have the enzymatic capacity – via 17α-hydroxylation – to convert corticosterone to cortisol (Fig. 3.1) (Prunet et al., 2006). Circulating levels of corticosterone in fish are often much lower than those of cortisol

(Høgåsen, 1998) and, as previously mentioned, comparing the concentrations (pmol/mg protein) of radiolabeled intermediates in the control (0 µg/L FLX) and control plus air-stress samples, indicates that exposure to acute stress results in higher concentrations of intermediates leading to cortisol as the pathway end product (versus corticosterone). It is unlikely that any alterations in 17-OHase activity in converting corticosterone to cortisol significantly affected cortisol production in this experiment. If there were stressor- or FLX-mediated alterations, then differences in corticosterone concentrations would have been observed across the microsomal samples. Whether the up-regulation of corticosteroidogenesis observed in FLX-exposed microsomes is a direct result of FLX exposure or occurs due to the SSRI's modulation of serotonergic activity in trout remains to be determined.

Interestingly, cortisol, the major glucocorticoid in teleosts (Mommensen et al., 1999; Høgåsen, 1998), was not traceable in any of the microsomal samples. This is likely due to the 90 min incubation period being inadequate for allowing sufficient conversion of ¹⁴C-P5 to cortisol. It is possible that 11β-OHase activity did not change regardless of stressor or FLX exposure. However, unchanged or down regulated steroidogenic activity in rainbow trout and experimental error are both unlikely, as plasma samples taken from trout exposed to the different waterborne FLX treatments (see Section 2.2.3) contain cortisol concentrations that reflect differences observed in the metabolism of ¹⁴C-pregnenolone.

To the best of my knowledge, this study is the first to investigate how cortisol biosynthesis in a teleost can be altered by exposure to an SSRI-type pharmaceutical. The experimental design of the study allowed me to achieve my research objective of determining if an SSRI can alter steroidogenesis in teleost fish, yet it still presented several

limitations and opportunities for more in-depth research of this topic. The most notable limitation was the use of ^{14}C -P5 substrate. Using P5 as the initial substrate eliminates the first and rate-limiting step of corticosteroidogenesis – that being the enzymatic translocation and conversion of cholesterol to P5 (Geslin and Auperin, 2004; Papadopoulos, 2004). A number of studies examining the effects of pharmaceuticals and endocrine disruptors on steroidogenesis in fish have found alterations in the expression of StAR and PBR (Arukwe, 2008; Gravel and Vijayan, 2006; Mimeault et al., 2005). Therefore, it could not be determined if FLX-mediated alterations in corticosteroidogenesis occur via alteration of the translocation and conversion of cholesterol into P5. However, given that P5 substrate was equally available in all microsomal samples in this study, if FLX only impeded the functions of the enzymes involved in cholesterol transport and conversion, then P5 metabolism and steroid production in head kidney microsomes from all FLX treatments would be relatively equal. FLX and the other SSRIs have demonstrated the ability to alter the metabolic functions of P450 enzymes in the liver (e.g. CYP2D6) (DeVane, 1999). The CYP enzymes share similar amino acid sequences, which results in similarities in the enzymatic functions of these proteins (Hemeryck and Belpaire, 2002). Therefore, it is likely that the CYP enzymes expressed in the head kidney will be influenced by SSRI exposure, with the degree of change likely depending on both drug dose and the metabolic status of the animal (Lam et al., 2002).

Another limitation to this study is that conclusions regarding FLX's effects on head kidney steroidogenesis can only be based on differences in steroid metabolite concentrations. FLX-mediated differences in enzyme kinetics can only be speculated, as enzyme concentrations were not measured. According to Michaelis–Menten kinetics, enzyme-

catalyzed reactions are saturable therefore, enzymatic rate does not demonstrate a linear response to substrate concentration (Lehninger et al., 2005). An equal amount of initial substrate (i.e. ^{14}C -P5) was incubated with each microsomal sample, but FLX-induced alterations in enzyme-substrate affinities, level of enzymatic activity and expression could have occurred but were not measurable in this experiment.

In teleosts, there are multiple hormones and chemical messengers that can influence cortisol production and secretion (Wendelaar Bonga, 1997). As this study evaluated *in vitro* corticosteroidogenesis, any modifications to these additional factors resulting from FLX exposure could not be determined. This study's results do show that FLX can impact corticosteroidogenesis, however the degree to which this pathway is altered could still be further influenced by alterations to these other hormones.

The findings of this study do hold biological significance as 0.54 $\mu\text{g/L}$ FLX is an environmentally relevant concentration (Brooks et al., 2005) and other human pharmaceuticals have been found to affect steroidogenesis in fish (Gravel and Vijayan, 2006; Mimeault et al., 2005). Furthermore, the steroidogenic pathway for cortisol is similar to those for the production of reproductive hormones and mineralocorticoids in other vertebrates (Bowen, 2001; Lin et al., 1995). Therefore, these results reveal the potential for FLX to target and alter multiple endocrine and/or physiological systems in teleosts.

3.4 Conclusions

SSRI-type pharmaceuticals are highly prescribed pharmaceuticals that are continuously entering aquatic systems, where they bioconcentrate in organisms like teleost fish (Smith et al., 2010; Brooks et al., 2005). Previous studies have shown that certain pharmaceuticals can impair hormone steroidogenesis in fish, by targeting the enzymes

involved in steroidogenic pathways (Han et al., 2010; Hontela, 2006). The results of this study have demonstrated that FLX exposure has the potential to alter steroid metabolism in rainbow trout head kidney microsomes. Steroidogenic enzymes are involved not only in the production of cortisol, but also other steroid hormones including testosterone and estradiol (Han et al., 2010; Mimeault et al., 2005), implicating that FLX's effects will extend beyond the teleost stress response. This is the first study to examine how FLX affects corticosteroidogenesis in teleosts and has provided a basis for understanding this SSRIs effects on steroidogenic pathways. However, further investigation is necessary in order to fully elucidate how FLX impacts steroid metabolism and production.

Chapter 4

Summary and Conclusions

4.0 General Conclusions

The endocrine stress axis and the production of cortisol are necessary to enable teleost fish and other vertebrates to regain homeostasis when confronted with physical, chemical and/or other forms of stress (Barton, 2002). However, chronic exposure of teleosts to stressors and/or compromised activity of the HPI axis are maladaptive and can jeopardize the physiology and overall fitness of the fish (Barton, 2002; Norris, 2000). There is significant evidence for the presence of endocrine disrupting chemicals in aquatic environments (Khetan and Collins, 2007; Hontela, 2006; Kolpin et al., 2002) and that at least some of these chemicals disrupt the functioning of the HPI axis in teleost fish (Hontela, 2006; Leblond et al., 2001; Brodeur et al., 1997).

I used experimental methods previously employed for evaluating cortisol production, cytotoxicity (Mimeault et al., 2007; Leblond et al., 2001) and steroidogenesis, to evaluate the effects of the pharmaceutical FLX, an SSRI, on the stress response in a teleost fish species. I hypothesized that FLX exposure would alter HPI axis activity – and ultimately, cortisol production – in rainbow trout and further predicted that *in vitro* cortisol production would be higher in FLX-exposed interrenals, and that FLX would up regulate corticosteroidogenesis.

The results of this research demonstrated that FLX exposure does alter cortisol production in trout, but not as entirely predicted. Induction of cortisol production (*in vitro*) by ACTH (1 IU/mL) was lower in head kidney (interrenal) cells isolated from trout previously exposed in their tank water to FLX at 0.54 and 54 µg/L compared to non-exposed trout. Cells isolated from FLX-exposed trout subjected to the 30 sec air-stress showed a similar trend in

cortisol production as their unstressed counterparts. Cell viability was notably lower in cells exposed (*in vitro*) to 400 μ M FLX and norFLX, which may account for the low cortisol production observed at this concentration. Although the head kidney cell isolation has been used successfully in past studies (Mimeault et al., 2007; Leblond et al., 2001), steroidogenic cells are still reported to account for just over 0.01% of total head kidney cells in rainbow trout (Hontela et al., 2008) making it possible that a low yield of interrenal cells is responsible for the low ACTH-stimulated cortisol production observed in this *in vitro* experiment.

This suggests that waterborne FLX exposure can compromise the teleost stress response by altering the interrenal response to corticotrophic (i.e. ACTH) stimulation. Waterborne exposure to 54 μ g/L FLX resulted in significantly higher plasma cortisol concentrations in both unstressed and air-stressed rainbow trout. These effects may have been mediated through altered serotonin concentrations resulting from FLX within the CNS.

This study also showed that plasma ACTH concentrations in unstressed trout were significantly lower in fish exposed to 54 μ g/L FLX (versus control and low-dose treated fish) potentially indicating that high cortisol concentrations under these same conditions was exerting negative feedback on the pituitary component of the HPI axis.

4.1 Prospective Future Research

While the results obtained from this research study have demonstrated that FLX can impact the cortisol stress response in teleosts, they do not fully elucidate the mechanism(s) by which FLX induces these alterations in production.

Future research should include the repetition of the *in vitro* and steroidogenic experiments, as additional replicates will validate these experimental findings. An exposure

time of 12 days to FLX concentrations of 0.54 µg/L and 54 µg/L resulted in altered corticosteroidogenesis, plasma cortisol and ACTH concentrations in rainbow trout; however, the observed effects differed at each concentration. FLX-exposure doses between 0.54 and 54 µg/L should also be tested so that a LOEC for specific endocrine changes to the teleost stress axis, resulting from FLX, could be established. Furthermore, a study examining how FLX impacts the teleost stress response when a fish is exposed to different types of stressors (i.e. confinement, predation, etc.) should be conducted, as there is evidence indicating that the degree of activation of the HPI axis is dependent on the type of stressor (Arends et al., 1999).

An analysis of gene expression of the receptors and proteins involved in the HPI axis should also be conducted. Quantification of CRF, POMCA, POMCB, StAR and PBR mRNA levels will show if FLX affects hormone production at points of the stress axis upstream of cortisol formation. The expression and kinetics of the CYP enzymes involved in converting cholesterol to cortisol should also be analyzed, as this would determine if FLX alters the activity of steroidogenic enzymes, providing a better understanding and explanation for the distribution and concentrations of the steroid intermediates obtained in the present study. Another option would be conducting similar experiments using a SSRI other than FLX. Doing so, would allow us to determine how similar each SSRI is in regards to effects on the teleost stress axis and begin to address the question of mixtures that to this point has not been done.

A final follow-up experiment should involve the assessment of brain serotonin levels in fish that result from FLX (or SSRI) exposure. Previous studies on both mammals and fish have shown that serotonin concentrations influence HPA/HPI axis activity (Zhang et al.,

2004; Khan and Deschaux, 1997; Winberg et al., 1997) and measuring 5-HT would allow us to better understand how SSRIs affect 5-HT concentrations in fish during times of stress.

4.2. Perspectives

This research specifically focused on the endocrine disrupting effects of FLX on the teleost stress response, a topic that to date has received little to no attention. Currently, there are over 3000 registered pharmaceutical ingredients available for prescription, including painkillers, antibiotics, contraceptives, impotence drugs and antidepressants like the SSRIs (Schultz and Furlong, 2008). SSRI-type pharmaceuticals are highly prescribed to human patients worldwide, continuously enter aquatic systems, with no effective means of removal (Alonso et al., 2010), where they deposit and bioconcentrate in organisms including teleost fish (Smith et al., 2010; Brooks et al., 2005; Mennigen et al., 2010). A large number of studies have demonstrated that the SSRI, FLX, has endocrine disrupting effects in teleosts (Gaworecki and Klaine, 2008; Mennigen et al., 2008; Stanley et al., 2007; Lister et al., 2009; Mennigen et al., 2009), and many other studies have provided evidence that altering serotonin concentrations in vertebrates alter the production of stress hormones and/or an animal's response to these hormones (Heisler et al., 2007; Zhang et al., 2004; Fritsche et al., 1993). In teleosts, cortisol has multiple and fundamental physiological roles, in addition to helping maintain homeostasis (Gilmour, 2005; Wendelaar Bonga, 1997).

In a biological context, the increased plasma cortisol concentrations observed in this study at high FLX exposures, could compromise osmoregulation (McCormick, 2001), immune function (Laflamme et al., 2000) and reproductive capacity in teleosts (Schreck et al., 2001). These physiological changes would result in changes to fish behaviors overall and could lead to reduced fish population sizes and a general decline in the adaptability and

survival capacity of fish ultimately affecting fitness (McCormick, 2001; Barton et al., 1987). Activating the endocrine stress response by using a standardized air-stress provides an indication of the potential hormonal response of teleosts to acute stressors in natural environments where SSRIs are present. Inducing cortisol production in isolated head-kidneys using a corticotropic agent like ACTH can provide an indication of how interrenal integrity is affected by FLX or other SSRIs, which will ultimately determine how well teleosts can cope with and respond to stressors (Barton, 2002; Wendelaar Bonga, 1997).

In an ecological context, these biological consequences will alter fish-influenced processes and properties of ecosystems, including productivity, nutrient cycling, trophic interactions, vegetation and biodiversity (McIntyre et al., 2007; Hilderbrand et al., 2004). Salmonids in particular, are also commercially valuable fish that provide food and even income to human communities (Guido, 2011). In North America, salmonids support commercial and recreational fishing industries, producing thousands of jobs and grossing billions of dollars in personal income (FAO, 2010; Oregon Rivers Council, 1992). Therefore, alterations in teleost corticosteroidogenesis resulting from FLX exposure could also produce economic losses.

In addition to contributing to the body of literature available in this area of research, the results from this study could also help in educating the general population on the safe uses and disposal of pharmaceuticals and perhaps encourage political powers to invest in upgraded water treatment facilities and services. The FLX concentrations used in this study were higher than environmental concentrations of FLX, but within range of total SSRI concentrations (Metcalf et al., 2010; Brooks et al., 2005). The SSRIs may vary in structure and pharmacokinetics, but they have all been ultimately designed to exert similar effects,

specifically, to alter brain serotonergic activity so that deficits in serotonin concentrations are remediated (CSM, 2004; Hiemke and Härtter, 2000). As such, their combined effects on non-target aquatic organisms like teleosts, are likely to display both similarities to the physiological effects observed in this study, and potential ‘additive’ effects at higher concentrations.

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