The serotonergic system as a target of neuroendocrine disruption for the pharmaceutical fluoxetine in the brain of goldfish (*Carassius auratus*)

by

Jan Alexander Mennigen

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Abstract

Serotonin stimulates reproduction and inhibits feeding/growth in the neuroendocrine brain of goldfish. The objective of this thesis is to study the effects of selective serotonin reuptake inhibitor pharmaceuticals (SSRIs) on these systems, as SSRIs, such as fluoxetine, are detected in effluent and bioconcentrate in the fish brain. Genes of the serotonin system were cloned to identify molecular conservation, seasonal expression, and tissue distribution. The serotonin transporter, the target molecule of fluoxetine, was highly conserved and ubiquitously expressed in goldfish. Seasonal changes of hypothalamic gene expression of the serotonin transporter support a role in the seasonal modulation of both processes. Fluoxetine injection experiments were used to assess effects on reproductive endpoints and to identify molecular mechanisms in the neuroendocrine brain. Fluoxetine inhibited serum estradiol concentrations in female goldfish and decreased isotocin mRNA abundance in the hypothalamus and telencephalon. Isotocin injections stimulated circulating estradiol concentrations, providing a causal link. Evidence for an involvement of serotonin in isotocin regulation was investigated using immunocytochemistry and 5-HT_{1A} receptor agonists and antagonists. A close proximity of serotonin fibers and isotocin cell bodies and fibers was found in the telencephalon and pituitary, respectively. Injection of a 5-HT_{1A} receptor antagonist inhibited isotocin mRNA expression in the telencephalon. Identified gene targets were investigated in waterborne fluoxetine exposures, including environmental concentrations. Waterborne fluoxetine led to a reduction in basal and pheromone-stimulated milt volume in male goldfish. Gene expression evidence indicated a central inhibitory effect of fluoxetine through the decrease in follicle-stimulating hormone in the pituitary and isotocin mRNA abundance in the telencephalon. Feeding rate and weight decreased in fluoxetine-injected goldfish, indicating an anorexigenic effect. Fluoxetine induced changes in

the gene expression of the feeding peptides neuropeptide Y, corticotropin-releasing Factor, and cocaine- and amphetamine-regulated transcript-I in the hypothalamus and telencephalon. Waterborne exposures of fluoxetine validated the anorexigenic effect and was correlated with increased expression of corticotropin-releasing factor mRNA, an anorectic peptide. The thesis provides evidence for disrupting effects of fluoxetine on neuroendocrine control of reproductive function and feeding/growth in goldfish, partially at environmental concentrations. The thesis provides the framework for the investigation of existing aquatic contaminants which modulate the serotonin system.

Résumé

. La sérotonine joue un rôle essentiel dans la régulation des axes neuroendocriens, stimulant l'axe reproducteur et inhibant l'alimentation et la croissance. L'objectif de cette thèse est d'étudier les effets d'une classe de produit pharmaceutique inhibiteurs de la recapture de la sérotonine (ISRS) sur ces systèmes. De récentes recherches ont montré que les ISRS, tels que la fluoxétine, se retrouvent dans les effluents et sont bio-accumulés dans les tissus du poisson et particulièrement dans le cerveau. J'ai cloné les molécules cibles du système de la sérotonine chez le poisson rouge ainsi que identifié leurs conservation au niveau moléculaire, leurs patrons d'expression au cours des saisons et leurs distribution dans les tissus. Le transporteur de la sérotonine, molécule cible de la fluoxétine s'est trouvée être très conservé. Les changements d'expression au cours des saisons dans l'hypothalamus du transporteur de la sérotonine suggèrent un rôle de la sérotonine dans la modulation saisonnière de ces processus. Cette information a été utilisée ensuite pour étudier les effets de dérèglements potentiels de la fluoxétine sur l'axe reproducteur et l'axe de l'alimentation/croissance. La fluoxétine a diminué la concentration d'œstradiol chez la femelle du poisson rouge, un effet qui est corrélé de façon significative avec la diminution d'ARNm de l'isotocine dans le telencéphalon. L'étude a ensuite montré que l'isotocine augmente la concentration *in vivo* d'œstrogène. Cet effet semble ne pas être relié à l'action centrale de l'isotocine sur la libération de LH. Le lien entre l'isotocine et la sérotonine a été étudié grâce à des expériences immuno-histochimiques en plus des agonistes et antagonistes 5-HT_{1A}. Les résultats ont révélé une proximité des fibres de sérotonine et des corps cellulaires d'isotocine et des extrémités de la synapse dans certaines parties du telencéphalon et de la glande pituitaire, respectivement. L'injection de 5-HT_{1A} antagoniste du récepteur a inhibé l'expression d'ARN de l'isotocine dans le telencéphalon. Les gènes identifiés ont été étudiés par

exposition à la fluoxétine dans l'eau notamment à des concentrations environnementales. La fluoxétine à des niveaux environnementaux a réduit le volume basal de la semence ainsi que celui stimulé par phéromones. L'expression génique indique clairement un effet inhibiteur central de la fluoxétine, surtout par la diminution d'hormones stimulatrices du follicule ainsi que de l'ARNm de l'isotocine. La fréquence de prise alimentaire ainsi que le poids se sont révélés être affecté négativement chez les poisons sous injection de fluoxétine, suggérant des propriétés anorexigènes de la fluoxétine chez le poisson rouge. Au niveau de l'expression génique de neuropeptides Y, les transcrits du facteur de libération de la corticotropine, ceux du trancrit regulé par la cocaïne et les amphétamines renforcent cette idée et suggèrent une action du telencéphalon. Les expositions dans l'eau valident aussi l'effet anorexigène de la fluoxétine, ce qui est corrélé avec l'augmentation de l'expression du facteur de libération de la corticotropine, un peptide anorexigénique. Plus précisément, cette thèse démontre l'effet de la fluoxétine sur le dérèglement des fonctions reproductrices chez le mâle à des concentrations comparables à celles de l'environnement. Dans l'ensemble, cette thèse pose les bases de l'étude de multiples contaminants qui agissent sur le système de la sérotonine.

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List of commonly used abbreviations in this thesis (in alphabetical order)

17,20P	17,20β-dihydroxy-4-pregnene-3-one
5-HIAA	5-hydroxyindol acetic acid
5-HT	serotonin
5-HTR	serotonin receptor
AA	amino acid
ACTH	adrenocorticotropic hormone
bp	base pairs
bw	body weight
CART-II	cocaine- and amphetamine regulated transcript
CRF	corticotropin-releasing factor
CNS	central nervous system
DA	dopamine
DO	3,4-Dihydroxyphenylacetic acid
DMSO	dimethyl sulfoxide
DOI	(+/-)-1-(2.5-dimethoxy-4-iodophenyl)-2- aminopropane
E_2	17ß-estradiol
EDTA	ethylenediaminetetraacetic acid
ERα	estrogen receptor α
ERß	estrogen receptor ß
FLX	fluoxetine
FSH	follicle stimulating hormone
FSHR	follicle stimulating hormone receptor
GABA	gamma-amino butyric acid
GH	growth hormone
GHR	growth hormone receptor
GnRH	gonadotropin-releasing hormone
GO	gene ontology
GSI	gonadosomatic index
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
hpf	hours post fertilization
HPLC	high performance liquid chromatography
HVA	homovanilic acid
i.p.	intra-peritoneal
IPTG	isopropyl-beta-D-thiogalactopyranoside
i.c.v.	intra-cerebroventricualar
IT	isotocin
Lep	leptin
Lepr	leptin receptor
LH	luteinizing hormone
LHR	luteinizing hormone receptor
lx	lux
MAO	monoamine oxidase
MHPG	3-methoxy-4-hydroxyphenylglycol

MOA	mode of action
MPT	alpha-methylparatyrosine
MS-222	tricaine methanesulfonate
NE	norepinephrine
NFLX	nor-fluoxetine
NPY	neuropeptide Y
OH-DPAT	8-hydroxy-*N*,*N*-dipropyl-2-aminotetralin
PCR	polymerase chain reaction
$PGF_{2\alpha}$	prostaglandin $F_{2\alpha}$
POA	preoptic area
PVN	nucleus paraventricularis
RIA	radioimmunoassay
ROS	reactive oxygen species
RT	reverse transcriptase
SSRI	selective serotonin reuptake inhibitor
Slc6a4	serotonin transporter
T	testosterone
TRH	tryptophan hydroxylase
VT	vasotocin
WAY100635	N-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-N-(2-pyridyl)cyclohexanecarboxamide
X-GAL	bromo-chloro-indolyl-galactopyranoside

Chapter 1: General Introduction

Partially based on Mennigen et al., 2010. Pharmaceuticals as neuroendocrine disruptors: Lessons learned from fish on Prozac™. Journal of Toxicology and Environmental Health Part B: Critical Reviews, accepted.

1.1. Thesis outline and rationale

The overall goal of this thesis was to investigate sub-lethal disrupting effects of the human pharmaceutical fluoxetine (FLX) on the neuroendocrine-controlled processes of reproduction, feeding, and growth in goldfish. The neurotransmitter serotonin (5-Hydroxytryptamine, 5-HT) is anatomically and functionally largely conserved across vertebrate species, and has a stimulatory role in reproduction and an inhibitory role on feeding and growth in goldfish, which is modulated via serotonergic pathways in the neuroendocrine brain. The human pharmaceutical FLX is routinely detected in waste water treatment plant (WWTP) effluents and also bioconcentrates in wild fish, particularly in the brain. Fluoxetine is the active compound of the antidepressant Prozac™ and acts as a selective serotonin reuptake inhibitor (SSRI) in humans. It is therefore specifically designed to block the 5-HT transporter mediated re-uptake of 5-HT in the synaptic cleft, resulting in an increase in extracellular 5-HT concentrations in the brain. Given the conservation of the 5-HT system in vertebrates, I formulated the following hypotheses, which were addressed by the experiments described in subsequent chapters and appendices of this thesis, as outlined below.

(1) The human pharmaceutical FLX disrupts the neuroendocrine-controlled processes of reproduction and feeding/growth in goldfish, at least partially through similar neuroendocrine pathways as have been identified in mammals.

(2) Environmentally relevant waterborne FLX concentrations are capable of disrupting the physiological processes of reproduction and feeding in exposed goldfish.

To address these two principal hypotheses, I cloned key genes of components of the serotonergic system in the goldfish neuroendocrine brain, in order to study the evolutionary conservation of components of the 5-HT system between fish and mammals at the molecular level. Next, I studied the seasonal expression patterns of these components of the 5-HT system in the neuroendocrine brain of goldfish, in order to correlate their seasonal expression pattern with the known seasonality of the neuroendocrine control of reproduction and feeding (Chapter 2). This helped me to identify suitable exposure windows to investigate the potential FLX-induced disruption of reproduction and feeding and growth. The first experiment investigated the effect of a pharmacological dose of FLX (5 µg/g bw) in sexually recrudescent female goldfish to characterize any potential disruptive effects on the reproductive axis in fish and correlate these effects with any underlying changes in gene expression in the neuroendocrine brain, in order to gain insight into potential mechanisms (Chapter 3). As the neuropeptide isotocin (IT) emerged as a key target of FLX in the neuroendocrine brain (Chapter 3), I further investigated its potential mechanistic role in the observed disruption of the reproductive axis in female goldfish through FLX: I first studied the potential interaction of 5-HT and IT in the neuroendocrine brain of goldfish to establish whether the disruptive effect of FLX on IT gene expression is mediated directly via the serotonergic system. Functional studies regarding the role of IT in reproduction were entirely lacking in teleost fish, so I investigated the function of IT in the neuroendocrine control of reproduction (Chapter 4). Following these studies, I exposed sexually mature female and male goldfish to waterborne FLX concentrations in the environmental (0.54 µg/L) and supra-environmental (54 µg/L) range. These exposures were conducted in order to investigate whether the disruptive effects on the reproductive axis at the molecular and physiological level that were identified in the injection experiments (Chapter 3) also occurred in a more

environmentally realistic waterborne exposure scenario (Chapter 5, Appendix 1). In an additional experiment, FLX injections (5 µg/g bw) were administered in sexually regressed goldfish that exhibit maximal growth rates. The objective was to identify potential anorexigenic properties of FLX, as well as potential underlying molecular mechanisms (Chapter 6). Following the identification of anorexigenic effects of injected FLX in goldfish, a waterborne FLX exposure (0.54 µg/L and 54 µg/L) was used to investigate whether the identified disruptive effects of FLX on feeding would also occur in a more environmentally realistic waterborne exposure scenario (Chapter 6). In the final chapter, key findings regarding FLX-induced disruption of reproduction, feeding, and growth are discussed within the context of the growing body of literature describing FLX effects in fish, with emphasis on underlying neuroendocrine mechanisms presented in this thesis. In this context, the environmental relevance of the findings presented in this thesis is discussed, as well as emerging directions regarding future study of the effects of FLX on the neuroendocrine regulation of fish physiology (Chapter 7).

1.2. Goldfish as a model for the neuroendocrine system and its disruption

Goldfish (*Carassius auratus*) are a member of the family *Cyprinidae,* which represents one of the largest vertebrate groups counting approximately 2,000 species. Goldfish have been used in physiological research as a model teleost species, in particular to study the neuroendocrine brain and its effects on circulating hormones (1,2). The goldfish is commonly used as a model species due to its relative ease of maintenance, and as a teleost, the unique evolutionary structure of its brain and pituitary: pituitary cells in teleosts are regionalized (3), and due to the lack of a median eminence in teleost fish (4), retrograde tract tracing techniques can be used to study the direct neuronal hypothalamic control of hormone release from the pituitary (5). Therefore, it is not surprising that two physiological systems controlled by the

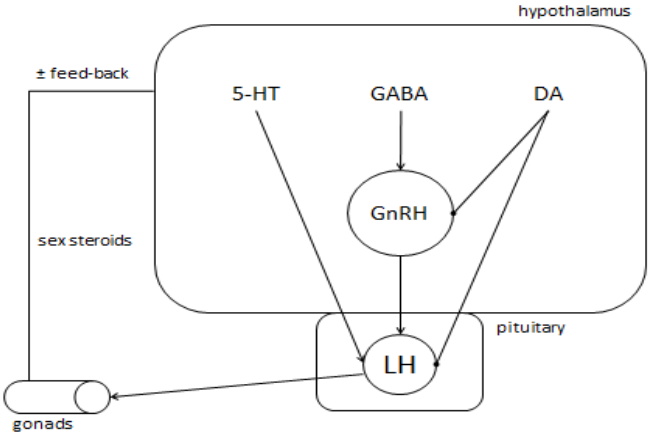
neuroendocrine brain, reproduction (1), and feeding/growth (2,6), have received particular attention and have been well characterized in this species.

In short, these systems are controlled seasonally - there is a distinct reproductive phase in sexually mature fish in May, followed by a growth phase in sexually regressed fish (1). These phases correlate with an increased release of Luteinizing Hormone (LH), and increased food intake and release of Growth Hormone (GH), respectively (1,2). The neuroendocrine systems that control reproduction and feeding are extensively studied in the goldfish model (1,6). The release of LH is regulated by a variety of hypothalamic factors, with stimulatory effects of ɣ-aminobutyric acid (GABA) and 5-HT, and inhibitory effects of dopamine (DA). These neurotransmitters act to stimulate or inhibit the release of Gonadotropin-releasing hormone (GnRH) in the hypothalamus, which subsequently stimulates the release of LH from the gonadotrophs in the pituitary. These neurotransmitters have also been demonstrated to have a direct effect on LH release from the gonadotrophs in the pituitary. Luteinizing hormone is subsequently secreted into the blood and acts on the gonads to stimulate gametogenesis and steroidogenesis. Sex steroids are then secreted into the blood and relay information to the hypothalamus in a feed-back loop (**Fig. 1.1A**).

The neuroendocrine control of feeding in goldfish is regulated by neuropeptides, which, in terms of the functional context of feeding, can be distinguished into two groups: peptides that stimulate feeding (orexigenic peptides) and peptides that inhibit feeding (anorexigenic peptides). Examples of orexigenic molecules include Neuropeptide Y (NPY) and Agouti-Related Protein (AgRP), while examples of anorexigenic peptides include CRF (Corticotropin-Releasing Factor), CART-I (Cocaine- and Amphetamine-Regulated Transcript I) and Leptin (**Fig. 1.1B**).

Finally, contaminants such as the pharmaceutical fluoxetine (FLX) are detected in the brain of wild cyprinid species, including the closely related common carp (*Cyprinus carpio*) (7); thus the goldfish represents a relevant model to assess the potential for neuroendocrine disruptive effects of FLX and other identified pharmaceuticals.

A



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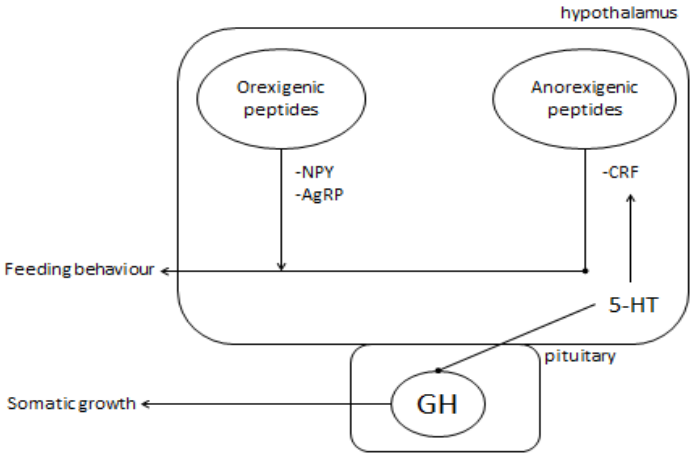


Fig. 1.1. *Schematic representations of the neuroendocrine control of the reproductive axis (A and feeding and growth (B) in goldfish. Pointed arrows indicate stimulatory effect, round arrows indicate inhibitory effect. The effect of 5-HT in both systems is shown. See text for detailed explanations.*

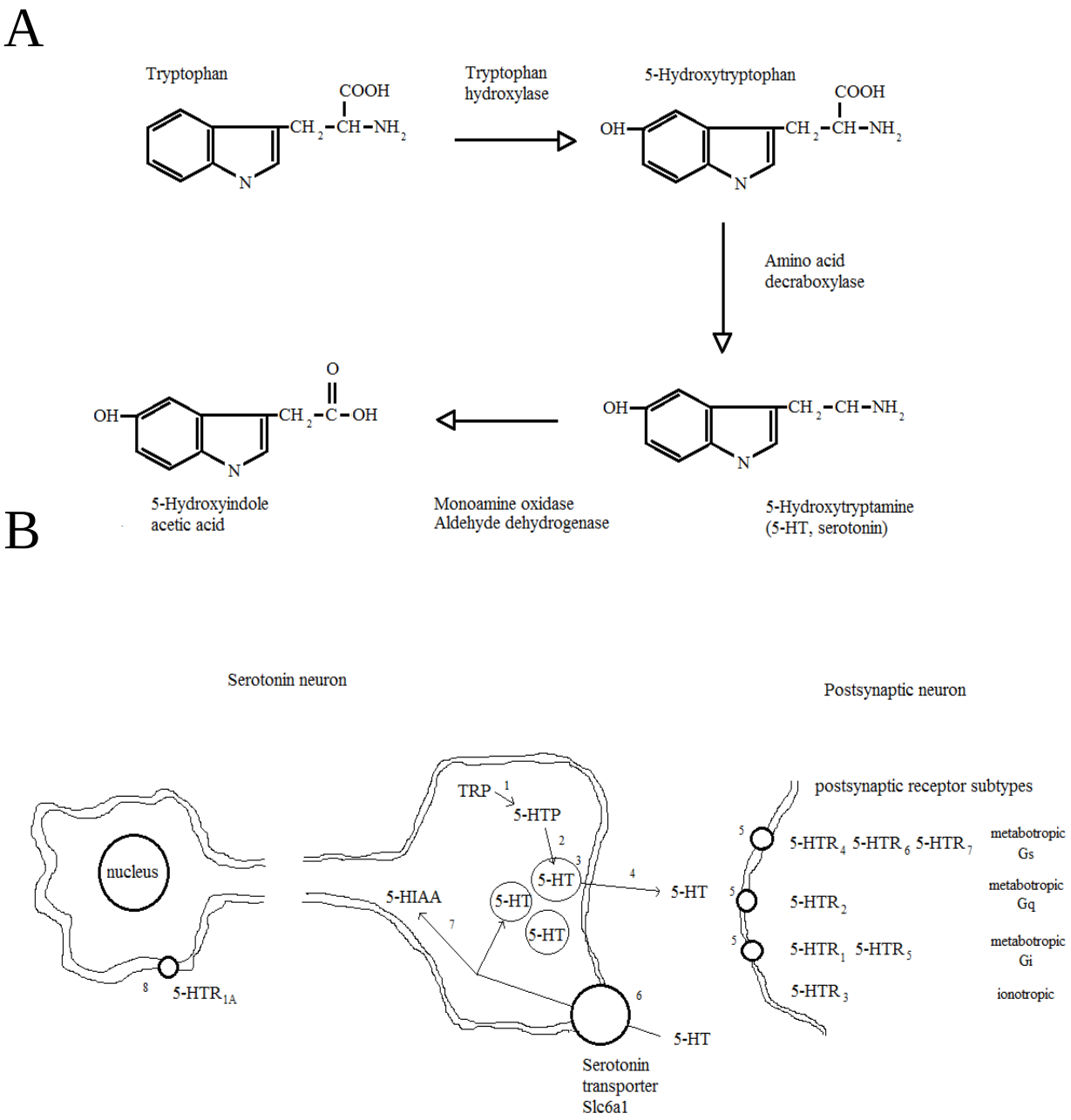
1.3. The serotonergic system in mammals and fish

The amine 5-hydroxytryptamine (5-HT) was initially isolated and identified by Rapport and colleagues in 1948 (8). Present in the serum, the chemical was found to have powerful effects on smooth muscle contraction, which led to the description of the compound as serotonin. It is now known to exist in nearly every biological organism studied, including plants, invertebrates and vertebrates (9); this wide distribution indicates an evolutionarily ancient origin. In vertebrates, high concentrations of 5-HT are found in the brain, gut, kidney, lung, and testes, indicating a ubiquitous distribution and function of this compound. However, most research has focused on the brain, where serotonergic innervation branches out from the raphe nuclei in the brain stem of vertebrates (10). In the serotonergic neurons, 5-HT is produced through the conversion of the essential amino acid tryptophan, first into 5-hydroxytryptophan by the rate-limiting enzyme in 5-HT synthesis, tryptophan hydroxylase (TRH), and subsequently into 5-HT by the enzyme amino acid decarboxylase (**Fig. 1.2A**). Serotonin is stored in vesicles that are released at the synapse following neuronal membrane depolarisations. Serotonin mediates its function by binding to a variety of 5-HT receptors (5-HTR, generally classified into seven families) on the post-synaptic membrane (11). Of these receptors, all families except for the 5-HTR_3 family are metabotropic receptors and utilize G-protein coupled signalling pathways that are of the G_s, G_q and G_i sub-types (**Fig. 1.2B**). The 5-HTR_3 is ionotropic and controls a Cl^- influx channel (11).

The 5-HTR_{1A} is found not only on post-synaptic neurons, but also on pre-synaptic perikarya (12), where it mediates the negative feedback regulation of 5-HT synthesis. Serotonin is actively taken up by the 5-HT transporter, which operates in a Na^+-dependent manner (11). Degradation of 5-HT occurs through the catalytic action of the enzymes monoamine oxidase (MAO) and aldehyde dehydrogenase to yield 5-hydroxyindolacetic acid (5-HIAA) (11). The

rate-limiting step in the degradation pathway is modulated by MAO A in mammals (11), and MAO in fish, where only one isoform of the enzyme exists (13).

The 5-HT system of the central nervous system (CNS) is well-conserved morphologically in vertebrates (10), including in fish species such as the stickleback (*Gasterosteus aculeatus*) (14) and the goldfish (15). As in mammals, serotonergic innervation is found in the neuroendocrine brain of goldfish (15). In contrast to mammals, the serotonergic system in goldfish includes perikarya in the posterior hypothalamus, from which innervation extends anteriorly to the nucleus ventromedialis thalami. Perikarya are also found more caudally in the nucleus posterior periventricularis, the nucleus recessus lateralis and the nucleus recessus posterioris in goldfish. Many immunoreactive fibers were observed in the telencephalon, including within the nucleus preopticus and the nucleus anterior periventricularis of the goldfish. Serotonergic fibers are also located in certain regions of the hypothalamus, such as the infundibular recess, from where they converge to enter the pituitary gland, predominantly in the proximal pars distalis. The anatomical distribution of the serotonergic system in the fish neuroendocrine brain is therefore indicative of modulatory effects of 5-HT on the pituitary gland, which controls physiological systems by modulation of hormonal output.



1 Tryptophan hydroxylase is the rate-limiting enzyme in 5-HT synthesis and converts Tryptophan (TRP) to 5-Hydroxytryptophan (5-HTP)
2 Amino acid decarboxylase convets 5-HTP to 5-HT
3 Serotonin is stored in vesicles
4 Depolarization of the membrane results in fusion of the vesicle with the membrane and release of 5-HT
5 Serotonin activates potsynatptic receptors
6 Serotonin is actively transported back into the serotonin neuron
7 Serotonin is degraded by the rate-limiting enzyme Monoamine oxidase and Aldehyde dehydrogenase
8 Serotonin activates the auto-receptor 5-HTR1A to inhibit 5-HT synthesis

Fig. 1.2. *Biochemical pathway of 5-HT synthesis and degradation (A). Schematic representation of the serotonergic neuron and its postsynaptic target (B). Based on information from Jacobs and Azmitia (11).*

1.4. Serotonin and the regulation of the neuroendocrine system in mammals and fish

Serotonin plays a key role in the regulation of specific neuroendocrine pathways in mammals such as the rat (*Rattus norvegicus*), and the macaque monkey (*Macaca mulatta*), including pathways controlling reproduction, stress, regulation of appetite, water-balance and behavioural functions (11,16,17).

The vertebrate reproductive endocrine axis is stimulated by the release of GnRH from the hypothalamus, which in turn stimulates the release of LH and FSH (Follicle-Stimulating Hormone) from gonadotrophs in the anterior pituitary. Luteinizing Hormone and FSH are subsequently released into the blood from which they reach the gonads to control maturation, steroidogenesis, and sperm or egg release in males and females, respectively (18). Serotonin exerts its reproductive effects in mammals in both the neuroendocrine brain and pituitary. Serotonin has stimulatory effects on LH release at least partially within the proximity of the median eminence of the hypothalamus (19), where synaptic contacts between serotonergic neurons and GnRH neurons exist (20). The stimulatory effects of 5-HT on LH release appear to depend on sex steroid signaling as estradiol (E_2), an estrogen molecule, is required for 5-HT to stimulate LH release (19). *In vivo* studies confirm expression of several 5-HT receptor subtypes with differential effects on GnRH release in immortalized GnRH neurons (GT1-7). For example, selective activation of the 5-HTR_{1A} decreases release of GnRH, while 5-HTR_{2C} and 5-HTR_4 activation increases release of GnRH (21). Evidence for a direct serotonergic stimulation of LH release via 5-HTR_{3A} in the pituitary exists from studies using the mouse (*Mus musculus*) immortalized gonadotroph-derived LβT2 cell line (22).

The hypothalamus in rats contains neurons located in the paraventricular nucleus (PVN) and the arcuate nucleus that release neuropeptides involved in the control of food intake (23).

In rats, 5-HT inhibits food intake by stimulation of anorexigenic factors, such as CRF, as well as inhibition of orexigenic factors, including NPY by 5-$HTR_{1B,}$ or 5-HTR_{2C} signaling (24). Water balance in vertebrates is regulated by the nonapeptides of the Vasotocin family and to some extent by nonapeptides of the oxytocin family, both of which are synthesized in the magnocellular and parvocellular neurons of the preoptic area (POA) (25). From the POA, neurons extend to the neurohypophysis (posterior pituitary) (25). These nonapeptide hormones are released in response to plasma osmotic pressure and secreted into the blood stream from the neurohypophysis. Peripherally, they act to control water excretion and blood pressure (25). Serotonin receptors are expressed on both vasopressin and oxytocin neurons of mammalian magnocellular neurons (26) and stimulate mRNA abundance and release of these nonapeptide hormones (16).

The vertebrate stress axis is stimulated by hypothalamic release of CRF, which subsequently acts to release adrenocorticotropic hormone (ACTH) from the anterior pituitary. In mammals, ACTH is released in the blood to stimulate cortisol release from the adrenals (27). Serotonin stimulates the stress axis mainly by direct interaction with the CRF neurons (27), which receive serotonergic innervation in the PVN (27). These effects are mediated by 5-HTR_{1A}, 5-HTR_{2A} and 5-HTR_{2C}, which were found to be expressed on CRF neurons in the PVN of the rat (26,28). This finding is substantiated by an observed increase in *crf* mRNA through stimulation of these receptors in the rat (16). There is also evidence of serotonergic activation of release of ACTH at the level of the corticotrophs in the pituitary (27). Several behaviours are also mediated by 5-HT-modulated release of neuropeptides. For example, oxytocin release has a central role in reproductive and feeding behaviours in the rat (29). Corticotropin-releasing factor is involved in the regulation of locomotory behaviours in the rat (30).

Serotonin is equally involved in the modulation of several neuroendocrine systems in teleost fish. For example, 5-HT is involved in the neuroendocrine stimulation of reproduction in the goldfish and Atlantic croaker (*Micropogonias undulates*) by increasing LH release (31-34). Intraperitoneal (i.p.) injection of 2.5-20 µg 5-HT/g body weight (bw) in goldfish of both sexes produced a dose-dependent increase in circulating LH concentrations between 0.5 h and 2 h, but not at 3 h post injection (31). This effect was most pronounced in sexually mature fish. Interestingly, injection of 5-HT into the third ventricle of the goldfish brain did not change serum LH concentrations, which provides evidence for a mechanism at the level of the pituitary (32). This mechanism of action was further validated using goldfish *in vitro* pituitary incubations where 5-HT dose-dependently increased LH release into the medium at concentrations between 0.2-5 µM (33). The increased LH release, but not basal LH concentration, was inhibited by the 5-HTR_2 antagonist ketanserin in both studies, arguing for a 5-HTR_2-like receptor mediated effect (32,33). In Atlantic croaker, i.p. injections of 5-HT alone (20 µg/ g bw) did not increase LH concentration, but did significantly enhance GnRH-induced (20 ng/g bw) LH release (34). Combined treatment of GnRH and 5-HT elicited increased *in vitro* LH release in pituitaries of mature Atlantic Croaker between 6 h and 18 h (34). These effects were abolished by ketanserin, indicating a role for the 5-HTR_2-like receptor, mirroring findings in goldfish (34). The role of 5-HT in enhancing GnRH-induced LH release appears to be highly dependent on circadian and seasonal rhythms. Maximal enhancement occurred in the mid-dark phase of a 12h:12h day/night cycle and in sexually mature croakers (31). The stimulatory effect of 5-HT on basal and GnRH-stimulated LH release was increased by DOI, a 5-HTR_2 agonist and decreased by LY53,857 maleate, a 5-HTR_2 antagonist (31). An immunohistochemical study localized 5-HT and GnRH neurons in close proximity to each other within the olfactory bulb, POA and posterior pars

distalis of the pituitary. These results indicate potential sites of 5-HT regulation of GnRH neurons in these areas of the croaker neuroendocrine system (35).

Intracerebroventricular (i.c.v.) injections of 10 μg/g bw 5-HT in goldfish significantly decreased feeding 2 h post-injection, an effect that was at least partially mediated by CRF, as the concurrent injection of an antagonist, α-Helical $CRF_{[9-41]}$, resulted in partial inhibition of the effect (36). It is also interesting to note that 5-HT suppresses Growth Hormone (GH) release in goldfish *in vivo* (32), which is likely mediated via a $5\text{-}HTR_{1A}$-like receptor at the level of the pituitary (33). Serotonin is also involved in stimulating the stress axis in rainbow trout (*Oncorhynchus mykiss*) and the Gulf toadfish (*Opsanus beta*) by $5\text{-}HTR_{1A}$ signaling in the neuroendocrine brain (37,38). Neuroendocrine changes in 5-HT are furthermore implicated in reproductive behaviours in the saddleback wrasse (*Thalassoma dupperey*) (39), aggression in rainbow trout (40), and locomotion in Chinook salmon (*Oncorhynchus tshawytscha*) (41).

These studies taken together support the conservation of important neuroendocrine functions for 5-HT between fish and mammals. However, while similar 5-HT receptor subtypes appear to be involved in most responses in mammals and fish (including the stimulatory effects on the reproductive axis mediated by $5\text{-}HTR_2$-like receptors or stimulatory effects on the stress axis mediated by $5\text{-}HTR_{1A}$ receptors), other specific 5-HTR subtypes remain to be fully characterized in fish in order to obtain a more detailed comparative picture of 5-HT signaling in the neuroendocrine brain when compared to mammalian models.

The serotonergic system in fish as in mammals is modulated by a variety of environmental factors, that are integrated into a vast array of physiological endpoints, which is in line with its proposed phylogenetically ancient nature and its proposed role of maintaining homeostasis of physiological systems (9). The serotonergic system in fish is a target for several

aquatic environmental stressors and may therefore disrupt the natural modulation of 5-HT by seasonal and environmental influences, including photoperiod, temperature, sex steroid feedback and social status, and subsequently regulated central and peripheral systems, as the blood brain barrier is permeable to 5-HT in fish (42). Serotonin modulated systems in fish include reproduction (32,31), feeding/growth (36,29), digestion (43), stress (37), social hierarchies and aggression (44), the immune system (45), osmoregulation (46), locomotion (41), learning (47) and neurogenesis (48). Sensory processes including vision (49), olfaction (50), the detection of pressure (51) and salinity (52) are modulated by, or evoke changes in, the serotonergic system. (**Fig. 1.3**). The neuroendocrine processes described in the previous paragraph may be disrupted by aquatic stressors acting on the serotonergic system. Compounds and adverse environmental conditions known to affect the 5-HT system in fish include metals such as mercury (53), lead (54), silver (55), polychlorinated-biphenyls (PCBs) (54), ammonia (56), hypoxia (57), and as investigated in this thesis, selective 5-HT reuptake inhibitors (SSRI) pharmaceuticals designed to target the 5-HT system of humans (**Fig. 1.3**).

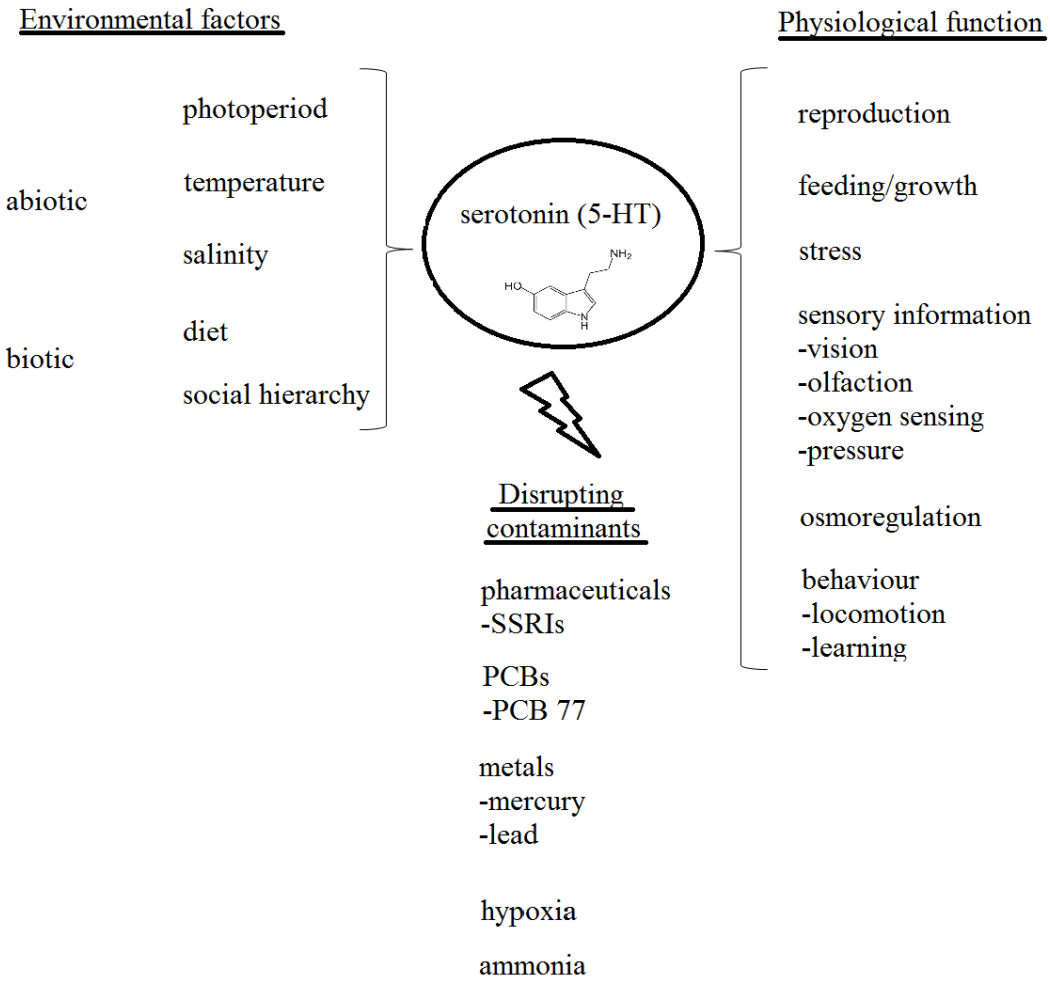


Fig. 1.3. *A simplified diagram of the integrative role of 5-HT in coordinating physiological effects in fish in response to environmental stimuli and disruption of the central 5-HT system by environmental contaminants.*

1.5. The pharmaceutical FLX

In 1987, FLX was the first SSRI to be marketed and it has been used by more than 40 million patients worldwide under the patent name Prozac™, which expired in 2001 (58). After the expiry of its patent (Prozac™) in 2001, FLX remains one of the most heavily prescribed generic SSRIs in the U.S., with more than 23 million prescriptions in 2007 (59); it also has significant sales numbers in less developed countries such as India (60). Fluoxetine is prescribed as a treatment for several mental illnesses, including depression, anxiety, obsessive compulsive disorder, bulimia, and recently under the new patent Sarafem™, for treatment of premenstrual dysphoric disorder. Daily FLX doses range from 20 to 60 mg, depending on the patient and circumstance. Fluoxetine is a racemic mixture of R-FLX and S-FLX enantionmers (**Fig. 1.4**), which display similar affinities for the 5-HT transporter (61). Following absorption, FLX is mainly bound to plasma proteins (>94%) and has a half-life in the blood of approximately 45 h for FLX and 210 h for its principal metabolite nor-fluoxetine (NFLX) (61). Typically, brain concentrations of FLX are several-fold higher than average blood concentrations (90-200 ng/mL), in keeping with the lipohilic properties of the drug (61). Repeated administration results in non-linear disposition kinetics and higher blood concentrations, which correlate poorly with clinical outcome (61). Fluoxetine is metabolized by first-pass metabolism in the liver by cytochrome P450 CYP 2D6 to yield NFLX, an equally potent SSRI (61). Both FLX and its metabolite NFLX are eventually excreted in the urine as active compounds, and as acid and conjugated FLX and NFLX metabolites, including glucoronidates (61) .

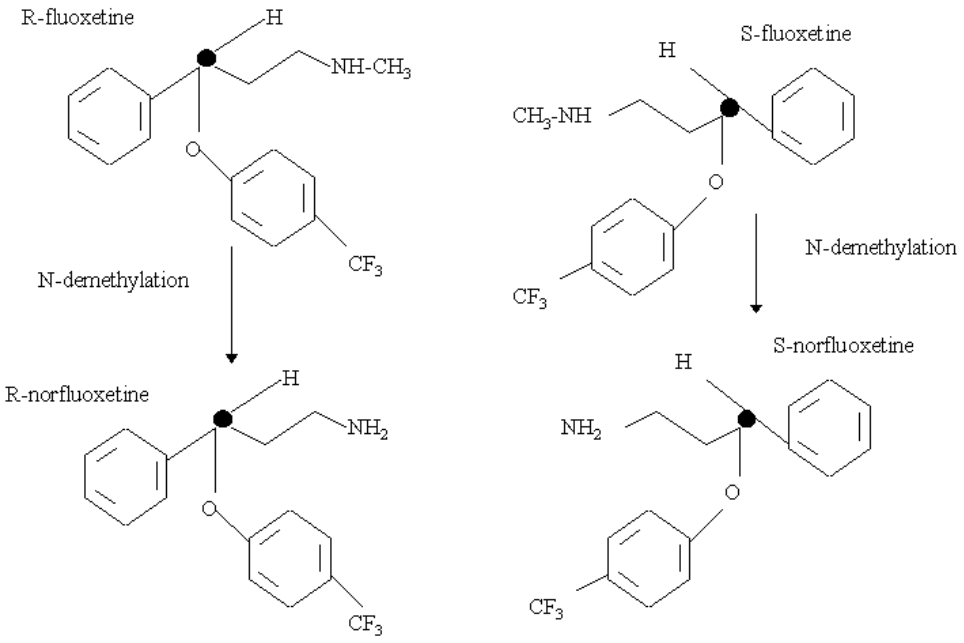


Fig.1.4. *Racemic nature of FLX and its cytochrome 450 II D6 mediated conversion into NFLX enantiomers.*

1.6. Fluoxetine as an environmental contaminant

Pharmaceuticals, including FLX, are increasingly detected in aquatic environments. Fluoxetine is regularly detected in several waterways (62-65) receiving sewage effluent from wastewater treatment plants (WWTPs). While studies targeting effluent dominated streams routinely detect FLX and its equally active metabolite NFLX, a survey study (66) drawing from a sample size of 139 random streams reported a relatively low occurrence frequency of FLX detection (1.2%). This reflects the input of FLX in the aquatic environment, as the presence of the drug is due primarily to its excretion by patients, who excrete approximately 11% of ingested FLX as unchanged parent compound, 7% as its metabolite NFLX and 8% as conjugated FLX and NFLX (61). However, improper disposal and leakage from production facilities are also generally considered as points of entry for pharmaceuticals into the environment (67) and may play some role in the increasing presence of FLX in the aquatic environment.

Fluoxetine and other SSRIs are found to pass through WWTPs in varying quantities, depending on the characteristics of the sewage, weather, and design and operation of the treatment plant (68-70). Reported WWTP removal rates for FLX are between 23% and 91% for FLX and 79-89% NFLX respectively (68,69) and treatment does not involve degradation of FLX, as the compound is found in extremely high concentrations (100-4700 µg/kg) in biosolids of WWTPs (71). Common treatments such as chlorination and dechlorination (72), as well as microbial treatments performed under nitrifying and denitrifying conditions (73,74) are not efficient in removing or degrading FLX from sewage. Ozone treatment (15 mg/L) of sewage was found to prevent bioconcentration of various SSRIs and their metabolites in brook trout (*Salvelinus fontinalis*), implicating a degrading effect of ozone treatment on SSRIs and their metabolites (75). Conversely, and similarly to other pharmaceuticals in WWTPs, the inactive

excreted glucuronidated metabolite of FLX may be cleaved by microorganisms to reform the active parent compounds FLX or NFLX (76).

Fluoxetine is resistant to hydrolysis and photolysis in a lab setting with half-lives of ≥ 100 d in buffered aqueous solutions at pH 5, 7, and 9. In synthetic humic acid, the half-life of FLX was determined to be 21 d at a pH of 7 (73). In surface waters, photolysis of FLX is accelerated, likely due to presence of radicals, which aid the processes (77). However, this process is limited in the environment, as FLX adsorbs to sediments, which may act as a reservoir from which FLX can be re-released into the water compartment (64,78).

Concentrations of FLX and single SSRI compounds in water bodies receiving effluent are typically below 1 µg/L (62,63,65,66). For example, the average concentration of FLX in a study of 139 streams was reported at 0.01 µg/L (66) while the highest concentration of FLX in an aquatic system reported to-date is 0.54 µg/L (65). Concentrations of citalopram, another SSRI, reached 0.62 µg/L in a stream receiving hospital effluent in Trømso, Norway (79). Overall concentrations of combined SSRIs were measured at 3.2 µg/L in close proximity to a waste water effluent and 0.5 µg/L 100 m downstream of the effluent in the Grand River in Ontario, Canada, and similar concentrations of combined SSRI loads have been described in separate streams including the Mississippi River in Minnesota, U.S. and Boulder Creek in Colorado, U.S. (63,64,80). Surprisingly, FLX and other SSRI pharmaceuticals bioconcentrate in fish in spite of a moderate octanol-water partition coefficient (K_{ow}) value of about 4 in the case of FLX (81). However, bioconcentration of FLX does not appear to be solely related to lipid content; Ramirez and colleagues (7) failed to identify a significant relationship between FLX bioconcentration and lipid content in various fish species. This is likely due to the fact that FLX ionizes at ambient pH values, which results in higher actual bioconcentration factors than predicted based on K_{ow}

values (81). A lower biotransformation capacity in the liver of fish compared to mammalian model systems, such as the rat, has been put forward as an additional hypothesis (82). Regardless of the actual mechanism, a key factor in FLX bioconcentration is considered to be the FLX concentration in the water and the type of treatment at the WWTP (83). Bioconcentration factors for FLX are reported to be 8.8-100 at pH 7 and 260 at pH 9 (84,85). Recent studies detected FLX and other SSRIs, as well as their metabolites, in wild-caught fish of several orders native to North American streams, including *Cypriniformes*, *Perciformes* and *Siluriformes* (64,83,86,87). Bioconcentration was particularly high in brain tissues, with concentrations typically around 0.01 µg/g for FLX, NFLX and other individual SSRI compounds (64,86). In comparison to other SSRIs, such as citalopram, FLX and NFLX appear to bioconcentrate more readily in the fish brain, as indicated by comparisons between water and fish brain concentrations measured in Boulder Creek, Colorado, U.S. (64).

While these FLX concentrations lie well-below concentrations that cause lethality, as determined by LC_{50} values, for example 0.55 mg/L in mosquito fish (*Gambusia affinis*) and 1.6 mg/L in rainbow trout, the bioconcentration of FLX in the fish brain has raised concerns over potential sub-lethal effects of FLX on neuroendocrine modulation of physiological processes in this vertebrate group. As many of these processes are modulated by 5-HT in the brain in fish (88 and **Fig.1.3**), the principal target of FLX and other SSRIs, the potential for FLX to disrupt neuroendocrine function especially deserves attention.

1.7. Evidence for FLX-induced neuroendocrine disruption in mammals

Fluoxetine and the pharmaceutical class of SSRIs in general are reported to disrupt neuroendocrine systems in mammals, ostensibly by increased 5-HT concentrations and altered 5-HT receptor signaling, which occur in the case of chronic exposure (89). These changes can result in alterations of hormonal profiles. For example, oxytocin release and mRNA abundance was reduced in chronic FLX treatment due to 5-HTR_{1A} desensitization following prolonged receptor activation because of increased 5-HT concentrations at the synapse (89). Therefore, the different effects of FLX on neuropeptides are at least partially a result of the indirect action on 5-HTR by chronically increased 5-HT concentrations.

Interactions between 5-HTR subtypes are also possible. For example, heterologous 5-HTR_{2A}-induced desensitization of 5-HTR_{1A} has been reported in the neuroendocrine system of the rat, resulting in the alterations of circulating ACTH and oxytocin concentrations (26). These SSRI-induced disruptions of hormone release are correlated with disturbances in physiological endpoints. For example, the SSRI-induced disruption of the reproductive hormone oxytocin is correlated with the disruption of sexual behaviours in mammalian models (90). Due to the role played by 5-HT in multiple neuroendocrine systems, several of which are disrupted by FLX, it may be difficult to establish the exact mechanism of FLX-induced disruption of a particular endpoint. For example, recent studies addressed the question of whether reproductive dysfunction was due to the disruption of the reproductive axis by FLX itself or whether it is a secondary effect due to the anorexigenic properties of the drug (91). Therefore, while particular endpoints in mammals regulated by 5-HT and disrupted by FLX provide for candidate systems when investigating FLX-induced disruptions in the neuroendocrine brain of teleost fish, they too may be difficult to fully separate. Furthermore, while serotonergic endpoints may be targeted due

to a potential conservation of the serotonergic system between fish and mammals, the nature of the modulation may differ, as waterborne exposures of FLX are shown to decrease 5-HT concentrations in fish brain, an effect opposite to the observed increase in 5-HT in the hypothalamus of FLX-injected rats (92).

1.8. Evidence for FLX-induced neuroendocrine disruption in fish

Several excellent reviews (65,81) provide a strong case for the need for ecological risk assessment of SSRIs. The presence of FLX in the aquatic environment is considered to trigger a risk assessment under European Union guidelines (81) and both of the aforementioned reviews identify the need to investigate more sensitive sub-lethal endpoints of FLX action in fish. In light of the need for further investigation of sub-lethal effects of FLX on fish, this thesis investigates the specific disruption of the 5-HT modulated processes of reproduction and feeding and growth, focusing especially on the effects of FLX exposure on the neuroendocrine brain.

Chapter 2: Seasonality of the serotonergic system in the neuroendocrine brain of goldfish and evolutionary conservation of the 5-HT transporter

Partially based on Mennigen et al., 2010. Waterborne fluoxetine disrupts the reproductive axis in male goldfish, Carassius auratus. Aquatic Toxicology, 100 (4): 354-364.
Mennigen et al., 2010. Pharmaceuticals as neuroendocrine disruptors: Lessons learned from fish on Prozac™. Journal of Toxicology and Environmental Health Part B: Critical Reviews, accepted.

2.1. Introduction

The physiology of goldfish and other teleost fish is highly seasonal, particularly when considering the two key neuroendocrine systems investigated in this thesis, reproduction and feeding/growth (**Fig. 1.1**). The effects of 5-HT on reproduction and feeding/growth in goldfish, outlined in Chapter 1, are indicative of a conserved effect of the neurotransmitter 5-HT across vertebrates. A stimulatory effect of 5-HT on reproduction (32,31) and an inhibitory effect of 5-HT on feeding (36) are included in the effects that are conserved among vertebrates. The stimulatory effect of 5-HT on reproduction appears to be most pronounced in the reproductive phase, as indicated by the maximally stimulating effects of 5-HT on LH release in goldfish and Atlantic croaker in this period (32,31). During the reproductive period , 5-HT concentrations and turn-over have been shown to increase in the neuroendocrine brain (hypothalamus and telencephalon) of several fish species, such as the rainbow trout (93) and the catfish (*Heteropneustes fossilis*) (94). These increases in 5-HT concentrations are correlated with, and indeed caused by, both external stimuli, such as photoperiod (95) and temperature (96), as well as internal stimuli, such as sex steroids (97,98,99). For example, goldfish exposed to a photoperiod of 16L:8D (light hours:dark hours) for 2 weeks experienced a significant increase in hypothalamic 5-HT concentration in the dark phase compared to the light phase, which is in in contrast to the absence of such changes in goldfish exposed to 8L:16D for 2 weeks (95). Temperature has been shown to have an effect on 5-HT concentration in tilapia (*Oreochromis*

mossambicus) fish; tilapia maintained at a temperature of 32 °C for 3 weeks had significantly decreased hypothalamic 5-HT concentrations when compared to fish kept at 26 °C for 3 weeks. Implantation of 20 mg/kg methyl-testosterone significantly reduced 5-HT turn-over (measured as the ratio of 5-HIAA/5-HT) in the neuroendocrine brain (telencephalon and hypothalamus) of immature male rainbow trout after 3 weeks. Ovariectomized recrudescent female catfish revealed an increase in hypothalamic 5-HT concentration after 3 weeks, an effect which was reversed by administration of 1 µg/g E_2 (99) .

Because all of the aforementioned factors represent important cues for reproduction in goldfish (1,100,101), it has been speculated that 5-HT may partially mediate seasonal information to coordinate seasonal reproductive events. In the case of photoperiod, which is the principal cue for reproduction in goldfish (1), it has for example more specifically been speculated that 5-HT integrates the photoperiod information via a retino-hypothalamic pathway to coordinate this seasonal event (95). While this has not been directly proven in fish, the idea is consistent with reported effects in higher vertebrates, where both external cues, such photoperiod (102), as well as internal cues, such as the sex steroid hormone environment (19), are involved in stimulatory effects of 5-HT in the reproductive system (19).

Furthermore, in goldfish, 5-HT inhibits feeding behaviour (36) and GH release (33), a finding consistent with its role in higher vertebrates (11). These findings correlate well with the observed growth phase in regressed goldfish, which coincides with a decrease in neuroendocrine 5-HT concentration following the reproductive phase (93,2).

In light of the previously mentioned findings, the hypothesis was therefore formulated, that seasonal changes in mRNA abundance of key genes involved in 5-HT function underlie the seasonal effect of 5-HT on the neuroendocrine control of reproduction and feeding/growth in

goldfish. Given the described function of 5-HT in both systems in vertebrates, including goldfish, I predicted that these changes would be indicative of a heightened 5-HT concentration in the reproductive phase, and a decreased 5-HT concentration in the feeding/growth phase.

The serotonergic system consists of a multitude of enzymes as well as multiple receptors, which belong to 7 distinct 5-HTR families that appear to be conserved in vertebrates (11,103). In order to characterize components of the goldfish 5-HT system, I focused on integral components, which were chosen based on the following literature describing the role of genes of the 5-HT system in the neuroendocrine brain in fish.

Tryptophan hydroxylase (TRH) is the rate-limiting enzyme of 5-HT synthesis. In rainbow trout, as in mammals, it appears to be unsaturated under physiological conditions and has a particularly high ability to convert tryptophan into 5-hydroxytryptophan in the hypothalamus (104), as determined by injections of tryptophan and subsequent measurement of 5-hydroxytryptophan. Tryptophan hydroxylase is crucial to the 5-HT-mediated stimulation of the reproductive axis in the Atlantic croaker, as shown by its specific disruption by hypoxia (57) and PCBs (105), which could be rescued by administration of 5-HTP, the product of TRH catalyzed conversion of tryptophan. Similarly, TRH inhibition by 100 µg/g para-chlorophenylalanine (p-CPA) resulted in a significant decrease in serum LH, an effect that was nullified by 20 µg/g 5-HTP in female ovariectomized catfish (106). In the early stages of development in the tilapia brain, E_2 increases 5-HT concentrations, partially through a significant increase in TRH activity (107). Monoamine oxidase exists only as a single isoform in teleost fish. This situation is in contrast to mammals, where MAO A and MAO B isoforms degrade 5-HT and DA, respectively (13). In teleost fish, the enzyme appears to be able to catalyze both biochemical reactions, however, the overall biochemical profile, including substrate binding, appears to be related more

to the MAO A (serotonergic type) isoform in mammals (108). Its physiological role in controlling 5-HT concentration has been established in zebrafish, where inhibition of MAO resulted in a hyper-serotonergic phenotype, characterized by increased 5-HT but not DA or NE concentrations. Zebrafish, in which MAO activity was inhibited by deprenyl, revealed 5-HT-dependent behavioural alterations, including increased locomotion and heart rate; these observed behaviours were abolished by concurrent depletion of 5-HT by p-CPA (109). In goldfish, MAO activity has been shown to be regulated by both photoperiod and temperature, with temperatures over 20 °C having a permissive effect on the stimulating action of long photoperiods (16L:8D) (110). Monoamine oxidase has also been implicated in the mediation of feedback-effects of estradiol (E_2) on hypothalamic 5-HT concentrations in female goldfish, as demonstrated by inhibited MAO activity in hypothalamic slices exposed to 100 ng E_2 for 8 h *in vitro* (111).

The 5-HT transporter (*slc6a4*) was chosen for the investigation of its seasonal hypothalamic mRNA abundance, because it regulates 5-HT availability in the brain by mediating reuptake of released 5-HT molecules in vertebrates (**Tab. 2.1**). Acute co-administration of FLX and 5-HT in sexually mature goldfish resulted in increased serum LH concentrations when compared to goldfish injected with 5-HT alone, implicating the goldfish 5-HT transporter as a contributing target to the 5-HT mediated stimulation of serum LH in goldfish (32). As the expressed protein product of *slc6a4* is the primary target of SSRIs, including FLX, I used the nucleotide sequence of the partially cloned goldfish *slc6a4* for comparison with other vertebrate sequences in an effort to predict conserved binding capacities to FLX and SSRIs in general. The seasonal mRNA abundance of *slc6a4* was used to subsequently design the seasonal time-frame for experiments investigating effects of FLX on reproduction and feeding and growth.

With regards to 5-HTR, I investigated the profiles of 5-HTR_{1A} and 5-HTR_{2C}, as these

receptors are the most studied in a variety of vertebrate taxa. To this day, they remain the only 5-HT receptors pharmacologically studied in fish (32,31), with established functions in the regulation of the neuroendocrine systems of interest in this thesis. For example, a $5HTR_2$-like receptor type, and to a lesser extent the $5\text{-}HTR_{1A,}$ have been shown to mediate the effects of 5-HT on LH release in goldfish and Atlantic croaker (33,34,25). There exists some evidence for the involvement of both receptor types in the regulation of GH release from studies using *in vitro* incubations of goldfish pituitaries (112). Both receptors are also most studied in terms of their involvement in neuroendocrine responses to SSRIs, such as FLX, in mammals. These effects include FLX-induced desensitization on $5\text{-}HTR_{1A}$ signalling and subsequent hormone release (113) and a described direct antagonism of FLX on the $5\text{-}HTR_{2C}$ receptor (114).

Taken together, this evidence supports a role for the gene products of the rate-limiting enzymes of 5-HT synthesis (*trh*) and degradation (*mao*), as well as the 5-HT transporter (*slc6a4*) and the 5-HT receptors (*5-ht1a* and *5-ht*2c) in the neuroendocrine control of reproduction and feeding/growth. Because these compounds and 5-HT concentrations in the hypothalamus are regulated by several external and internal cues, which are important for the seasonal coordination of both processes, I investigated the seasonal mRNA abundance of these compounds. This investigation allowed me to better design the subsequent experiments of FLX exposures, in which effects might depend on the *a priori* status of the neuroendocrine 5-HT system in goldfish at any given season. Potential sexual dimorphisms at the molecular level have been described for the hypothalamic 5-HT system of some fish species; for example, a higher *trh* expression in the hypothalamus of developing male tilapia compared to female tilapia has been observed within 20 days post hatching (115). In light of this potential for sexual dimorphism, I investigated both male and female goldfish. This approach was chosen because FLX exposures were conducted in

both genders when investigating FLX effects on the reproductive axis in this thesis. In order to correlate potential changes in the hypothalamic mRNA abundance of genes related to the 5-HT system with the seasonality of reproduction, the gonadosomatic index (GSI) was assessed as an index of reproductive status of the animals, and weight as a measurement of growth status.

2.2. Material and Methods

2.2.1. Cloning of genes encoding components of the serotonergic system in Carassius auratus

Goldfish (Aleong's International, Mississauga, ON, Canada) were dissected and whole brain tissue was immediately frozen at -80 °C. Total RNA was extracted using Quiagen RNeasy kit (Quiagen Mississauga, ON, Canada). First strand cDNA was synthesized using Superscript ™ II enzyme (Invitrogen, Burlington, ON, Canada) as per manufacturer's protocol. In brief, first strand cDNA synthesis was done using 2 µg total RNA from goldfish whole brain in a reaction tube containing 200 ng random primers. The reaction was heated to 70 °C for 10 min and quickly chilled on ice before briefly centrifuging the sample. 4 µL of 5X reaction buffer (Invitrogen), 2µL 0.1M DTT, 1 µL 10 mM dNTPs and 1 µL RNAse inhibitor were added, gently mixed, and heated at 42 °C for 2 min. 1 µL Superscript ™ II RNAse H^- Reverse Transcriptase (Invitrogen) was added and the reaction was allowed to continue at 42 °C for 50 min. The reaction was inactivated for 15 min at 70 °C and then stored at -20 °C until used.

Primers were constructed using ClustalW (EMBL-EBI; http://www.ebi.ac.uk.clustalw/) and Primer 3 programs (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Primer construction used the most highly conserved regions of the genes from vertebrate species, whose sequences were retrieved from Genbank (http://www.ncbi.nlm.nih.gov/genbank). PCR amplification was carried out using the Mastercycler ® gradient Thermal Cycler (Eppendorf, Westbury, NY, USA). General PCR conditions used were as follows: 1-2 µL cDNA template in 36.3 µL water, 5 µL PCR reaction buffer, 1.5 µL $MgCl_2$, 1 µL dNTPs (10mM), 2 µL forward (FW) and reverse (RV) primers (10 mM each), and 0.2 µL Taq®

DNA Polymerase (Invitrogen). The initial denaturation step was done at 95 °C for 4 min to activate the Taq® enzyme. This step was followed by 35 cycles with a denaturation step at 95 °C for 30 s, an annealing step ranging between 50-60 °C (depending on the primer set used) for 45 s, and an extension step at 72 °C for 10 min. Amplification products were identified on a 1% agarose gel and ligated either directly from the PCR reaction or excised and purified from the band on the agarose gel using Qiaquick® Gel extraction Kit (Quiagen). Ligation was done using the 2.1 TOPO® vector (Invitrogen).

One shot chemically competent *Escherichia coli* (Invitrogen) were transformed and plated onto LB agar plates containing ampicillin, X-gal, and IPTG. Positive colonies were selected and an additional round of PCR was performed using M13 primers to ensure the correct insert was present before sequencing. Colonies were grown overnight in LB broth containing ampicillin, and plasmids were purified using the Wizard® Plus SV Minipreps DNA purification system (Promega, Madison, WI, USA). Approximately 10 µL of plasmid was sent to either the CAREG sequencing facility at the University of Ottawa or the Canadian Molecular Research Services (CMRS; Ottawa, Canada) for sequencing. Partial sequences for goldfish reverse transcribed mRNA were cloned using the following primers:

Tab. 2.1. *Primers used to clone genes of the 5-HT system in goldfish. Letters different from regular bases (A,C,G,T), indicate degenerate primers.*

Gene	Primer sequence 5'3' (FW)	Primer sequence 3'5' (RV)	Genbank accession
trp1	TGCMGAGAGTACCTRAAGAACC	GTGGTGATGAKRCACTCCTG	EF490969
trp2	TGTGTGTTTTGGTGGATTG	CAGCTTTTGCACATTCTCGT	EU003451
mao	TGGTACGTCAAGCAGTGCGG	ATCTTCCTCCTCGATCACCA	EF490970
slc6a4	GTCCAGGCTTTGGTCTTCTC	CAGACTGGGACCAGCATCCT	EF490971
5-htr1a	AGACCACGGGTCCACCATCT	TCCGCGATTTTAGCTTTCTC	EF493019
5-htr2b	CGCACTGGTCTGGCTAATCT	AGTCCCCAAATCCTTCTCGT	EF493017
5-htr2c	CATGGTGGTCACGTATTGTCTC	AAGTAGGTGGAGCTGGAGGTT	EF493018
5-htr3	GGCTGCACACAAACTAAAGACA	ATGAGGTGGAAGGGGAAGAG	EF490968
5-htr5	ACGTGGGTGCTCTCTTCAGT	AGCCGCTCTCCTCTCTTTCT	EF493015
5-htr7	AAAGGAATGGAAGCCAGCAGA	ATGAGGTGGAAGGGGAAGAG	EF493016

2.2.2. Phylogeny of the 5-HT transporter in vertebrates

Sequence similarity between cloned partial goldfish mRNA sequences and full length zebrafish (*Danio rerio*) and human (*Homo sapiens*) mRNA sequences obtained from Genbank was established using BLASTX (http://blast.ncbi.nlm.nih.gov/Blast.cgi), converting the obtained nucleotide sequence into a translated amino acid (AA) sequence and comparing it to the protein data base of Genbank. The phylogenetic analysis is based on amino acid sequences retrieved from ENSEMBL (http://uswest.ensembl.org/index.html) using the algorithm by Dereeper and colleagues (116). This algorithm specifically uses the MUSCLE algorithm for multiple sequence alignment, the PhyML program for tree-building, and the Tree-Dyn program for the rendering of the tree. Numbers indicate percentage values for 1000 bootstraps, with 1 representing 100%. ENSEMBL and Genbank (NCBI) nomenclatures for Slc6a4 sequences are as follows: Lizard (*Anolis carolinensis,* ENSACAG00000011633), frog (*Xenopus laevis*, ENSXETG00000023265), dolphin (*Delphinus delphis*, ENSTTRG00000016339), rat (ENSRNOG0000003476), human (*Homo sapiens*, ENSG00000108576), turkey (*Meleagris gallopavo,* ENSMGAG00000006010), chicken (*Gallus gallus,* ENSGAL00000004246), finch (*Taeniopygia guttata*, ENSTGUG00000006501), medaka (*Oryzias latipes*, ENSORLG00000013932), fugu (*Takifugu rubipres*, ENSTRUG00000008917), stickleback (ENSGACG00000006192), goldfish (EF490971), zebrafish A (ENSDARG00000061165), zebrafish B (ENSDARG00000057017), aphid (*Acyrthosiphon pisum,* XP_001944311), silkmoth (*Bombyx mori*, NP001037436.1), and fruitfly (*Drosophila melanogaster*, NP523846.2). This analysis is based on AA sequences retrieved from ENSEMBL and aligned using ClustalW alignment software (www.http://www.ebi.ac.uk/Tools/clustalw2). Protein structure was derived from human annotations retrieved from UNIPROT (http://www.uniprot.org/uniprot/P31645).

2.2.3. Tissue distribution of slc6a4 mRNA abundance and seasonal variation in the hypothalamus

For the tissue distribution experiment, sexually mature male and female fish (n=2 each) were sacrificed and hypothalami dissected and pooled for each gender before storage at -80 °C. The mRNA was extracted and transcribed into cDNA as previously described (2.2.1). PCR primers for *slc6a4* were utilized in a standard PCR reaction as described, and amplified products were analysed using a 1% agarose gel. The experimental design of this seasonal study is described in detail by Zhang and colleagues (117). Briefly, fish were ordered from a commercial supplier (Aleong's International, Mississauga, ON, Canada) and allowed to acclimate for 4 weeks. Tissues from male (n=15) and female (n=15) fish were collected at the beginning of every other month, resulting in six distinct seasonal time points that cover a year[1]. Two or three hypothalami were pooled and stored at -80 °C prior to storage, resulting in an n=6 per gender and seasonal timepoint. Additionally, both overall fish weight and gonadal weight were obtained to calculate the GSI.

2.2.4. RNA Extraction and cDNA synthesis

Goldfish were dissected and whole brain tissue was immediately frozen at -80 °C. Total RNA was extracted as previously described (see 2.2.1)

2.2.5 Real-time RT-PCR

Real-time RT-PCR primers were designed using the cloned and sequenced goldfish gene sequences deposited at Genbank. The program used was Primer 3 with the following adjustments of parameters to decrease the likelihood of primer dimer formation and thus improve performance in real-time RT-PCR: the amplicon length was limited to a size ranging between

[1] The tissue collection over the year was shared among Dr. Vicki Marlatt, Dr. Dapeng Zhang and myself in Dr. Trudeau's lab, University of Ottawa, ON, Canada.

100-300 basepairs (bp). The primer size was adjusted to 20 bp (±2 bp). Melting temperature was set at 60 °C (±1 °C). The primer's GC content was limited to 50% (±5%). The maximally allowed self-complementarity of the primers was adjusted to a value of 3, the maximum allowed self-complementarity at the 3' end of the primers was adjusted to 0. In cases where the program did not generate primers under these stringent conditions, parameters were relaxed in the following order: self complementarity, melting temperature and GC content. Of the primers designed by the programme, those that did not contain consecutive four consecutive guanine or cytosine bases, those that avoided guanine and cytosine repeats in the 3' end, and those that did not show a string of thymidine bases at the 3' were preferentially used. Primer sequences used for the real-time RT-PCR including the reference gene *ß-actin* are listed in **Tab. 2.2**. Each PCR reaction contained the following final concentrations: 20 ng first-strand cDNA template, 1X QPCR buffer, 5 mM $MgCl_2$, 150–600 nM gene specific primer, 0.5X SYBR green (Invitrogen), 200 µM dNTPs, 1.25U HotStarTaq (Invitrogen), and 20 µM ROX reference dye in a 25 µl reaction volume. The thermal cycling parameters were an initial 1 cycle Taq activation at 95 °C for 15min, followed by 40 cycles of 94 °C for 15 s, 58 °C–60 °C for 5 s, 72 °C for 30 s, and a detection step at 80 °C for 8 s. Dilutions of cDNA pooled from randomly selected individual samples (containing both male and female cDNA samples from different seasonal time points) were used to construct a relative standard curve for each primer set. In all cases, only standard curves with slopes between −3.2 and −3.5, with R^2 values of 0.98 were used. After the reaction was complete, a dissociation curve was produced starting at 55 °C and ending at 95°C (+1 °C/30 s). Real-time RT-PCR was carried out using a MX4000® Multiplex Quantitative PCR system (Stratagene, La Jolla, CA, USA), and data were analyzed using the MX4000 Software Package. Standard curves relating relative initial template mRNA abundance to fluorescence and

amplification cycle were generated using the amplified PCR product from the described pool of individual samples as a template, and were used to calculate relative mRNA abundance in individual samples. These values were normalized to relative *ß-actin* mRNA, which had been analyzed the same way as the target. The *ß-actin* mRNA abundance did not significantly change with season or gender. After each run, a random subset of PCR products (total n=4) from control and treatment groups were pooled and purified using a Minelute Reaction Cleanup kit (Qiagen, Mississauga, ON, Canada) and sequenced with a CEQ™8000 Genetic Analysis System (Beckman Coulter, Mississauga, ON, Canada) to confirm specificity of the assays.

Tab. 2.2. *Primer sequences and conditions used for seasonal gene expression analysis using real-time RT-PCR in goldfish hypothalamus tissues.*

Gene	Primer sequence 5'3' (FW)	Primer sequence 3'5' (RV)
trp1	TGATGAGGCACTCCTGTTTG	GTACAGACACTCGCCACTTGTT
mao	TGGTACGTCAAGCAGTGCGG	ATCTTCCTCCTCGATCACCA
slc6a1	GTCCAGGCTTTGGTCTTCTC	CAGACTGGGACCAGCATCCT
5-htr1a	AGACCACGGGTCCACCATCT	TCCGCGATTTTAGCTTTCTC
5-htrt2c	CATGGTGGTCACGTATTGTCTC	AAGTAGGTGGAGCTGGAGGTT
ß-actin	ACTACTGGTATTGTGATGGACTCC	CGGTCAGGATCTTCATCAGGTAG

2.2.6. Statistical analysis

Data on mRNA abundance were tested for normality and homoscedasticity using the Shaprio-Wilk test and Levene's test, respectively. In instances when data failed to meet these assumptions, they were either transformed to meet the conditions before running a univariate 2-way ANOVA, or, in the cases were transformations were not possible, were transformed to ranks prior to ANOVA analysis before using the Kruskal Wallis Scheirer-Ray-Hare extension to investigate interaction between factors non-parametrically. Appropriate post-hoc tests were

performed and adjusted according to Bonferroni to control for false type one error rate. All statistical analysis was conducted using SPSS version 17.0 (IBM, Chicago, USA).

2.3. Results

2.3.1. Phylogenetic analysis of the 5-HT transporter in vertebrates

The obtained partial sequences from goldfish reverse transcribed mRNA reveal a relatively high conservation when compared to another cyprinid (as seen in **Tab. 2.3)**, the zebrafish, for which the genome is fully sequenced. With respect to human 5-HT-related AA sequences derived from the gene sequences, the highest conservation was found for *trh1* and *slc6a4*, with a lesser degree of conservation for *mao* and *5-htr1a* and *5-ht2c*, respectively.

Tab. 2.3. *Derived AA sequence similarity between partially cloned goldfish reverse transcribed mRNA sequences and zebrafish or human sequences, respectively. BLAST X was used for comparisons.*

gene *Carassius auratus*	similarity *Danio rerio*	similarity *Homo sapiens*	length AA
trp1	94%	84%	191
trp2	96%	88%	169
mao	92%	66%	105
slc6a4	98%	84%	109
5-ht1a	90%	79%	108
5-ht2b	87%	59%	105
5-ht2c	87%	40%	87
5-ht3	92%	78%	221
5-ht5	92%	73%	164
5-ht7	90%	69%	208

When comparing *slc6a4* derived AA sequences across the vertebrates, a clear distinction among the vertebrate classes can be made (**Fig. 2.1**). Nevertheless, when considering particular AAs with a role in FLX and SSRI binding (**Tab. 2.4**), a higher degree of conservation between fish and mammals can be found than between mammals and birds, for example, in spite of the

fact that their overall *slc6a4* derived AA sequence appears to be more similar (**Tab. 2.5**). The main residues responsible for this conservation are located in transmembrane domain 3 and the extracellular loop 4, as indicated the aligned AA sequences (**Fig. 2.2**).

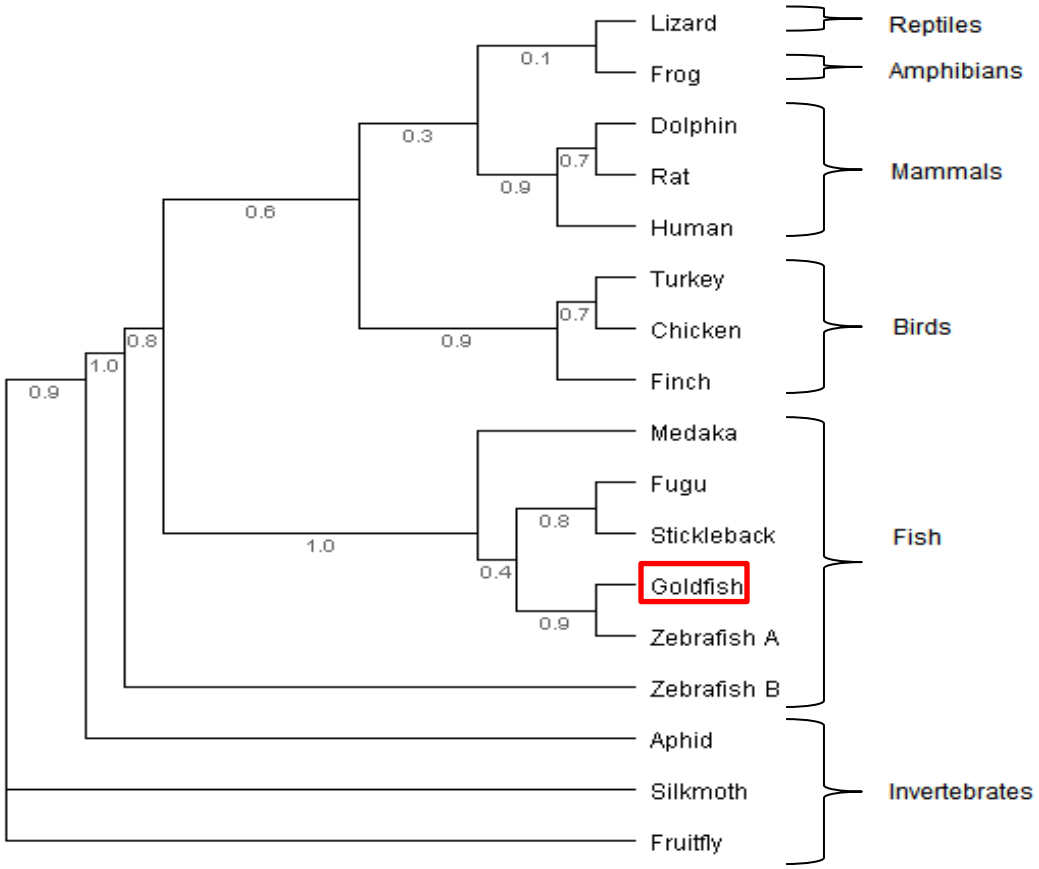


Fig 2.1. *Phylogenetic analysis of the 5-HT transporter, Slc6a4 across vertebrate species using invertebrates as an outgroup. Maxiumum likelihood analysis was used using the program of Dereeper and colleagues (116) Values indicate bootstrap values (n=1000), with 1 representing 100%.*

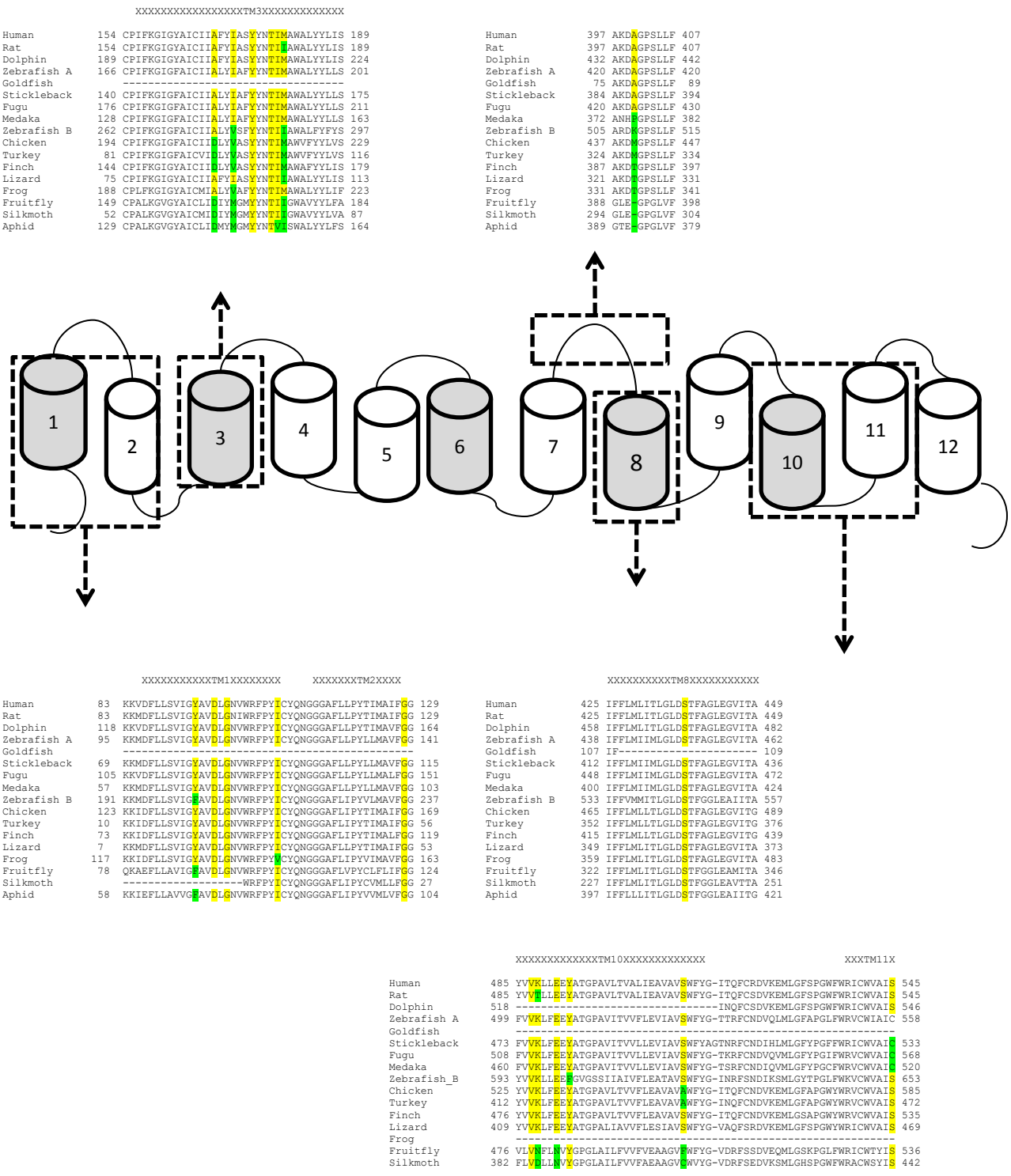


Fig 2.2. *Conservation of SSRI binding residues in 5-HT transporters from vertebrates and invertebrates using Clustal W. Yellow highlight indicates conserved AA compared to the human sequence, green highlight indicates different residues compared to the human sequence.*

Tab. 2.4. *Identified AA residues in the human 5-HT transporter involved in SSRI binding.*

Citation	Type of Study	SSRI studied	Identified human Slc6a4 AA residues involved in SSRI binding
118	Mutagenesis	Citalopram Paroxetine FLX	Met^{180}, Tyr^{495}
119	Mutagenesis	Citalopram Paroxetine	Ala^{169}, Ile^{172}
120	Mutagenesis	FLX Citalopram	Tyr^{95}, Ile^{172}
121	Modelling	Citalopram	Asp^{98}
122	Modelling Mutagenesis	Citalopram	Tyr^{95} Asp^{98} Gly^{100}, Ile^{172} Ser^{438}
123	Mutagenesis	Citalopram Paroxetine	Gly^{100}, Gly^{128}, Tyr^{178}
124	Modelling Mutagenesis	FLX Sertraline	Ile^{179}, Tyr^{175}, Ile^{108}, Glu^{493},Lys^{490},V^{489},Ala^{401}
125	Mutagenesis	Citalopram FLX	Ser^{513}
126	Review	Citalopram	Asp^{98} ,Ser^{545}

Tab. 2.5. *Serotonin transporter sequence identity at the AA level, comparing total sequence identity with identity in AA residues involved in SSRI recognition.*

Vertebrate	Conserved AA compared to human Slc6a4 [average %]	Conserved AA with known function in SSRI binding compared to human Slc6a4 [average %]
Mammals (n=2)	89	94
Fish (n=5)	69	95
Birds (n=3)	80	75
Reptiles (n=1)	85	89
Amphibians (n=1)	72	76
Invertebrates (n=3)	55	35

2.3.2. Tissue distribution of the 5-HT transporter mRNA

Amplification of the reverse transcribed tissue mRNA pools using specific *slc6a4* primers revealed a ubiquitous detection of the amplified *slc6a4* mRNA in all tissues investigated (**Fig. 2.3**). These tissues include the brain parts of the telencephalon and hypothalamus, both of which form the neuroendocrine brain. The tissues in which *slc6a4* mRNA was detected also include peripheral tissues of importance in the endocrine systems of reproduction and growth, including the pituitary and testes. While only the male tissue distribution is shown here, the female distribution of *slc6a4* mRNA paralleled that of the males.

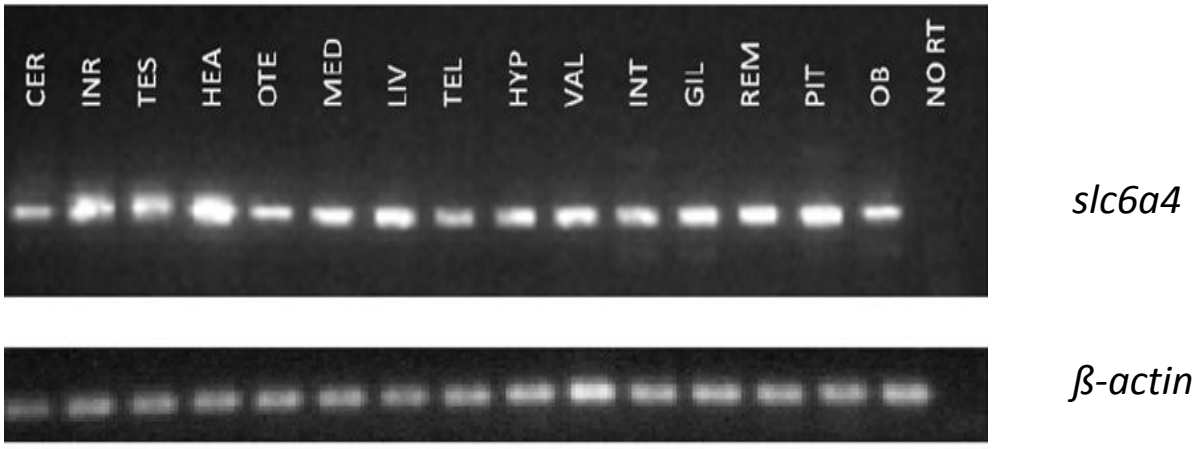
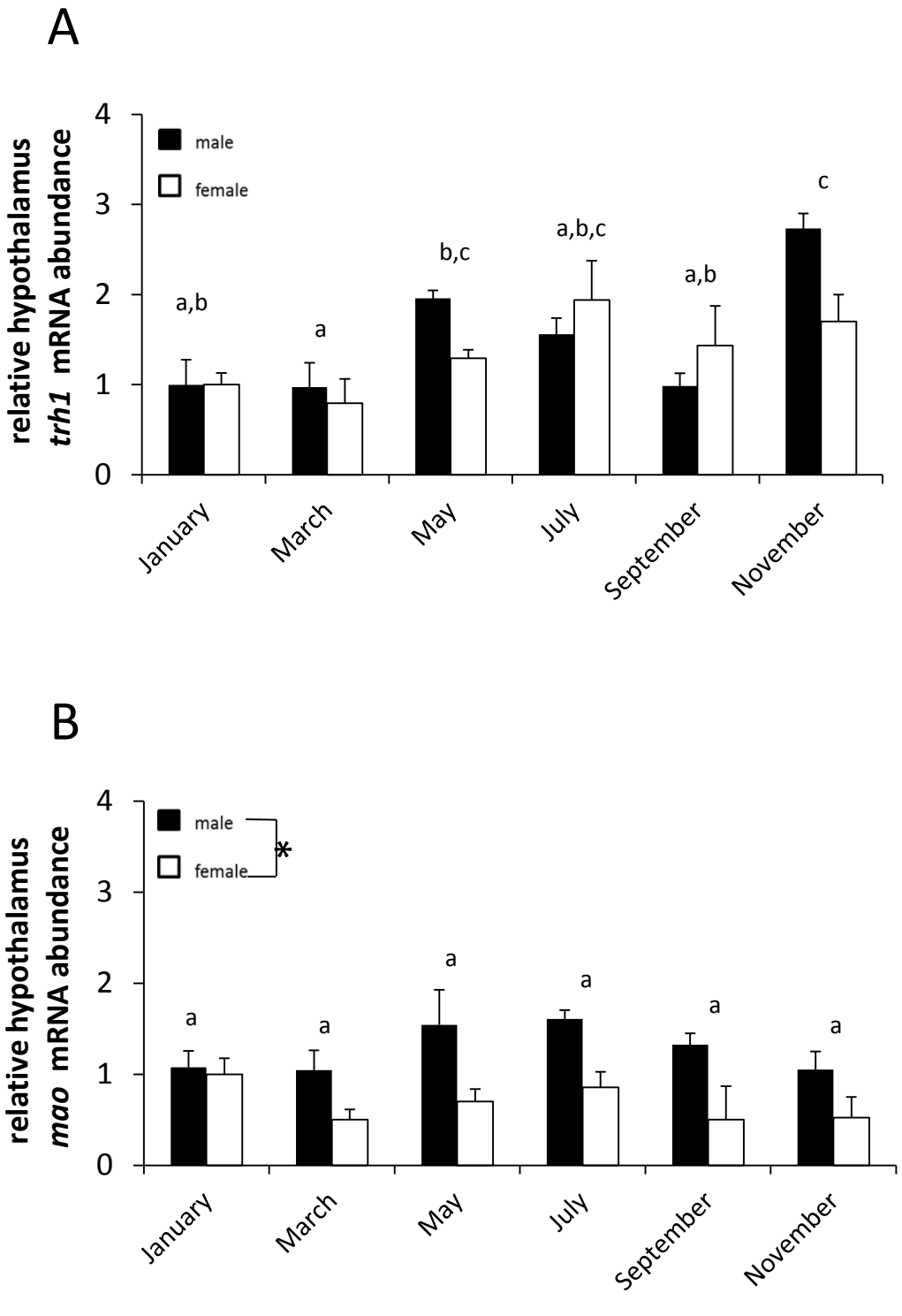


Fig. 2.3. *Tissue distribution of reverse transcribed slc6a4 mRNA in male goldfish (n=2). Shown at the bottom is the distribution of the ß-actin control gene.CER=cerebellum, INR=inter-renal, TES=testis, HEA=heart, OTE=optic tectum, MED=medulla, LIV=liver, TEL=telencephalon, HYP=hypothalamus, VAL=Vagal lobe, INT=intestines, GIL=gill, REM=whole brain;PIT=pituitary, OB=olfactory bulb, NO RT=no reverse transcriptase control. Distribution in female goldfish was also found to be ubiquitous but is not represented here.*

2.3.3. Seasonal mRNA abundance of components of the serotonergic system in Carassius auratus

The mRNA abundance of 5-HT related genes reveals seasonal variations, for example in the case of *trh1* mRNA abundance (**Fig. 2.4A**). Tryptophan hydroxylase 1 shows a significant effects in mRNA abundance (df=11; F=2.8; $p \leq 0.05$), with effect of season (df=5; F=4.9; $p \leq 0.05$) but no effects of gender (df=1; F=0.6; $p > 0.5$) or interaction between gender and season (df=5; F=0.6; $p > 0.05$). A significant increase of *tph1* mRNA abundance in November when compared to mRNA abundance in January, March and September ($p \leq 0.05$). Significant differences in mRNA abundance were observed for *mao* mRNA abundance (df=11; F=3.57; $p \leq 0.05$). No seasonal differences (df=5; F=1.35; $p > 0.05$), but gender differences were observed with a higher *mao* mRNA abundance in male fish (**Fig.2.4B**; df=1; F=21.53; $p \leq 0.01$), however no significant interaction between season and gender were observed (df=5; F=1.15; $p > 0.05$) with a significantly higher expression of *mao* in male fish compared to female fish in May and September ($p \leq 0.05$). The changes in the *slc6a4* mRNA abundance (df=11; F=4.6; $p \leq 0.05$) (**Fig. 2.4C**), are significant only with regard to season (df=5; F=7.4; $p \leq 0.01$), but not with regard to gender (df=1; F=1.0; $p > 0.05$) or and interaction between season and gender (df=5; F=2.0; $p > 0.05$) The seasonal pattern shows a significant increase in *slc6a4* mRNA abundance in March and May compared to January ($p \leq 0.05$), followed by a significant decrease between May and July ($p \leq 0.05$). Significant changes in hypothalamic *5-htr1a* abundance were observed (df=11; F=3.9; $p \leq 0.05$; **Fig. 2.4D**), with a significant effect of season (df=5; F=8.1; $p \leq 0.05$), but no effects of gender (df=1; F=0.8; $p > 0.05$) or the interaction between season and gender (df=5 F=0.3; $p > 0.05$). The *5-ht1a* mRNA abundance was significantly elevated in November compared to abundances between March and September ($p \leq 0.05$). A significant difference in *5-htr2c* mRNA abundance (df=11; F=2.1; $p \leq 0.05$; **Fig. 2.4E**) can be observed with an effect of

season (df=5; F=3.5 p ≤0.05), but no effects of gender (df=1; F=0.23; p >0.05) or the interaction between season and gender (df=5; F=0.7; p >0.05).



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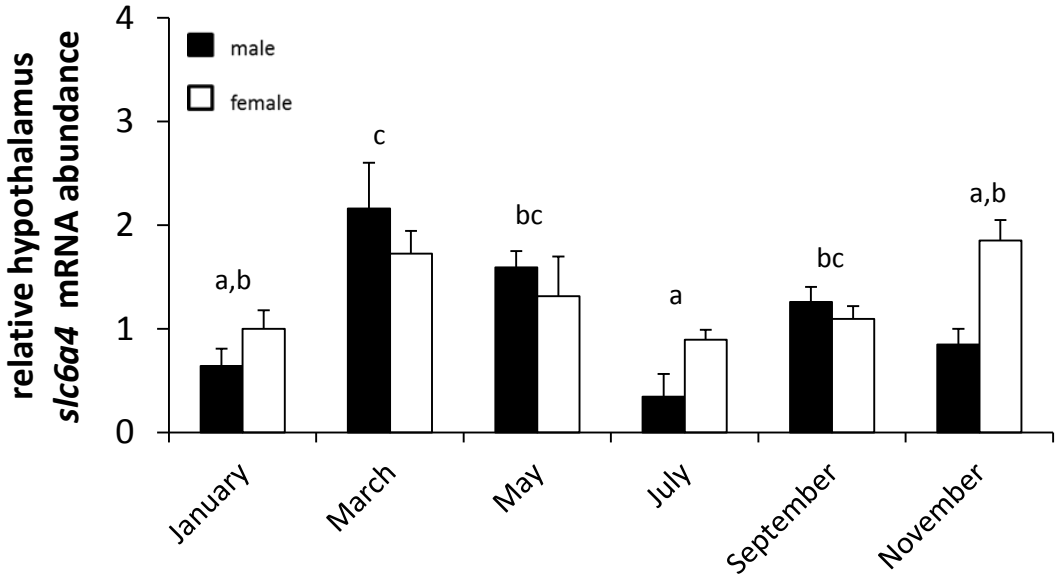
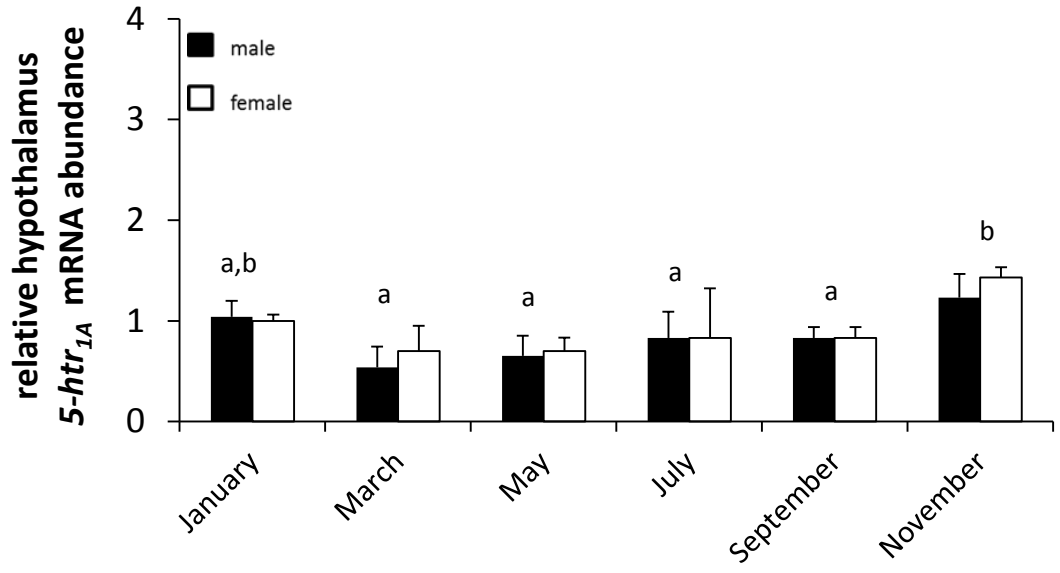

relative hypothalamus
slc6a4 mRNA abundance
4
3
2
1
0
male
female
a,b
c
bc
a
bc
a,b
January
March
May
July
September
November

D


relative hypothalamus
5-htr1A mRNA abundance
4
3
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male
female
a,b
a
a
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March
May
July
September
November

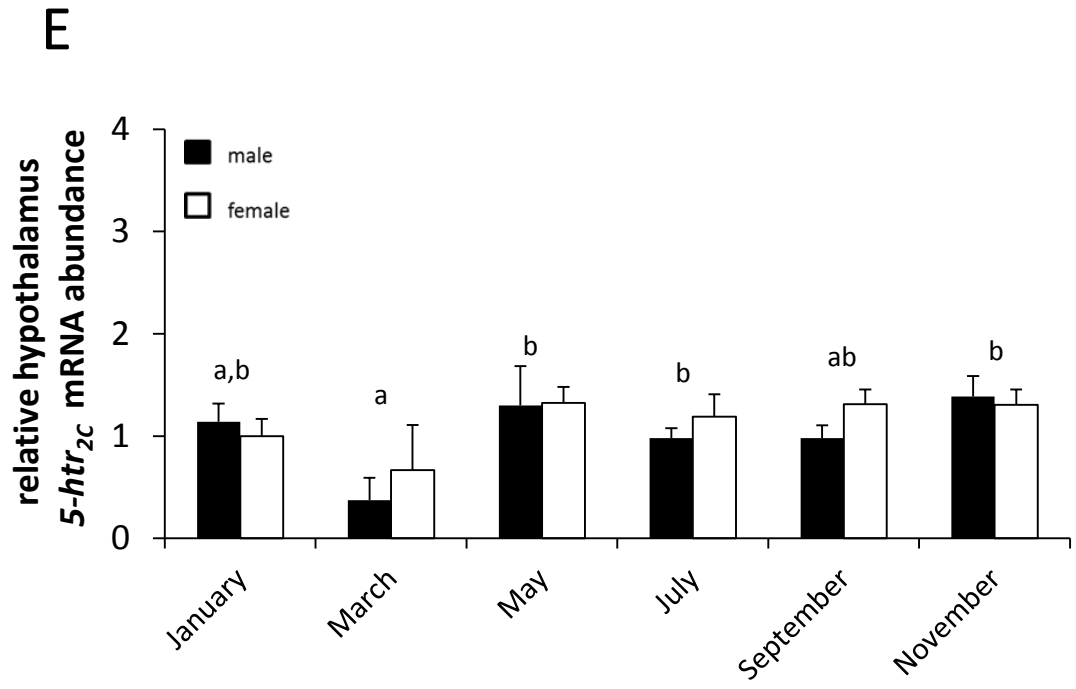


Fig. 2.4. *Relative seasonal hypothalamus mRNA abundance (±S.E.) of trh1 (A), mao (B), slc6a4 (C), 5-htr1a (D) and 5-htr2c (E) in male (black bars) and female (white bars) goldfish. An n=6 was used for each individual time point for each male and female goldfish. Data was analyzed using a two-way ANOVA followed by Bonferroni adjusted post-hoc tests. Same letters signify homogenous groups; different letters indicate a significant seasonal differences at p ≤0.05. Asterisk indicates a significant gender difference.*

2.3.4. Seasonal profile of the GSI and body weight

The seasonal profile of the GSI (**Fig. 2.5**) is modulated by season (df=5; H=15.4; p ≤0.01), but not gender (df=1; H=0.01; p ≤0.01). An interaction between month and gender was also not found (df=5; H=9.16; p >0.05). Post-hoc analysis revealed that GSI was significantly elevated in March and May when compared to other months. Body weight (df=11; F=6.9; p ≤0.01; **Fig. 2.6**) was found to be modulated by season (df=5; F=12.6; p ≤0.01), but not gender (df=1; F=0.08; p >0.05). An interaction between season and gender was detected (df=5; F=3; p ≤0.05).

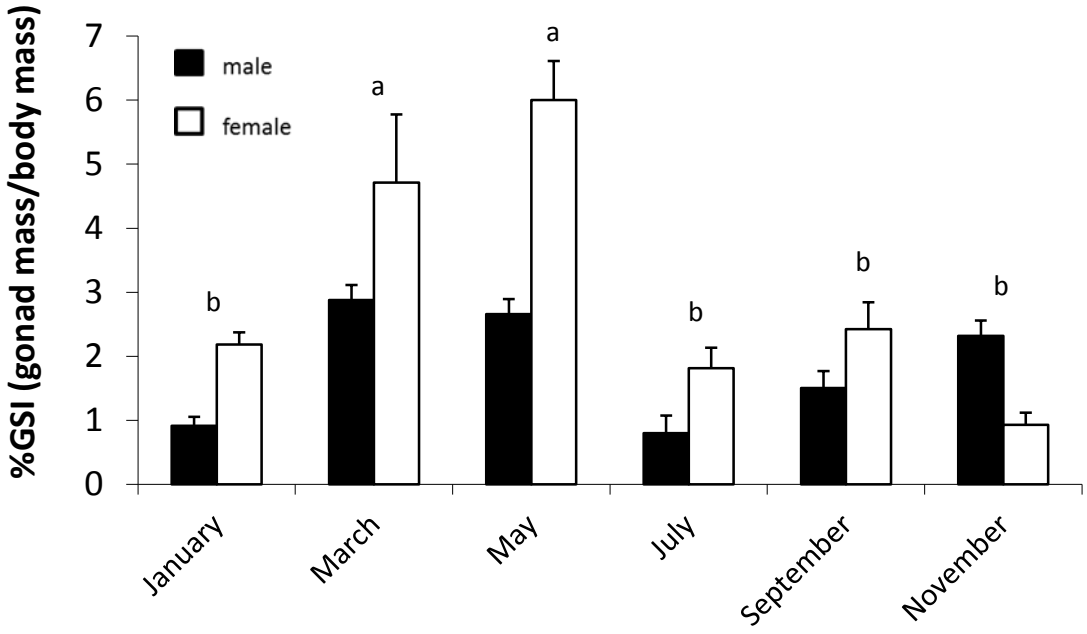


Fig. 2.5. *Mean seasonal values of the GSI ±S.E (calculated as percent value of gonad weight per total bw) in male (black bars) and female (white bars) goldfish. An n=15 was used for each individual time point and gender. Data was not normally distributed and analyzed using a Kruskal-Wallis test with Ray-Scheirer-Hare extension. Same letters indicate a homogenous group, different letters indicate a statistical difference at p ≤0.05 in a Bonferroni adjusted post-hoc test for season. Partially adapted from Zhang and colleagues (117).*

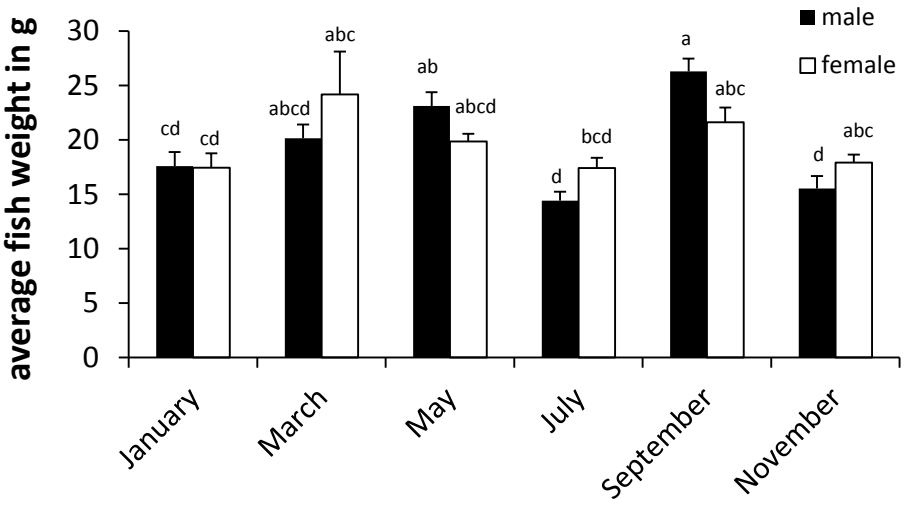


Fig. 2.6. *Mean seasonal weight in male (black bars) and female (white bars) goldfish. Data was log-transformed to fit a normal distribution and analyzed using a two-way ANOVA with Bonferroni adjusted post-hoc test. Different letters indicate a significant difference at p ≤0.05).*

2.4. Discussion

2.4.1. Phylogenetic conservation of slc6a1 – implications for the effects of the FLX target in fish

The concern regarding an ecotoxicological impact of FLX in fish is based on the assumption that there exists a conservation of both the serotonergic system and the specific SSRI target, the 5-HT transporter, between humans and non-target species. In mammals, FLX is metabolized into NFLX by cytochrome P450 II D6 (CYP2D6) in the liver (61). Both compounds act by inhibiting synaptic 5-HT reuptake through the specific 5-HT transporter, with *in vitro* inhibitory constants (K_i) between 14 and 309 nM (61). Fluoxetine is a racemic mixture of R-FLX and S-FLX, and the S-enantiomers of both FLX and NFLX exhibit higher potencies in inhibiting 5-HT reuptake (1.6-fold and 22-fold, respectively) in comparison to the R-enantiomers (61). **Fig. 1.1** indicates that the 5-HT transporter, the target molecule of FLX and other SSRIs, is found in all vertebrate classes. When comparing sequences more specifically, by screening for AA known to be important for SSRI binding to the Slc6a4 (**Tab. 2.4**), it becomes evident that in comparison to humans, fish have more conserved residues involved in SSRI binding than species that are evolutionary more closely related to humans, such as birds (**Tab. 2.5**). Based on this observation, it is reasonable to speculate that fish 5-HT transporters should bind SSRIs with similar pharmacokinetics to human 5-HT transporters (**Fig. 2.5**). Indeed, the only studies in fish measuring 5-HT reuptake in the presence of SSRIs (127,128) revealed k_i values in the low nM range (2.99 nM-204.4 nM), which is highly similar to rats (2.76 nM-163 nM). Some SSRI compounds, such as sertraline, showed an even higher affinity to the zebrafish Slc6a4 than the human Slc6a4 when these transporters were transiently transfected into a human embryonic kidney cell line (128).

The mRNA of the 5-HT transporter in goldfish, *slc6a4*, was detected by PCR in samples of the telencephalon and hypothalamus, indicating *slc6a4* mRNA abundance in the neuroendocrine brain. Studies in zebrafish also show a distinct distribution of mRNA of *slc6a4a* and *slc6a4b* in the brain, which confirms detection in specific areas of the neuroendocrine brain. *In situ* hybridization reveals an early developmental onset (24-h post-fertilization; hpf) of expression of *slc6a4a* in the raphe nucleus. Expression of *slc6a4a* is visible in the pretectic diencephalic cluster and pineal organ at 72 hpf. In the adult zebrafish brain, the expression pattern for *slc6a4a* remains similar to the larval expression stage, however, expression of *slc6a4b* becomes visible in the PVN of the hypothalamus bordering the third ventricle. Overall, combined expression of *slc6a4a* and *slc6a4b* reflects the known patterns of serotonergic innervation in the zebrafish brain (127,129).

The effect of FLX on 5-HT concentrations, which is mediated by the serotonin transporter, was assessed in FLX-exposed hybrid striped bass (*Morone saxatilis x M. chrysops*) and sheepshead minnow (*Cyprinodon vareigatus*): the concentration of 5-HT in the brain decreased by 14-29% (from 31 pg 5-HT/µg protein to 26-22 pg 5-HT/µg protein) in hybrid striped bass exposed to FLX concentrations of 23.2 µg/L, 51.4 µg/L and 100.9 µg/L for 3 d, when compared to 5-HT concentrations in the brain of control hybrid striped bass (130). Serotonin concentrations in the brain of bass exposed to FLX remained significantly decreased in fish given a 6 d recovery period (130). In sheepshead minnow exposed to 30 µg/L FLX, a 53% decrease (from 49 ng 5-HT/g to 23ng 5-HT/g) in the 5-HT concentration of the whole body is measured after 48 h when compared to control minnows (131). Conversely, studies using FLX injections of doses comparable to studies using mammalian models typically lead to an increase in 5-HT concentrations: for example, 5-HT concentrations increased 36% (10pg 5-HT/g protein

to 13.6pg 5-HT/g protein) in the hypothalamus and 26% (7.6pg 5-HT/g protein to 9.6pg 5-HT/g protein) in the telencephalon of goldfish that were repeatedly injected with 5 µg/g bw FLX, when compared to control (132). In gulf toadfish implanted with 25 µg/g and 50 µg FLX /g bw, plasma 5-HT concentrations increased by 23% (from 3.0 ng 5-HT/mL to 3.8 ng 5-HT ng/mL) and 26% (from 3.0 ng 5-HT/mL to 3.7 ng 5-HT ng/mL), respectively after 1 d (133). These differences in serotonergic responses are unexpected, and emphasize the need for an investigation of low, environmentally relevant FLX concentrations, which are generally not tested in mammalian models. This is supported by the fact that injection studies in fish, using FLX concentrations similar to studies in mammals, show an increase in 5-HT concentrations. Further research of FLX-induced changes in the brain 5-HT concentration is clearly needed to investigate dose-responses and potential species differences. While the described differential responses of 5-HT to FLX challenges in fish may further depend on various parameters, such as dose, time, administration protocol and the tissue analyzed, the previously discussed studies unequivocally show an effect of FLX on the serotonergic system in fish. The detection of the fish 5-HT transporter mRNA in the neuroendocrine brain of fish, as well as the ability of FLX to modulate 5-HT concentrations in fish brain, make this system an important candidate for studying disruptive effects of FLX. The tissue distribution of *slc6a4* mRNA, however, shows that mRNA of goldfish *slc6a4* is detected in peripheral tissues, including pituitary and testis. This finding has implications for the investigation of the effects of FLX on reproductive endpoints (as described in Chapters 5 and 6), because SSRIs have been shown to be capable of modulating peripheral 5-HT concentrations in trout (134). Therefore, altered endocrine endpoints in the reproductive axis and the growth axis may stem not only from FLX actions in the neuroendocrine brain, but also from direct FLX actions on peripheral targets.

2.4.2. Seasonal variations in mRNA abundance of genes related to the 5-HT system: Implications for the neuroendocrine disruption of reproduction and feeding/growth by FLX in fish

Seasonal changes in the hypothalamus mRNA abundance of 5-HT related genes are pronounced in the hypothalamus of goldfish. In addition, the mRNA abundance of several targets, for example, *5htr2c* and *slc6a4*, reflects the predicted changes and supports evidence for a role of 5-HT in the seasonal regulation of the neuroendocrine control of reproduction and feeding/growth. For example, the *5-htr2c* mRNA abundance significantly increases over the span of the reproductive period, resulting in a significant difference between mRNA abundance in in March and July. This is in line with the postulated role of $5HTR_2$-like receptors in mediating the stimulating effects of 5-HT on LH release, which is particularly potent in sexually mature goldfish in this reproductive phase (32,33), as well as in sexually mature Atlantic croaker (31). Furthermore, the observed seasonal profile fits the observed increased binding capacity of ketanserin, a $5\text{-}HTR_2$ agonist, in trout hypothalamus preparations, where the binding capacity appeared to be positively correlated with increased 5-HT concentrations in the POA of the telencephalon and the hypothalamus. As previously described, increased 5-HT concentrations are found in this spawning period (4-6). The seasonal expression data suggest that the changes in *5ht2c* mRNA abundance may play a role in regulating 5-HT stimulated LH release in goldfish. However, the agonists and antagonists (ketanserin, LY538557-maleate and ±DOI hydrochloride) used in the studies cannot selectively differentiate between $5\text{-}HTR_{2A}$ and $5\text{-}HTR_{2C}$ subtypes in mammals or fish, especially considering the relatively low conservation of these receptors in fish when compared to mammals (**Tab. 2.3**). The utilization of more specific agents should allow for a verification of the role each specific subtype plays in fish. The 5-HT transporter mRNA abundance increases prior to the spawning period, and decreases sharply afterwards. This pattern correlates well with the described seasonal differences in 5-HT, which include increased

hypothalamic 5-HT concentrations in the neuroendocrine brain and the pituitary of spawning fish (93,135). Therefore, the changes observed may implicate the regulation of mRNA abundance of the 5-HT transporter as a key factor in regulating the seasonal change in 5-HT concentrations in fish. One potential mechanism for this regulation may be the action of testosterone (T), as concentrations of this sex steroid are known to increase in sexually mature goldfish (101), and were found to increase *slc6a4* mRNA abundance in hypothalamic nuclei of male rats (136). Future studies of SSRI binding, using methodology similar to that established for fish brain by Gould and colleagues (137), would be useful in verifying whether these changes translate into differences in hypothalamic Slc6a4 protein expression. However, for the scope of this thesis, the temporal correlation of mRNA abundance of *slc6a4* and activation of the reproductive axis and feeding/growth provides useful information for the seasonal exposure windows of FLX exposures presented in the following chapters.

Monoamine oxidase activity is important in regulating seasonal 5-HT concentrations in goldfish, as it responds to photoperiod and temperature changes (138), which also change goldfish hypothalamic 5-HT content (95). However, this regulation does not appear to be mediated by seasonal changes at the gene expression level, but may be attributed to post-transcriptional modifications and regulatory mechanisms controlling protein concentrations or enzyme activity. With respect to differences in mRNA abundance between male and female fish, the mRNA abundance of *mao* was the only gene to exhibit significant gender differences. A sexual dimorphism for MAO has recently been described in the rat, where *mao* mRNA abundance is positively regulated by SRY, located on the y chromosome (139). It is unknown whether a comparable situation exists in fish. However, in spite of the fact that sexual dimorphisms in the hypothalamic 5-HT system have been described in some fish species, for

example, there is a higher mRNA abundance of *trh* in the developing brain of male tilapia (115), and a more abundant 5-HT innervation in the posterior/prepacemaker nucleus in the male weakly electric fish (*Apteronotus leptorhynchus*) (140), true sexual dimorphisms are difficult to establish (141). Furthermore, no functional differences have been found between male and female goldfish in terms of the 5-HT modulation of LH and GH (32,33). Therefore, although a sexual dimorphism of the neuroendocrine 5-HT system would have important consequences with respect to the regulation of physiological endpoints by SSRIs, such as the documented gender dependent SSRI regulation of the stress axis in rats (142), evidence from the seasonal mRNA abundance of 5-HT components is overall probably not indicative of a sexual dimorphism at the gene expression level. Nevertheless, I subsequently investigated both male and female goldfish in waterborne FLX exposures designed to investigate reproductive parameters (Chapters 5 and 6).

2.5 Conclusions

In summary, the determined partial AA sequence for goldfish 5-HT transporter derived from the cloned reverse-transcribed mRNA suggests that the human pharmaceutical FLX is likely to bind to the goldfish 5-HT transport with high affinity. The sites of mRNA detection include the neuroendocrine brain, the site where 5-HT acts to stimulate reproduction and inhibit growth and feeding season-dependently. The seasonal mRNA abundance of *slc6a4* correlates well with these described roles and implies that the mRNA abundance of this transporter is an important element in the seasonal regulation of 5-HT signalling. As such, the 5-HT transporter may be particularly susceptible to FLX disruption, which by altering 5-HT homeostasis may interfere with this temporally controlled effect of 5-HT on reproduction and feeding/growth. Additionally, *slc6a4* mRNA was detected in peripheral tissues, which warrants the investigation

of direct effects on the periphery, because reproductive endpoints, such as sex steroid concentrations, are not solely dependent on neuroendocrine-controlled hormone release, but also depend on the hormonal effects on peripheral tissues, such as the gonads. These factors aided in the experimental designs devised for the FLX exposures (Chapters 3, 5 and 6).

Chapter 3: The effects of pharmacological FLX injections on the reproductive axis in female goldfish

Partially based on: Mennigen et al., 2008. The effects of fluoxetine on the reproductive axis in female goldfish, Carassius auratus*. Physiol. Genomics 35: 273-82.*

3.1 Introduction

This study investigated the effects of pharmacological FLX injections on the reproductive axis in female recrudescent goldfish. The neuroendocrine regulation of reproduction in goldfish has been well studied and is influenced by numerous neurotransmitters and neuropeptides (143), as described in Chapter 1 of this thesis. Briefly, the monoamines 5-HT and norepinephrine (NE) stimulate GnRH release from the brain preoptic-anterior hypothalamic region in female goldfish *in vitro*, while DA exhibits potent inhibition of GnRH and LH release (144). A stimulatory role for 5-HT in the neuroendocrine regulation of reproduction is also well-established in fish, as noted in Chapters 1 and 2. For example, an increase in 5-HT turnover and a concurrent decrease in DA have been observed during the peri-ovulatory period in rainbow trout (93). Neurons for 5-HT and GnRH have been reported in close proximity to each other in the POA of the hypothalamus in the Atlantic croaker (35). Khan's and Thomas' (31) study of the same species provided evidence for the involvement of a 5-HTR_2-like receptor subtype mediating the 5-HT potentiation of GnRH-induced LH release in post-vitellogenic, but not regressed, fish. Serotonin was shown to stimulate LH release in sexually mature goldfish *in vivo*, an effect potentiated by the acute co-administration of FLX (32). Based on this extensive evidence for a serotonergic regulation of the reproductive axis, I hypothesized that FLX would alter the function of the reproductive axis in female goldfish and that these changes would at least be partially correlated with alterations in the 5-HT regulation of neuroendocrine function in the hypothalamus.

I addressed this hypothesis by measuring several endpoints to establish a mechanistic framework for subsequent waterborne FLX exposures. As an indicator of reproductive function, serum sex steroid concentration of E_2, as well as its targets in the neuroendocrine brain, the three teleost nuclear estrogen receptors, ERα, ERß and ERß2, were measured. Concentrations of the neurotransmitters 5-HT, DA, and NE, together with their primary metabolites, were measured using High Performance Liquid Chromatography (HPLC) in the hypothalamus and telencephalon, the two major brain regions involved in neuroendocrine function. Changes in the hypothalamus were also investigated by analysing changes in the transcriptome in response to FLX treatment, using microarray and real-time RT-PCR.

A preliminary transcriptome analysis established that IT mRNA was significantly reduced in the hypothalamus of FLX-treated fish. Isotocin is the fish homolog of the mammalian nonapeptide oxytocin (145), which plays an important role in mammalian reproductive behaviour (146) and physiology (147).I therefore investigated a specific involvement of IT in the reproductive axis of teleost fish. While studies in fish reveal a role for IT in reproductive behaviour (148), the impact of IT on the endocrine axis of reproduction has not been investigated. Given the changes detected in *it* mRNA in hypothalamus and telencephalon, and the concurrent decrease in E_2 concentrations in FLX-injected fish, I hypothesized that IT affects serum concentrations of reproductive hormones in teleost fish. Specifically, I predicted that IT would have a stimulatory effect on circulating E_2 concentrations, as a concurrent decrease in *it* mRNA and circulating E_2 was observed in FLX-injected female goldfish. A second experiment was therefore conducted to establish whether a functional link existed between the observed neuroendocrine changes and the reproductive endpoints.

3.2 Material and Methods

3.2.1 Animals and experimental design

Common female goldfish were purchased from a commercial supplier (Aleong's International, Mississauga, ON, Canada) and maintained at 18 °C under a natural simulated photoperiod. The experiment was conducted in December, with lights (50 lx) synchronized to Ottawa, ON, Canada day length (07.00 h–17.00 h). Fish were fed and maintained on standard flaked goldfish food. Goldfish were anesthetized with 3-aminobenzoic acid ethylester (MS-222) prior to all handling and dissection procedures. Care was taken to standardize all handling, injection, and sampling protocols. All procedures were approved by the University of Ottawa Protocol Review Committee and followed standard Canadian Council on Animal Care guidelines on the use of animals in research. Sexually recrudescent female goldfish with body weights ranging from 15 to 51 g were i.p. injected with FLX (Sigma-Aldrich, Oakville, ON, Canada). Fifty fish were injected twice a week over the course of 17 d, for a total of five injections of 5 µg FLX/g bw (**Fig. 3.1**) with an injection volume of 1 µL/g. This concentration represents a low pharmacological concentration in the mammalian literature, where typically concentrations of 10 mg/kg are used in rat studies (149). This concentration was chosen as it is the lowest injected dose at which mammalian studies revealed an impact on reproductive behaviour (146). Furthermore, a study that investigated aggression and arginine vasotocin (*vt*) mRNA abundance in the POA of male bluehead wrasses (*Thalassoma bifasciatum*) used a similar FLX concentration of 6 µg/g bw (150). The length of the treatment was chosen to evaluate the more long-term effects that are known to be important in SSRI action (149) and that are a likely scenario in the environment given the pseudo-persistency of this drug in aquatic systems (151). Goldfish were injected twice a week to account for the 2 d to 6 d half-life of FLX *in vivo*, as

described in similar experiments in mammalian models (152). Fluoxetine was dissolved in physiological saline (0.6% NaCl). The control group (n=50) received an equivalent volume of saline. One day after the final injection, goldfish were killed by spinal transection and brain tissues were rapidly dissected, immediately frozen on dry ice, and stored at -80 °C until analyzed. I also performed experiments to determine the effects of IT on circulating sex steroid concentrations. Sexually regressed female goldfish with body weights ranging from 17 g to 48 g were injected with 1 µg/µL IT (ChemImpex, Intl., Wood Dale, IL) dissolved in dimethyl sulfoxide (DMSO). The injection was given at 1 µL/g bw, and the fish were killed 12 h after the injection. Control groups received an equivalent volume of DMSO. The dose of the neuropeptide was chosen on the basis of other studies investigating effects of neuropeptides on LH release in goldfish, as well as doses used to investigate the effects of the homolog oxytocin on LH release in mammals (153,154). The time between injection and sampling (12 h) was chosen to maximize the time window because oxytocin effects on LH in mammals are known to be relatively slow (154).

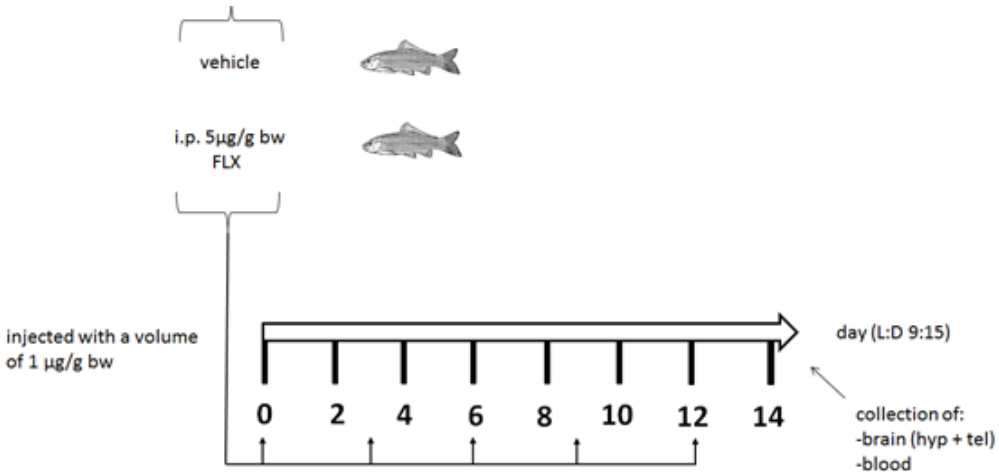


Fig. 3.1. *Schematic representation of the experimental design*

3.2.2 High Performance Liquid Chromatography

The HPLC protocol[2] described by DiBattista and colleagues (155) was used. Frozen individual telencephali and hypothalami (n=7) were placed into 1 mL of a solution containing 0.1 mM Na_2EDTA, 0.3 mM monochloracetic acid, 50 mL methanol, 12.5 pg/µL internal standard DHBA, and distilled water and was kept on dry ice. Samples were sonicated and total protein content assessed with the BCA protein assay reagent kit (Pierce, Rockford, IL), using an ICK Multiscan/MCC 348 (Titertek, Huntsville, AL) plate reader. Samples were centrifuged at 12,000 rpm for 3 min in a 4 °C microcentrifuge and supernatants were transferred into HPLC microvials. Brain monoamines were then quantified by high performance liquid chromatography (HPLC) using electrochemical detection. The HPLC consisted of a solvent-delivery system (Waters590/WaterPump, Mississuaga, ON, Canada), an autoinjector (Waters712WISP), a reverse-phase column 1100 series ZorbacXDB/C8 4.62 column with a length of 150 mm (Agilent Technologies, Mississauga, ON, Canada) kept at 30°C and a 5100A Coulochem detector (ESA, Bedford, MA, USA) with two electrodes at oxidizing potentials of –330 mV and +350 mV. The mobile phase consisted of 1.3 g/L heptanesulphonic acid sodium salt, 0.1 g /L disodium ethylene tetracycline, and 7.3 ml triethyloamine adjusted to pH 2.45 with orthophosphoric acid. Sample monoamine levels were indexed to standard solutions of known concentration, corrected for recovery of the internal standard, and expressed relative to total tissue protein content. Chemicals used in standards were purchased from Sigma-Aldrich. Data were log-transformed to fit a normal distribution and were analyzed by Student's *t*-test with SYSTAT v10 (Systat Software, San Jose, CA) run on Windows XP.

[2] The HPLC analysis was conducted by Jerzy Kulczycki in Dr. Anisman's lab at Carleton University, Ottawa, ON, Canada.

3.2.3 Radioimmunoassay (RIA) for LH

Blood (100–300 µL) was collected from anesthetized goldfish by caudal puncture, both from FLX-injected (n=10) and saline-injected control (n=10) animals at the end of the experiment. Blood from the second experiment was also collected from IT-injected (n=15) and DMSO-injected control (n=15) animals 12 h after the IT injection. Blood samples were allowed to clot and serum was harvested by centrifugation (10 min at 3,000 rpm) and stored frozen (-80 °C) until analyzed. The double-antibody RIA protocol of Zhao and colleagues (153) was used to analyze circulating LH concentrations[3]. Data from both experiments were tested for normality with SYSTAT v10 and were not normally distributed. A Mann-Whitney U-test was used to test for significant differences.

3.2.4 Radioimmunoassay for T and E2

Sex steroids were extracted and T and E_2 concentrations were assessed by RIA according to the protocol of McMaster and colleagues (156). Two hundred µL of the original serum samples used in the LH assay were used to extract sex steroids (n=10). Data were tested for normality with SYSTAT v10 and were not normally distributed. The Mann-Whitney U-test was used to test for significant differences in plasma E_2 concentration of serum samples taken from female goldfish at the end of the FLX injection experiment. The detection limit (0.031 ng/mL) was defined as the first E_2 concentration significantly different from the zero point on the standard curve (ANOVA followed by Tukey's post-hoc test). This value was assigned to samples that were below the detection limit. The remaining plasma samples from those used for the LH measurements in the second experiment (n=15) were pooled for a total of five samples (n=5), each representing three individual females. Two hundred µL were obtained from these samples

[3] The actual assay was run by Dr. E Zhao in Dr. Trudeau's lab at the University of Ottawa, ON, Canada.

to extract and measure the effects of IT on T and E_2. All samples were above detection limits. Data were not normally distributed, and were therefore analyzed as described above.

3.2.5. RNA extraction and cDNA synthesis

Total RNA was isolated using the TRIzol method (Invitrogen, Burlington, ON, Canada) following the manufacturer's protocol. Total RNA was extracted from eight pools consisting of 2-3 individual hypothalami or telencephali from either control or FLX-injected female goldfish. Total RNA concentration was evaluated with the GeneQuant spectrophotometer (Amersham Pharmacia Biotech, Oakville,ON, Canada). To remove genomic DNA, samples were treated with DNase and an RNAeasy Micro kit (Qiagen, Mississauga, ON, Canada). The cDNA was prepared from 1 µg total RNA and 200 ng random hexamer primers (Invitrogen) with Superscript II RNase H^- reverse transcriptase according to the manufacturer (Invitrogen). Each 20 µL reaction was diluted five-fold in nuclease-free water and used as the template for the real-time RT-PCR assays.

3.2.6. Multiplex real-time RT-PCR and SYBR Green assays

The Mx4000Multiplex Quantitative PCR System (Stratagene, La Jolla, CA) was used to amplify and detect the transcripts of interest. Primers were synthesized by Invitrogen, and gene-specific dual-labeled fluorescent probes were purchased from IDT (Toronto, ON, Canada). The multiplex reaction for the reference gene *β-actin* in combination with *erα*, *erß1*, and *erß2* was previously validated in the Trudeau lab[4] (157). Data were normalized to *ß-actin*, because its mRNA abundance did not change significantly with FLX treatment in the hypothalamus or telencephalon ($p > 0.05$). A SYBR Green assay was developed to validate differentially regulated

[4] This assay was developed and optimized by Dr. Vicki Marlatt and Kate Crump, MSc in Dr. Trudeau's lab at the University of Ottawa, ON, Canada.

candidate genes identified by the microarray in the hypothalamus. Pooled samples were used for microarray analysis (n=3), while additional, independent samples (n=8) were used for the SYBR Green assay. Each PCR reaction was run with parameters described by Martyniuk and colleagues (158), with primer concentrations of 150 nM for all genes validated. Primers (**Tab. 3.1**) were designed with Primer3 and ordered from Invitrogen. Data were analyzed with the MX4000 software package. Standard curves relating the initial relative mRNA abundance to fluorescence and amplification cycle were generated by using the amplified PCR product as a template and were used to calculate relative changes in mRNA abundance in each sample. *β-actin* mRNA was used as a normalizing gene in the SYBR Green assays because its mRNA abundance did not significantly change with treatment. If data were normally distributed or could be transformed to fit a normal distribution, a two tailed t-test was used to test for statistical differences in gene expression. If data were not normally distributed (SYSTAT v10), so significant changes in gene mRNA abundance were evaluated with the Mann-Whitney U-test.

Tab. 3.1. *Primer sequences used in real-time RT-PCR assays.*

Gene target	Primer sequence 5'3' (FW)	Primer 5'3' sequence (RV)	Gen bank
it	ATCTTGGCTACTGGCAGCTT	GTATCTGCTGTGGTGAAGGT	AF322651
gpx-4b	CCAAAGGCTACAAGGCAGAG	TTGATCCGAAAGGCTACAGG	NM_001030070
atpif1	GTGGCCCATATCAGGAGAAA	ACTGTCCAGGGTGAATGGAA	NM_001089521
arl6ip5	GTGGGCAGGAGAGAACAAAG	TCCACACAAGGACAACAGGA	NM_001006096
slc1a1	AGAGCGGAGAAGCAGAGATG	ATGCTGGAGACGATGAGAGG	HM138690.1
slc3a6	CACTGCAACAACACCTGGAA	TAGAGCACCACGATGACCAC	EF371919.1
epd-I	TGAGCGGAACAATGAAAGTG	TCAGACTCGTGAGTGGCATC	X14134.1
pcna	TGATGGATCTGGATGTGGAG	TGCTGGTCTGTGAGAGCTTG	AF140608
ß-actin	ACTACTGGTATTGTGATGGACTCC	CGGTCAGGATCTTCATCAGGTAG	AB039726

3.2.7. Microarray hybridizations

The production and use of our goldfish-carp cDNA microarray was previously described and validated (157,158); for a detailed description of the microarray, see Williams and

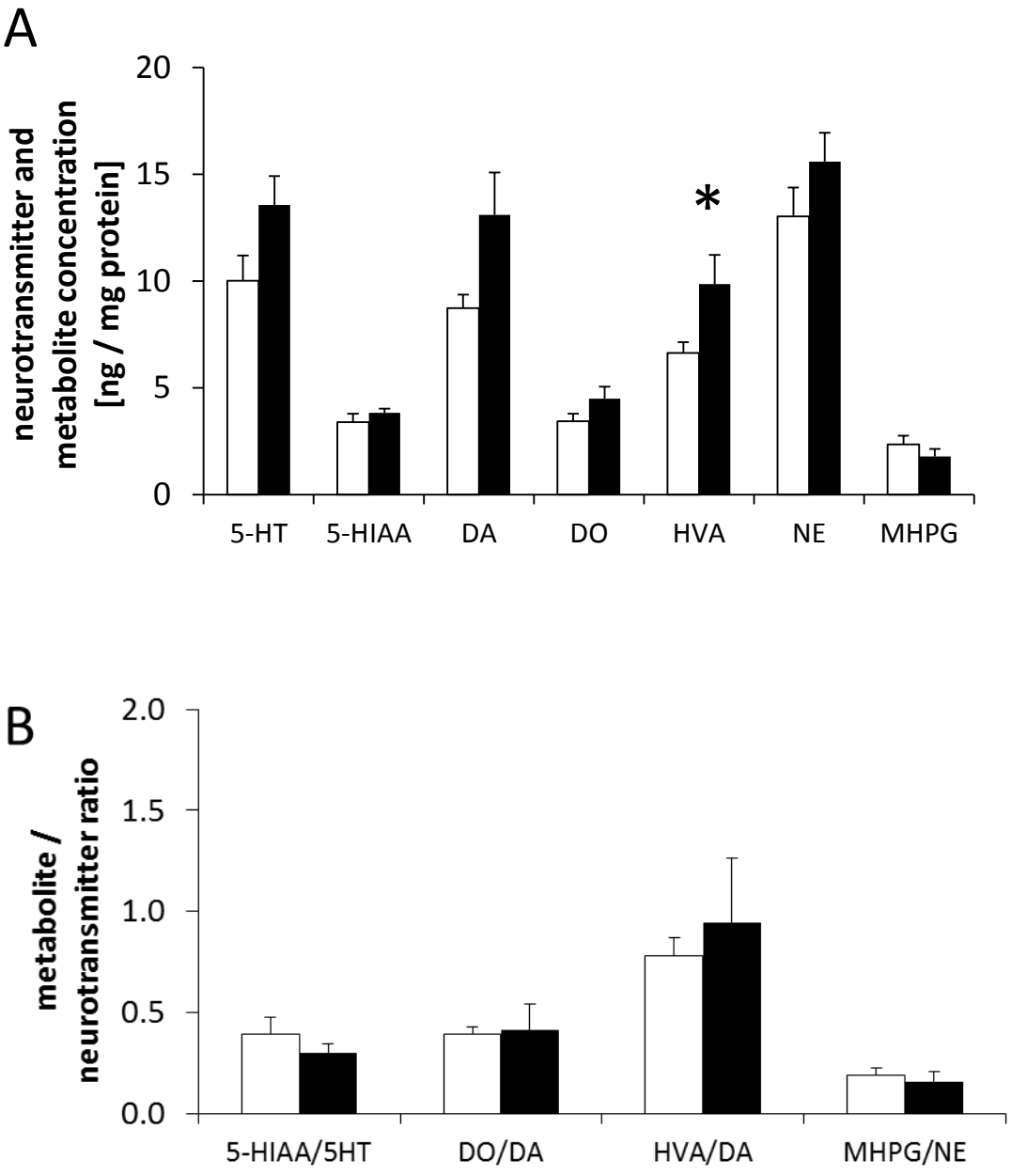
colleagues (159). A total of four microarray hybridizations were performed for hypothalamic tissue, and served as the initial screen for the effects of FLX on the transcriptome of the neuroendocrine brain. I compared three independent treatment samples (containing 4 or 5 pooled hypothalami) to a collective pool of control samples (representing n=30 control fish). This experimental design allows for less technical variation because only one reference is handled, while maintaining biological variation of the treatment samples (160). I also performed a replicate of one treatment pool using the opposite dye (dye-swap design). The hybridization and scanning protocols used were previously described (158,161). In brief, hybridizations were performed according to the protocol supplied by Genisphere (Hatfield, PA). Microarrays were scanned at full-speed 10 μm resolution with the ScanArray 5000 XL system (Packard Biosciences, PerkinElmer, Woodbridge, ON, Canada), using both red and blue lasers. Images were obtained with ScanArray Express software using automatic calibration sensitivity varying photomultiplier (PMT) gain (PMT starting at 65% for Cy5 and 70% for Cy3) with fixed laser power at 80% and the target intensity set for 90%. Microarray images were analyzed with QuantArray (Packard Biosciences, PerkinElmer), and raw signal intensity values were obtained for duplicate spots of genes. Raw intensity values for all microarray data and microarray platform information were deposited in the NCBI Gene Expression Omnibus database (platform accession no. GPL3735; series accession no. GSE5420). Alien Spot Report Genes 1-10 (Stratagene) wre used to normalize the microarray data and the significance analysis of microarrays (SAM) method was used (162) for microarray analysis[5]. Candidate genes identified as being differentially regulated were further analyzed with the GOSSIP package in BLAST2GO (163) for Gene Ontology (GO) categorizations.

[5] The microarray data were normalized and analyzed by Huiling Xiong in Dr. Xia's lab at the University of Ottawa, ON, Canada.

3.3 Results

3.3.1 Fluoxetine increases homovanillic acid (HVA) concentration and norepinephrine turnover in the neuroendocrine brain

Concentrations of 5-HT ($p=0.07$) and DA ($p=0.06$) in the hypothalamus showed a marginal, non-significant increase of 35% and 50%, respectively, in FLX-treated animals (**Fig. 3.2A**). The DA metabolite HVA increased significantly in FLX-treated animals ($p \leq 0.05$). There were no significant changes in other neurotransmitters or metabolites measured. The ratio [metabolite]/[parent monoamine] was used as an index of brain monoaminergic activities. Calculation of this index indicated no significant changes in monoamine turnover in the hypothalamus **(Fig. 3.2B)**. There was an observed non-significant trend for an increase in 3-methoxy-4-hydroxyphenylglycol (MHPG) concentration and a concurrent trend for a decrease in NE content in the telencephalon (**Fig. 3.2C**), resulting in a significant increase in NE turn-over in the telencephalon ($p \leq 0.01$; **Fig. 3.2D**).


A
neurotransmitter and metabolite concentration [ng / mg protein]
20
15
10
5
0
*
5-HT
5-HIAA
DA
DO
HVA
NE
MHPG
B
metabolite / neurotransmitter ratio
2.0
1.5
1.0
0.5
0.0
5-HIAA/5HT
DO/DA
HVA/DA
MHPG/NE

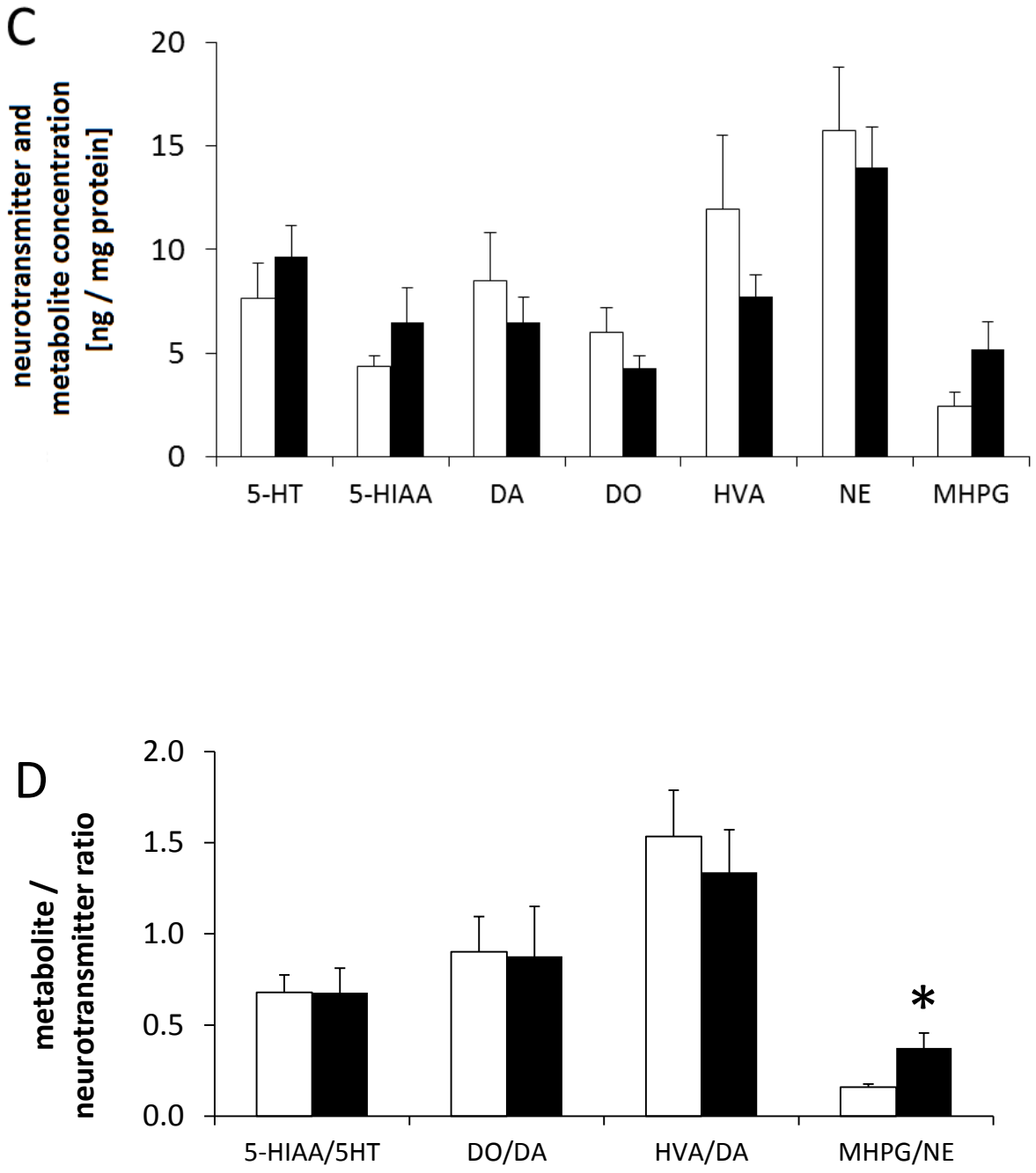


Fig. 3.2. *Neurotransmitter and metabolite concentrations in the hypothalamus (A) and the telencephalon (C). Turn-over ratios for monoamines in the hypothalamus (B) and the telencephalon (D) are shown. An n=7 was used for both tissues for the analysis of monoamines and metabolites. White bars indicate saline-injected control animals and black bars indicate FLX-injected animals. Individual monoamines and ratios were compared using a t-test. Asterisks indicate a significant difference at p ≤0.05.*

3.3.2 Fluoxetine suppresses E_2 concentration without altering LH concentration in the serum

Serum LH concentrations were not affected by FLX treatment in female fish used for the neurotransmitter and microarray analysis (**Fig. 3.3A**). In contrast, E_2 concentration was significantly decreased ($p \leq 0.05$) by approximately three-fold in the serum of FLX-treated fish (**Fig. 3.3B**).

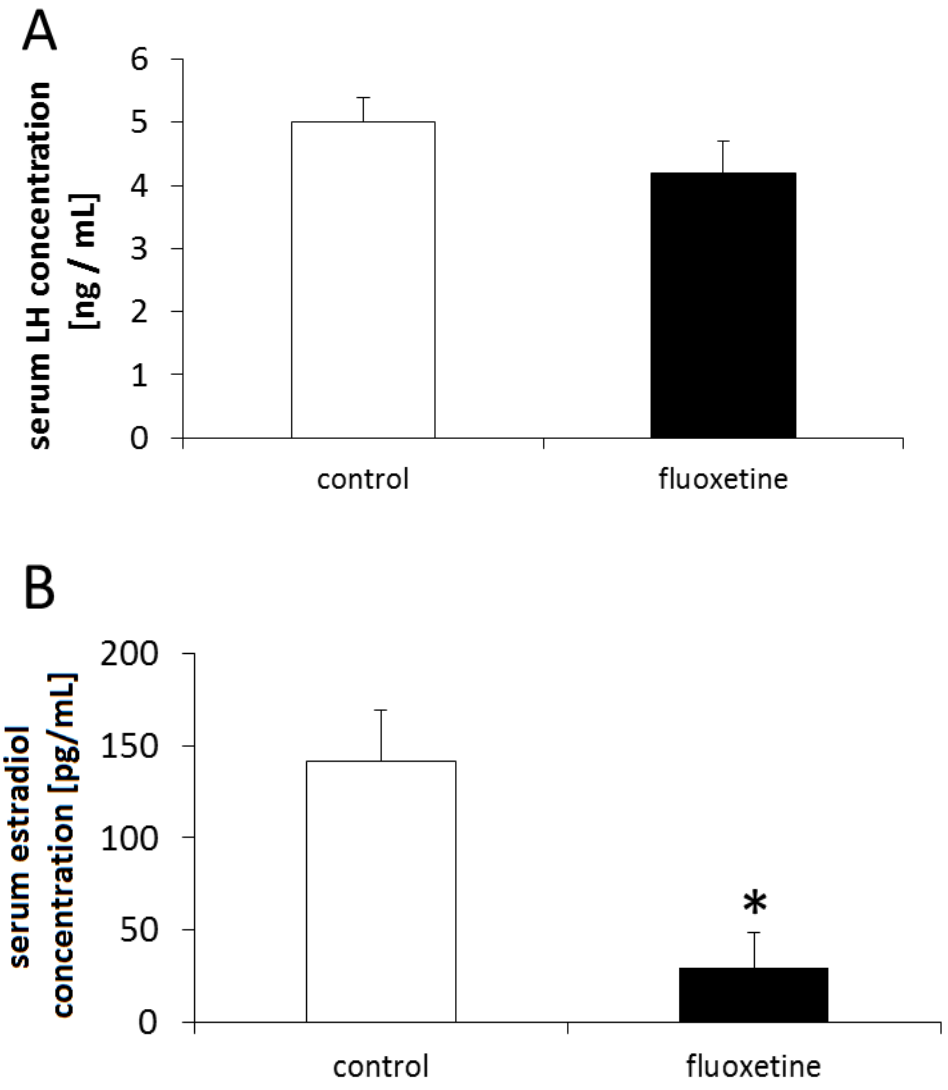
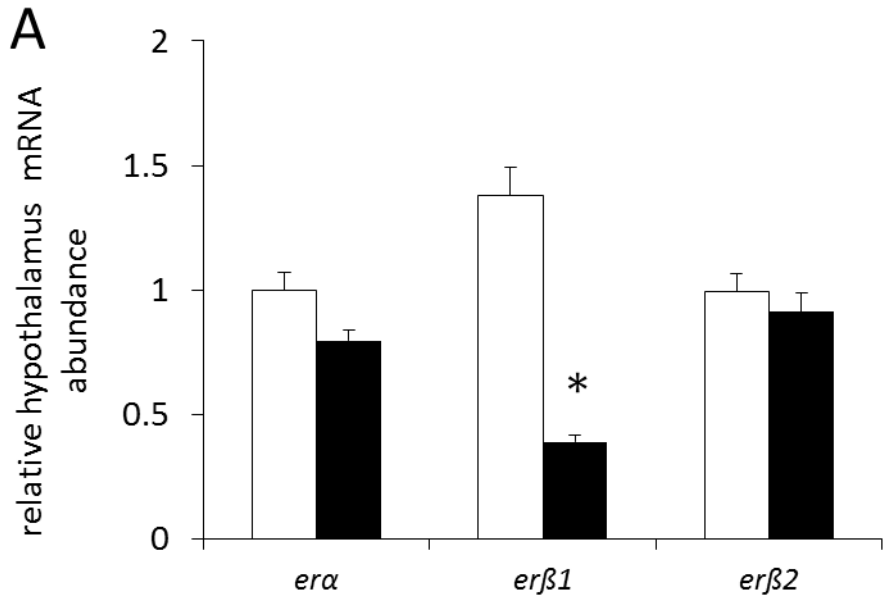


Fig. 3.3. *Serum concentrations (±S.E.) of LH (A) and E_2 (B) in control (white bar) and FLX- treated (black bar) female goldfish. An n=10 serum samples was used for LH and E_2 analysis. Data normally distributed was analyzed by a two-tailed t-test, data that were not normally distributed were analyzed using a Mann-Whitney U test. Asterisk indicates significant difference at $p \leq 0.05$.*

3.3.3. Fluoxetine decreases relative mRNA abundance of erα and erß1 in the neuroendocrine brain

Because FLX decreased serum E_2 concentration, I determined whether the E_2 system in the neuroendocrine brain was also affected. In both hypothalamus and telencephalon, *erß1* mRNA abundance was decreased significantly in FLX-treated fish (**Fig. 3.4A-B**). Abundance of *erß1* mRNA decreased 4-fold in the telencephalon ($p \leq 0.01$) and 3.5-fold in the hypothalamus ($p \leq 0.01$). Abundance of *erα* mRNA (**Fig. 3.4A**) also decreased significantly in the telencephalon, by 1.7-fold ($p \leq 0.01$). Fluoxetine did not significantly change *erß2* mRNA abundance in either tissue (**Fig. 3.4A-B**).



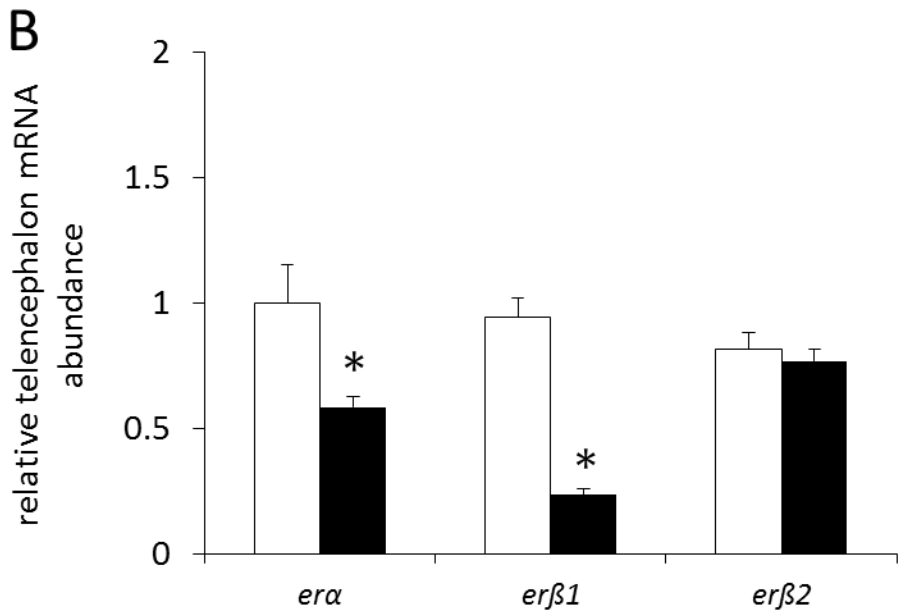


Fig. 3.4. *Relative mRNA abundance (±S.E.) of E_2 receptor isoforms in goldfish hypothalamus (A) and telencephalon (B). An n=8 samples were used for either tissue. White bars indicate saline-injected control animals and black bars indicate FLX-injected fish. Data normally distributed was analyzed by a two-tailed t-test, data that were not normally distributed were analyzed using a Mann-Whitney U test. Asterisk indicates significant difference at p≤0.05.*

3.3.4 Microarray and real-time RT-PCR identified the mRNA of the neuropeptide IT as being decreased by FLX

A microarray and real-time RT-PCR assays were used to identify FLX-regulated target genes in the hypothalamus to investigate the potential neuroendocrine mechanisms involved in the observed effects of FLX on circulating E_2. Several candidate target genes were identified for which mRNA abundance was regulated by FLX treatment (a total of 183). Twenty mRNA targets were increased in FLX-injected fish; 17 of these had q-values below 5, while 3 additional targets had q-values between 5 and 7.5. Of the 17 targets with q-values below 5, 12 could be annotated using the BLASTX homology search. 1 of the 3 targets with q-values between 5 and 7 could be annotated. There were 163 gene targets whose mRNA was identified as being decreased by FLX treatment. Of these targets, 70 had q-values <5, and 93 were between 5 and 7.5. Fifty-one of the 70 targets could be annotated, while 43

targets of the group of 93 could be annotated using BLASTX homology search. The remaining targets either did not retrieve any significant results in a homology search (referred to as expressed sequence tags, ESTs), or in some cases were redundant hits and removed from the list. An enrichment analysis containing both sets of annotated targets (increased and decreased mRNA) using the GOSSIP program of BLAST2GO (163) was computed, but no significant over-representation of any GO (gene ontology) group was found in comparison to the known distribution of categories on the microarray. Therefore, results are represented as multi-level GO terms at a cut-off level of three targets per GO term class. The results of a GO term-based classification of identified targets with overall mRNA abundance (both increased and decreased) are shown in **Fig. 3.5**. They represent GO categories of cellular localization (A), molecular function (B) and biological process (C) of the targets, respectively. Genes in numerous processes were affected and included signal transduction, various metabolic pathways, organogenesis, and reproduction. A full list of targets whose mRNA abundance was increased or decreased can be found in **Tab. 3.2**. As this study investigated mechanisms of FLX on reproduction, I chose several targets, based on the most important categories of overall identified targets, keeping in mind potential mechanisms linking FLX-induced changes in the hypothalamus to the observed inhibition of the reproductive endpoint, serum E_2 concentration. Therefore, I specifically validated targets related to neurotransmitter release, whose importance in regulating the neuroendocrine control of reproduction in teleosts was described earlier. These targets included mRNA abundance of targets involved in DA and glutamate metabolism. As well, I chose the reproductive hormone IT to further verify its decreased mRNA abundance with real-time RT-PCR in the hypothalamus and telencephalon. Isotocin mRNA was significantly reduced with FLX treatment in both the hypothalamus, by 6.4-fold ($p \leq 0.01$; **Fig. 3.6A**), and the telencephalon, by 4.8-fold ($p \leq 0.01$; **Fig. 3.6B**). As several mRNA targets were implicated in

neuroplasticity, ependymin (*epd-I*) and two targets involved in oxidative stress and apoptosis, *atpif-1*and *gpx-4b,* were also assessed.

Tab. 3.2. Relative hypothalamic gene expression changes identified by microarray analysis. The identified changes in the mRNA abundance of the transcriptome are separated into targets with increased mRNA abundance (A) and decreased mRNA abundance (B). The list reveals the identity of all targets that could be annotated using BLASTX homology search. The line represents the statistical cut-off value (q-value <5) used for the original presentation in the published manuscript (Mennigen *et al*., 2008). Targets below this line were identified as having significantly altered mRNA abundance with a cut off value of (5<q<7.5) and are additionally reported to complement the characterization of the transcriptomic response. All targets are ordered by original q-value. Targets in bold represent targets whose mRNA abundance was subsequently validated using real-time RT-PCR.

Gene	fold-change	Gen bank ID
H+ lysosomal acessory protein	+1.4	NM173265.1
ATPase mitochondrial inhibitory factor 1	**+1.2**	**NM_001089521.1**
angiopoietin 1 receptor precursor	+1.3	AF_053633.1
ribosomal protein SA/ laminin receptor I	+1.3	NM_201052.1
cellular retinol binding protein II	+1.2	NM_001002307.1
titin	+1.3	BC090540.1
vacuolar protein sorting 35	+1.3	NM_001025517.2
lactate dehydrogenase a	+1.3	NM_131246.1
IGF1 receptor subtype B	+1.3	AY144592
eukaryotic translation initiation factor 4 gamma	+1.5	XM_689398.4
apolipoprotein a IV	+1.3	NM_001079861.1
ependymin II	+1.3	JO49861
alpha globin	+1.3	M25643

B

Gene	fold-change	Gen bank ID
leucine rich repeat (in FLII) interacting protein 1a	-1.4	BC067562.1
myelin basic protein	-1.7	AY860977.1
similar dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 2 (DYRK2)	-1.5	BX465210.7
glutaminase	-1.7	XM_001345063
novel protein containing lectin c domain	-1.9	BX548256.7
novel protein similar to vertebrate sorbin and SH3 domain containing family	-2.1	BX546482.15
transmembrane transporter 27 (Mg++)	-1.6	NM_001139458
pra1 family protein 3 = ADP-ribosylation factor-like protein 6-interacting protein 5	**-1.6**	**NM_001002517**
protein phosphatase 3, catalytic subunit, alpha	-1.3	NM_001198550

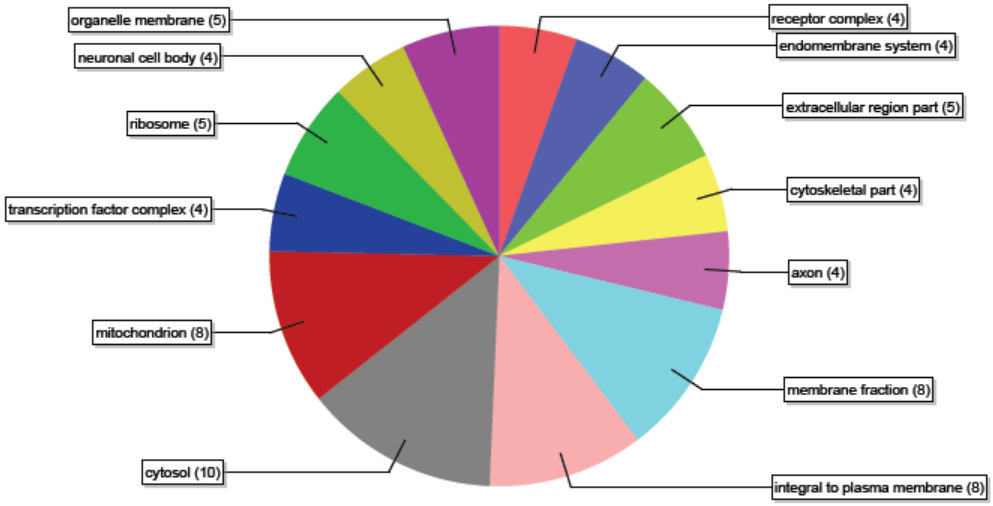
Tab.3.2 continued

Gene	fold-change	Gen bank ID
dopamine transporter	**-1.4**	**EF371919.1**
solute carrier family 1 (neuronal/epithelial high affinity glutamate transporter)	**-1.3**	**NM_001002666**
vitamin k epoxide reductase subunit 1-like 1	-1.4	EU589035.1
16S Ribosomal RNA	-1.6	AP009047.1
family with sequence similarity 82, member A2	-1.4	BC163078.1
muscarinic receptor 2	-1.3	AY72059.1
Bardet-Biedl syndrome 4	-1.3	NM_001077466.1
ependymin 1	-1.4	X14134.1
protachykin 1 precursor	-1.2	U61272.1
septin 7 isoform 2	-1.3	BC125858.1
SH3 and cysteine rich domain 3	-1.4	NM_001003505.1
nuclear receptor subfamily 2, group F, member 6b	-1.3	BC155820.1
activating transcription factor 4b2	-1.4	NM_00103192.1
natural killer enhancing factor	-1.8	EF424251
gamma glutamyl-cyclotransferase	-1.5	NM_213005
proliferating cell nuclear antigen	-1.6	BC064299.1
glutathionperoxidase 4b	-2.0	FJ656212.1
TSC22 domain family, member 3	-1.2	CR381633.6
dihydrolipoamide dehydrogenase	-1.4	AY391421.2
vacuolar protein sorting-associated protein 28 homologue	-2.2	NM_200590
ribosome biogenesis protein nsa2 homologue	-1.3	NM_199568.1
apolipoprotein a-4	-1.3	FJ170106.1
isotocin precursor	-1.4	NM_178291
fizzy related protein homologue	-1.4	XM_002660706.1
netrin-4	-2.3	BC163582.1
ribosomal proteinl23	-1.3	NM_200732.1
a-chain fructose bisphosphate aldolase	-1.3	U36777.1
hclsi associated protein x-1	-1.4	NM_001002337.1
reticulon 1a	-1.3	BC090486.1
ring finger protein 13	-2.2	BC065620.1
apolipoprotein eB	-1.3	FJ170108.1
14kDa apolipoprotein	-1.5	FJ170111.1
Tif2	-1.8	AY210802.1
fibrinogen beta chain	-1.5	XM_002660368.1
liver basic fatty acid protein	-1.6	EU363801.1
fxyd domain ion transport regulator 6	-2.0	XR045226.2
interleukin 6 signal transducer	-1.8	AB018216.1
fidgetin-1 like	-1.5	NM-00112875.11
ribosomal protein l26	-1.5	AF401580.1
nin1 rpn12 binding protein 1 homologue	-1.3	NM_001016830.2
hydroxypyruvate isomerase	-2.0	NM_200334.1
RNAse K	-1.4	NM_001045405.2
Desmin	-1.4	NM_130963.1
ankyrin repeat domain containig protein 57	-1.3	NM_0231016.3
tubulin alpha 1 chain like	-1.3	NM_194388.2
choline/ethanolamine phosphotransferase 1	-1.6	NM_01109717.1
Iron-sulfur cluster assembly 2 homolog, mitochondrial precursor	-1.3	NM_194279.2
novel protein similar to the vetrebrate sal-like family	-1.5	NM_001080609.1

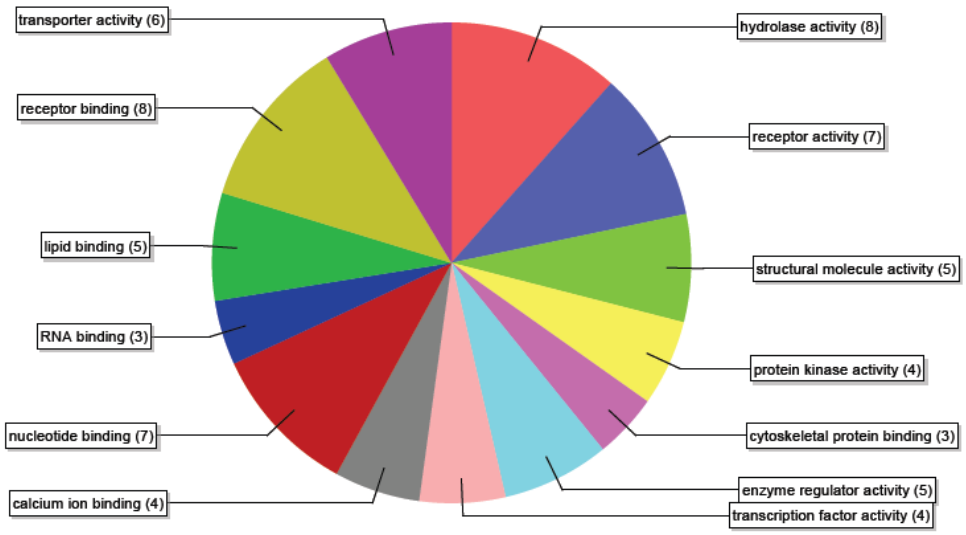
Tab.3.2 continued

Gene	fold-change	Gen bank ID
synaptogyrin 2b	-1.4	XM_691842.3
glucose 6 phosphatase	-1.7	NM_001003512.1
immunoglobulin superfamily containing leucine-rich repeat 2	-1.3	NM_001161535.1
serpin peptidase inhibitor, clade I (neuroserpin)	-1.5	NM_01122752.1
membrane magnesium transporter I	-1.4	NM_001106970.1
cytochrome c type heme-lyase	-1.7	NM_001031275.1
heterogeneous nuclear riboprotein g	-1.5	NM_001025663
ATPase, H+ transporting, lysosomal, V0 subunit c	-1.3	NM_173255.1
NEDD8-conjugating enzyme Ubc12 (ubc12)	-1.6	NM_001173918.1
Suppressor of Ty6 homologue	-1.3	NM_065568.2
Ribosomal protein L8	-1.6	NM_200713.1
H2 class 2 hisotcompatibility antigen gamma chain	-1.3	X13044.1
myeloid cell leukemia sequence 1b	-1.3	NM_194394.2
heatshock protein 90	-1.4	NM_131328.1
synapse protein (SNAP-25) processed pseudogene	-1.6	L22975.1
novel protein similar to synaptotagmin 1	-1.3	XM_001489395.2
carnosine dipeptidase 1	-1.4	NM_001089952.1
MARCKS-like 1	-1.2	NM_213133
beta synnuclein	-1.8	NM_200969.1
dolychyl-diphosphooligosaccharide protein glycosyltransferase subunit 4	-1.6	NM_001134690.1
maguk p55 subfamily member 3-like	-1.3	XM_003131358.1
14-3-3 protein beta alpha	-1.3	AK292717
Clusterin	-1.7	NM_200802.1
rna binding motif protein 10	-1.3	NM_005676.3
seizure 6-like protein 2 like isoform 2	-1.2	NM_001114099.1
s-100 calcium binding beta	-1.2	NM_002666232.1
lysine (K)-specific demethylase 6B, b	-1.2	NM_001080424.1
jumonji domain containing protein 3	-1.3	NM_001030178
calcium/calmodulin-dependent protein kinase IV	-1.4	NM_001793.3
clathrin 1 heavy chain	-1.9	NM_019299.1
tryptophan dioxygenase	-1.4	NM_022403.1
serine palmtoyltransferase II	-1.3	BC005123.2
g kinase anchoring protein 1	-1.3	NM_053665.1
MHC class I antigen	-1.6	NM_131704.1
creatine kinase M3-CK	-1.2	NM_001824.3
dystrobrevin alpha	-1.3	NM_010087.3
zona pellicuda glycoprotein2	-1.2	NM_131330.1

A


organelle membrane (5)
neuronal cell body (4)
ribosome (5)
transcription factor complex (4)
mitochondrion (8)
cytosol (10)
receptor complex (4)
endomembrane system (4)
extracellular region part (5)
cytoskeletal part (4)
axon (4)
membrane fraction (8)
integral to plasma membrane (8)

B


transporter activity (6)
receptor binding (8)
lipid binding (5)
RNA binding (3)
nucleotide binding (7)
calcium ion binding (4)
hydrolase activity (8)
receptor activity (7)
structural molecule activity (5)
protein kinase activity (4)
cytoskeletal protein binding (3)
enzyme regulator activity (5)
transcription factor activity (4)

C

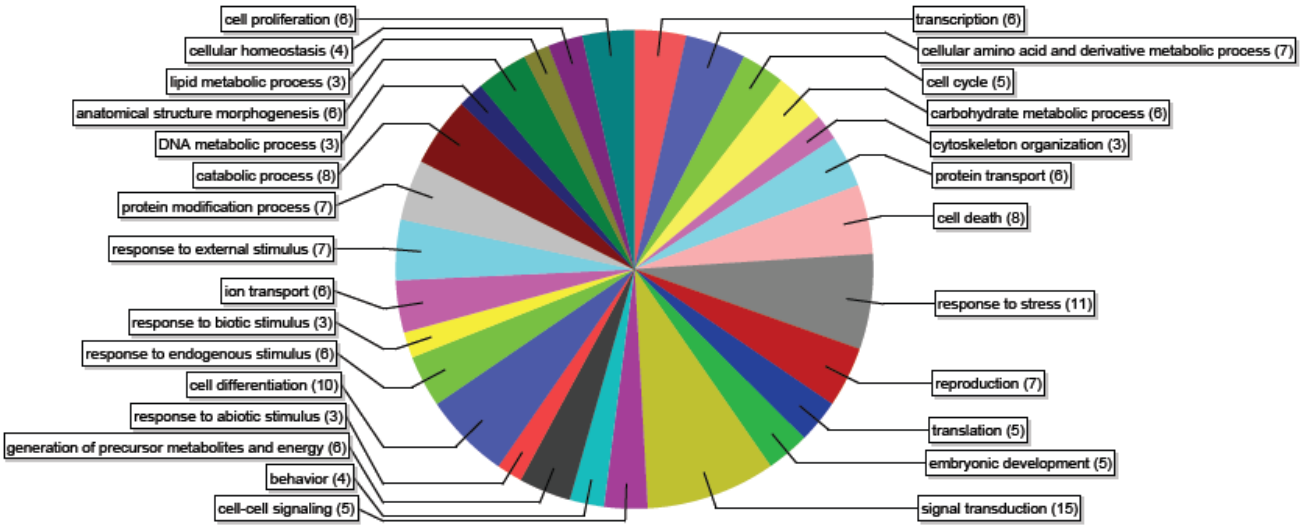
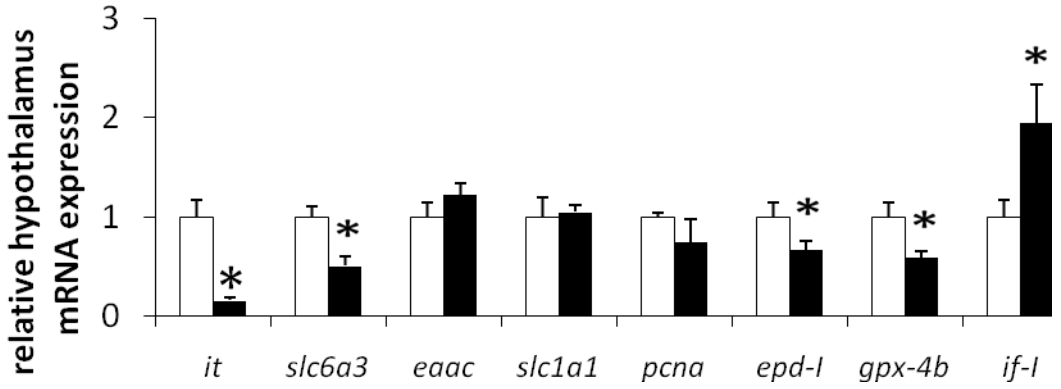


Fig. 3.5. *Categorization of differentially expressed genes into, cellular compartment (A) molecular function (B), and, biological processes (C) as determined by BLAST2GO, using GO annotations tools. Number in brackets identifies the number of genes classified as belonging to a particular category.*

A



B

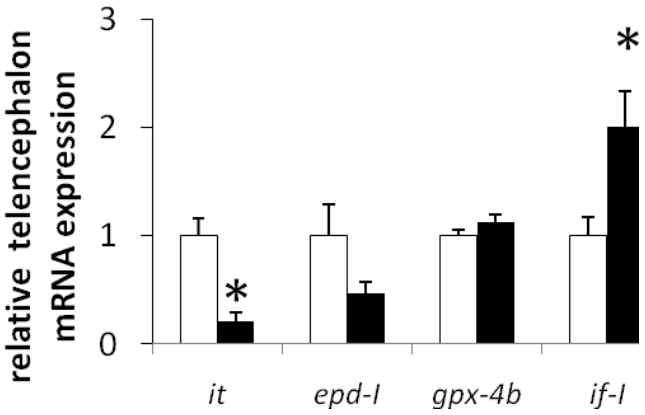
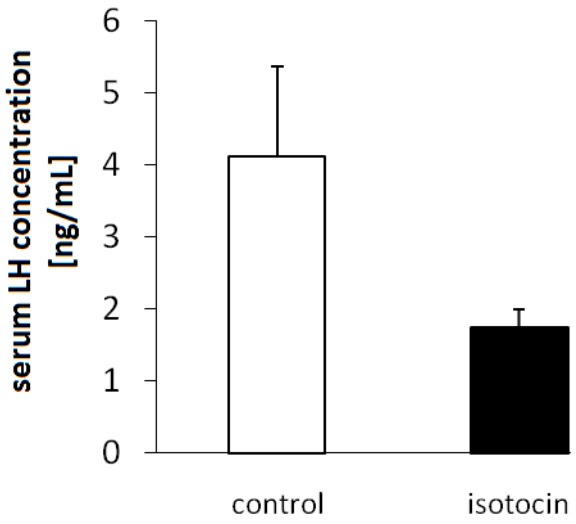


Fig. 3.6. *Validation of changed relative mRNA abundance (+S.E.) in differentially expressed genes identified in microarray analysis in the hypothalamus (A) and telencephalon (B) using real-time RT-PCR. An n=8 hypothalamus and telencephalon samples were used. Abbreviations are it=isotocin, slc6a3= dopamine transporter, eaac= addicsin, slc1a1= glutamate transporter, pcna= proliferating cell nuclear antigen, epd-I= ependymin I, gpx4b= glutathione peroxidase 4b, if-1=atpase inhibitory factor 1. White bars indicate saline-injected control fish and black bars indicate FLX- injected fish. Data normally distributed was analyzed by a two-tailed t-test, data that were not normally distributed were analyzed using a Mann-Whitney U test. Asterisk indicates significant difference at* $p \leq 0.05$.

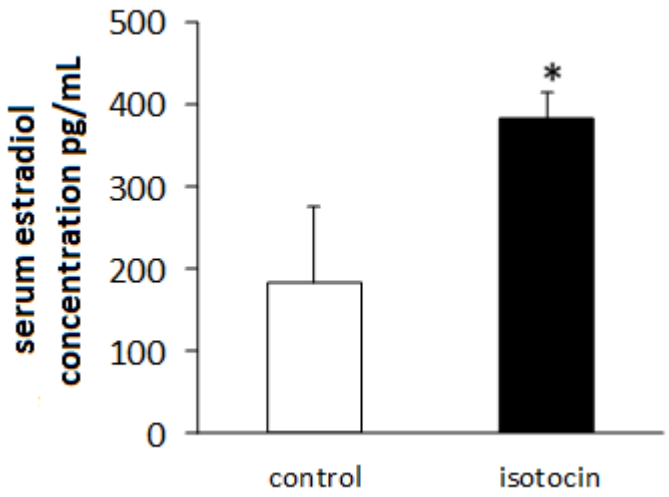
3.3.5 Isotocin increases serum E_2 concentration

I was interested in establishing a probable causal link between the transcriptome analysis results and the observed decrease in E_2 concentration following FLX injection. Therefore, in order to investigate whether IT has an effect on circulating E_2, LH, T, and E_2 concentrations were assessed in IT-injected animals. There was no change in serum LH concentrations 12 h after injection compared with the control fish (**Fig. 3.7A**). However, compared with the control group, a 2-fold increase ($p \leq 0.05$) in E_2 (**Fig. 3.7B**), and a concurrent 3-fold decrease ($p \leq 0.05$) in T (**Fig. 3.7C**), and was observed in IT-injected females. The calculated E_2 to T ratio increased by 5-fold ($p \leq 0.01$) in IT-injected fish (**Fig. 3.7D**).

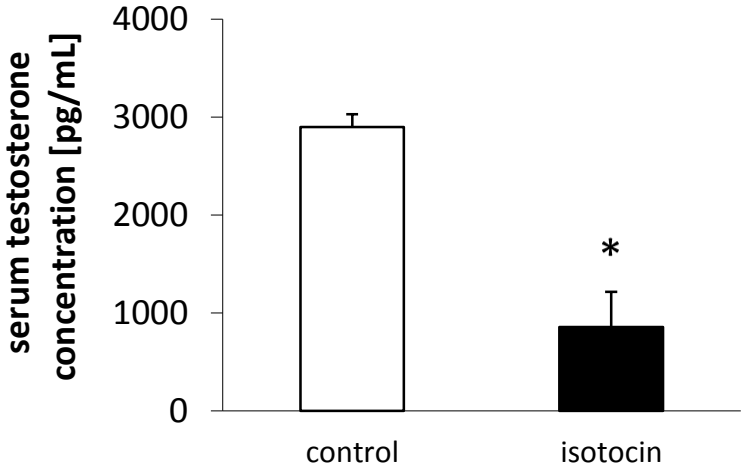
A



B


serum estradiol concentration pg/mL
500
400
300
200
100
0
*
control
isotocin

C


serum testosterone concentration [pg/mL]
4000
3000
2000
1000
0
*
control
isotocin

D

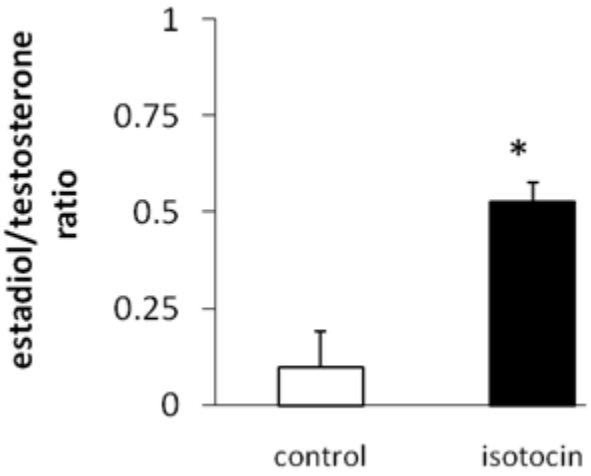


Fig. 3.7. *Effects of i.p. injected IT on serum concentrations (±S.E.) of LH (A), E_2 (B) and T (C). Additionally the E_2/T ratio is shown (D). An n=15 was used for the LH assay and an n=5 for the E_2 and T assays, respectively. White bars represent control fish and black bars indicate fish injected with 1 µg/µL g^{-1} bw IT. Data were not normally distributed and analyzed using a Mann-Whitney U-test. Asterisk indicates a significant difference at $p \leq 0.05$.*

3.4 Discussion

The results support the hypothesis that repeated injections of FLX have the capacity to disrupt the reproductive axis in recrudescent female goldfish, as indicated by the decrease in serum E_2 concentration. Furthermore, this study provides evidence that the disruptive effects of FLX on the reproductive axis may at least be partially mediated at the level of the neuroendocrine brain. Evidence suggests an involvement of the neuropeptide IT in this process, because FLX induced a significant decrease in *it* mRNA that correlates with the observed significant decrease in serum E_2 concentration and because administration of IT elicited an

increase in serum E_2 concentrations. A detailed discussion of the disruptive effects of potential underlying neuroendocrine mechanisms is presented in the following paragraphs.

3.4.1 Fluoxetine reduces serum E_2 concentration and decreases mRNA abundance of E_2 receptor isoforms in the neuroendocrine brain

Fluoxetine injections into female goldfish significantly decreased circulating E_2, as previously reported in female rats chronically treated with the SSRI fluvoxamine (48). Accompanying the decrease in serum E_2 was a decrease in *erß1* mRNA abundance in both the hypothalamus and the telencephalon, and a decrease in *erα* mRNA abundance in the telencephalon. It is, however, unclear whether these decreases are directly related to the decreased serum E_2 concentrations. The effect of E_2 on ER subtypes is not well defined, but E_2 does affect goldfish in an ER receptor subtype- and tissue-specific manner (161).

Mice deficient in ERß showed disruptions of the reproductive system (164), implicating ERß as an important factor in regulating the reproductive axis. For example, homozygous *erß* -/- knockout mice exhibit subnormal fertility (165) and female *erß* -/- knockout mice had infrequent pregnancies, reduced litter size, and reduced ovulation (166), possibly due to morphological abnormalities observed in the brain of *erß* -/- knockout mice (167). Mice knock-out models for *erα* revealed an even more severe disruption of the reproductive axis, including severe disruptions in the feed-back control of E_2 on the hypothalamo-pituitary gonadal axis (reviewed in 168). The abundance of *erß2* mRNA did not change with FLX treatment in either tissue, the hypothalamus or telencephalon, in the goldfish. The significant reduction of *erα* and *erß1* abundance in the hypothalamus of FLX-treated fish in this study likely had an impact on the ability of the female brain to respond to either locally produced neuronal E_2 or circulating E_2, contributing to the anti-reproductive actions of FLX.

3.4.2. Modulation of the dopaminergic and noradrenergic pathways

Monoamine neurotransmitter concentrations, as well as concentrations of their metabolites known to be involved in modulating LH release in the neuroendocrine brain of goldfish (143,144) were assessed by HPLC to investigate possible mechanisms responsible for the observed decrease in circulating E_2. While moderate changes should be interpreted with caution, a 35% increase in 5-HT concentration in the hypothalamus was observed in FLX-injected goldfish, consistent with the described function of FLX as an SSRI. A significant increase in HVA may reflect changes in DA activity and is concurrent with the observed trend of a 50% increase in DA concentrations in the hypothalamus of FLX-treated fish. However, it should be noted that DA concentrations increased only marginally ($p=0.06$) and future experiments using a larger sample size are required to validate a potential involvement of DA in the observed effects. A 1.3-fold decrease in the abundance of the DA transporter mRNA was observed with the microarray and validated using real-time RT-PCR, providing molecular evidence for FLX effects on the DA system in the hypothalamus. Fluoxetine injections do increase extracellular NE and DA concentrations (92), as well as glutamate concentrations (169), in addition to 5-HT concentrations in the hypothalamus of the rat. The seasonal stage of reproduction in some teleosts is linked to increased brain 5-HT concentrations (51) and decreased DA concentrations (51), and potential interactions between both systems in regulating neuroendocrine control of reproduction in some teleosts are hypothesized (170). In rats, 5-HT modulates DA concentrations via 5-HTR_{1A} and 5-HTR_{2C} (171,172), a mechanism that is associated with efficacy (173) and adverse side effects of SSRIs (174).

Dopamine is the principal inhibitor of LH release in teleost fish (143,144), while 5-HT and NE stimulate LH release (143,144) in at least some teleost fish, including goldfish (32).

However, the trends in the monoaminergic systems assessed in FLX-injected female goldfish in this study were generally not significant and not correlated with changes in plasma LH concentrations. The lack of expected changes in LH could be due to the inability of FLX to elicit changes in the 5-HT, but given the duration of the exposure may also be due to desensitization events at the site of postsynaptic 5-HTRs (175), or antagonism, as FLX does inhibit 5-HTR_{2C} receptors (114) and may be part of the 5-HTR_2-like mediated action of 5-HT on LH (31,33). More detailed time course studies and different doses of FLX should be used to address these possibilities. Microarray analysis did furthermore not identify GnRH mRNA as being affected. Overall, these data do not support the GnRH-LH pathway as a major contributor to the observed decrease in E_2 concentrations.

3.4.3 Fluoxetine modulation of mRNA abundance in the hypothalamus: generation of a hypothesis regarding the role of hypothalamic mediation of FLX-induced endocrine disruption

Hypothalamic tissue was chosen for microarray analysis in order to investigate other potential mechanisms for the observed decrease in E_2, as the hypothalamus contains the major hypophysiotrophic nuclei. Interestingly, enrichment analysis did not show a significant over-representation of any GO term group in either the induced or down-regulated group of genes. This likely reflects the widespread actions of 5-HT in the CNS where it is known to modulate various physiological systems, including feeding, thermoregulation, sleep/arousal, nociception, modulation of motor systems and neurogenesis (11). Indeed, 5-HT is speculated to "hold a pivotal role in the homeostasis of the brain, which is consistent with function of 5-HT throughout evolution, and explains the large number of biological systems and behavioural activities associated with serotonergic function" (176, p.33).

Hypothalamic genes affected by FLX fall into a large number of categories, a fact that may also be related to the observation that a variety of neurotransmitters and metabolites could change in FLX-treated fish. This effect was observed in the hypothalamus of FLX-treated rats (92), and it was speculated that changes in numerous neurotransmitter systems explain the FLX-modulated changes in mRNA abundance of numerous transcripts in these animals (10). However, in this study only HVA concentration in the hypothalamus, as well as NE turnover in the hypothalamus changed significantly. Other 5-HT independent-effects are postulated for FLX, including the release of the neurosteroid allopregnanolone at low SSRI doses that are ineffective in inhibiting 5-HT reuptake (177). Allopregnanolone was shown to modulate GABA signalling via allosteric modulation of GABA receptors (177). Some effects of FLX may be partially explained by the potential direct effects on cell membranes due to the amphipathic nature of FLX, as tested in the yeast system, which does not contain 5-HT transporters (178). More specifically, these direct effects include effects of FLX on cell polarity (178) and mitochondrial function (179). When a similar multiple injection protocol is employed over the same period, the abundance of most identified transcripts is decreased by FLX in mammalian models. For example, a microarray analysis also reported a more pronounced decrease in mRNA abundance of the hypothalamic transcriptome in a FLX injection protocol using rats and mice (149,180). Additionally, similar to the goldfish study, the transcripts could be categorized into multiple functional classes based on gene ontology annotations.

The following section introduces some general patterns and functions of specific targets, which were subsequently used to guide the experiments investigating molecular neuroendocrine pathways altered in waterborne FLX exposures in Chapters 5 and 6.

3.4.3.1 Fluoxetine regulates genes with roles in neuroplasticity

Since its market introduction and the focus on its actions on the monoamine system, FLX's therapeutic efficacy has been reported to be at least partially related to its neuroplastic actions mainly within the hippocampus (181); some of these actions are 5-HT independent, but others are 5-HT-dependent and display onset after 14 d of treatment (176,182). While initial evidence focused on neurogenesis, and neurotrophic and anti-apoptotic effects in the hippocampus (183), recent literature also provides evidence for FLX effects on neuroplasticity that are not involved in neurogenesis. Rather, these effects on neuroplasticity involve remodelling of neuronal architecture (184), for example, by inhibition of dendritic branching and synaptogenesis (185,186), and indeed, inhibition of mitotic cell divisions (187). These effects appear to be conserved, as they were found in both rats and invertebrates (186). Consistent with these recent findings, our microarray screen revealed specific target gene mRNAs that suggest inhibitory processes that underlie neuroplasticity in the hypothalamus. For example, FLX decreased mRNA abundance of *pcna* (proliferating cell nuclear antigen), a marker of neurogenesis, and its directional change in mRNA abundance was confirmed in real-time RT-PCR. Similarly, the mRNA abundance of *epd-I*, which is involved in synaptic plasticity in the goldfish brain (188), was significantly decreased by FLX using both microarray and real-time RT-PCR.

More specifically, analysis of gene targets whose mRNA abundance was decreased in FLX-injected goldfish identified several target genes involved in the centrosome, a structure important in spindle tubule organization during mitosis. These included *nin-1*, *bbs-4*, *septin 7* and *fidgetin-1*, all of which have functions in cell cycle progression and neuronal growth

(189,190,191). Therefore, the spindle formation in mitotic neuronal cells may represent a specific target of FLX in the hypothalamus.

Apoptosis may also be an important factor in proliferating cell numbers and be a factor in neuroplastic restructuring. The mRNA abundance in the hypothalamus of FLX treated fish was increased for *atpif-1* and decreased for *gpx-4b*. These genes are involved in the slowing the creation (192) and buffering (193) of reactive oxygen species (ROS), which are known to trigger neuronal apoptotic signalling (194). Fluoxetine was shown to act directly on mitochondria (195) and to create ROS in liver tissues of fish (196). Therefore, an increase in apoptosis may contribute to FLX-induced changes hypothalamic neuroplasticity. Another potential target for FLX is the cytoskeleton, due to its role in shaping neuronal plasticity. This phenomenon may indeed underlie the neuroplastic actions of FLX in vertebrates (186,197,198) and invertebrates (186). In support of this idea, the microarray screen identified decreased mRNA abundance of *titin* and *α-tubulin* genes. In spite of the fact that these speculative mechanisms require specific testing, the overall transcriptomic response points to an inhibition of hypothalamic neurogenesis in FLX-treated female goldfish. This finding may have potential impact on the neuroendocrine control of reproduction, as reproductive hormones, including the identified target IT, have been shown to be very plastic in female fish, where peak innervation and immunopositive cells are detected in the POA during the reproductive season (199).

3.4.3.2. Gene targets involved in neurotransmitter homeostasis in the hypothalamus

Several of the identified target genes with decreased mRNA abundance are involved in neurotransmitter homeostasis in the hypothalamus, which, as previously described, do function in controlling neuroendocrine regulation of reproduction by altering LH release in teleosts, including goldfish (1). Gene targets involved in both DA as well as glutamate metabolism were

identified in the microarray screen, supporting reports of altered DA (92) and glutamate concentrations in the hypothalamus of rats injected with FLX (169). The mRNA abundance of the DA transporter, *slc6a3*, was decreased, which correlates with the observed trend of a 50% increase in hypothalamic DA concentrations. However, an inhibitory effect of DA on serum LH concentrations which may account for the observed decrease in serum E_2 concentrations was excluded as a potential mechanism, owing to the fact that serum LH remained unaltered in FLX injected fish. Dopamine, however was previously shown to inhibit hypothalamic mRNA abundance of *it* via a D_2 mediated mechanism in goldfish (200). Decreased mRNA abundance of components of the glutamate system, including glutaminase (*gls*), the glutamate transporter (*slc1a1),*and addicsin (*arl6ip5)* (a positive allosteric modulator of *slc1a1* activity; 201) support the notion that astroglial cells may be specific targets for SSRIs (202,203) since they are crucially involved in glutamate metabolism in the brain. However, neither the decreased mRNA abundance of *slc6a4* nor *arl61p5* could be validated using real-time RT-PCR.

3.4.3.3 Fluoxetine decreases the mRNA abundance of IT, an important reproductive neuropeptide

The principal target in our investigation was IT, a neuropeptide with reproductive function, which is highly conserved in vertebrates and belongs to the oxytocin gene family (204). Several FLX-induced mechanisms observed in this experiment may explain the more than 5-fold decrease in *it* mRNA abundance in the telencephalon and hypothalamus. For example, oxytocin neurons are co-localized with *erß* mRNA and protein in mammals (205), and indeed, oxytocin mRNA abundance is increased by ER-β and its recruited cofactor TIF-2, through binding to an E_2 response element (ERE) downstream of the oxytocin gene (206,207). Although this mechanism has not been directly verified in fish, evidence implies that this mechanism is

conserved between mammals and fish. Venkatesh and colleagues created transgenic rats containing the puffer fish *it* gene and its upstream regulatory elements, and showed that the regulation of the *it* gene is functional in rats, implicating not only a conserved evolution of the oxytocin gene, but also of its regulatory and transcriptional control (208). Furthermore, conserved regulatory elements were identified upstream of the *it* gene, including an E_2 response element in carp (209). The goldfish microarray screen used in this study found the mRNA abundance of several components of the ER-β signalling complex were down-regulated, including *erß* itself, its cytosolic binding partner *hsp90*, and also its nuclear co-transcription factor *tif-2*. Other potential pathways that may be involved in the observed decrease in *it* mRNA in FLX-injected goldfish include the neurotransmitters 5-HT and DA, both of which were elevated, albeit not significantly, in the hypothalamus. In rats, 5-HTR_{2C} is the main serotonergic pathway for stimulating oxytocin mRNA abundance in the hypothalamus, although 5-HTR_{1A} and 5-HTR_{2A} are also reported to stimulate oxytocin mRNA abundance and release (16). Interestingly, the 5-HTR_{2C} is directly blocked by FLX with affinities similar to FLX affinities reported for its main target, the 5-HT transporter (114). Additionally, a down-regulation and desensitization can be observed in numerous 5-HTRs, including 5-HTR_{2C} in response to FLX (175). I identified a decrease in mRNA abundance for *magukp55*, which binds specifically to 5-HTR_2 to reinforce downstream Ca^{2+} signalling (210), which may provide a mechanism for desensitization in the signaling pathway. In the mammalian literature, ample evidence exists in support of a FLX-induced desensitization of oxytocin release via 5-HTR_{1A} (211) and 5-HTR_{2A} (212), likely through reductions in specific hypothalamic G protein abundance involved in the downstream signalling pathways of the receptors.

Hypothalamic concentration of the dopamine metabolite HVA increased in FLX-injected fish in our study, a finding that is consistent with a decreased mRNA abundance of the DA transporter. Increased DA concentrations may lead to decreased *it* mRNA abundance, as earlier work in goldfish demonstrated that *it* mRNA abundance is decreased by the D_2-receptor agonist quinpirole (200). Albeit speculative at this point, since FLX failed to increase DA concentrations in the hypothalams, these mechanisms (**Fig. 3.8**) merit further investigation in the regulation of the FLX-induced decrease in mRNA abundance of *it* in the neuroendocrine brain.

The innervation of IT-containing neurons in fish originates from the magnocellular neurons of the POA that innervate the pituitary as well as hypothalamic and extrahypothalamic areas, indicating a role for IT as both a neuromodulator and a hormone, as reported for oxytocin in mammals (213). This assertion is strengthened by the fact that centrally administered IT does modulate reproductive behaviours (148), and that IT can be measured in the blood (214), from where it may act on peripheral IT receptors located on the gonads, which have been identified in the white sucker, *Catostomus commersoni* (215). Therefore, IT could potentially act at several points within the reproductive axis, similar to the situation in mammals. For example, the IT homologue oxytocin stimulates GnRH-mediated LH release in rats (147) and increases ovarian E_2 production from porcine cells (216).

Seasonal changes in whole brain IT peptide concentrations were observed in the female, but not the male three-spined stickleback (217), with the lowest brain concentrations found in recrudescent fish (December) and the highest in pre-spawning fish (July), suggesting a distinct role for IT in the reproductive cycle of female fish. An increased *it* mRNA abundance in the early reproductive phase (May) was also detected in the preoptic nucleus in female masu salmon (*Oncorhynchus masou*) and precedes increased IT protein content in August and November

(218). In ordinary male masu salmon, *it* mRNA abundance and IT protein concentration in the POA occur earlier in the season, with peaks in January and May, respectively (219). In precocious male salmon, the pattern resembles the one described for female masu salmon (218,219). Female, but not male Japanese medaka (*Oryzias latipes*) show a significant decrease in abundance of VT- and IT-immunoreactive neurons following spawning (199). Thus, in teleosts there is significant evidence that IT may be an important regulator of reproductive processes, although this finding is less documented than investigations of the effects of the mammalian homolog oxytocin on reproduction. In this study, I observed a significant reduction in *it* mRNA abundance after FLX injection. Decreased transcription of the related arginine vasotocin (*vt*) gene was also reported in the brains of FLX-treated male bluehead wrasse (150).

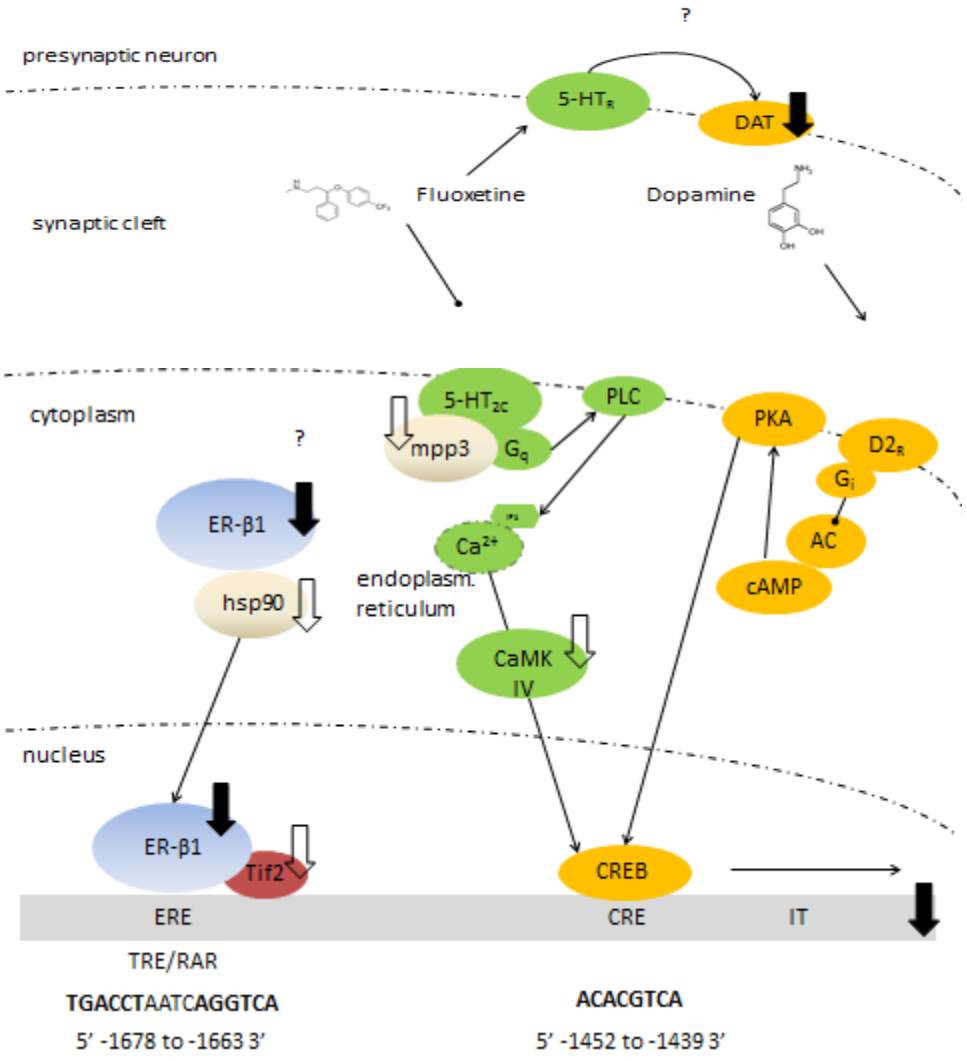


Fig. 3.8. *Mechanistic model of the inhibitory effects of FLX on it gene expression in the goldfish neuroendocrine brain (telencephalon and hypothalamus); see text for explanation. Black arrows indicate decreased gene expression detected by both microarray and real-time RT-PCR, and white arrows indicate detected decreased mRNA abundance in microarray, but not validated by real-time RT-PCR.*

3.5. Isotocin injection does not change serum LH concentration, but increases serum E_2

Fish that were FLX-injected in the first experiment showed no significant changes in circulating LH, yet E_2 concentrations decreased. Based on comparative literature, I hypothesized that IT may have a role in the reproductive axis in goldfish, and, based on the observed concurrent decrease of *it* mRNA abundance and serum E_2, I made the more specific prediction that IT would increase serum E_2 concentration. To test this hypothesis, goldfish were injected with IT. No significant effects were observed on LH release, while serum E_2 concentrations increased concurrent with a decrease in T concentration after 12 h. It is therefore unlikely that decreased E_2 following FLX treatment is related to effects on circulating LH. While no specific assay exists to measure serum FSH concentrations in teleost fish (220), this pathway may play a role in the action of FLX, particularly since FSH is reported to be involved in FLX-induced disruptions of the reproductive axis in mammals (221). For the specific time points assessed, our results suggest that IT may act directly on the fish ovary to regulate E_2 production, as observed in porcine granulosa cells *in vitro* (216). While this is plausible, the role of IT in the reproductive axis requires further testing and this was pursued in more detail in Chapter 4 of this thesis.

3.6. Conclusions

The injection experiment reported in this chapter demonstrates that FLX has the potential to impact the reproductive axis in goldfish. The underlying neuroendocrine mechanisms, such as ER-ß receptor mRNA abundance, may have contributed to the observed decrease in mRNA abundance of the reproductive neuropeptide IT in the neuroendocrine brain. While evidence from these experiments did not support an LH-dependent mechanism, the second experiment did provide a functional link between IT and serum E_2 concentrations. As the physiological function

of IT in teleost fish is largely uncharacterized, additional studies were undertaken to investigate the 5-HT-dependent regulation of *it* mRNA and the effects of IT on the reproductive neuroendocrine axis (Chapter 4). The experiments presented in this chapter raise the possibility that SSRIs are potential endocrine disruptors and provide the mechanistic foundation needed to test the hypothesis that environmentally relevant concentrations of FLX disrupt reproduction in fish by affecting the neuroendocrine pathways identified (Chapter 5).

Chapter 4: Investigation of the interaction of 5-HT and IT in the neuroendocrine brain and pituitary, and effects of IT on LH release

4.1. Introduction

The neuropeptide IT was identified as a key target for FLX, as its mRNA abundance in the neuroendocrine brain (hypothalamus and telencephalon) was decreased by repeated FLX injections (Chapter 3). Furthermore, IT injections revealed a stimulatory effect of IT on serum E_2 concentrations, implicating a potential link between the observed decrease in hypothalamic *it* mRNA abundance and the observed decrease in serum E_2 concentrations in FLX- injected fish (Chapter 3). The following experiments were therefore designed to firstly, investigate the hypothesis that the IT system is a direct target of 5-HT in the neuroendocrine brain and secondly, to test the hypothesis that the stimulatory action of IT on the reproductive axis is mediated by stimulating LH release from the pituitary.

Isotocin was initially discovered in three different teleost species: the pollock (*Pollachius virens)*, the European hake (*Merluccius merluccius)* and the pout *(Gadus luscus)* (222). Isotocin (4-Ser, 8-Ile oxytocin) is conserved across teleost species and belongs to the evolutionary line of oxytocin, which is present in mammals (204) .The oxytocin lineage is believed to have arisen from a *vt* gene duplication event early in vertebrate evolution, as only a *vt*-like gene is present in lamprey (204). In teleost fish, the distribution of the IT system is well characterized, both in the CNS (223-225) and in peripheral organs (215,226).

Evidence of central sources of IT stem from studies conducted in the European eel (*Anguilla anguilla)*, in which IT and the related neuropeptide VT were identified in the preoptic tract and the pituitary gland (223). In the teleost POA, magnocellular and parvocellular isotocinergic neurons form a network through specialized contacts called puncta adhaerentia,

which exist between adjacent plasma membranes (224). A co-localization of GnRH and IT has been reported in ventral parvocellular neurons of the POA in the dwarf gourami (*Colisa lalia*) (227). From the POA, isotocinergic projections extend to several brain areas, including the olfactory bulb, optic tectum, the nucleus of the solitary tract, and the hypothalamus (228). Brain regions receiving isotocinergic projections in the hypothalamus include the nucleus recessus lateralis, the saccus vasculosus and inferior lobe of the hypothalamus (228,229). From the hypothalamus, the isotocinergic innervation extends dorsally to innervate the pituitary (229). Because of its close proximity to highly vascularized areas in the neural lobe of the pituitary (229), IT is released into the blood stream as a circulating hormone in the low nM concentration range (214). Numerous peripheral tissues, among them the ovary, liver, and intestine, were shown to express the G-protein coupled isotocin receptor (*itr*) in the white sucker (*Catostomus commersoni)* and Amargosa pupfish (*Cyprinodon nevadensis amargosae*) (215,226). Functional expression of the white sucker ITR receptor in frog oocytes revealed an activating effect in the nM range, representing physiological circulating IT concentrations (214).

Anatomical evidence therefore suggests a high degree of conservation of the isotocinergic system when compared to the oxytocin system in mammals. This suggests that IT neurons in goldfish may be the target of serotonergic innervation similar to the situation in the rabbit (*Oryctolagus cuniculus*;230), especially since 5-HT positive fibers have been described in the POA and pituitary of the goldfish (15). The anatomical distribution of IT in goldfish also raises the potential of an isotocinergic action on components of the HPG axis involved in the stimulation of LH release. However, in contrast to the role of oxytocin in mammalian reproduction, the effects of IT on the physiology of reproduction in fish remain poorly understood.

To address these questions, I used immunocytochemistry, and 5-HTR_{1A} specific agonists and antagonists, in order to investigate potential direct interactions of 5-HT and IT in the POA and pituitary. The 5-HTR_{1A} was chosen as a target, as it has been shown to stimulate oxytocin mRNA abundance and release in the rat (16). Furthermore, this receptor has been implicated in FLX-induced desensitization of oxytocin release in several studies in the rat (211,212,231). To study specific effects of IT on the HPG axis, I initially obtained partial goldfish coding sequences of *it* and *itr* through cloning. I used these sequences to identify seasonal hypothalamic *it* mRNA abundance and tissue specific mRNA distribution, in order to gain insight into possible targets of IT with regard to reproduction in goldfish. I subsequently studied the effect of IT on LH release in female fish *in vivo* and *in vitro,* using i.p. injection of IT, and IT incubation of dispersed pituitary culture, respectively. The use of female fish was justified by the fact that the initial FLX-induced microarray screen, which identified *it* mRNA as a target molecule in the FLX-induced disruption of the reproductive axis, was conducted in female fish. Additionally, sex differences in the isotocinergic system have been characterized in fish (148,199,217,232). These differences pertain especially to seasonal abundance of IT innervation (199) or IT protein content in the brain (217). Both IT innervations and IT protein content in the brain are most abundant during the reproductive stage in female, but not male fish.

4.2. Materials and Methods

4.2.1 Experiments investigating neuroendocrine interaction between 5-HT and IT in goldfish

4.2.1.1 Immunocytochemistry

Four sexually mature female goldfish (20-26 g) were purchased from a local supplier and maintained at the University of Ottawa aquatic facility. The animals were anesthetized with benzocaine and perfused with saline solution (0.8% NaCl in PBS pH 7.4), followed by 50 mL of Bouin´s fixative. Both solutions were administered via infusion pump at a flow rate 2 mL per min into the anterior conus of the heart. In all cases, the brains were carefully removed and fixed overnight in Bouin´s fixative at 4 ºC and then submitted to routine histological procedures and embedded in paraffin (233). Sections (5 µm) were mounted on gelatin-coated slides and dried overnight at 22 ºC. Anatomical localization in the forebrain was determined by comparisons with the goldfish brain atlas (234) and an atlas of the goldfish pituitary (3), as seen in **Fig. 4.1**. Following this, slides were de-paraffinized in xylene and re-hydrated with a graded series of ethanol in decreasing concentrations (100-70%). Sections were washed with PBS and then blocked with PBS containing 1% BSA and 0.3% Triton X-100 for 35 min at ambient temperature. Both oxytocin (T-5021; host guinea pig, polyclonal, antigen sequence H-Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH_2; Peninsula Labs, Santa Carlos, CA, USA) and 5-HT antibodies[6] (S5545; host rabbit, polyclonal, antigen 5-HT creatinine sulphate; Sigma-Aldrich, Oakville, ON, Canada) have previously been validated in fish (235,236). A control for specificity was also performed with guinea pig anti-oxytocin (1:250) and rabbit anti-5-HT antiserum (1:250), by pre-absorbing with 10 µM IT (Chem-Impex, Wood Dale, IL, USA) and 10 µm 5-HT (Sigma-Aldrich, Oakville, ON, Canada), respectively, at 22 ºC overnight. For co-localization, tissue sections were incubated overnight at 22 ºC with polyclonal rabbit 5-HT

[6] The 5-HT antibodies were kindly provided by Michael Jonz, University of Ottawa, ON, Canada.

antiserum (1:250) and oxytocin antiserum (1:250) diluted in PBS. On the following day, sections were rinsed (two 10 min changes) with PBS, and incubated at 22 ºC with secondary antibodies Alexa Fluor 594 (red) goat-anti guinea pig IgG (H+L) (Invitrogen, Eugene, Oregon) for IT, and, for 5-HT, Alexa Fluor 488 (Green) goat anti-rabbit IgG H+L (Invitrogen). Both antibodies were used in a 1:100 dilution in blocking solution. The sections were rinsed twice with PBS, and mounted with mounting fluid (Light Diagnostics, Salt Lake City, UT, USA). Photographs of brain and pituitary sections were captured using the Nikon Eclipse E7000 and the Image Pro Plus software (Media Cybernetics, Bethesda, MD, USA). The digital images were assembled in Photoshop (Adobe System Inc., San José CA, USA) adjusting only slightly brightness and contrast parameters that visually did not alter perceived fluorescence quality or quantity.

4.2.1.2. The effect of 5-HTR$_{1A}$ modulation on it mRNA abundance in the telencephalon

Common female goldfish were purchased from a commercial supplier (Aleong's International Inc., Mississauga, ON, Canada) and maintained at 18 °C under a natural simulated photoperiod. Fish were fed and maintained on standard flaked goldfish food. Goldfish were anesthetized using MS-222 for all handling and dissection procedures. Care was taken to standardize all handling, injections, and sample protocols. Goldfish were divided into four tanks of n=14 fish each. Average fish weight was 21g ± 1g (S.E.) and not different between tanks ($df=3$; $F=0.97$; $p>0.5$). Fish were injected with either saline vehicle (control), a 5-HTR$_{1A}$ agonist (8-hydroxy-2-(di-*n*-propylamino)-tetralin = OH-DPAT; Tocris, Ellisville, MO, USA), a 5-HTR$_{1A}$ antagonist (WAY; [O-methyl-3H]-N-(2-(4-(2-methoxyphenyl)-1-piperazinyl) ethyl)-N-(2-pyridinyl) cyclohexanecarboxamidetrihydrochloride = WAY600135; Tocris, Ellisville, MO, USA), or a combination of both. Injections were staggered between groups and administered

within 30 min of each other. The concentrations used were 0.5 µg/g bw for OH-DPAT and 1 µg/g bw for WAY600135. Solutions were prepared for an injection volume of 1 µL/g bw. The concentrations were based on a previous study in rats, which investigated the effect of either drug, or the combination of both, on oxytocin secretion (237). Furthermore, similar concentrations have been shown to be effective in fish, as exemplified by the antagonistic action of the 5-HTR_{1A} agonist and antagonist on sexually dimorphic communication signals in the weakly electric fish (*Apteronotus leptorhynchus*) (238). Fish were sacrificed 6 h after the injections, as gene expression changes in the neuroendocrine brain of goldfish in response to receptor agonists and antagonists have been shown to occur in this timeframe. Telencephalic tissue was harvested and pooled, resulting in a n=7 for each treatment group. Tissues were subsequently stored at –80 °C until further processing. Data were transformed to fit a normal distribution and analyzed using a two-way ANOVA and Tukey's post-hoc test.

4.2.2 Experiments investigating the effect of IT on the HPG axis

4.2.2.1 Cloning and mRNA tissue distribution of goldfish it and its receptor, itr

Primers for the goldfish *it* gene have been previously published (132), and primers for the *itr* were designed using Primer3 (http://frodo.wi.mit.edu). Primer sequences used are listed in **Tab 4.1.** Primers were subsequently used to amplify *it* and *itr* amplicons using cDNA templates from female goldfish tissues. The RNA isolation, cDNA synthesis, and PCR reactions were run as previously described (132). Separate reactions using *β-actin* primers were used as positive control. A no RT negative control obtained during cDNA synthesis was run with the reactions. A

1% agarose gel was run under the conditions previously mentioned and a photograph taken using ChemiDoc and Quantity One Software, version 4.5.2 (BioRad, Mississauga, Ontario).

Tab. 4.1 *Primer sequences used to determine tissue distribution of it mRNA and itr mRNA.*

Gene target	Primer sequence 5'3' (FW)	Primer 3'5' sequence (RV)	Genbank
it	ATCTTGGCTACTGGCAGCTT	GTATCTGCTGTGGTGAAGGT	AF322651
itr	ATCATCCCCGTAGCCATTCT	AACGTCATTTTCACCGTCCT	FJ618543
ß-actin	ACTACTGGTATTGTGATGGACTCC	CGGTCAGGATCTTCATCAGGTAG	AB039726

4.2.2.2. Phylogenetic analysis of the ITR

The partial coding sequence obtained from the cloned *itr* was phylogenetically mapped using full coding sequences of *vt* and oxytocin receptor families retrieved from Genbank (http://www.ncbi.nlm.nih.gov/genbank). Sequences from representatives of all vertebrate families were included, except for reptiles, for which no receptor sequences were available. Translated protein sequences were submitted to PhyML online (239) (www. http://www.atgc-montpellier.fr/phyml), and a phylogenetic tree was created using the algorithm described by Dereeper and colleagues (116) with 1000 bootstraps.

4.2.2.3 Seasonal pattern of hypothalamic it mRNA abundance

Using the previously described samples from the seasonal study (Chapter 2; ref. 117), I investigated the seasonal mRNA expression profile using the *it* primers listed in **Tab. 4.1.** The *it* real-time RT-PCR assay was conducted as previously described (Chapter 3 and ref. 132). Data was normally distributed and analyzed using a two-way ANOVA with Bonferroni's post-hoc test for season.

4.2.2.4 Animals and in vivo injections of IT

Common female goldfish were purchased from a commercial supplier (Aleong's International Inc., Mississauga, ON, CAN) and maintained at 18 °C under a natural simulated photoperiod. Fish were fed and maintained on standard flaked goldfish food. Goldfish were anesthetized MS-222) for all handling and dissection procedures. Care was taken to standardize all handling, injections, and sample protocols. Sexually regressed female goldfish (n=75) were divided into 5 groups of 15 fish each. Following the results of a preliminary dose-response study, fish received 1 mM IT injections. Fish were sacrificed 2 h, 6 h and 12 h after injections to investigate time courses of LH serum concentration. These time points were chosen based on the observed IT stimulated increase in E_2 12 h after injection (Chapter 3). The rationale was that if the observed effect on E_2 was LH dependent, a previous, IT-induced elevation of LH should occur in a shorter timeframe. Saline injected control groups were used for every time point. In all cases, injection volumes administered were 1 µl/g bodyweight. Fish were sacrificed 2 h after the injection and blood was taken through caudal puncture and kept at 4 °C overnight. Samples were centrifuged for 10 min at 3000 rpm and 4 °C, and serum was collected and stored at -80 °C until further processing.

4.2.2.5 Static incubation of goldfish pituitary cells

I was interested to find out whether IT also had direct effects on pituitary cells. Short-term culture experiments of primary dispersed goldfish pituitary cells[7] were performed for sexually recrudescing (December) and sexually mature (May) fish. More than 100 pituitaries were collected from male and female goldfish, and immediately put in ice-cold dispersion

[7] The pituitary cell incubations were performed in collaboration with Dr. Zhao at the Trudeau lab, University of Ottawa, ON, Canada.

medium (M199 with Hank's salts, 25 mM HEPES, 26.2 mM sodium bicarbonate, 0.3% bovine serum albumin, 100,000 U/L penicillin, 100 mg/L streptomycin, pH 7.2). Pituitaries were washed with dispersion medium at room temperature 3 times, before being diced into 1 mm fragments. Fragments were then dispersed in medium containing 2.5 mg/mL trypsin II for 35 min and shaken at room temperature, followed by 5 min in a dispersion medium containing a trypsin inhibitor (2.5 mg/mL) and 5 min in a dispersion medium containing 10µg/mL DNAse II. Fragments were washed in calcium-free medium (Calcium-free Hank's Balanced Salts, 25 mM HEPES, 26.2 mM sodium bicarbonate, 0.3% bovine serum albumin, 100,000 U/L penicillin, 100 mg/L streptomycin, pH 7.2) containing 2 mM, 1 mM and 0 mM EGTA in sequence. Cells were then filtered through a nylon mesh and centrifuged at 1500 rpm for 10 min. Cells were resuspended in calcium-free medium and counted using a hemocytometer.

Cells were distributed in n=8 wells per treatment group and kept in plating medium (M199 with Earle's salts, 25 mM HEPES, 26.2 mM sodium bicarbonate, 1% horse serum, 100,000 U/L penicillin, 100 mg/L streptomycin, pH 7.2) at 28 °C and 5% CO_2 under saturated humidity for at least 12 hours. Prior to the incubation, cells were transferred to testing medium (M199 with Hank's salts, 25 mM HEPES, 26.2 mM sodium bicarbonate, 0.1% bovine serum albumin, 100,000 U/L penicillin, 100 mg/L streptomycin, pH 7.2) for 1 h before treatments were added. Treatments were negative control (testing medium), 1 nM IT, 10 nM IT, 100 nM IT, and positive control (100 nM sGnRH). Concentrations were chosen based on the known affinity of the teleost ITR (215). After 12h of incubation, plates were centrifuged at 500 *g* for 5-10 min, and supernatants were collected for subsequent LH analysis.

4.2.2.6 Radioimmunoassays for LH

LH concentrations were determined using a well-established RIA described by Peter and colleagues (240). The specific binding for the LH assay was 40.6% and the detection limit was 0.32 ng/mL. Samples were diluted in barbitone buffer at dilutions of 1:5 for serum and 1:100 for culture medium. The data for LH assays of the *in vivo* experiments was not normally distributed, and was analyzed using a Kruskal-Wallis test with a Scheirer-Ray-Hare extension to assess interaction effects. Comparisons between control and IT-injected groups were made using Bonferroni-adjusted Mann-Whitney U tests. Data were analyzed using SPSS, version 17.0. Data for LH assay of the pituitary experiments *in vitro* were transformed to fit normal distribution. A univariate ANOVA with Bonferroni's post-hoc test was used for statistical analysis.

4.3. Results

4.3.1. Serotonergic fibers are found in close proximity to IT cells in the POA of the telencephalon and neuronal lobe of the pituitary in female goldfish

Transversal sections of the POA and sagittal sections of the pituitary were used in the collocalization study (**Fig. 4.1**). Immunoreactivity for IT (**Fig. 4.2**; red colour) was observed in the POA and in the pituitary. Dense populations of magno- and parvocellular IT perikaya were found in the POA (**Fig. 4.2**A), with emanating fibers that could be traced to the pituitary stalk. In the pituitary, immunoreactive fibers could be located especially in the pars nervosa, but immunoreactivity of fibers was also found in the proximals pars distalis and the pars intermedia (**Fig.4.2**E).

Serotonergic immunoreactivity (**Fig. 4.2**; green colour) was observed in several parts of the telencephalon and diencephalon, but also in the pituitary gland. Specifically, numerous perikaya were observed along the wall of the third ventricle. These cell bodies were observed

most anteriorly in the ventral POA (**Fig.4.2**C), and appeared to be more abundant posteriorly in the nucleus ventromedialis. These perikaya appear to be contacting the cerebrospinal fluid, but no traceable fibers were seen emanating from these cells. Many immunoreactive fibers were detected in the telencephalon and in the POA (**Fig.4.2**C), however their origin was difficult to determine. In the pituitary gland, 5-HT immunoreactive fibers were observed, especially in the proximal pars distalis (data not shown), but stained fibers were also observed in the pars nervosa *(***Fig.4.2**G*)*, pars intermedia (**Fig.4.2**G) and rostral pars distalis (not shown).

Evidence for synaptic contacts was found for individual magnocellular IT perikaya in the dorsal POA, as exemplified in **Fig.4.2**D. Evidence for points of contact between 5-HT innervation and IT varicosities was found in parts of the pars nervosa of the pituitary (**Fig.4.2**H).

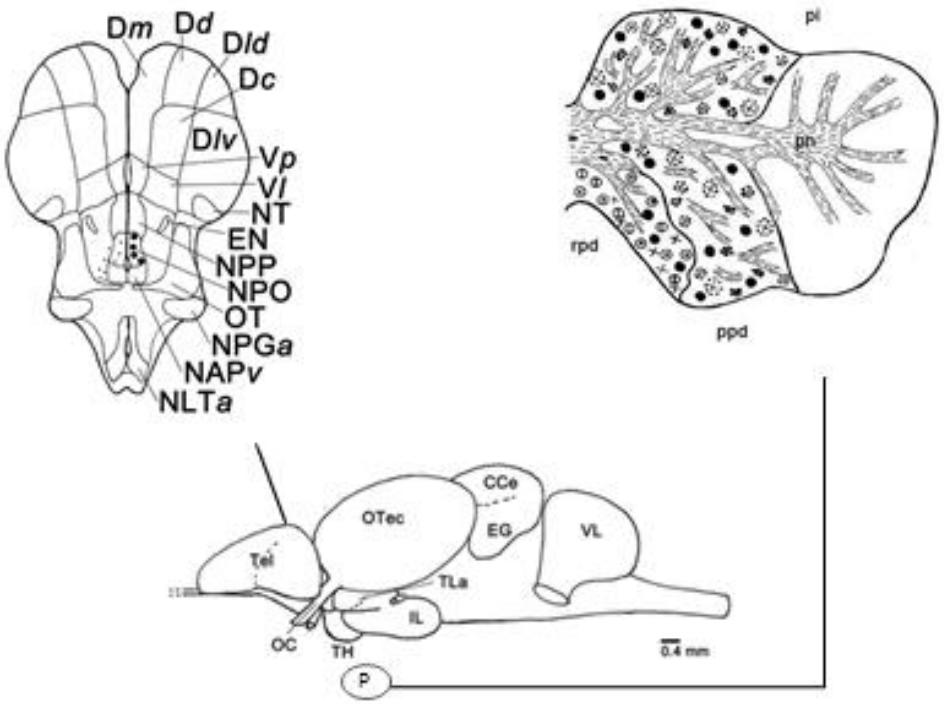


Fig. 4.1. *Schematic diagram of a goldfish brain.Tel= telencephalon, Otec=optic tectum, OC=optic chiasm, TH= thalmaus, P=pituitary, IL=infundibulum, CCe=cerebellum, EG=emminentia granularis , TLa=Torus lateralis, VL=vagal lobe. Depicted is a saggital section of the goldfish dorsal telencephalon, including the nucleus preopticus (NPO=POA), as described by Peter and Gill (234). Dm=area dorsalis telencepali pars medialis, Dd=area dorsalis telencephali pars dorsalis, Dld=area dorsalis telencephali pars dorsalateralis, Dc=area dorsalis telencephali pars centralis, Dlv=area dorsalis telencephali pars lateroventralis, Vp=area ventralis telencephali post-commisural part, Vl=area ventralis telencephali nucleus intermediatis, EN=nucleus entopeduncularis, NPG=nucleus praeglomerularis, NLT=nucleus lateral tuberis, NPP=nucleus preopticus paraventricularis, NAPv=nucleus anterior paraventricularis, NT=nervus terminalsis, Ot=optic tectum. Black points in the POA indicate IT cell bodies, lines indicate innervation. A saggital section of the pituitary gland is shown, as is a clear zonation of trophic cells, as described by Kaul and Vollrath (3). Rpd=rostral pars distalis, ppd=proximal pars distalis, pi=pars intermedia, pn=pars nervosa. Isotocin immunoractive fibers and cells were identified in pars nervosa and to some extent, the rostral pars ditalis.*

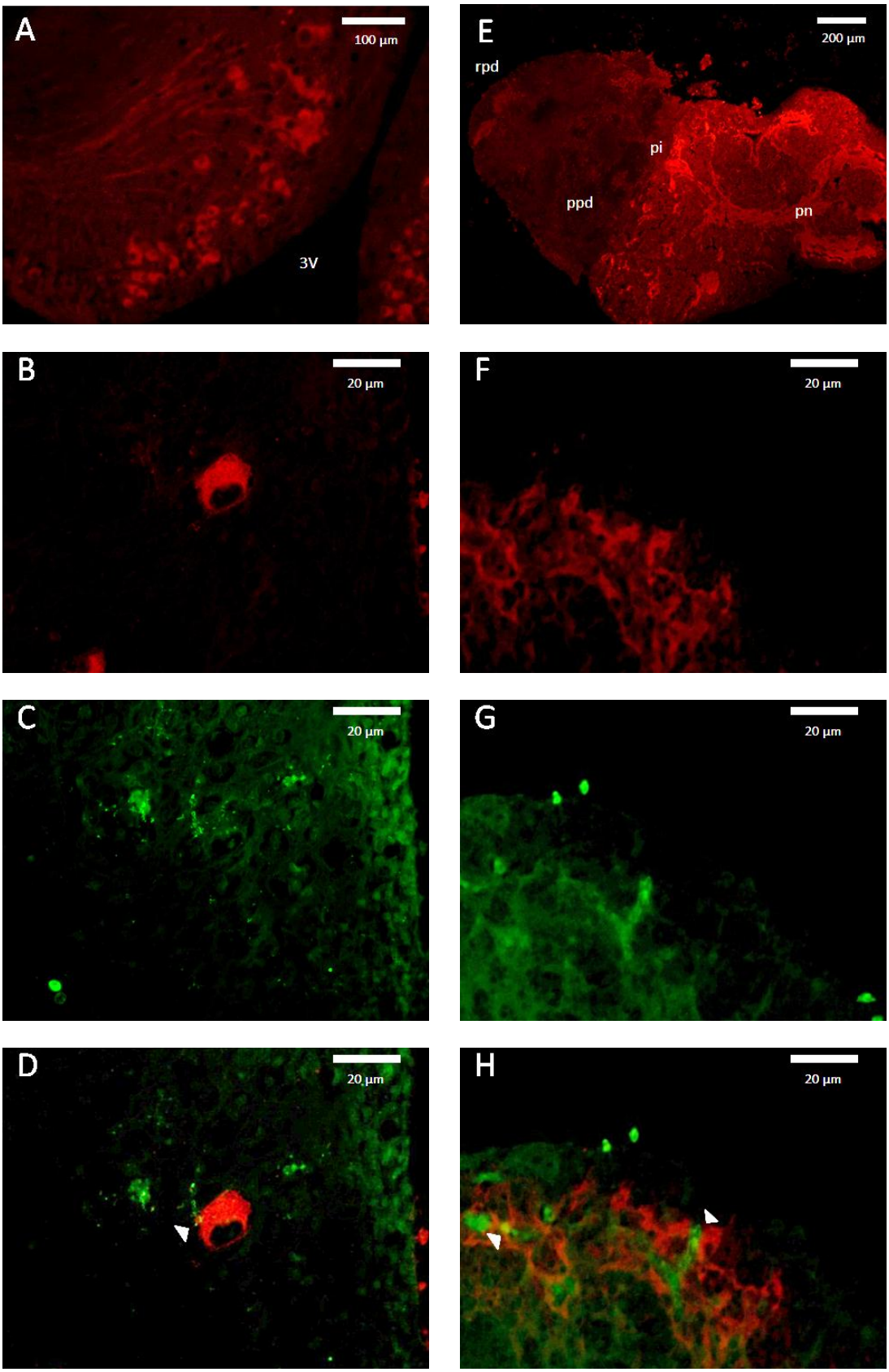


Fig. 4.2. *Representative double labelling immunocytochemistry using 5-HT (green) and IT (red) antibodies in the POA (A-D) and pituitary gland (E-H) of sexually mature female goldfish. A total n=4 fish were analyzed. See text for detailed explanations.*

4.3.2 5-HTR$_{1A}$ regulates it mRNA expression in the telencephalon

Two-way univariate analysis of variance (ANOVA) revealed significant changes in *it* mRNA abundance (**Fig. 4.3**, df=3; F=8.61; p ≤0.01). No effects were observed for the agonist (df=1; F=0.5; p >0.05), but significant effects were observed for the antagonist (df=1; F=25.5; p ≤0.05). No interaction between agonist and antagonist was detected (df=1; F=0.01, p >0.05). Post-hoc analysis revealed significantly decreased *it* mRNA abundance (p ≤0.05) in groups which were injected with the 5-HTR$_{1A}$ antagonist, either alone or in conjunction with OH-DPAT.

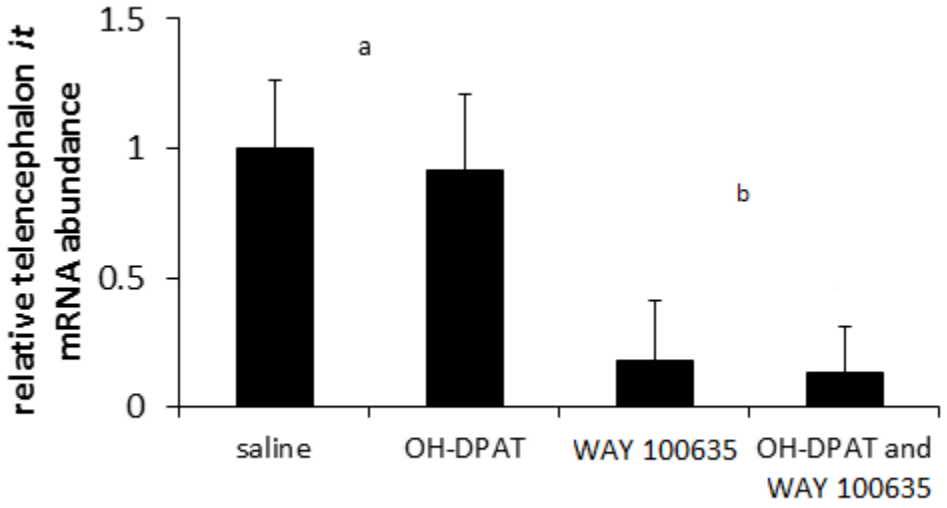


Fig. 4.3. *Relative it mRNA abundance (±S.E.) in the telencephalon of fish injected with saline, the 5-HTR$_{1A}$ receptor agonist OH-DPAT, the 5-HTR$_{1A}$ receptor antagonist WAY100635, or a combination of agonist and antagonist. An n=7 of telencephalon tissue was used. Data wastransformed to fit normal distribution and analyzed using a 2 two-way ANOVA. Different letters indicate a significant post-hoc difference for the drug WAY600135 at (p≤ 0.05).*

4.3.3 Cloning and phylogeny of the goldfish itr

The phylogenetic tree (**Fig.4.4**) shows that the cloned partial sequences of the goldfish ITR group with the oxytocin receptor family, confirming the specificity of the cloned fragment.

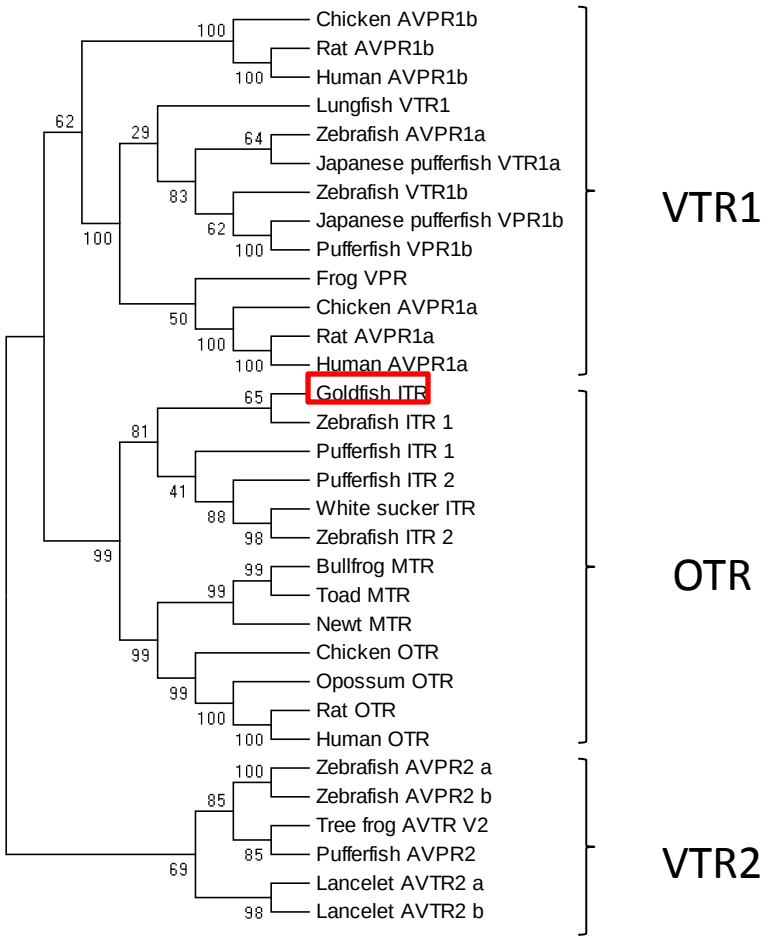


Fig. 4.4. *Phylogenetic tree of the oxytocin receptor family, including the cloned fragment for the goldfish itr. Numbers represent percentage values for bootstrap analysis (n=1000).*

4.3.4. The mRNA of the itr is detected in all components of the HPG axis

The tissue distribution, as determined by PCR analysis and gel electrophoresis, is shown in **Fig. 4.5**. The mRNA of *it* was found to be restricted to central brain tissues, such as the optic tectum, telencephalon, hypothalamus, but was also found in the pituitary. The mRNA of the *itr* was detected in several brain parts, including the telencephalon and hypothalamus. Peripheral organs showing *itr* expression include the pituitary and ovary. *β-actin* served as a control gene. No mRNA for either *it*, its receptor, or *β-actin* was detected in the no-RT control, indicating that there was no genomic DNA contamination.

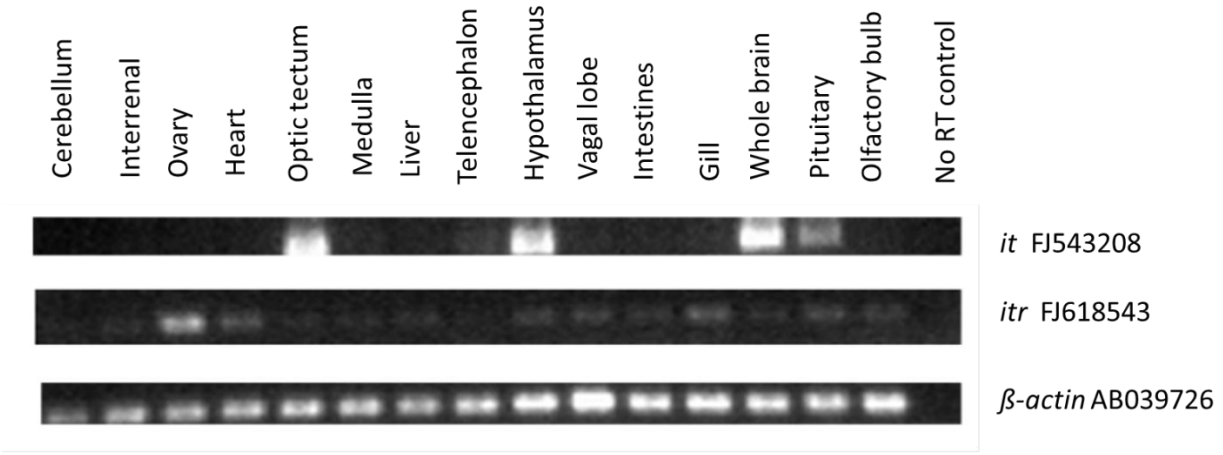


Fig. 4.5. *Tissue specific expression of it mRNA and itr mRNA in a pooled sample of female goldfish (n=2), as determined by PCR and agarose gel electrophoresis. The bottom row shows the ß-actin control mRNA expression.*

4.3.5. The it mRNA is most abundant in the hypothalamus and telencephalon of sexually mature goldfish

Two-way univariate analysis of variance of the mRNA abundance of *it* (**Fig. 4.7**; df=11; F=2.87; p ≤0.01) reveals a clear seasonal effect (df=5; F=5.46; p ≤0.01), with no gender difference (df = 1; F=1.33; p >0.05). I found no interaction effect between both factors (df=5; F=0.59; p >0.05) In the hypothalamus of both male and female sexually mature fish, there is a significant increased *it* mRNA abundance in May compared to any other time point (p ≤0.05).

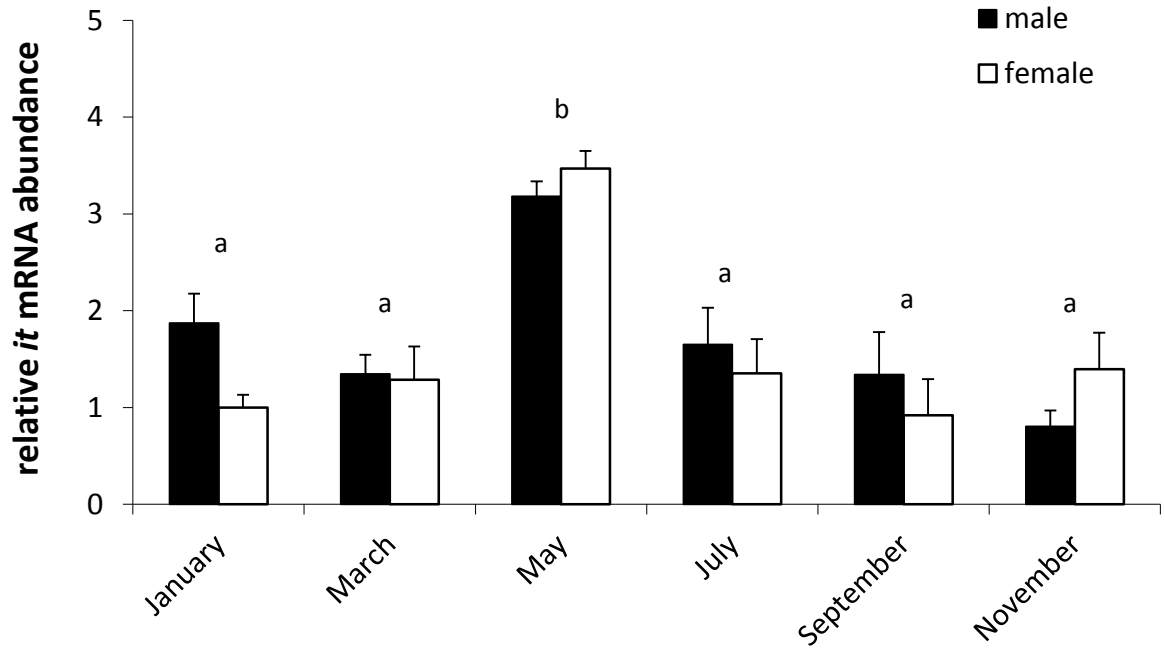


Fig. 4.6. *Seasonal mRNA abundance of it mRNA in the hypothalamus of male and female goldfish. An n=6 hypothalamus tissues were used. Data was normally distributed and analyzed using a two-way ANOVA. Different letters indicate a significant seasonal difference, as revealed by Bonferroni's post-hoc analysis for season.*

4.3.6. Isotocin does not modulate serum concentration of LH in vivo

The time course experiment investigating the effect of i.p. injected IT on serum LH (**Fig. 4.8**; df=5; H=11.05; $p \leq 0.01$) shows an effect of time (df=2; H=21.88; $p \leq 0.01$), but neither an effect of IT (df=2; H=0.87; $p > 0.05$), nor an interaction between IT and time (df=2; H=5.00; $p > 0.05$).

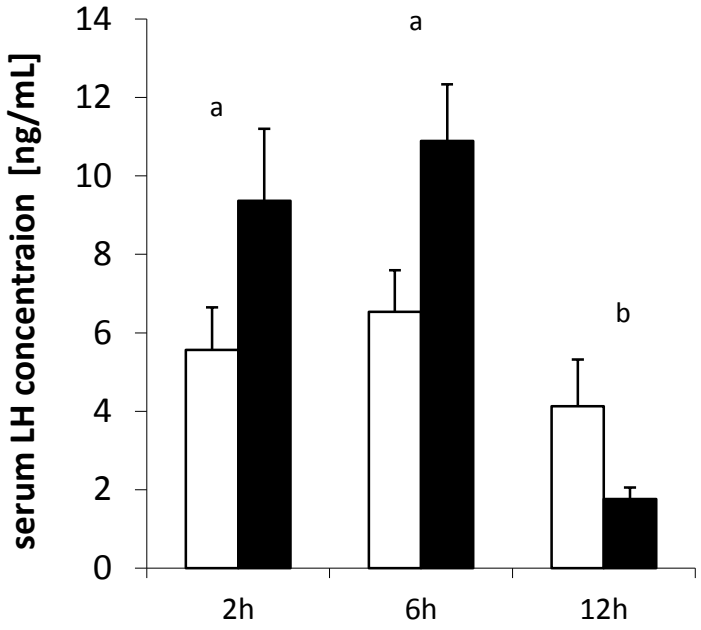


Fig. 4.7. *Serum LH concentrations (±S.E.) of female goldfish i.p. injected with saline (white bars) or 1µg/µL g^{-1} IT (black bars). An n=15 serum samples were analyzed for each group and time point. Data was not normally distributed and analyzed using Kruskal Wallis analysis with Ray-Scheirer-Hare extension to assess interaction of time and treatment non-parametrically. Different letters indicate significant difference of time ($p<0.05$).*

4.3.7 Isotocin stimulates LH release from dispersed pituitary cells in vitro

The medium concentrations of LH analyzed by a one-way ANOVA (df=5; F=12.67; $p \leq 0.01$) showed significant increases in LH, which were elicited by 100 nM IT ($p \leq 0.05$) and 100 nM GnRH, either alone ($p \leq 0.05$), or in conjunction with 100nM IT ($p \leq 0.05$). The combination of 100 nM IT and 100 nM GnRH significantly increased LH concentration compared to control ($p \leq 0.05$), but was not different from LH concentration measured for either peptide individually. No effects of IT were found in pituitary incubations from recrudescent fish (data not shown).

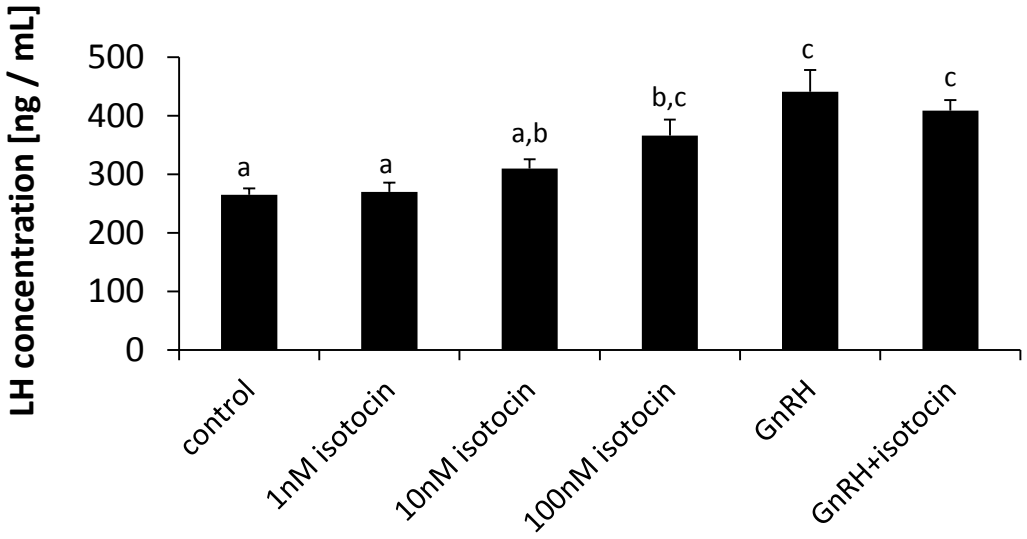


Fig. 4.8. *Concentrations (±S.E.) of LH secreted in the medium of dispersed pituitary cells of sexually mature fish exposed to a control medium, IT (1nM-100nM) and 100nM GnRH for 12h. An n=8 wells were used for each treatment. Data was transformed to fit a normal distribution and was analyzed using a one-way ANOVA analysis. Different letters indicate a significant difference at p ≤0.05 using Bonferroni's post-hoc test.*

4.4. Discussion

These results support the hypothesis that 5-HT may directly act on the IT system in the neuroendocrine brain of the goldfish. With regard to the hypothesis that there is a specific IT action on LH release in the HPG axis, the results show that the principal action of IT on the reproductive axis in goldfish is not likely mediated by a neuroendocrine stimulation of LH release. The results are discussed in detail in the following paragraphs.

4.4.1 Evidence for a direct influence of 5-HT on the central IT system in goldfish

Following the observed decrease of *it* mRNA in the neuroendocrine brain in response to i.p. injections of FLX, a SSRI (Chapter 3), I hypothesized that there is a direct link between the 5-HT system and the IT system in the neuroendocrine brain of goldfish. Immunocytochemistry revealed potential sites of synaptic contact between 5-HT fibers and IT cell bodies in the POA, and IT synaptic terminals in the pars nervosa of the pituitary. These findings are similar to studies conducted in the rat and rabbit (230,241). More specifically, in the rat, magnocellular neurons of the anterior fornical nucleus were reported to be in close proximity to 5-HT positive varicosities (230). The pars intermedia of the pituitary in the rabbit revealed immunopositive staining for both 5-HT and oxytocin. Therefore, the immunocytochemical evidence presented in this chapter argues for a conserved 5-HT innervation of this neuropeptidergic system, and suggests that 5-HT may modulate synthesis and release of IT in goldfish, similar to the described effects of 5-HT on oxytocin mRNA abundance and protein release in the rat (16).

To further investigate the potential effect of 5-HT on *it* mRNA abundance, I utilized a 5-HTR_{1A} agonist and antagonist. This receptor was specifically investigated, as it is known to play a role in 5-HT stimulation of oxytocin mRNA abundance and oxytocin release in the rat (16). Furthermore, numerous reports have causally linked FLX-induced desensitization of the

5-HTR_{1A} and decreased oxytocin mRNA abundance and release in mammals (89,113,212). Because *it* mRNA was decreased in the neuroendocrine brain in female goldfish injected with FLX (Chapter 3), this pathway was further studied in order to investigate whether this effect may be mediated by a similar pathway in goldfish.

In my experiment, the agonist failed to alter *it* mRNA abundance in the telencephalon, while the antagonist significantly decreased 5-HT mediated *it* mRNA abundance. While the reduction of telencephalic *it* mRNA abundance in goldfish is similar to effects observed in the rat following 5-HTR_{1A} antagonist treatment (16), the lack of a stimulatory effect of the 5-HTR_{1A} agonist on telencephalic *it* mRNA abundance is surprising, and in contrast to studies in the rat (16). The lack of a stimulatory effect of the agonist (OH-DPAT) does not appear to be related to a lack of effect of the agonist in fish. This agonist has been used for injections of comparative concentrations in several fish studies (37,242), as well as for *in vitro* studies in goldfish (243,112), and is therefore biologically active. For example, administration of 0.4 µg/g OH-DPAT in rainbow trout resulted in a significant increase in serum cortisol 30 min after injection (37). This agonist appears to also be active in goldfish, as *in vitro* studies revealed that OH-DPAT stimulates cAMP, and inhibits neurite outgrowth in goldfish retina (243). Administration of 0.1 µM and 1 µM OH-DPAT stimulated LH release in dispersed goldfish pituitary cells *in vitro* (112). Therefore, it is likely that OH-DPAT is active in goldfish. However, because the 5-HT concentration and turn-over is elevated in sexually mature fish (93,94); this may result in a maximal saturation and stimulation of the 5-HTR_{1A}, preventing any additional stimulation by the agonist OH-DPAT. A high serotonergic tone in these fish at this time of the year may also explain the magnitude of the (5-fold) reduction of *it* mRNA abundance in the telencephalon of goldfish treated with the 5-HTR_{1A} antagonist WAY 600135.

Interestingly, the data obtained from FLX-injected goldfish (Chapter 3) and the antagonist experiments are quantitatively similar. Both pharmacological manipulations resulted in approximately 5-fold decrease in telencephalic *it* mRNA abundance. In rats, long term FLX administration results in blunted responses of OH-DPAT stimulated oxytocin release (113,212,244), which may be sustained after FLX discontinuation (89). Mechanistically, this effect has been linked to the desensitization of hypothalamic 5-HTR_{1A}, which involves a reduction in hypothalamic protein concentrations of G_i and G_o proteins, but not of $5HTR_{1A}$ proteins (212,245).

The evidence from this chapter provides support for a conserved stimulatory action of 5-HT on the IT system in goldfish, which is at least partially mediated through the 5-HTR_{1A}. This finding has implications for the proposed mechanism of action of FLX on the IT system in fish, and future time course studies investigating FLX-induced desensitization to 5-HTR_{1A} receptor agonists or antagonists should provide further evidence for this conserved mechanism of action of FLX in the neuroendocrine brain of goldfish.

4.4.2 Isotocin does not elicit significant release of LH

Evidence from my previous study in goldfish, which revealed a stimulatory role of IT on serum E_2 (Chapter 3), led me to investigate the effects of IT on the reproductive axis in goldfish in more detail. The partial *itr* coding sequences I obtained from partially cloned goldfish cDNA confirmed specificity of the cloned fragment, as the ITR groups with the oxytocin receptor family, rather than with the VT receptor family, in a phylogenetic analysis of the translated AA sequence. This analysis is in line with earlier phylogenetic analysis of nonapeptide receptors (226,246), in which distinct receptor lineages were found to largely reflect ligand evolution.

The *itr* was expressed in the hypothalamus, pituitary and ovary, indicating that all tissues of the HPG axis are potential targets for modulation by IT. These results confirm previous teleost studies in which evidence for *itr* mRNA was found in the brain, pituitary and ovary (215,226,10). In the hypothalamus, the seasonal changes in mRNA abundance of *it* further suggest an effect of IT on the goldfish reproductive axis, as peak mRNA abundance was found in sexually mature goldfish in May. This observation is supported by findings in the female three-spined stickleback (*Gasterosteus aculeatus*), where brain IT protein concentrations peak in July, the reproductive season of this long-day breeder (217). In female Japanese medaka *(Oryzias latipes)*, IT neuron numbers sharply decline following spawning (199). While 5-HT may be implicated in this seasonal control, recent work in goldfish (200) has also implicated a role for other neurotransmitters, which is consistent with their role in reproduction: MPTP and MPT-induced depletion of dopamine, the main inhibitor of the neuroendocrine regulation of reproduction in teleosts, resulted in an increase in hypothalamic *it* mRNA abundance. Similarly, the injection of the $GABA_A$ receptor agonist muscimol increases *it* mRNA abundance in the hypothalamus.

I therefore investigated potential effects on LH release, both *in vivo* and specifically *in vitro* in the pituitary, where expression of the *itr* was observed. No changes in serum LH concentrations in IT-injected fish were observed *in vivo,* and only mild stimulation of LH release in IT-incubated pituitary cells was observed *in vitro.* These findings suggest a mild stimulatory action of IT on LH release in teleosts. A stimulatory role of the IT homolog oxytocin on LH release has been reported in mammals, both *in vivo* and *in vitro* (247). These effects, however, appear to be limited. For example, oxytocin-stimulated LH release is dependent on permissive

steroid action in the rat (248), and several studies failed to identify clear stimulatory effects of oxytocin on LH in mammals (249).

These findings suggest that the effects of IT on reproduction in teleosts are more likely mediated outside of the LH pathway in the neuroendocrine brain and the pituitary. The first possibility is a role for IT as a neuromodulator in the brain, which is in line with the detected *itr* receptor mRNA abundance in various brain areas of the goldfish, and the described effects of *it* injection reproductive behaviour in the midshipman (*Porichthys notatus*) (148). In goldfish, IT injections have also been shown to evoke social approach behaviour (250). Similar effects of central oxytocin have been extensively described in mammals (146,251,252).

Secondly, because IT is released from the pituitary and can be measured as a circulating hormone in fish (253), it may act on the ITR expressed in the ovary. In male rainbow trout, IT has been shown to significantly enhance T release from rainbow trout testis *in vitro* (254). In male African catfish (*Clarias gariepinus*), injection of oxytocin increased the amount of strippable milt. Comparable fish studies in female fish are scarce, but Venkatesh and colleagues report that in the viviparous guppy (*Poecilia reticulata*), IT induces premature parturition (255). *In vitro* studies of oxytocin incubated early luteal cells of the pig ovary report stimulatory effects of oxytocin incubation on E_2 secretion. Therefore, a similar mechanism may be responsible for the observed increase in serum E_2 concentrations in IT-injected female goldfish, reported in Chapter 3.

4.5 Conclusions

The investigation of IT supports a conserved stimulatory effect of 5-HT on this neuropeptide in the neuroendocrine brain. Furthermore, evidence suggests that the observed FLX-induced decrease in neuroendocrine *it* mRNA abundance (Chapter 3) may be caused by desensitization events of the 5-HTR_{1A}, similar to the situation in mammals. This hypothesis requires further testing. Physiologically, the main reproductive effects of IT appear to involve only a mild stimulation of the gonadotrophs, and may rather be related to neuromodulatory action in the brain and/or peripheral effects of the circulating hormone directly on the gonads. The latter hypothesis may explain the observed stimulatory effect of IT on serum E_2 (Chapter 3).

Chapter 5: Effects of waterborne FLX on the reproductive axis in sexually mature male goldfish

Partially based on: Mennigen et al. 2010. Waterborne fluoxetine disrupts the reproductive axis in sexually mature male goldfish, Carassius auratus. Aquat Toxicol. 100(4):354-64.

5.1. Introduction

To investigate the hypothesis that reproductive function in sexually mature goldfish is disrupted by waterborne FLX exposures, separate experiments using the same basic exposure design were conducted for both sexually mature female and male goldfish. Because the neuroendocrine brain of the male goldfish appears to be more responsive to waterborne FLX challenges, the results of this particular study are provided here in Chapter 5, while the results for the female exposure can be found in Appendix 1 of this thesis.

While some effects of pharmacological and waterborne studies of FLX reveal a disruption of the reproductive axis in female fish (132,256), similar to the effects observed in pharmacological studies in mammals, in particular females (91), the potential disruption of the reproductive axis in male fish has received less attention, in spite of the description of disruptive effects of FLX in the reproductive system of male rats (257).

I used goldfish in this study, in order to determine the effects of waterborne FLX on the reproductive axis in male fish. Goldfish are a well-characterized model for the neuroendocrine control of reproduction (1), and were also especially important for the discovery of steroid- and prostaglandin-derived vertebrate sex pheromones (258). Briefly, the male goldfish reproductive axis is principally active in May (1); seasonal regulation occurs through exogenous cues (e.g. photoperiod), including pheromone signalling. Exogenous signals are integrated at the level of the POA by GnRH neurons that project to the pars distalis of the pituitary to regulate the synthesis and release of LH, FSH and GH (1). These hormones bind to their corresponding

receptors in the testes and regulate spermatogenesis and steroidogenesis (259). In male goldfish, the neuroendocrine axis is acutely activated by at least two well-characterized female sex pheromones, 17,20β-dihydroxy-4-pregnene-3-one (17,20P) and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), as described by Stacey and colleagues (258). 17,20P is the 'priming pheromone' that initiates physiological changes within the reproductive axis, while secondarily released $PGF_{2\alpha}$ stimulates reproductive behaviours (258). The activation of both the endocrine axis and behaviours is critical to reproductive success, as demonstrated by the influence of both systems on milt release (260). In this study, sexually mature male goldfish, which show the highest mRNA abundance of hypothalamic *slc6a4a* (Chapter 2), were used in the exposure. Measured physiological endpoints included serum concentrations of the pituitary hormones LH and GH, as well as pituitary mRNA abundance of the specific *fsh-β* subunit, as protein measurements of serum FSH are only available in salmonid species (220). However, mRNA abundance of *fsh-β* is found to correlate well with circulating FSH concentrations in rainbow trout (220). The relative mRNA abundance of the nonapetide hormones, *it* and *vt*, was measured in the telencephalon of the neuroendocrine brain, where isotocinergic and vasotocinergic innervation originates (224). In the gonads, I measured mRNA abundance of the LH, FSH, and GH receptor genes (*lhr*, *fshr*, *ghr*). Gonadosomatic index, milt release and serum concentrations of the sex steroid hormone T were measured as reproductive endpoints. An increase in gonadal aromatase mRNA abundance (*cyp19a*) was observed with fish exposed to 54 μg/L FLX treatment, therefore, I measured *cyp19a* mRNA abundance and Cyp19a activity, serum E_2 concentrations, and hepatic mRNA abundance of the estrogenic marker *erα*. More specifically, these parameters were measured for control fish and fish exposed to either concentration of FLX only, but not in the pheromone-

stimulated groups. The study was designed to test the hypothesis that FLX disrupts basal and pheromone-induced endpoints of the reproductive axis.

5.2. Materials and methods

5.2.1. Animals and experimental design

In April 2009, sexually mature male goldfish were purchased from a commercial supplier (Aleong's International Inc., Mississauga, ON, Canada) and maintained at 18 °C in dechlorinated tap water under a photoperiod simulating April day length in Ottawa (L:D 13 h:11 h). Fish had an average weight of 18.3 ± 1.3 g (S.E.), which was not significantly different across treatment groups ($df=8$; $F=0.97$; $p>0.05$). Fish were acclimated to holding conditions for 3 weeks prior to the exposure. Fish were fed standard flaked goldfish food (Martin Mills, Elmira, ON, Canada). Fish (n=108) were separated into nine aerated 70 L tanks in groups of 12. For all tanks, 2/3 of water volume was changed every second day (**Fig. 5.1**). This experimental design was based on preliminary data reported by Paterson and Metcalfe (84) and Gaworecki and Klaine (130), who estimated the half-life of FLX in populated fish tanks of about 3 d. As the estimated half-life of FLX in fish is 9.4 d (84), bioconcentration is expected to occur in this experimental design. The tanks consisted of a vehicle control group receiving ethanol (Commercial Alcohols Inc., Brampton, ON, Canada, purity ≥99%), and two separate tanks exposed to nominal FLX (Sigma–Aldrich, Oakville, ON, Canada, purity ≥98%) concentrations of 0.54 μg/L and 54 μg/L in a static renewal exposure. The 0.54 μg/L concentration represents the highest reported FLX concentration in the aquatic environment, and was measured in treated waste water effluent (65). This concentration was chosen in order to simulate concentrations of combined SSRI loads in the environment, which lie between 0.5 μg/L and 3.4 μg/L (63). Two additional groups were

pretreated with the same FLX concentrations prior to receiving pheromone treatment of either $PGF_{2\alpha}$ or 17,20P (both Sigma–Aldrich, Oakville, ON, Canada, purity ≥99%) 6 h before the end of the 14 d exposure. Two groups were not exposed to FLX, but received only pheromone treatments of either $PGF_{2\alpha}$ or 17,20P 6 h before the end of the exposure[8]. Nominal tank concentrations of pheromones were 4.3 nM 17,20P and 3 nM $PGF_{2\alpha}$, based on findings by Sorensen and colleagues (261). Ethanol concentration in the tanks was 0.28×10^{-6} % (v/v), well below the acute concentration of ethanol (1%, v/v) found to affect the 5-HT system in zebrafish brain (262). At the end of the 14 d exposure, fish were anesthetised using 0.1 g/L MS-222 (Syndel Laboratories, Vancouver, BC, Canada), and weighed. Following measurement of milt release, blood was collected by caudal puncture, stored on ice, and kept at 4 °C overnight. The next morning, serum was harvested by centrifugation at 5000 *g* for 10 min. Serum was stored at −80 °C until processing for RIA. Anesthetised fish were sacrificed by spinal transsection and neuroendocrine brain tissue (telencephalon and hypothalamus), pituitary, liver, and gonadal tissues were dissected and immediately placed on dry ice before storage at −80 °C until RNA isolation.

In a second exposure (**Fig. 5.1**) designed identically with respect to photoperiod (season), FLX concentrations, and the steady state exposure protocol, actual water concentrations of FLX were measured. In this experiment, a total of 30 sexually mature goldfish were used - there was one control group and two FLX-exposed groups (0.54 μg/L and 54 μg/L) containing n=10 fish each. The analytical method used to measure concentrations of FLX in water has been previously described by Paterson and Metcalfe (84).

Briefly, samples were prepared by filtering the 500 mL samples of tank water through a

[8] Pheromone was added by Wudu Lado of Dr. Vance Trudeau's lab, University of Ottawa, ON, Canada.

glass fibre filter, which was pre-extracted with acetone and hexane and collected in a beaker using a vacuum pump. The pH of the filtered water was adjusted to 2.5-3.0 by addition of concentrated HCl. Samples were then spiked with 100 µL of the internal standard Fluoxetine-D5 (Sigma-Aldrich, Oakville, ON, Canada) in methanol to achieve final concentrations of 200 ng/mL. The solid phase extraction (SPE) enrichment was performed with Waters OASIS MCX SPE cartridges (6 cc, 150 mg; Waters Inc., Milford, MA, USA). The cartridges were first conditioned with 6 mL methanol and aspirated to dryness, followed by addition of 6 mL water. Care was taken to not let the cartridges dry before samples were loaded. Six samples were extracted simultaneously (2 control samples, 2 samples from tanks with a nominal concentration of 0.54 µg/L and 2 samples from tanks with a nominal concentration of 54 µg/L) and the sample flow rate was adjusted to 2-3 ml/min. Once the samples were filtered through the cartridges the beakers was rinsed with about 10 mL water, which was subsequently loaded onto the cartridges. The cartridges were aspirated to dryness, then washed with 2 mL 0.1 M HCl in water. Following this washing step the cartridge was aspirated to dryness with vacuum, this time for a period of 30 min. Following this, the cartridges were washed with 3 × 3 mL of DCM at a flow rate of 1 mL/min, letting the cartridges aspirate to dryness between the washing steps.. After the final wash, the cartridges were again aspirate to dryness for about 30 min with vacuum. The analytes were eluted from the cartridges with 3 × 3 mL of a solution of 5 % NH_4OH in methanol (5v/ 95v), which was prepared daily. The eluent was collected in a centrifuge tube and evaportated to dryness under analytic grade nitrogen at room temperature. Samples were then reconstituted in 400 µL methanol and transferred to an autosampler vial with an insert for LC-MS/MS analysis. The analysis[9] was performed with the Applied Biosystems/Sciex Q-Trap mass

[9] Mass spectrometry analysis of samples was conducted by Hongxia Li, Trent University, Peterborough, ON, Canada.

spectrometer and an Agilent 1100 HPLC system. The HPLC column used was a Dynatec C-18 column (150 × 2.1 mm i.d.) operated at a mobile phase flow rate of 0.2 ml/min. The mobile phases were: A (10 mM ammonium acetate in water) and B (10 mM ammonium acetate in 95 % acetonitrile /water (v/v)). The elution gradient was established as follows: mobile phase B was increased from 5 % to 100 % within 12 min and then held for 6 min, before being decreased to 5 % by 2 min and held for 15 min. The mass spectrometer was operated with the APCI source in positive ion mode. Nitrogen was used as the nebulizer, drying, curtain and collision gases. Detection was by tandem mass spectrometry in multiple reaction monitoring (MRM) mode. The APCI source operational parameters were: TEM: 300°C; CUR:10; GS1: 60; GS2:5; CAD: 5; NC:3 and ihr: ON. The dwell time for each precursor-product ion transition was set to 200ms. The retention time for FLX was 15.6 min, while declustering potentiation was 22V, the entrance potential 4V, the collision cell entrance potential 12V and the collision energy 28eV. A total of 12 water samples (500mL) were collected at random time points from control, 0.54 µg/L FLX, and 54 µg/L FLX treatments. Testis samples (n=5 per treatment) were taken at the end of the second exposure and used for histological assessments.

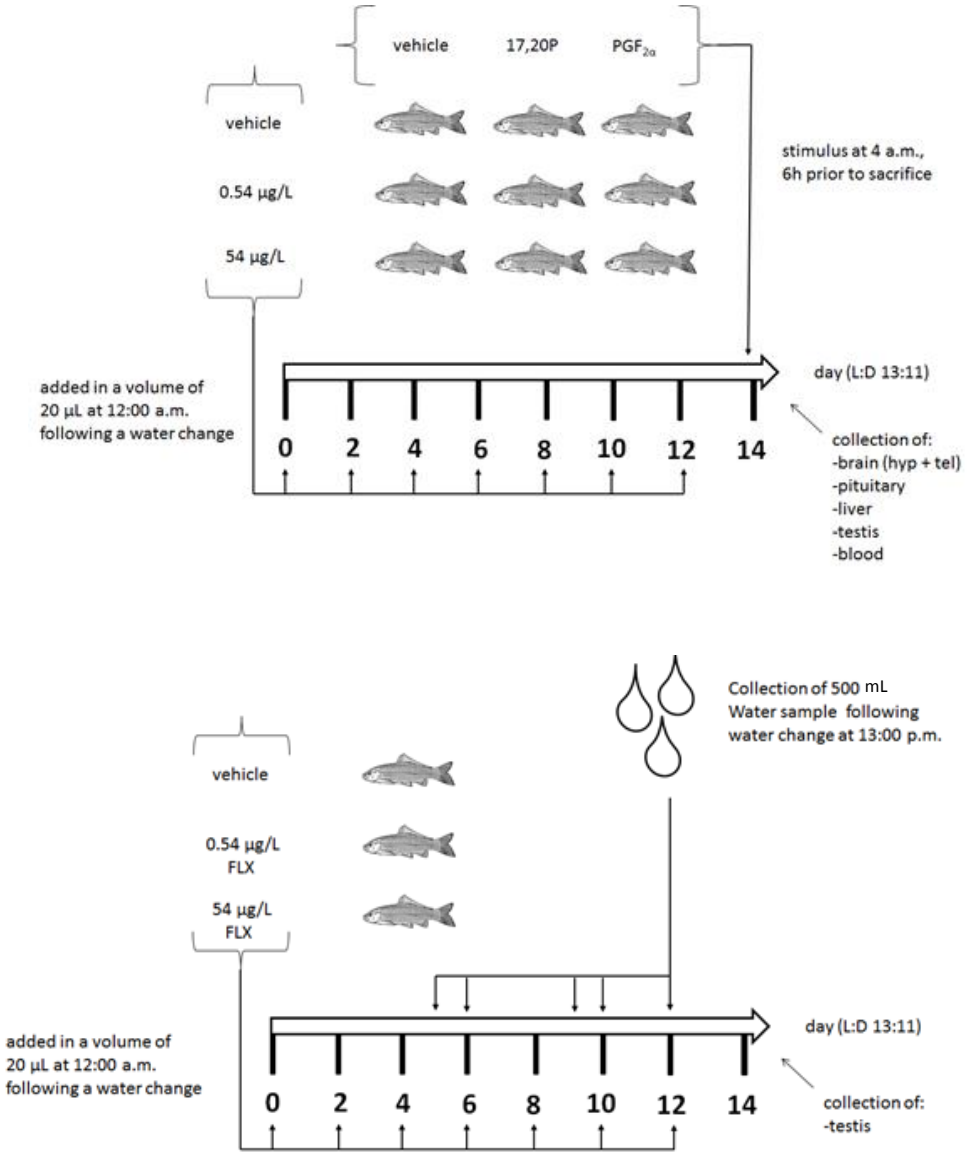


Fig. 5.1. *Schematic diagram of the experimental design*

5.2.2. Milt release

Milt was collected[10] from all fish prior to exposure to avoid *a priori* differences in milt measurements, as described by Zheng and Stacey (260). Milt was then collected under mild MS-222 anesthesia at 7 d, and again at the end of the 14 d exposure. Milt was collected into pre-

[10] Milt data collection was performed by Wudu Lado of Dr. Trudeau's lab, University of Ottawa, ON, Canada.

weighed hematocrit tubes by gently squeezing the abdomen from the mid-ventral region posterior to the gonopore. After milt collection, the hematocrit tube was weighed and the milt volume calculated, as described by Dulka and colleagues (263). Milt data were not normally distributed and median milt values are reported, according to the method used by Dulka and colleagues (263).

5.2.3. Radioimmunoassays for LH, GH and sex steroids

Radioimmunoassays for LH and GH are established for goldfish (240,264). The specific binding for the GH assay was 37.7% with a detection limit of 2.5 ng/mL. For the LH assay, specific binding was 40.6% and the detection limit was 0.32 ng/mL. Steroid extraction and RIAs for T and E_2 were performed according to methods described by McMaster and colleagues (156). Testosterone and E_2 antibodies were obtained from Medicorp (Montreal, QC, Canada). Beckman-Coulter Ready Safe Scintillation Cocktail (Beckmann-Coulter, Mississauga, ON, Canada) was added to the extracted samples prior to reading in a LS 6500 Scintillation Counter (Beckmann-Coulter). The assay detection limits were 0.32 ng/mL for T and E_2.

5.2.4. RNA extraction, cDNA synthesis, and real-time RT-PCR

Total RNA isolation, cDNA synthesis, and real-time RT-PCR assays were performed as described by Mennigen and colleagues (132). For the telencephalon and pituitary, two individual samples were pooled prior to RNA isolation to ensure sufficient material. For testis and liver tissues, individual samples were assessed. In all cases, a sample size of n=6 was used for cDNA synthesis and real-time RT-PCR assays. The mRNA abundance data were normalized to the reference gene *18S*. Standard curves for real-time RT-PCR mRNA abundance assays were obtained from serial dilutions of a randomly pooled subset of sample cDNA. Only standard

curves with slopes between −3.2 and −3.5 and R^2 values of ≥0.99 were used. Specific primer sets used are listed in **Tab. 5.1**.

Tab. 5.1. *Primer sequences used for real-time RT-PCR analysis.*

Gene target	Primer sequence 5'3' (FW)	Primer 3'5' sequence (RV)	Genbank
lh-β	GAGGGCTGTCCAAAATGTCT	AGCCACAGGGTAGGTGATGT	D88024
fsh-ß	ATGCGCTTCGTTGTTATGGT	AGCTGCCACATTCCTCACTT	D88023
gh	GTGGTGCTGGTTAGTTTGT	GCTCTTCTGTGTTTCATC	AF401272.1
it	GTATCTGCTGTGGTGAAGGT	ATCTTGGCTACTGGCAGCTT	AF322651
vt	TGCCTGCTACATCCAGAAC	GAGACCCGAGCAGACAA	HM140792
lhr	CCTCTGCATCGGTGTGTATC	AGAGCGTGTGTGATCGTTGT	HM347776
fshr	AGCATCTGCCTGCCAATG	GAAGTCGGTGAAGATGAGCA	HM347775
ghr	GACCCAGAAATCCCAAATCC	CAGGTGAAATCCCAAATCC	AF293417
cyp19a	GCTGCATTGGTTATGGGTTT	TGTCCTTCAGCATCAGTTTCC	AF324895
erα	GCAGGAGGGTTTGATTCTGAGA	CCATAATGATAGCCGGACGCA	AY055725
vasa	CAGCAGACAAGCCAGATCAA	CTGCCATCTCACATCCACAAG	AY773078
18S	AAACGGCTACCACATCCAAG	CACCAGATTTGCCCTCCA	AF047349

5.2.5. Aromatase activity assay

The aromatase activity assay[11] was conducted using radiometric methods, as described by Langlois and colleagues (265). An n=6 of testis samples was used for each treatment group.

5.2.6. Testis histology

Tissues were processed as described by Hogan and colleagues (266). Briefly, testis were dissected and stored in 70% ethanol for storage until histological processing. Individual testes were transversally cut into pieces of approximately 1 cm length using a razor blade, starting at the caudal end of the testis. Care was taken to maintain organization of the testis when placed in individual plastic tissue cassettes (Fisher Scientific, Ottawa, ON, Canada). At this time, individuals were assigned a random number to facilitate blind histological analyses. Dehydration and paraffin embedding occurred automatically using an automated Tissue-Tek VIP Vacuum

[11] The aromatase was conducted by Dr. Valérie Langlois and Paula Duarte-Guterman of Dr. Vance Trudeau's lab at the University of Ottawa, ON, Canada.

Infiltration Processor E150/E300 Series (Sakura Fintec Inc., Torrence, California, USA)[12]. Each dehydration step was conducted for 40 min at 40 °C and the following order of reagents was used: 70% ethanol, 80% ethanol, 90% ethanol, 95% ethanol, 100% ethanol (2 cycles), Xylene (3 cycles). Paraffin was used at 58 °C (4 cycles). Embedded tissues were subsequently transversally sectioned using a rotary microtome HM350 knife (Microm, Heidelberg, Germany) placed in a warm water bath and applied to microscopic slides and left to dry over night. Serial sections of testis (5 µm) were stained with hematoxylin and eosin. Briefly, slides were de-paraffinized and rehydrated using the following steps: Slides were maintained in xylene for 3 cycles of 3 min, 100% ethanol for 3 cycles of 1 min, 95% EtoH for 1 min and tap water for 30 s before being rinsed under tap water. Sections were then stained using hematoxylin (Richard Allan Scientific, Kalamazoo, MI, USA) for 2 min, rinsed under tap water for 30s, put in Clarifier II solution (Richard Allan Scientific) for 30s rinsed in tap water for 30 s, exposed to blueing agent for 1 min, rinsed under tap water for 1 min, put in 95% ethanol for 30 s and stained with eosin Y (Richard Allan Scientific) for 1 min 30 s. Sections are then dehydrated using 100% ethanol for 1 min (3 cycles) and xylene for 1 min (3 cycles). Tissues were then examined and images of the sections were captured using a Micro Publisher 3.3 Digital microscope camera (Qimaging Corp. Burnaby, BC). Developmental stages of spermatogenesis were assessed qualitatively according to the system described by Begtashi and colleagues (267).

5.2.7. Statistical analysis

Statistical analyses were performed using SPSS 17.0. Data were tested for normality and homoscedasticity. One-way analysis of variance or the Kruskal-Wallis tests were used to analyze the results in which only one factor was analyzed. If results were significant, appropriate

[12] I conducted the automated dehydration and paraffin embedding at Dr. Michael Wade's lab at Health Canada, Ottawa, ON, to facilitate simultaneous processing of multiple samples.

Bonferroni-adjusted post-hoc tests were used to evaluate significant differences between groups. significance for all tests was set at $p \leq 0.05$. As no full factorial design accommodating all factors is possible, no factors and their interaction effects were addressed, but data was analyzed by one-way ANOVA only.

5.3. Results

5.3.1. Waterborne concentrations of FLX

Measured mean concentrations of FLX were 0.375 ± 0.55 µg/L (S.E.) for the group exposed to a nominal concentration of 0.54 µg/L FLX, and 45 ± 5.2 µg/L (S.E.) for the group exposed to a nominal concentration of 54 µg/L FLX, validating the nominal exposure concentrations. No FLX was measured in control samples.

5.3.2. Fluoxetine inhibits basal and pheromone-stimulated milt release

Milt release measured after 7 d of the exposure (**Fig. 5.2A**) was significantly decreased in FLX-exposed fish (df=2; H=10.29; $p \leq 0.01$) when compared to control. A significant decrease in milt release was observed in fish exposed to FLX at 0.54 µg/L ($p \leq 0.05$) and 54 µg/L ($p \leq 0.01$). Milt release measured at day 14 (df=8; H=15.21; $p \leq 0.05$) changed across groups FLX inhibited both basal and pheromone-stimulated milt release. (**Fig. 5.2A**); basal release decreased significantly when compared to the control, only at the higher FLX concentration ($p \leq 0.05$), while both FLX concentrations decreased pheromone-stimulated milt release significantly when compared to the response of the respective pheromone alone ($p \leq 0.05$). These changes were not associated with changes in GSI (df=8; F=0.95; $p > 0.05$; **Fig. 5.8D**), or weight changes (df=8; F=0.61; $p > 0.05$; data not shown), as compared by one-way ANOVA. Average weight after 14 d was 17.6 ± 1.5 g (S.E.).

5.3.3. Serum T decreases and serum E_2 increases in FLX exposed fish

Testosterone concentrations (**Fig. 5.2B**) changed across treatment groups (df=8; F=11.54; $p \leq 0.01$). Bonferroni-adjusted t-tests revealed that stimulation with either pheromone significantly increased T compared to the control group ($p \leq 0.05$). FLX at 54 μg/L significantly decreased both basal and pheromone-treated T concentrations when compared to the control or pheromone alone, respectively ($p \leq 0.05$). Estradiol concentrations were assessed only for FLX-exposed fish and the control. An increase in serum E_2 (**Fig. 5.2B**) was observed for FLX (df=2; H=10.67; $p \leq 0.05$), which was statistically different from the control at both FLX concentrations (0.54 μg/L, $p \leq 0.05$; 54 μg/L, $p \leq 0.05$).

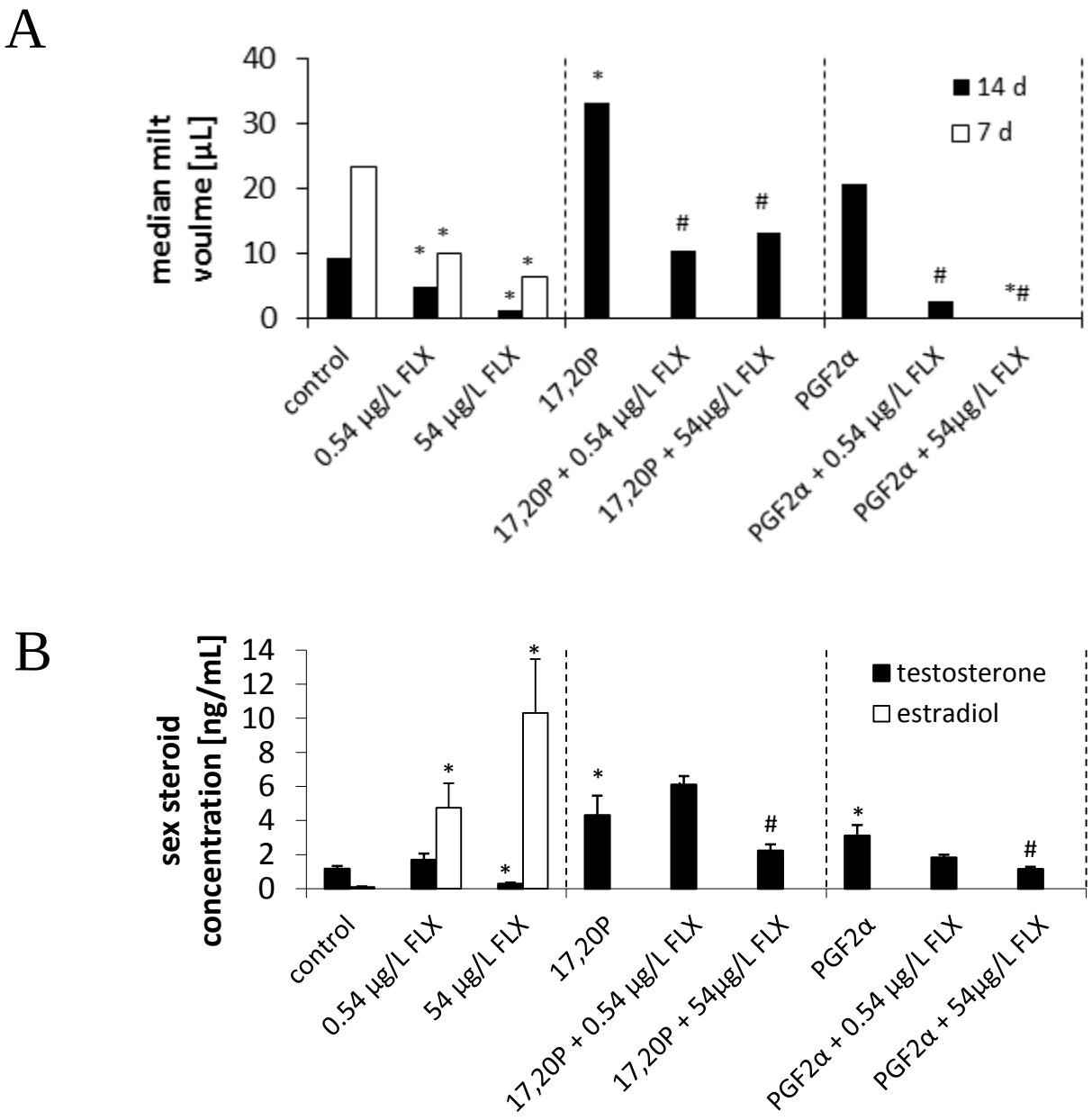
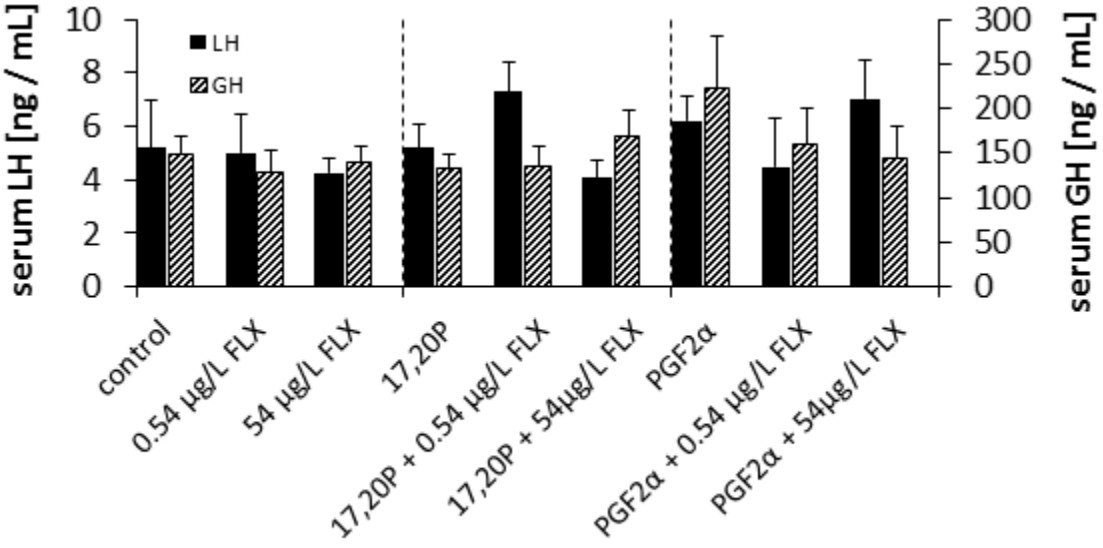


Fig. 5.2. *(A) Median milt volume at mid exposure (after 7d, white bars) and the end of the exposure following pheromone exposure (after 14d, black bars). Data were non-parametrically distributed and analyzed using a Kruskal-Wallis test which for the 14d data was followed by a Ray-Scheirer-Hare extension. Asterisk (*) indicates a significant difference between control and a particular treatment. The number sign (#) indicates a difference between a pheromone treatment and a pheromone treatment in combination with FLX. Statistical differences were set at p ≤0.05in Bonferroni-adjusted pre-determined comparisons using the Mann-Whitney U-test. (B) Serum sex steroid concentration (±.S.E.) at the end of the exposure after 14 d. An n=12 milt samples were collected for each group. Data for T was transformed to fit a normal distribution and analyzed using a two-way ANOVA. Bonferroni adjusted t-tests were used to indicate significant effects. Black bars represent serum T concentrations, white bars represent serum E_2 concentration. Different letters indicate significantly different values between groups, at p ≤0.05.*

5.3.4. FLX specifically decreases fsh-β mRNA abundance in the pituitary

Serum LH concentration (**Fig. 5.3A**) did not change significantly across groups (df=8; F=1.66; p>0.05). No changes were observed in serum GH concentrations (df=5; F=0.81; p >0.05). Similarly, no changes were observed in pituitary mRNA abundance of *lh-β* (df=5; F=2.26; p >0.05) or *gh* (df=8; F=2.79; p>0.05) (**Fig. 5.3B**). However, pituitary *fsh-β* subunit mRNA abundance (**Fig. 5.3B**) changed across treatment groups (df=8; F=7.36; p≤0.01). Bonferroni-adjusted comparisons revealed that 17,20P-treated fish had significantly increased *fsh-β* mRNA abundance when compared to the control (p ≤0.05), but this response was not apparent when either concentration of FLX and 17,20P were administered together, as indicated by the significant difference in fsh-ß mRNA abundance between 17,20P exposed fish and fish exposed to a combination of 17,20P and FLX. Additionally, a lack of difference between control and the groups exposed to 17,20P and FLX (p>0.05) was also observed.

A



B

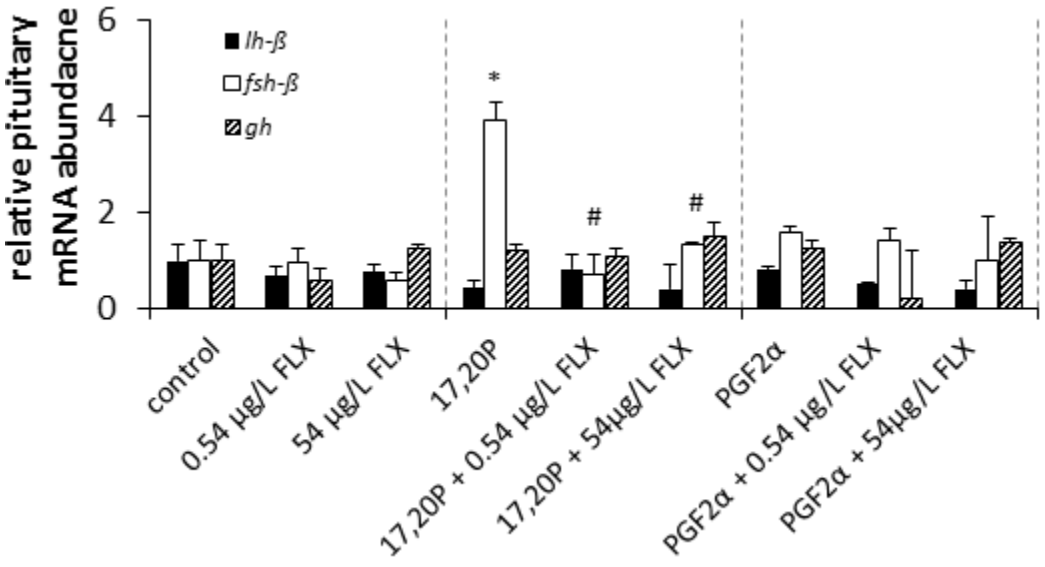


Fig. 5.3. *(A) Serum (n=12) concentrations (±S.E.) of LH and GH at the end of the 14d exposure. Black bars indicate serum concentrations of LH, hatched bars indicate serum concentrations of GH. (B) Relative pituitary mRNA abundance (±S.E.) of lh-ß (black bars), fsh-ß (white bars), and gh (hatched bars) at the end of the exposure after 14 d. An n=6 pituitary tissues was used for each group. Data was transformed to fit normal distribution and analyzed using a two-way ANOVA. Asterisk (*) indicates a significant difference between control and a particular treatment. The number sign (#) indicates a difference between a pheromone treatment and a pheromone treatment in combination with FLX. Statistical differences were set at p ≤0.05in Bonferroni-adjusted pre-determined comparisons using t-tests.*

5.3.5. Fluoxetine inhibits $PGF_{2\alpha}$ stimulation of it mRNA abundance in the telencephalon

Isotocin mRNA (**Fig. 5.4**) content in the telencephalon changed significantly across treatment groups (df=8; F=4.0; p ≤0.05). Bonferroni-adjusted comparisons by *t*-test revealed that the *it* mRNA abundance in fish exposed to both $PGF_{2\alpha}$ and 54 µg/L FLX was significantly lower when compared to fish treated with $PGF_{2\alpha}$ alone (p ≤0.05). Vasotocin mRNA abundance in the telencephalon (**Fig. 5.4**) changed significantly across treatment groups (df=5; H=6.2; p ≤0.05). When compared to control fish by pre-determined Bonferroni-adjusted Mann-Whitney U , *vt* mRNA abundance increased over control values in all animals treated with $PGF_{2\alpha,}$ and in fish exposed to 0.54 µg/L FLX in addition to 17,20P (p ≤0.05).

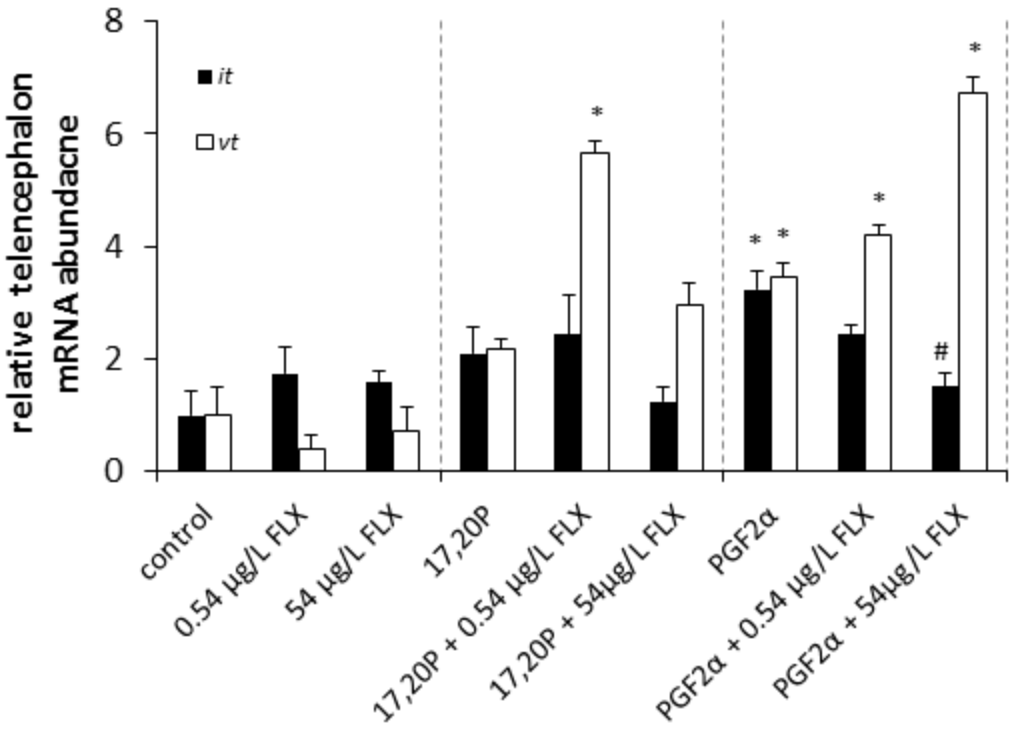


Fig. 5.4. *Relative telencephalon mRNA abundance (±S.E.) of it (black bars) and vt (white bars) at the end of the 14d exposure. An n=6 telenecephalon tissues were used for each group. Different letters indicate significantly different values at p ≤0.05. Data for it mRNA abundance was transformed to fit a normal distribution and analyzed by two-way ANOVA or non-paramteric Kruskal-Wallis test with Scheirer-Ray-Hare extension in order to investigate effects of FLX and either pheromone treatment respectively. Pre-determined Bonferroni adjusted t-tests and Mann-Whitney U tests in non-paramteric data distributions were used for comparisons. Asterisk (*) indicates significant difference (p ≤0.05) compared to control, number sign (#) indicates a significant difference to the respective pheromone group (p ≤0.05).*

5.3.6. Fluoxetine modulates testis and liver mRNA abundance

Significant changes were found for testicular mRNA abundance of *lhr* (**Fig. 5.5**) investigated in one-way ANOVA (df=8; F=8.67; p ≤0.01). Bonferroni-adjusted t-test comparisons revealed that addition of 17,20P did not alter *lhr* mRNA abundance compared with control (p >0.05), but a significant increase was found in the group exposed to 17,20P and 0.54 µg/L FLX (p ≤0.05), as compared with the control group and the group exposed to 17,20P alone. $PGF_{2\alpha}$ addition did not change *lhr* mRNA abundance, but 54 FLX µg/L, when combined with

$PGF_{2\alpha}$ increased *lhr* mRNA abundance when compared with both control ($p \leq 0.05$) and $PGF_{2\alpha}$ alone ($p \leq 0.05$) Significant changes across treatment groups were observed in the abundance of testicular *fshr* mRNA (**Fig. 5.5**) when analyzed in a one-way ANOVA ($df=8$; $F=3.83$; $p \leq 0.01$). $PGF_{2\alpha}$ treatment did not result in a significant increase in *fshr* abundance compared to control ($p > 0.05$), but the combination of 0.54 µg/L FLX with $PGF_{2\alpha}$ did result in a significant decrease, compared to $PGF_{2\alpha}$ alone. The abundance of *ghr* mRNA (**Fig. 5.5**) was not affected by any of the treatments as analyzed by one-way ANOVA ($df=8$; $F=1.43$; $p > 0.05$), respectively.

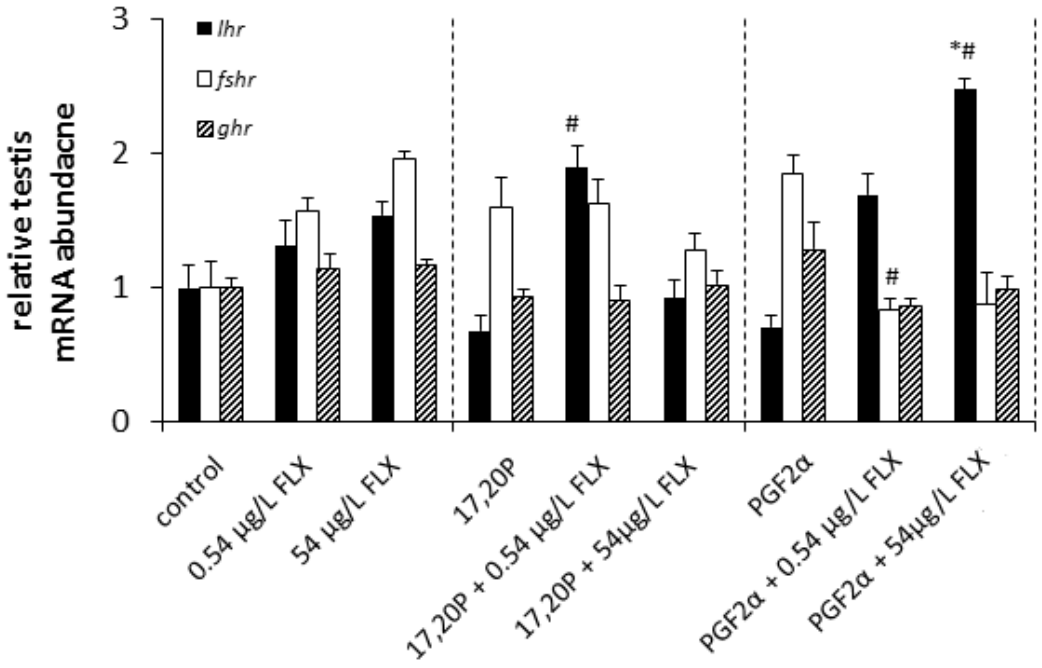
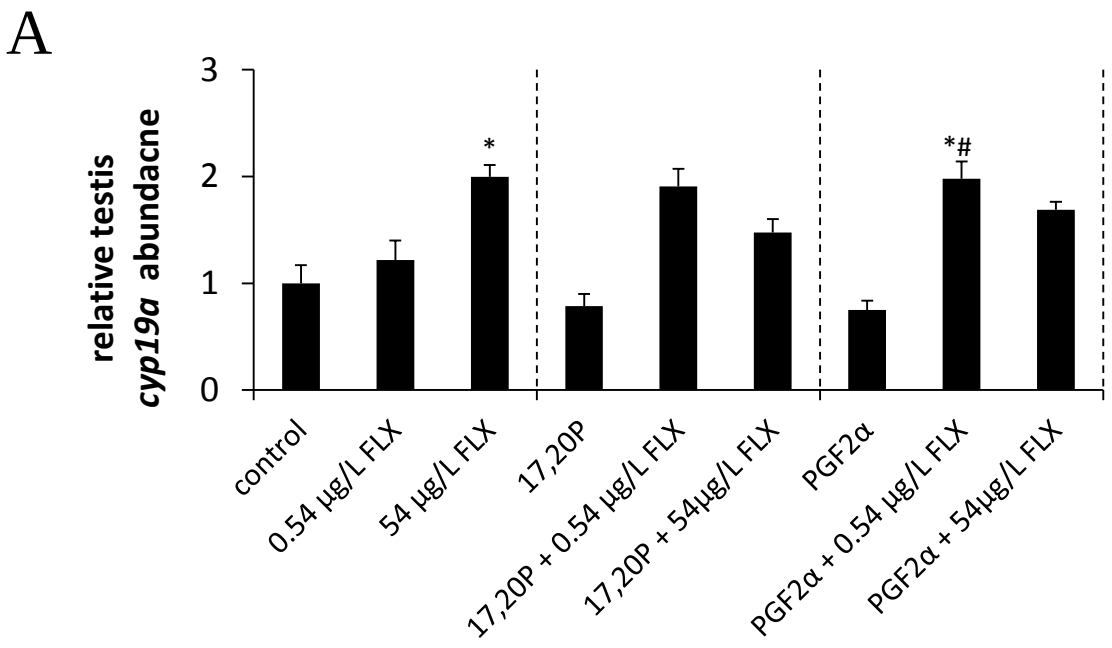


Fig. 5.5. *Relative testis mRNA abundance (±S.E.) of lhr (black bars) fshr (white bars), and ghr (hatched bars) at the end of the exposure (after 14 d). An n=6 testis tissues were used. Data was transformed to fit a normal distribution and analyzed by two-way ANOVA in order to investigate effects of FLX and either pheromone treatment respectively. Pre-determined Bonferroni-adjusted t-tests were used for comparisons. Asterisk (*) indicates significant difference ($p \leq 0.05$) compared to control, number sign (#) indicates a significant difference to the respective pheromone group ($p \leq 0.05$).*

Testicular *cyp19a* mRNA abundance (**Fig. 5.6A**) changed across treatment groups, when analyzed by one-way ANOVA (df=8; F=2.84; $p \leq 0.01$). Bonferroni-adjusted t-test comparison revealed that the mRNA abundance of *cyp19a* was significantly increased in the group treated with 54 µg/L FLX, when compared with control ($p \leq 0.05$). The cyp19a mRNA abundance also increased significantly in the group exposed to 17,20 and FLX compared to the group exposed to 17,20P alone. However, no significant changes in Cyp19a activity (**Fig. 5.6B**) were found (df=2; F=0.45; $p > 0.05$). Liver *erα* mRNA abundance (**Fig. 5.6C**) increased with the addition of FLX (df=2; F=7.16; $p \leq 0.01$), and a significant difference in liver *erα* was found between the 54 µg/L FLX group and control ($p \leq 0.05$; **Fig. 5.6C**).



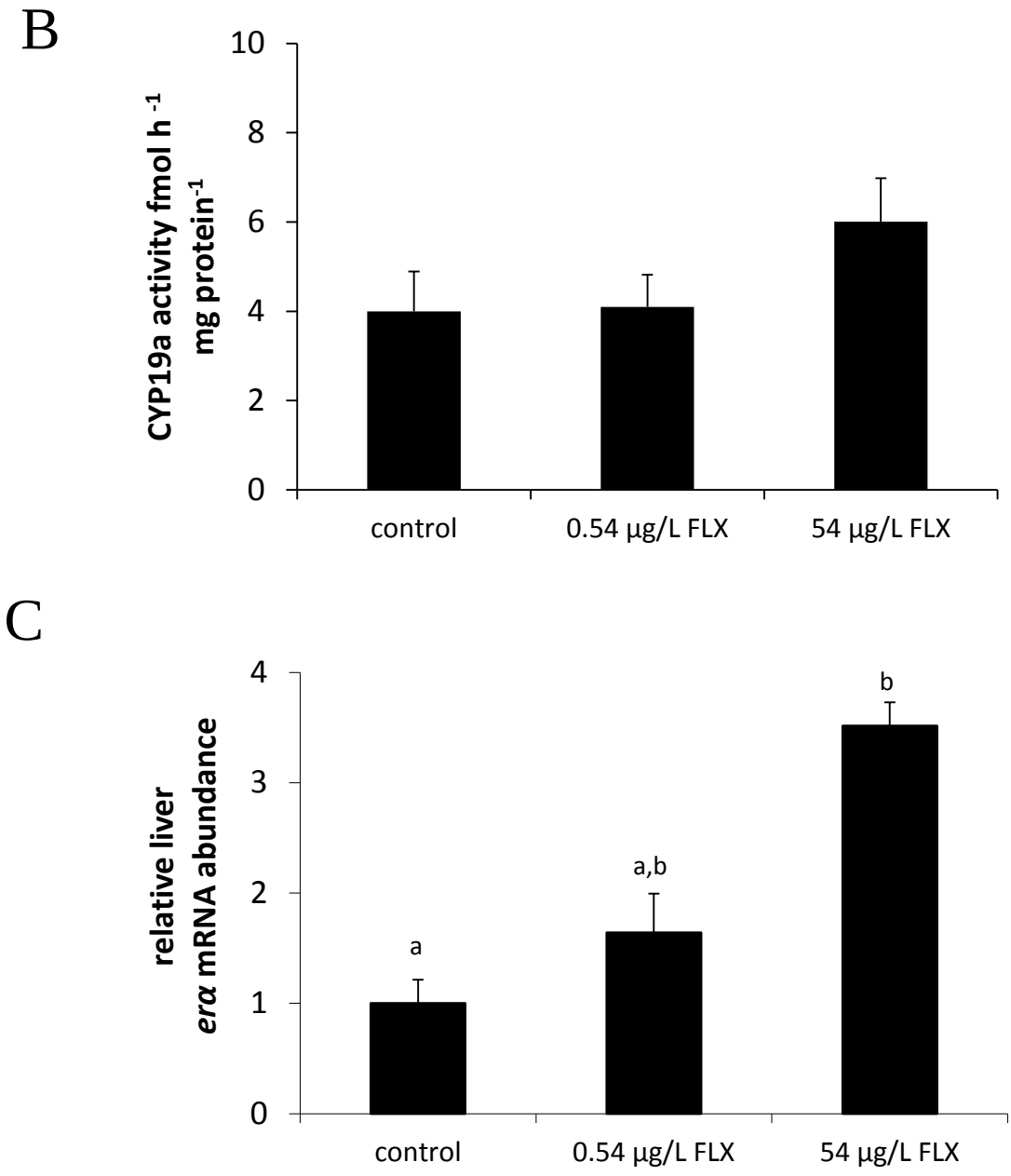
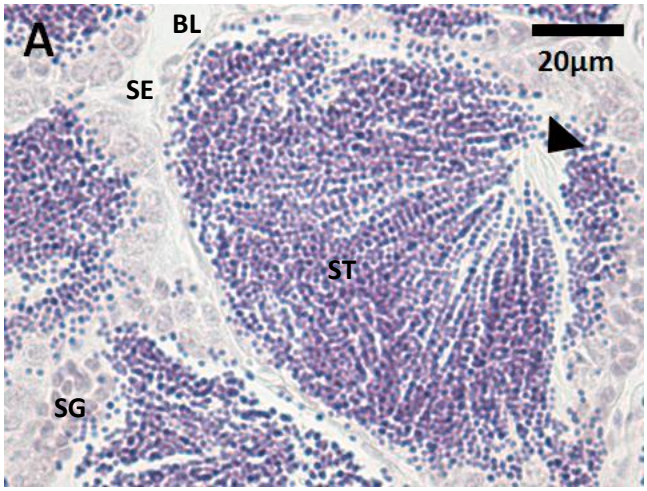


Fig. 5.6. *Investigation of estrogenic indices in male goldfish at the end of the 14d exposure. Relative testis mRNA abundance (±S.E.) of cyp19a (A) fit a normal distribution and was analyzed by two-way ANOVA in order to investigate effects of FLX and either pheromone treatment respectively. Pre-determined Bonferroni adjusted t-tests were used for comparisons. Asterisk (*) indicates significant difference ($p \leq 0.05$) compared to control, number sign (#) indicates a significant difference to the respective pheromone group ($p \leq 0.05$). CYP19a activity ± S.E. (B) was normally distributed and analyzed with a one-way ANOVA and Bonferroni adjusted post-hoc comparisons. Relative liver mRNA abundance ±S.E. (C) of erα at the end of the 14 d exposure was not normally distributed and analyzed with a Kruskal-Wallis test and Bonferroni-adjusted Mann-Whitney U comparisons. An n=6 of tissue samples were used in all cases.*

5.3.7. Fluoxetine does not alter testis histology

All developmental stages of spermatogenesis, including spermatozoa were present in all histology slides examined (**Fig. 5.7A-C**). This indicates that a 14 d exposure to FLX does not have any observable effects on testis morphology or size, as the GSI ($df=2$; $F=0.95$; $p>0.05$) did not change significantly over treatment period (**Fig. 5.7D**) While spermatogenesis in fish is a process which involves a longer time period than the 14 d used in this exposure, the 14 d time window is sufficient to investigate any direct cytotoxic effects of FLX, which have recently been described for human sperm *in vitro* (268). The mRNA abundance of *vasa* (**Fig. 5.7E**) did not change across groups when analyzed by one-way ANOVA ($df=8$; $F=1.2$; $p>0.05$).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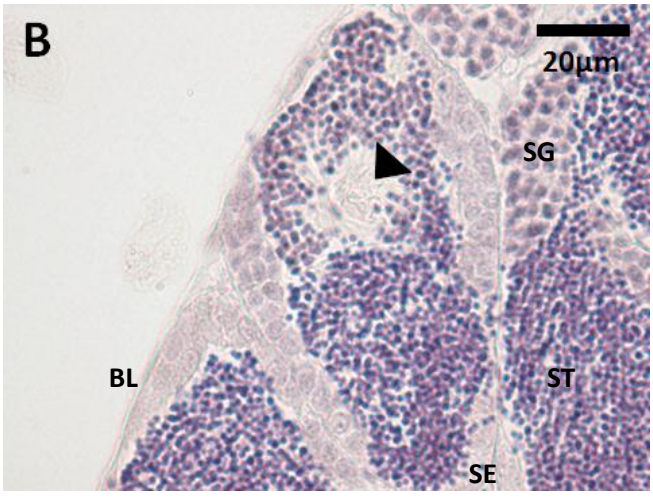
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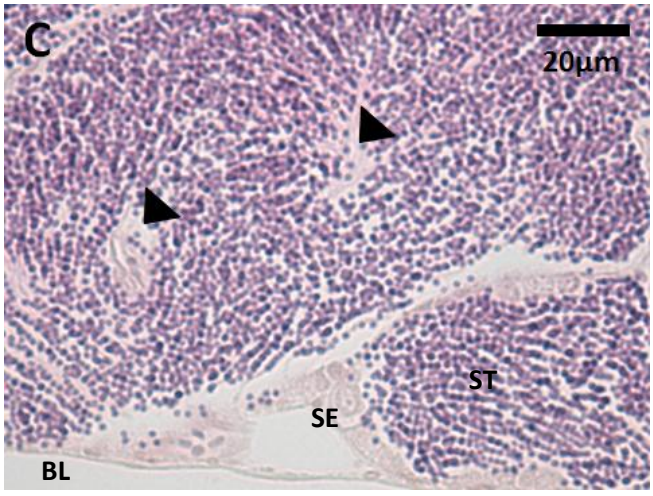
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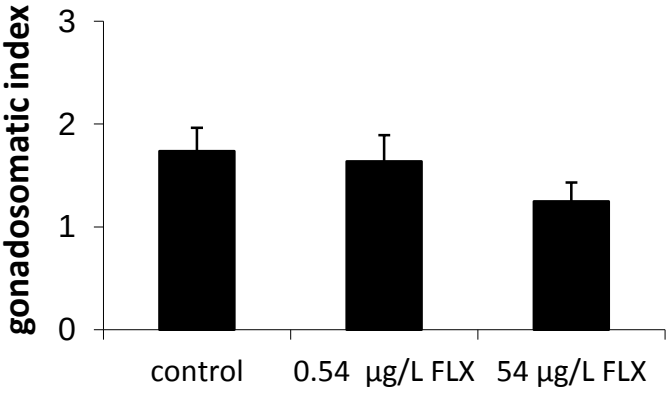


E

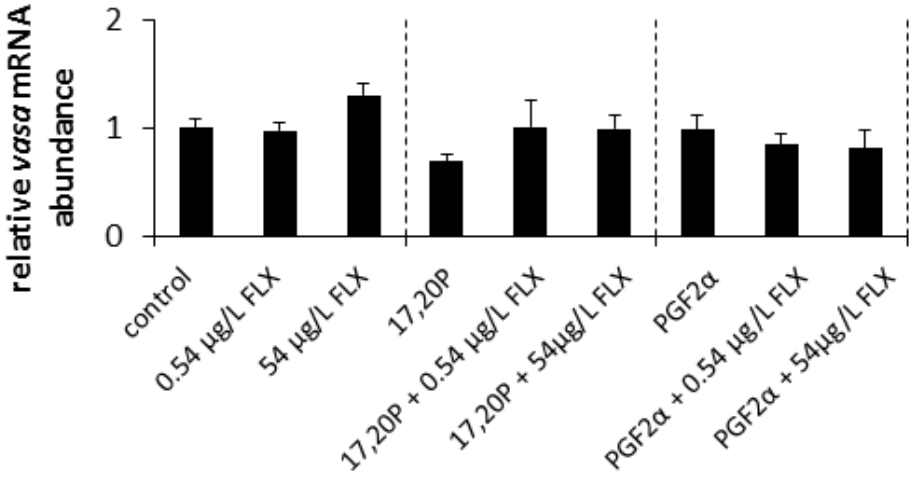


Fig. 5.7. *Investigation of testicular development indices in male goldfish at the end of the 14 d exposure: Representative, eosin- and haematoxylin- stained 5µm slices of testis of control fish (A), fish exposed to 0.54 µg/L (B), and 54 µg/L (C). A total n=5 fish testis per group were used for histological examination. The following abbreviations are used: BL= basal lamina; SE= Sertoli cells; SG= spermatogonia; ST= spermatid; black arrows indicate mature spermatozoa. Gonadosomatic index (±S.E.) at the end of the 14 d exposure (D) was normally distributed and analyzed by one-way ANOVA. Relative testicular mRNA abundance of vasa ±S.E (E) was normally distributed and analyzed by separate two-way ANOVAs to investigate effects of FLX and respective pheromones. An n=6 testis samples was used per group.*

5.4. Discussion

The results discussed throughout this chapter provide evidence for a specific disruption of the reproductive axis in male fish by waterborne FLX exposures. The nature of the response is dependent on the FLX concentration (0.54 µg/L FLX and 54 µg/L FLX) used in the waterborne exposure. A detailed discussion of the results is provided in the following paragraphs.

5.4.1. Potential for multiple sites of action of FLX on the reproductive axis in male goldfish

Interestingly, serotonergic innervation of mitral cells, which are involved in pheromone recognition in goldfish (269), is reported to exist in the olfactory bulb of the sea lamprey (*Petromyzon marinus*) (50). This finding provides the anatomical basis for a potential serotonergic modulation of pheromone signalling at the level of the olfactory bulb. As described in Chapter 1, 5-HT is also involved in stimulating the gonadotrophs to release LH (31,33), by acting in the hypothalamus and pituitary. The results presented in Chapter 2 show a ubiquitous *slc6a4a* mRNA abundance in goldfish tissues, including the testis. This provides molecular evidence for the testis as a target of peripheral circulating 5-HT in fish. In rainbow trout, peripheral 5-HT concentrations are reported to be modulated by SSRIs *in vivo* (134). This has implications for the potential mode of action of FLX, as it could modulate the reproductive axis by actions both in both brain and the testis.

5.4.2. Fluoxetine decreases milt and serum T concentration

Fluoxetine exposure in this study effectively decreased milt release and circulating T concentrations, without changes in body weight or GSI, at a concentration of 54 µg/L. Pheromone-stimulated milt release was decreased at either FLX concentration tested. Disrupted ejaculatory responses in humans taking SSRIs are a common side effect, and in some cases are

correlated with decreased T concentrations (270). Decreased circulating T and sperm counts were observed in rats injected for 60 d with 200 mg FLX/ d kg bw (221). Testosterone plays an important role in spermatogenesis and spermiation in fish, including goldfish (259,271); therefore, inhibited sperm release may be a secondary effect following changes in circulating T concentrations.

5.4.3. Fluoxetine inhibits 17,20P induction of fsh-β mRNA abundance

Given the peak mRNA abundance of *slc6a4a* in the hypothalamus of sexually mature fish and the known roles of 5-HT in controlling reproductive hormone release, I investigated potential modulation of the neuroendocrine function in the brain of fish exposed to waterborne FLX. No changes in circulating concentrations of LH and GH were detected at the FLX concentrations tested. This supports previous studies in goldfish using repeated FLX injections of 5 μg/g bw over a 14 d period – these studies also reported no changes in either plasma LH (132) or GH (272). However, increased LH 2 h after 17,20P exposure was correlated with subsequent milt increases after 12 h (260). Single blood sampling after 14 d of exposure did not capture peak LH concentrations, but the expected significant stimulatory effect of 17,20P on milt production was clear, confirming numerous other reports (260,263). However, in spite of an observed 2-fold increase in milt release in fish exposed to $PGF_{2\alpha}$, only the 3-fold increase in 17,20P-stimulated milt release represented a significant increase over control values. The findings indicate a specific inhibitory effect of FLX on *fsh-β*, in particular when fish were treated with the pheromone 17,20P. Decreased circulating FSH was reported in rats treated daily for 60 d with 200 mg FLX/kg (221). In male fish, FSH is crucial for steroidogenesis and spermiation (259). Recombinant goldfish FSH protein induces milt production in goldfish *in vivo* (273). Therefore, the observed effects of FLX on milt release and steroidogenesis in 17,20P-treated fish

may be at least partially attributed to inhibition of the *fsh-β* mRNA abundance. In teleost fish, gonadotrophs are innervated by 5-HT, which acts to stimulate gonadotropin release, likely via a 5-HTR_2-like receptor (33). In mammals, it is known that FLX is an antagonist to 5-HTR_{2C} (114), and it is possible that FLX may act to block 5-HT stimulated *fsh-β* mRNA abundance through a similar mechanism.

5.4.4. Fluoxetine differentially alters $PGF_{2\alpha}$ induced it and vt mRNA abundance in the telencephalon

Isotocin is a neurohypophyseal nonapeptide and the teleost homolog of mammalian oxytocin (204). In male fish, IT stimulates reproductive behaviours (148), testicular steroidogenesis (254) and milt release (274), making it a target in my study. Goldfish possess a dense isotocinergic neuronal network originating in the magnocellular preoptic area (224), an area central to the control of sexual behaviours and sperm release in goldfish (275). Interestingly, microarray analysis showed that the *it* transcript is down-regulated 80% in the telencephalon of FLX-injected female goldfish (132). In the current study, a significant increase in *it* mRNA abundance in $PGF_{2\alpha}$-stimulated animals was observed, which was dose-dependently reduced by FLX. My study links the primarily behavioural reproductive hormone IT to the reproductive releaser pheromone $PGF_{2\alpha}$, which is known to stimulate the behavioural components of goldfish reproduction (261). Studies investigating side effects of SSRIs in mammals strongly implicate oxytocin in FLX-induced disruption of ejaculation (90), likely through the process of desensitizing 5-HTR_{1A} mediated oxytocin release. Therefore, FLX may disrupt reproductive behaviours, stimulated by the releaser pheromone $PGF_{2\alpha}$ in goldfish, through a similar desensitization. Behavioural cues can stimulate milt release independently of the 17,20P-LH-dependent pathway in goldfish (260). Isotocin may be a component of this yet uncharacterized

pathway. IT may act within the CNS, to modulate socio-sexual behaviours, or peripherally, as depressed IT release may result in reduced stimulation of steroidogenesis and spermiation in the testis. Since hypophysectomy does not prevent milt increases through spawning stimuli (260), the first alternative should be investigated further by correlating behaviour and IT signalling.

Vasotocin is a related neurohypophyseal peptide and homolog of the mammalian hormone vasopressin (204). In the roughskin newt, *Taricha granulosa*, VT mediates reproductive behaviours (e.g., clasp motor reflexes) in response to stimulatory sex pheromone (276). A role for VT in pheromone signalling in fish is supported in this study, as $PGF_{2\alpha}$ increased *vt* mRNA abundance in the telencephalon. In the male bluehead wrasse, *vt* mRNA abundance in the telencephalon is positively correlated with territorial aggression (277). Both telencephalic *vt* mRNA and aggressive behaviours are decreased when these animals are exposed to FLX (277). Interestingly, I identified inverse trends for mRNA abundance of *it* (dose-dependent decrease) and *vt* (dose-dependent increase) in fish treated with FLX before receiving $PGF_{2\alpha}$. In goldfish, IT and VT have antagonistic effects on approach behaviours, more specifically, IT has stimulatory effects and VT has inhibitory effects (250). Therefore, FLX exposure may dose-dependently and differentially alter the nonapeptide response to the releaser pheromone $PGF_{2\alpha}$, which is believed to stimulate the reproductive axis in response to behavioural cues. Further studies exploring the roles of these nonapeptides in $PGF_{2\alpha}$-induced behaviours are warranted. Nevertheless, it appears plausible that FLX disrupts the integration of pheromone cues to control socio-sexual behaviours. This may have the secondary effect of reducing LH-independent milt release.

5.4.5 Potential peripheral effects of FLX

I investigated effects in the testes resulting from exposure to waterborne FLX, especially since I localized *slc6a4a* mRNA (Chapter 2) to the goldfish testis. It was recently reported that the mRNA abundance of *lhr*, *fshr* and *cyp19a* decreased dose-dependently in the ovaries of zebrafish exposed to waterborne FLX at concentrations between 0.32 μg/L and 32 μg/L (256). I observed effects of FLX on goldfish *lhr*, *fshr*, but not *ghr*. These effects indicate the possible disruption of testis function by FLX, but the changes may also be linked to responses to circulating hormone concentrations. Time course studies as well as *in vitro* incubations of testis are necessary to fully separate these possibilities.

In female fish, both LHr and FSHr are implicated in downstream regulation of Cyp19a, the key enzyme responsible for conversion of T into E_2 (278). I observed a significant stimulating effect of FLX on basal testicular *cyp19a* mRNA abundance and therefore investigated estrogenic parameters in fish not exposed to either pheromone. I found a decrease in circulating T in fish exposed to 54 μg/L FLX, and a concentration-dependent increase in circulating E_2 in male fish treated with FLX. This increase is biologically meaningful because liver mRNA abundance of *erα* also increased. It is well known that hepatic *erα* is under positive estrogenic regulation and has been proposed to be a good biomarker for E_2 exposure (161). To assess whether the increase in *cyp19a* mRNA abundance is functionally linked to the increase in E_2, I investigated gonadal Cyp19a activity in the testis and did not find a significant increase. Therefore, the increase in E_2 concentrations may be mediated by other mechanisms, for example, FLX inhibition of the liver cytochromes involved in steroid degradation. Increased E_2, at concentrations similar to the ones found in this study, are linked with decreased testicular function and sperm release in male goldfish exposed to estrogenic compounds (279). Therefore,

observed declines in sperm release in this study may be attributed to increased E_2 concentrations, and future studies should investigate the potential mechanisms responsible for this correlation.

5.4.6 Fluoxetine does not disrupt sperm cell development

Both T and FSH are involved in the early phases of spermatogenesis in fish (259). The observed decrease in both hormones may indicate a FLX-induced disruption at an early stage of gonadal development. Studies of FLX effects in rats, which showed a reduction in both FSH and T, revealed spermatogenic disruption at multiple developmental stages (221). As *vasa* is a well-defined spermatogenesis marker in goldfish (280), I investigated its mRNA abundance in testis, but found no changes. This finding was supported by a histological analysis of the gonads, where all spermatogenic stages, including spermatozoa, could be identified across all treatment groups. This indicates that the 14 d FLX exposure inhibited milt release rather than spermatogenesis.

5.5 Conclusions

The results discussed throughout this chapter indicate that environmental concentrations of FLX can disrupt critical components of male goldfish reproductive processes. Future research should address potential effects of SSRIs on sperm release in the testis of goldfish, especially with regard to $PGF_{2\alpha}$-induced socio-sexual stimulation of sperm release. With increasing use of SSRIs in several sectors of the population, the impacts of human sewage effluents containing these and other pharmaceuticals should be more closely monitored, and remediation efforts directed at removing them.

Chapter 6: The effects of repeated injections of pharmacological fluoxetine and waterborne fluoxetine on neuroendocrine control of feeding in female goldfish

Partially based on: Mennigen et al. 2009. Fluoxetine affects weight gain and expression of feeding peptides in the female goldfish brain. Regul. Pept. 155: 99-104.
Mennigen et al. 2010. Waterborne fluoxetine disrupts feeding and energy metabolism in the goldfish Carassius auratus. Aquat. Toxicol. 100:128-37.

6.1. Introduction

Serotonergic control of food intake and growth appears to be conserved amongst vertebrates (281). Goldfish provide an excellent model to study neuroendocrine control of food intake and growth, as several neuropeptides with functions in food intake are well characterized in this species (282). Moreover, the neuroendocrine control of GH secretion (283) and the stimulatory role of GH on somatic growth and food-intake (284) are well studied in goldfish. Food intake can readily be measured in goldfish (36) and goldfish have seasonally variable growth rates, with post-spawning, sexually regressed animals exhibiting the highest growth rates (2). The role of the neuroendocrine serotonergic system in the control of growth and food intake in fish is less well studied (36,285). It is known that 5-HT inhibits pituitary GH release in goldfish *in vitro* (33). Several studies report inhibitory effects of 5-HT on feeding in fish, but only one study addressed the underlying mechanisms on food intake and growth (36). It was demonstrated that increased CRF plays an important role in the mediation of the anorectic effects of 5-HT on feeding in goldfish (36). However, CRF antagonists co-administered with 5-HT resulted in only partial inhibition of the anorectic effect (36). This implies that other feeding-related neuropeptides may be involved in this response. In mammals, for example, NPY, melanocortin, orexin, CRF and CART-1 neurons are known to be modulated by 5-HT (286-288). While the orexic or anorectic actions of some of these peptides are evolutionarily conserved, studies on their regulation by 5-HT or other classical neurotransmitters in non-mammalian

vertebrates are generally lacking. This study tests the hypothesis that FLX acts as an anorexigenic agent in goldfish, and that the effects of FLX in goldfish are mediated by multiple 5-HT-controlled brain neuropeptide systems that are involved in controlling feeding behaviour. This study was designed in a similar approach as the previously described studies that investigate the effects of FLX on reproductive pathways (Chapters 3, 5 and Appendix 1). An initial injection protocol of pharmacological FLX concentrations (5 µg/g; similar to Chapter 3) was used to identify the effect of the drug on feeding and growth parameters and to investigate potential underlying neuroendocrine mechanisms. A secondary waterborne exposure (0.54 µg/L and 54 µg/L; similar to Chapter 5 and Appendix I) was conducted to investigate whether effects and mechanisms observed in the initial injection study occur at environmentally realistic FLX concentrations. Food intake and weight gain were assessed as physiological endpoints for feeding and growth. Both the mRNA abundance of the potentially 5-HT-regulated feeding neuropeptides and the key components of the somatotropic axis were assessed to study potential mechanisms. In particular, the genes of the orexigenic neuropeptide *npy* and the anorexigenic peptides *cart-1*, *crf* and tachykinin (*tac*) were analyzed for changes in mRNA abundance as their functions in goldfish food-intake are previously well-described (289-291). The *cart-1* isoform was specifically chosen based on evidence supporting involvement of the *cart-1* but not the *cart-2* isoform in goldfish food-intake (292). For *tac*, indirect evidence exists for an anorectic role, as *tac* mRNA abundance increases postprandially in the hypothalamus, implicating it as a potential anorexigenic satiety factor (293). Additionally, hypothalamic *tac* was identified as being affected by FLX-injection in female goldfish (132). With respect to the somatotropic axis, serum concentrations of GH were assessed, as these are known to be involved in feeding and weight gain in goldfish (284). As GH release in teleost fish is predominantly under tonic

inhibitory control (294), mRNA abundance of the most potent of the three identified somatostatins *in vitro*, somatostatin-2 (*sst-2*) (295), was measured. Further physiological evidence for a role of SST-2 in GH regulation in the context of food-intake and nutrition is provided by studies showing that hypothalamic *sst-2* in goldfish decreases by 60% postprandially (296).

In the second exposure, the FLX effects on feeding rate and growth were validated in waterborne exposures, and target mRNA of *npy*, *crf* and *cart-1* were analyzed in the hypothalamus. Additionally, in order to investigate the hypothesis that the observed changes in the reproductive system (described in Chapters 3, 5 and Appendix 1) are a consequence of potential anorexigenic effects of FLX, I investigated a potential effect on the mRNA abundance of the leptin gene (*lep*) and its receptor (*lepr*) in the second exposure. This target was chosen as it has been reported to integrate nutritional status and the activation of the reproductive axis in the neuroendocrine brain of mammals, in a mechanism that ensures sufficient energy supplies are available for the energetically costly activation of the reproductive axis (297).

6.2. Material and methods

6.2.1. Experiment I: FLX injections

6.2.1.1. Experimental design and animals

Growth hormone concentrations and growth rate vary seasonally and are highest in post-spawning, sexually regressed goldfish of both sexes, although growth rates are generally higher for females at this stage in June (2). Therefore experiments were conducted in June using female goldfish purchased from a commercial supplier (Aleong's International Inc., Mississauga, ON, Canada) and maintained at 18 °C under a simulated natural photoperiod (see Materials and

methods of Chapter 3 for details). Goldfish were anesthetised using MS-222 for all handling procedures; care was taken to standardize all handling procedures. After a 6 d period to obtain baseline food intake, fish were injected i.p. every third day with 5 µg FLX/g bw at an injection volume of 1 µL/g for a total of five injections (**Fig. 6.1A**). Fluoxetine (Sigma-Aldrich, Oakville, ON) was dissolved in physiological saline (0.6% NaCl). The rationale for the concentration and timing of FLX injections is discussed elsewhere (Chapter 3; ref. 132). Initial individual fish body weight ranged from 13 g to 34 g and care was taken to distribute fish of different weights evenly across the four control and 4 treatment tanks. One day after the final injection goldfish were anesthetised using MS-222 and euthanized by spinal transection. Brain tissues were dissected and frozen on dry ice immediately, just after food intake measurements, at 11:30 am. All experiments were approved by the University of Ottawa Animal Care Protocol Review Committee and follow guidelines established by the Canadian Council on Animal Care for the use of animals in teaching and research.

6.2.1.2. Measurement of food intake and weight

Goldfish were distributed into 4 control and 4 treatment tanks with n=4–5 fish per tank. The total fish weight per tank was 106.5 g (S.E. ± 2.1) with an average fish weight of 21 g for both control and treatment groups in each tank. Of the control tanks, one was used as a handling control, and 3 received an equivalent i.p. injection volume of 0.6% NaCl. Fish were fed an excess amount (4% of body weight) of Martin Proficient Classic Floating Trout Grower 3 mm pellets (Martin Mills Inc., Travistock, ON, Canada) at 11:00 am every day for the duration of the experiment (6 d baseline + 13 d injection period = 19 d). Food was retrieved from the tank 1 h after feeding and immediately dried in an oven at 70 °C. The difference in food intake for each tank (FI) was calculated as described by de Pedro and colleagues (36), using the formula

$FI = W_i - (W_r \times F)$, where W_i is the initial dry food weight, W_r is the remaining dry food weight and F is a correction factor determined by leaving pellets in an empty tank for 1 h before retrieving and drying the food. The factor F represents the quotient of initial food weight and retrieved dried food weight and was determined to be 1.14 ± 0.12 (n=4). Average baseline food intake values established from the first 6 d did not differ significantly among tanks. Food-intake data were normally distributed and analyzed using a two-way mixed ANOVA with repeated measures for one factor (days) using Systat v10 (Systat Software Inc., San Jose, CA). A Huynh-Feldt correction was applied to account for violations of sphericity. Differences were considered significant when $p \leq 0.05$. Fish weight was determined only at the beginning (day 1) and the end of the experiment (day 13 of the injection phase) to avoid additional handling stress. The weight difference was calculated as an average of the entire tank of 4–5 fish, for both control (n=4) and treatment (n=4) tanks. These differences were normally distributed and analyzed by *t*-test using Systat v10.

6.2.1.3. Radioimmunoassay for GH

Blood (100-300 µL) was collected from individual anesthetized goldfish by caudal puncture at the end of the experiment. The blood was allowed to clot and then centrifuged for 10 min at 3000 *g* to collect serum which was stored at −20 °C until GH analysis by RIA as previously described (Chapter 5). A total of 18 serum samples for each control and FLX-treated group was assayed. Data were normally distributed and analyzed by an independent samples *t*-test using Systat v10.

6.2.1.4. Total RNA extraction, cDNA synthesis and real-time RT-PCR

Differences in the relative mRNA abundance of neuropeptides between control and FLX-injected fish were assessed using real-time RT-PCR. Total RNA was extracted from tissues and cDNA synthesized as described previously (Chapter 2). A total of n=7 was used for control and FLX samples in both hypothalamus and telencephalon. Each brain tissue sample contained 2–3 pooled hypothalami or telencephali. PCR reactions and data analysis were conducted as previously described (Chapter 2). Significant changes in mRNA abundance were evaluated either with a t-test or a Mann-Whitney U test if the data were not normally distributed, using Systat v10). A p-value ≤ 0.05 was considered to constitute a significant difference. The following table (**Tab. 6.1)** contains the primer sequences used for real-time RT-PCR assays in experiment 1.

Tab. 6.1. *Primer sequences used for real-time RT-PCR assays in experiments 1.*

Gene	Primer sequence 5'3' (FW)	Primer sequence 3'5' (RV)	Genbank accession
npy	CTGGGGATGGGACTCTGTTT	TTCGTCTGCTTGGGAACTCT	M87297
crf	TTTCCACCACCGTATGAATGT	TTTGCCTAATGGCTTTGCTC	AF098629
cart-I	CCATGGAGAGCTCCAAACTC	TCTTGACCCTTTCCTGATGG	AF288810
tac	GGTTCAGGATGAGTGCCTTC	CCTCTTGGCTCCTTTTACCCC	U61272
sst-II	GAACCCGAATCACAGCTACAA	TCTTGACCCTTTCCTGATGG	U60262

6.2.2. Experiment 2: Waterborne FLX exposure

6.2.2.1. Experimental design and animals

A second exposure experiment[13] using a waterborne protocol was also undertaken with both male and female goldfish purchased as above in August 2008 and allowed to acclimate to the University of Ottawa Aquatic Care Facility for approximately one month. The experiment

[13] The actual exposure was conducted by Jeanette Sassine, MSc from Dr. Moon's lab, University of Ottawa, ON, Canada and was described in her MSc thesis (see Sassine, 2010) as well as in a joint publication (43).

was conducted in September; see Chapter 5 and Appendix I for details of FLX administration. Care was taken to distribute fish weights evenly across tanks with each tank receiving 12 fish; average fish weights (± S.E.) at the start of the exposure were 22.5 g ± 0.5 g (control), 18 g ± 0.2 g (0.54 µg/L FLX), 20.4 g ± 0.3 g (pair-fed I), 20.3 g ± 0.4 g (54 µg/L FLX), 21 ±0.4 g (pair-fed II), and 21.8 g ± 0.5 g (fasted) and were not statistically different (one-way ANOVA; df=5; $F=1.19$; $p > 0.05$). Fish were fed commercial trout feed (see above). Goldfish were anesthetised using MS-222 (see above). To track fish throughout the experiment, each fish was uniquely marked by differential clipping (1-3 total cuts) of the dorsal, pelvic and caudal fins. Tank 1, the control tank, was fed *ad libitum* daily as above. Food intake was calculated by feeding the tank ten pellets at a time; once the fish ate all ten pellets, they were given ten more with this continuing for 30 min or until the fish no longer ate the pellets. Two additional tanks, the 0.54 µg/L FLX and 54 µg/L tanks, were also fed *ad libitum* and intake was determined as for Tank 1. A 2 mg/mL stock concentration of FLX (Sigma–Aldrich, Oakville, ON, Canada) in methanol was prepared according to Gaworecki and Klaine (130). The volume of total methanol in each tank (70 L) was 19 µL in the 0.54 µg/L FLX and 1.89 mL in the 54 µg/L FLX tank; both final methanol concentrations are well below those reported to affect feeding or to be toxic (298).

Two pair-fed groups in addition to the FLX groups were fed the same amount of food as the respective FLX exposed groups. These pair-fed groups were not exposed to the drug. A sixth group, the fasted group, received no food or FLX for the duration of the 28-day experiment. The overall experimental design is summarized in **Fig. 6.1B**. Feeding rates were measured for each individual tank while weight gain was calculated by subtracting the initial weight from the final weight for each individual fish used in the experiment.

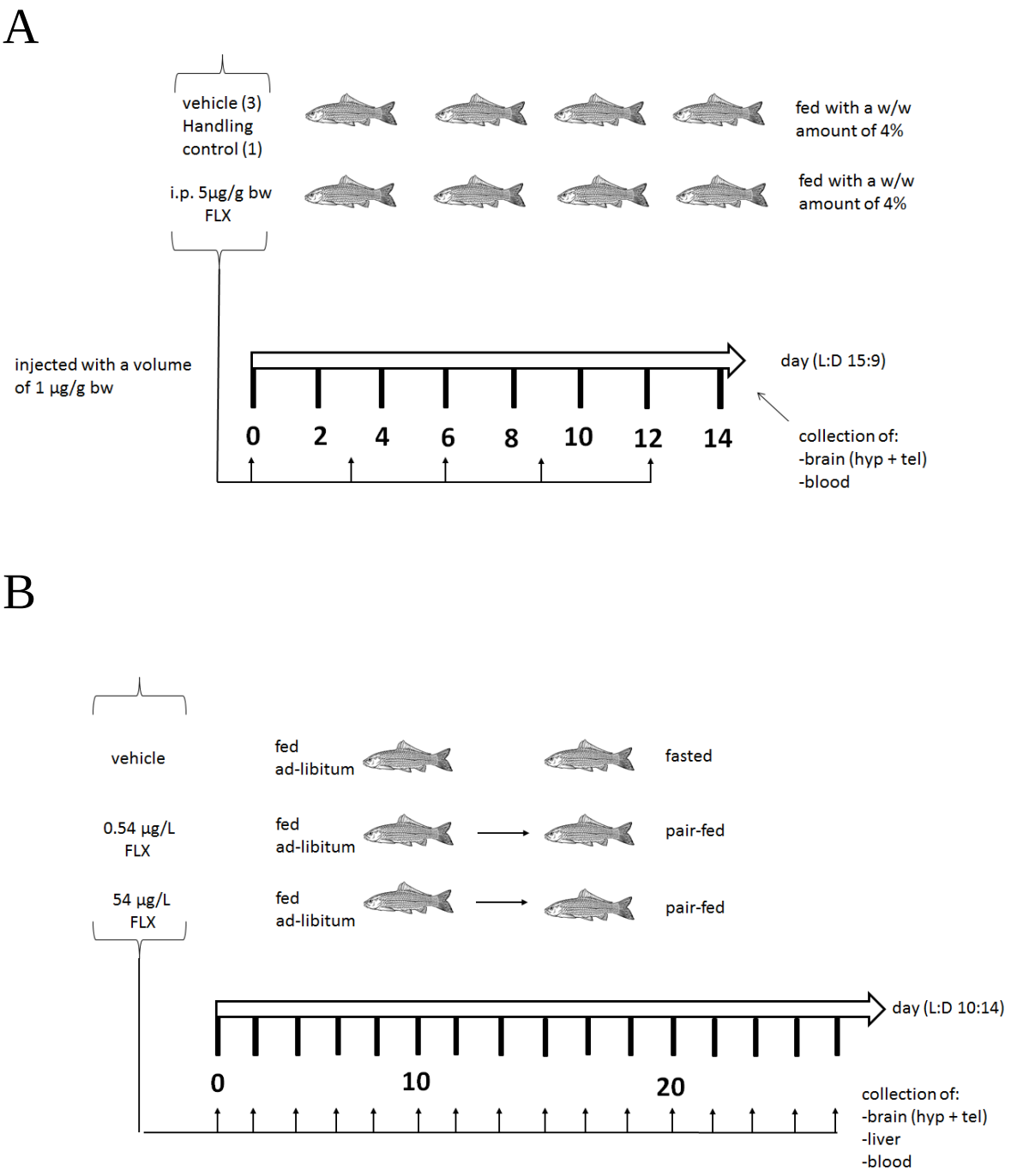


Fig. 6.1. *Schematic diagram of the experimental designs for the injection experiment using repeated injections of 5µg/g FLX (A) and the waterborne exposure using FLX concentrations of 0.54µg/L FLX and 54µg/L FLX (B).*

6.2.2.2. Radioimmunoassay for GH

No changes in serum GH were detected in the injection experiment described in this chapter or in the waterborne exposure investigating the reproductive axis (Chapter 5 and Appendix 1), therefore GH analysis was not conducted for the waterborne exposure study described in this chapter.

6.2.3.3. *Total RNA extraction, cDNA synthesis and real-time RT-PCR*

Total RNA was extracted from tissues and cDNA synthesized as described above in section 6.2.1.4. A total of n=7 was used for control and FLX samples for both hypothalamus and telencephalon. Each brain tissue sample contained 2–3 pooled hypothalami or telencephali. PCR reactions and data analysis were conducted as noted above. Data were either normally distributed or transformed to fit a normal distribution prior to analysis in a one-way ANOVA, followed by Bonferroni adjusted post-hoc tests. A p-value ≤ 0.05 was considered to constitute a significant difference. The following table (**Tab. 6.2)** contains the primer sequences used for real-time RT-PCR assays in experiment 2. In addition to primers targeting reverse transcribed mRNA of genes involved in feeding behaviour, the *lepr* and *lep* were also investigated. The *lepr* mRNA abundance was measured in hypothalamic tissue, as it represents the site at which the leptin hormone acts to stimulate the reproductive axis in mammals (297). In contrast to mammals, where leptin is expressed and released primarily from adipose tissues as a satiety signal (299), the liver is the primary site of mRNA and protein in fish (300) and was hence chosen for analysis of the mRNA abundance of *lep* in goldfish in this study.

Tab. 6.2. *Primer sequences used for real-time RT-PCR assays in experiment 2.*

Gene	Primer sequence 5'3' (FW)	Primer sequence 3'5' (RV)	Genbank accession
npy	CTGGGGATGGGACTCTGTTT	TTCGTCTGCTTGGGAACTCT	M87297
crf	TTTCCACCACCGTATGAATGT	TTTGCCTAATGGCTTTGCTC	AF098629
cart-I	CCATGGAGAGCTCCAAACTC	TCTTGACCCTTTCCTGATGG	AF288810
lep	TCCTCAGAATCAAAGATCAC	CCATAATAGACCCAAGACC	EU911004
lepr	TGTTCAGGGTCTTTGTTC	GCGTTCATTCAGTGTAGTT	EU911004

6.3. Results

6.3.1. FLX injection experiments

6.3.1.1. Fluoxetine decreases food intake and reduces weight gain in injected fish

The food intake in both groups of injected fish (**Fig. 6.2**) was not statistically different during the initial 6 d acclimation period where baseline food intake was determined (data not shown). Two-way mixed ANOVA analysis of food intake data following the injection protocol showed no effect of time within fish groups ($F=0.872$; $df=3.497$; $p >0.05$, Huynh-Feldt corrected, as the sphericity criterion was not met), but an interaction between time and treatment was noted ($df=2$; $F=3.497$; $p \leq 0.05$, Huynh-Feldt corrected). A significant effect was found between FLX-injected and saline-injected control groups ($df=1$; $F=19.034$; $p \leq 0.01$). Bonferroni-adjusted pairwise comparisons were used to determine statistical differences between treatments at any given time point. Differences on days 5, 6, 9, 11, and 12 were found to be significant ($p \leq 0.05$).

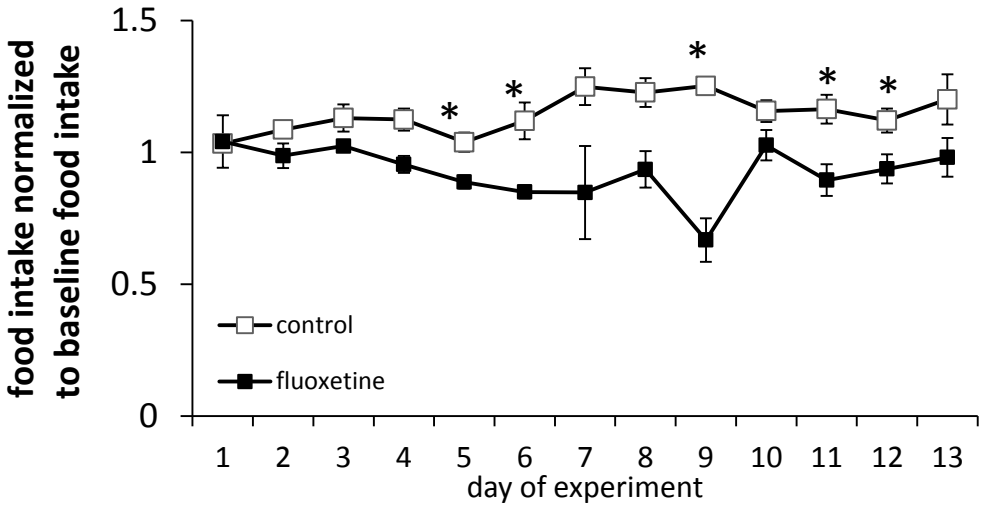


Fig. 6.2. *Average food intake per g of body weight of fish was normalized to baseline values measured for 7d prior to the injections in saline-injected (white squares) and FLX-injected (black squares) female goldfish. An n=4 tanks per treatment group were used to monitor food intake. Fluoxetine (5µg/g bw) and saline injections started on day 8 after 7 d of entrainment on the feeding regimen and were administered on day 1,4,7,10 and 13. Data was analyzed using a mixed two-way ANOVA, with a repeated measurement factor (food-intake) and a fixed factor (treatment). A Huynh-Feldt correction as sphericity was not met for repeated measurements. Asterisks denote significant difference between saline and FLX injected fish for a particular time point as determined by Bonferroni-corrected pairwise comparison ($p \leq 0.05$).*

A 52% decrease in body weight gain between FLX-treated and control animals was detected when comparing animals at the beginning of the baseline measurements (day 1) and at day 13 of the experiment (**Fig. 6.3**). The reduction in body weight gain was found to be on average 3.3 g per fish; control fish gained an average of 6.3 g and FLX-treated fish gained an average of 3.0 g ($p \leq 0.01$).

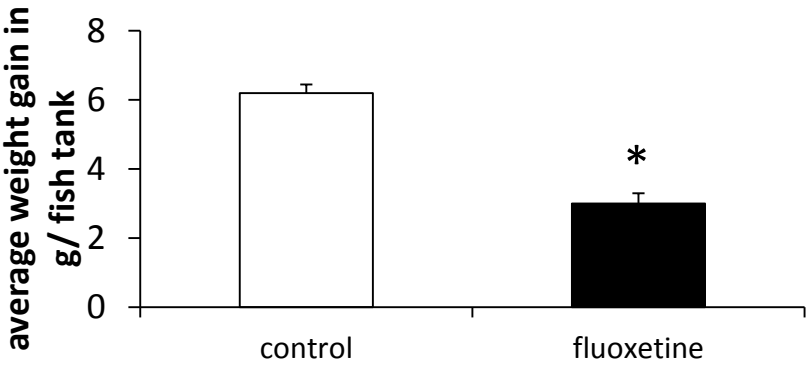


Fig. 6.3. *Average weight gain (±S.E.) in saline-injected (white bar) and FLX-injected (black bar) female goldfish. Average weights from n=4 tanks were used for each treatment. Data was normally distributed and analyzed using a two-tailed t-test. Asterisk denotes a significant difference (p ≤0.05).*

6.3.1.2. Fluoxetine does not alter circulating GH concentrations

Serum GH concentrations (**Fig. 6.4**) were not significantly different (p >0.05). Serum GH concentrations were 132 (± 10 S.E.) and 130 (± 10 S.E.) ng/mL in saline-injected and FLX-treated fish, respectively.

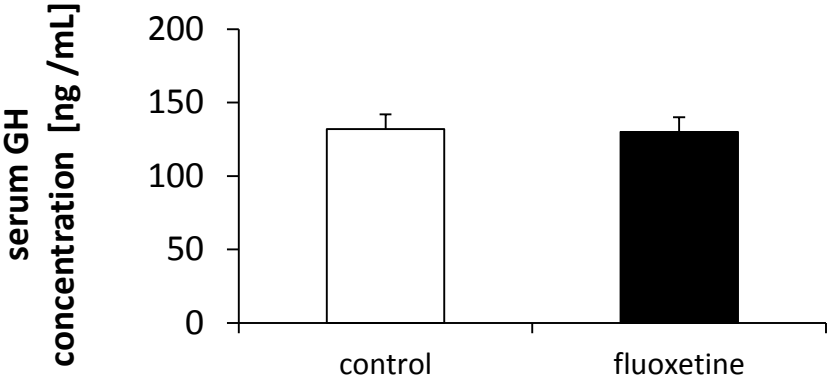


Fig. 6.4. *Relative serum growth hormone concentration (±S.E.) in saline-injected control fish (white bars) and FLX-injected fish (black bars). An n=18 serum samples were used for each group. Data was normally distributed and analyzed using a two-tailed t-test.*

6.3.1.3. Fluoxetine has tissue-specific effects on npy, crf and cart-1 mRNA abundance

There was no significant difference in *crf* mRNA abundance between saline-injected and the handling control group in either the hypothalamus or the telencephalon (**Fig. 6.5**). This suggested it is unlikely that any changes in *crf* mRNA abundance were related to either handling or injection stress. In the hypothalamus, there was a 2.3-fold increase in the mRNA abundance of *npy* ($p \leq 0.05$), and a 2.1-fold increase in the mRNA abundance of *crf* ($p \leq 0.05$) in FLX-treated females compared with the saline-injected controls (**Fig. 6.6A**). In contrast, hypothalamic mRNA abundance of *cart-1*, *tac* and *sst-2* was unchanged. A decrease in *npy* ($p \leq 0.02$) and an increase in *cart-1* ($p \leq 0.05$) transcript abundances were detected in the telencephalon with no changes detected for *crf*, *tac* or *sst-2* (**Fig. 6.6B**).

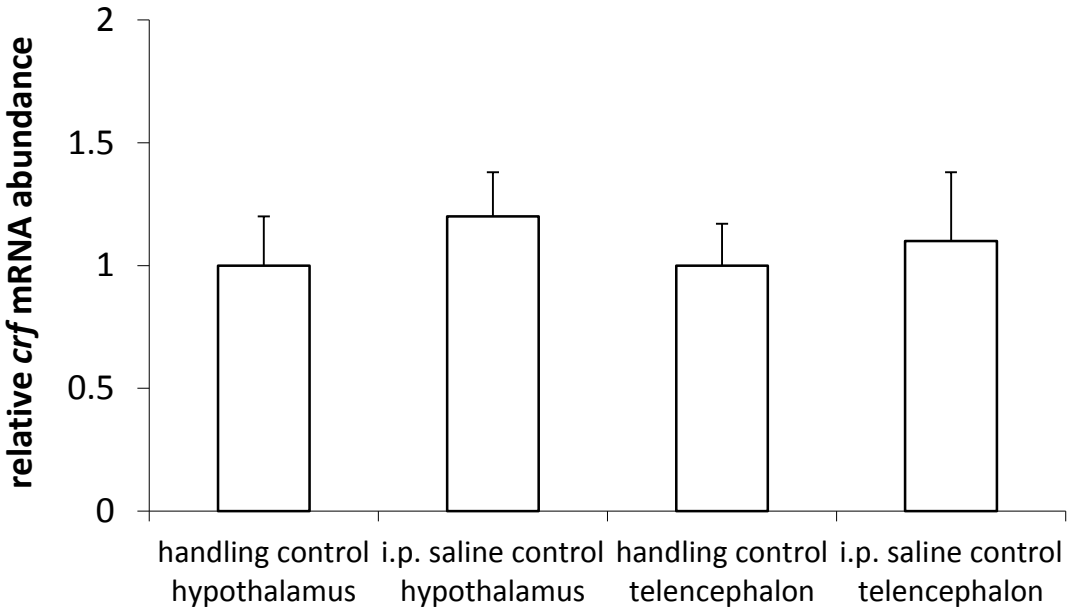
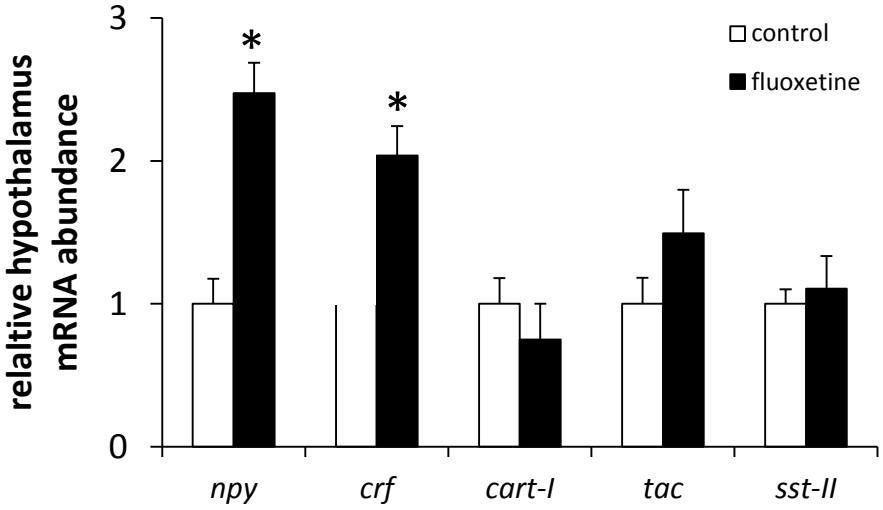


Fig. 6.5. *Relative crf mRNA abundance (±S.E.) in the hypothalamus (A) and telencephalon (B) of injected and unhandled control fish, an n=3 of hypothalamus and telencephalon tissue was used for each subgroup within the contral groups. Data not normally distributed and analyzed using a Mann-Whitney U test.*

A



B

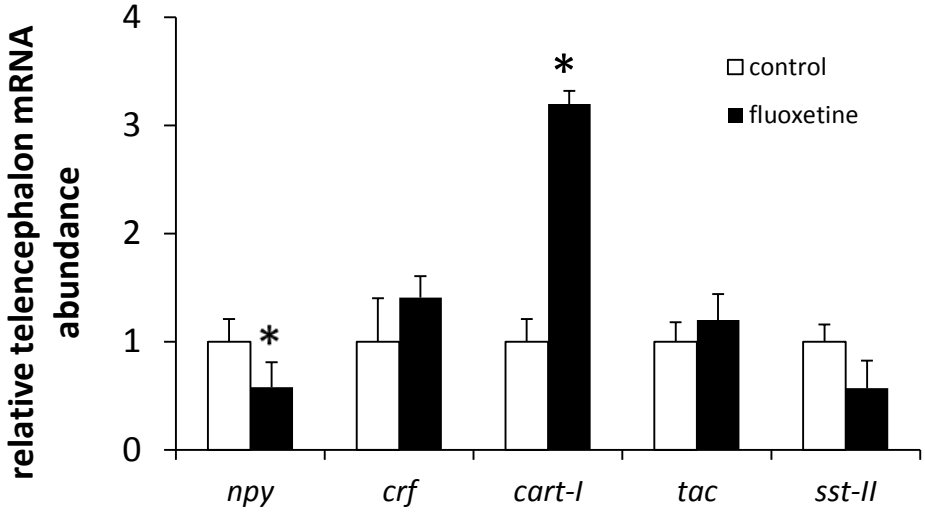


Fig. 6.6. *Relative neuropeptide mRNA abundance (±S.E.) in the hypothalamus (A) and telencephalon (B) of saline-injected control fish (white bars) and FLX-injected fish (black bars). An n=7 hypothalamus and telencephalon tissues were used for either treatment group.Data was analyzed using a Bonferroni-adjusted t-test when normally distributed and by Bonferroni-adjusted Mann-Whitney U test when non-parametrically distributed. In all cases, asterisks represent a significant difference at* $p \leq 0.05$.

6.3.2. Waterborne FLX experiments

6.3.2.1. Waterborne fluoxetine decreases food intake and weight gain dose-dependently

No replicate tanks were used in the waterborne exposure experiment; therefore no statistical analysis is available for the feeding data which was assessed for each tank (**Fig. 6.7**).

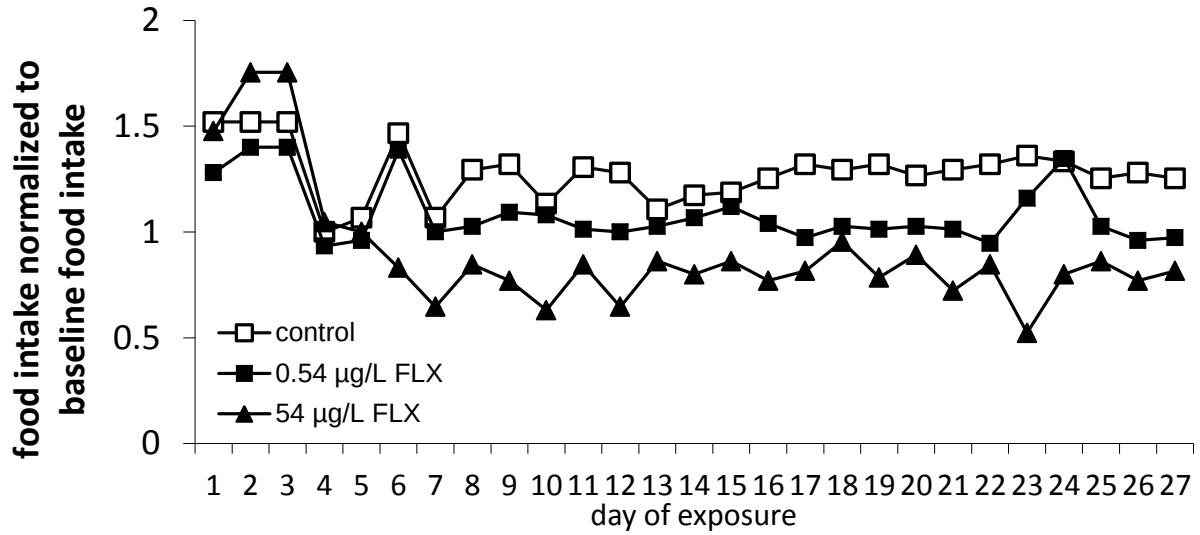


Fig. 6.7. *Daily measured food intake in control fish (white squares), fish exposed to FLX at 0.54 µg/L (black squares) and 54 µg/L (black triangles). No error bars are shown as data were collected for single tanks (n=1) per treatment only.*

Regarding changes in body weight ($df = 5$; $F = 32.90$; $p \leq 0.01$), control fish gained on average 1 g per fish which was significantly different from fish exposed to 54 µg/L FLX and the fasted fish, whose overall body weight decreased by 1 g per fish and 3 g per fish, respectively (**Fig. 6.8**). No differences in weight were observed between the FLX-exposed groups and the respective pair-fed groups.

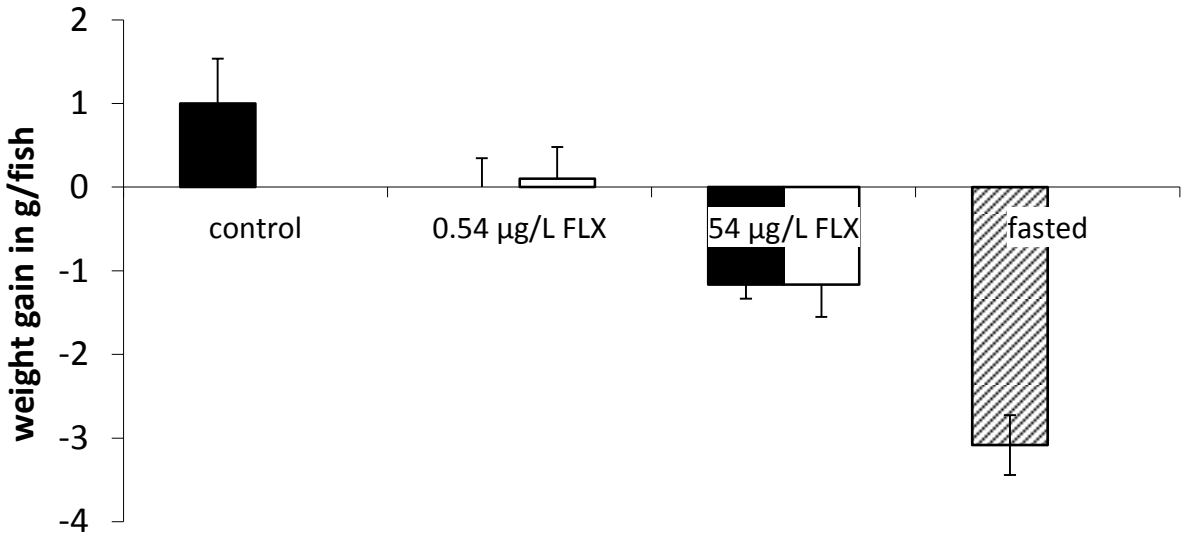


Fig. 6.8. *Mean (±S.E.) weight gain of n=12 fish per treatment group after 21 d in control fish, fasted, fish exposed to 0.54 µg/L FLX and 54 µg/L FLX, as well as the respective pair-fed groups for each FLX treatment or fasted fish. Ad libitum fed fish are indicated by black columns, pair-fed groups by white columns and fasted fish by the hatched column. Data was transformed to fit a normal distribution and analyzed using a one-way ANOVA, followed by Bonferroni-adjusted post-hoc tests. Different letters indicate a significant difference at p <0.05.*

6.3.2.2. Waterborne FLX modulates neuropeptide mRNA abundance in the neuroendocrine brain

Significant changes in transcript levels of hypothalamic feeding peptides were observed (**Fig. 6.9**). NPY mRNA abundance (**Fig. 6.9A**; df=5; F=4.67; $p \leq 0.05$)was lower in the group exposed to 0.54 µg/L FLX in comparison to its pair-fed group ($p \leq 0.05$) but this trend was exactly reversed in the 54 µg/L group ($p < 0.05$). The mRNA abundance of *crf* (**Fig. 6.9B**; df=5; F=5.26; $p \leq 0.01$) in the group exposed to 54 µg/L FLX was not different from control, but was significantly elevated compared with the respective pair-fed group ($p \leq 0.05$). The *crf* mRNA abundance in the respective pair-fed group and fasted group were significantly decreased compared to the control group ($p \leq 0.05$). The mRNA abundance of *cart-I* (**Fig. 6.9C**) did not change significantly (df=5; F=1.44; $p > 0.05$).

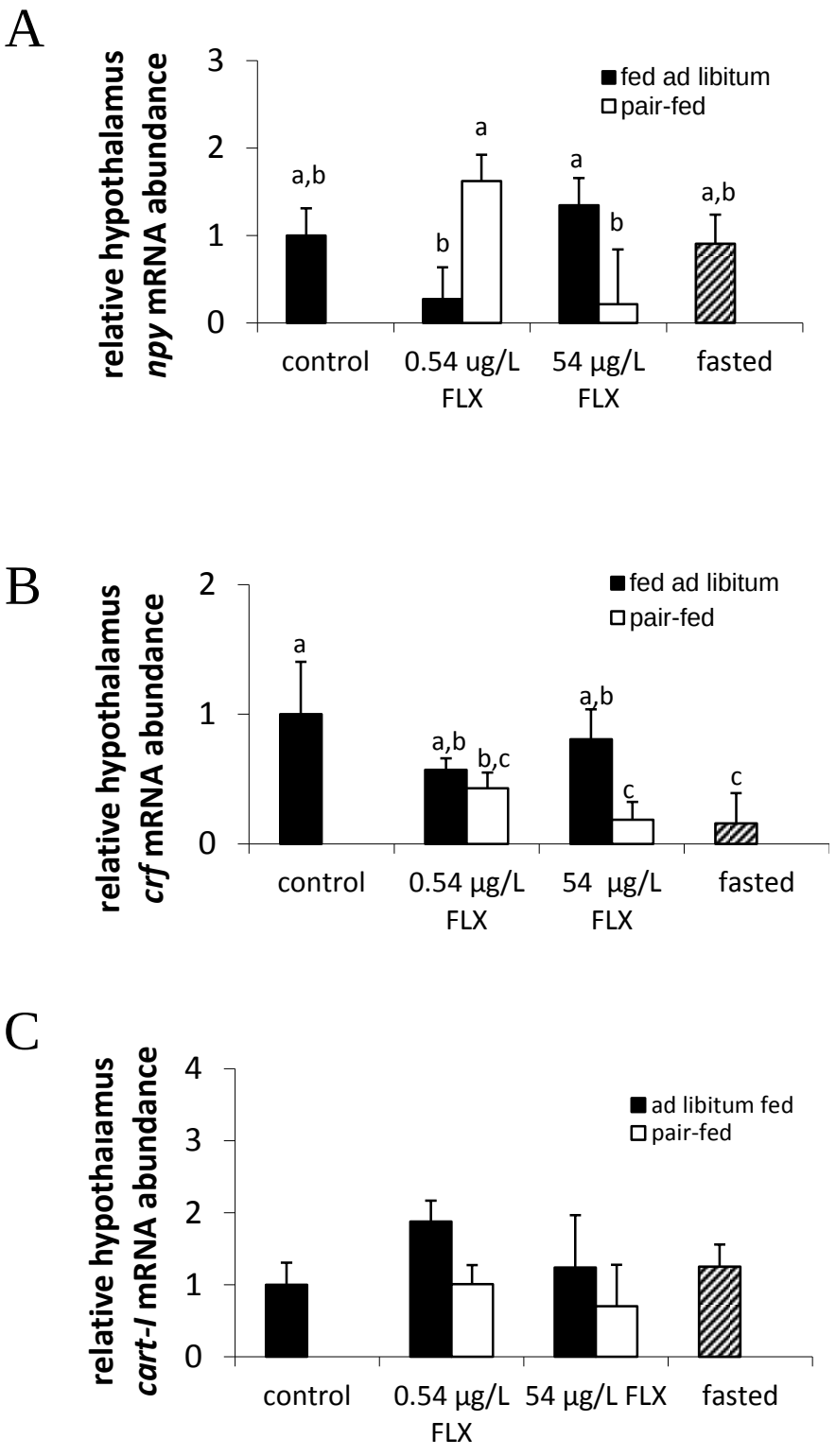


Fig. 6.9. *Mean hypothalamus mRNA abundance (±S.E.) of npy (A), crf (B), cart-I (C) across treatment groups. An n=7 separate hypothalamus tissue pools were used. Data were transformed to fit a normal distribution and analyzed using a one-way ANOVA, followed by Bonferroni-adjusted post-hoc tests. Different letters indicate a significant difference at p ≤0.05.*

6.3.2.3 Fluoxetine does not alter mRNA abundance of hepatic lep or the lepr in the hypothalamus

Neither *lep* mRNA abundance in the liver (df=5; F=0.57; p >0.05) nor the *lepr* mRNA abundance in the hypothalamus (df=5; F=0.54; p >0.05) changed significantly across treatment groups (**Fig. 6.10**).

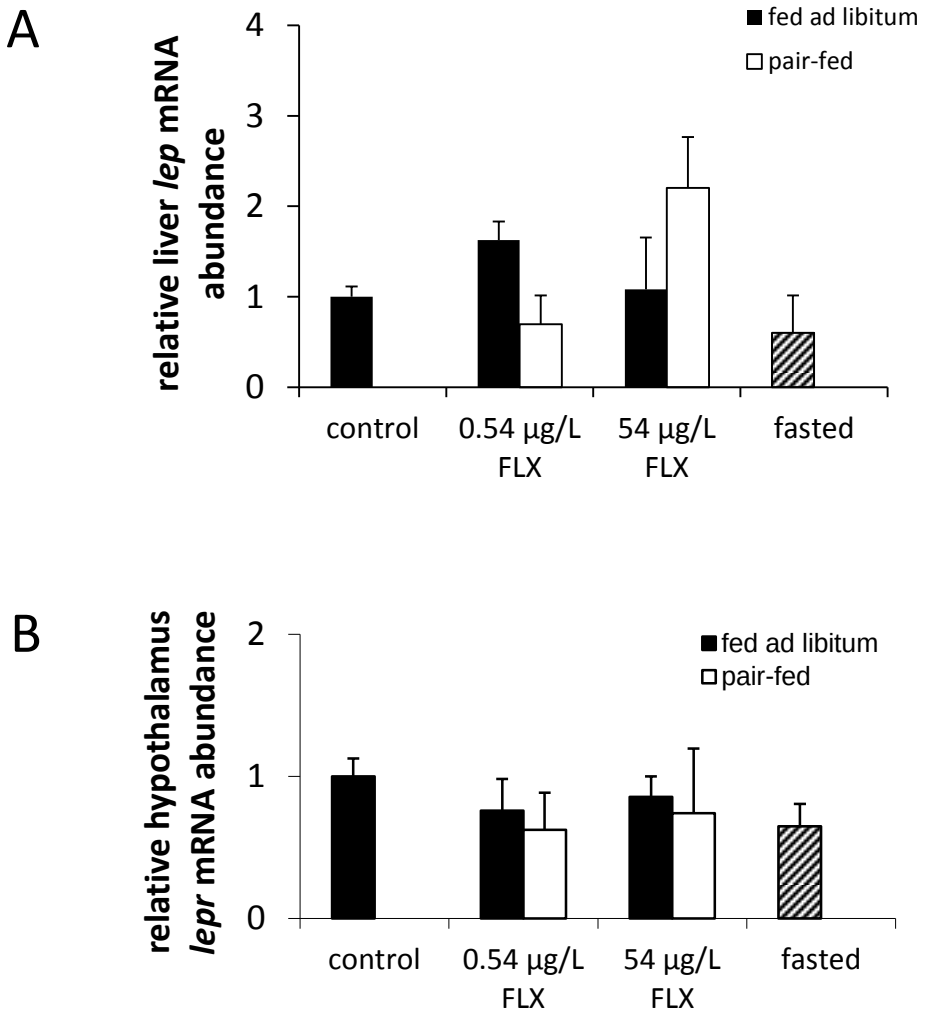


Fig. 6.10 *Mean (±S.E.) liver mRNA abundance of lep (A) and hypothalamic mRNA abundance of the lepr (B) across treatment groups. An n=7 individual liver tissues were used for each group. Data were transformed to fit a normal distribution and analyzed using a one-way ANOVA, followed by Bonferroni-adjusted post-hoc tests. Different letters indicate a significant difference at p ≤0.05.*

6.4. Discussion

The experiments described in this chapter support the hypothesis that FLX acts as an anorectic agent in goldfish, and that these effects may be at least partially mediated by changes in the hypothalamic mRNA abundance of feeding peptides. These changes were especially pronounced in the injection study and do not appear to occur at the environmentally relevant concentration (0.54 µg/ L) of FLX tested. The mRNA abundance of components of the leptin system is not regulated by waterborne FLX, which does not support the hypothesis that reproductive effects observed at lower doses are mediated by a primary action of FLX on nutritional status. A detailed discussion is presented in the following paragraphs.

6.4.1. Fluoxetine is an anorectic agent in goldfish

The initial injection study showed that FLX can disrupt food intake and reduce body weight gain in goldfish. These findings argue for similar anorectic effects of FLX in goldfish as observed in mammals (286). More specifically, this study paralleled findings in male rats where daily FLX injections of 5 mg/kg for a period of 44 d inhibited weight gain rather than decreased body weight in comparison to a saline-injected control group (146). Inhibition of weight gain was also reported in male rats injected with 5 mg/kg fenfluramine for 15 d; fenfluramine also acts by blocking 5-HT reuptake but it additionally releases 5-HT from pre-synaptic nerve terminals (301). The injection experiment was conducted in June, a period of maximum growth rates in female goldfish (2); therefore a body weight gain was expected. In fact, body weight gain in the saline-injected control goldfish (29%) was in the range previously reported over the same period in another study, where goldfish gained 44% body weight when fed a diet high in crude protein (302).

The waterborne FLX exposure revealed that a FLX concentration of 54 µg/L but not 0.54 µg/L could similarly lower feeding rate and elicit a significant decrease in weight gain. These findings are in line with studies which investigated the effects of FLX (303) and an additional SSRI, sertraline (304) on feeding rates and growth in fish. For example, FLX significantly decreased feeding rate in the fathead minnow (*Pimephales promelas*) at a concentration of 106 µg/L, and significantly decreased growth at a concentration of 53 µg/L (303). In the same species, Valenti and colleagues (304) reported an inhibition of feeding rate at sertraline concentrations between 15 and 250 µg/L, with lower sertraline threshold concentrations at higher pH values. In the same study, growth rate was decreased between sertraline concentrations of 30 and 250 µg/L. In both cases, an elevated pH value (8.5) resulted in a response at lower sertraline concentrations.

The qualitative differences noted in the anorexigenic action of FLX in the two experiments may be related to the feeding regimen employed as well as a seasonality effect, as growth rates in goldfish differ according to season (2). However, both studies clearly indicated that FLX can act as an anorectic agent in goldfish. As no previous study had investigated molecular mechanisms associated with these physiological endpoints, molecular mechanisms were first investigated in FLX-injected fish before investigating specifically identified molecular targets in the context of the second, waterborne exposure.

6.4.2. Fluoxetine injection affects mRNA abundance of the feeding-related peptides npy, crf and cart-1 but does not change the GH-axis in the neuroendocrine brain

The neuroendocrine brain in both mammals (23) and fish (6) modulates feeding behaviour by affecting neuropeptide concentrations that stimulate feeding (orexigenic peptides) or inhibit feeding (anorexigenic peptides). Tissue-specific changes in the *npy* mRNA abundance

in FLX-injected goldfish were observed, with an increase in the hypothalamus but a decrease in telencephalon. The changes in telencephalon *npy* mRNA abundance may be associated with the observed decrease in food intake and weight gain. NPY is a potent orexigenic factor in rats when injected directly into the PVN (305) and in goldfish when injected into the third brain ventricle (289). In rats, there is evidence for a decreased *npy* mRNA expression in the PVN in FLX-injected animals (306). Further evidence for serotonergic modulation of *npy* mRNA abundance comes from studies employing 5-HT agonists and antagonists. The mRNA abundance of *npy*, for example, is increased in the hypothalamus of rats i.p. injected with 10 mg/kg methylsergide, a 5-HT antagonist (24). Conversely, rats injected with mCPP, a $5HT_{1B}$ and $5\text{-}HTR_{2C}$ agonist, showed reduced *npy* mRNA abundance in the hypothalamus (24).

The observed increased hypothalamic *npy* mRNA abundance does not correlate with the decreased food intake or weight gain in FLX-injected goldfish in this study. This induction could represent a serotonin feedback response that desensitizes receptors, such as $5\text{-}HTR_{1B}$ or $HTR_{2C,}$ which decrease *npy* mRNA abundance, (24). The fact that in fenfluramine-injected rats *npy* mRNA abundance in the posterior arcuate nucleus was reduced after 1 d but returned to previous *npy* mRNA abundance after 15 d supports this hypothesis (301). Additionally, it is possible that NPY neuron populations in the hypothalamus and telencephalon express different 5-HTR subtypes and therefore respond differentially to a change in 5-HT concentration. Furthermore, the increased hypothalamic *npy* mRNA abundance may represent a starvation response, as *npy* mRNA abundance reaches its peak just prior to scheduled feeding times in both telencephalon and hypothalamus in goldfish (307). This possibility is supported by the finding that a reduction in ration size by 50% leads to increased *npy* mRNA abundance in both brain

areas in goldfish (307). Increased hypothalamic *npy* mRNA abundance could be involved in the recovery of food intake we observed at the end of the experiment.

When investigating *crf* mRNA expression, it is important to consider that CRF is a key factor in the stress axis, and that handling procedures themselves could therefore result in modulation of *crf* mRNA abundance in response to stress. However, this was not found to be the case as no changes were observed between the injected and unhandled goldfish in either the hypothalamus or telencephalon, excluding possible influences of handling stress on the observed *crf* mRNA responses. Injection of FLX in this study increased *crf* mRNA specifically in the hypothalamus. It was reported that i.c.v. CRF injections inhibited food intake in goldfish after 2 h (290). These findings are similar to studies in rats, where i.p. injection of 10 mg FLX/kg bodyweight increased *crf* mRNA in hypothalamic PVN neurosecretory cells after 30 min (308) and increased CRF secretion by 60% into hypophyseal portal blood (309). This effect in rats is likely mediated through 5-HT signalling via 5-HTRs of the 5-HTR_1 and 5-HTR_3 family, potentially located on CRF neurons in the PVN (310). The observed increase in *crf* mRNA abundance in FLX-injected fish supports an involvement of CRF in 5-HT-modulated feeding in goldfish reported by de Pedro and colleagues (36). In fish, the hypothalamic–interrenal axis is modulated by 5-HT at least partially through a 5-HTR_{1A} receptor (37), which led the authors to conclude that the serotonergic control of CRF neurons is likely to be evolutionarily conserved across vertebrates. Interestingly, a positive correlation between *npy* and *crf* mRNA abundance was described in the preoptic area in socially subordinate rainbow trout (311), which generally also exhibited high 5-HT concentrations (44). It is therefore possible that increased serotonergic signalling as a result of FLX treatment in this experiment induced both *crf* and *npy* mRNA in the hypothalamus. While NPY is an orexigenic peptide in different vertebrates (24,289), its

stimulatory activity on food intake is blocked by CRF in mammalian models (312). Therefore a simultaneous increase in CRF could potentially block any stimulatory effects that the increase in hypothalamic NPY might have on feeding.

The mRNA abundance of *cart-1* in the telencephalon, but not the hypothalamus, was increased in FLX-injected goldfish. CART fragments (AA55-102) injected i.c.v. in rats (313) and goldfish (291) potently inhibited feeding and NPY-induced feeding. Evidence for serotonergic control of *cart* mRNA abundance stems from studies in rats where *cart* mRNA abundance increased in mice administered 10 mg/kg CP9253, a 5-HTR_{1B} agonist that inhibits food intake (288). CART was cloned in goldfish and two genes, *cart-1* and *cart-2*, are found in this species (292). The *cart-1* gene was most abundantly expressed in the olfactory bulb and hypothalamus, regions important for food intake in fish. The mRNA abundance of *cart-1*, but not *cart-2*, showed postprandial increases in the hypothalamus and decreased expression in fasting (292). The 3.2-fold increase of *cart-1* mRNA abundance observed here in the telencephalon correlates with decreased NPY mRNA abundance, which raises the possibility that *cart*-1 may down-regulate *npy* mRNA abundance to suppress food intake at the level of mRNA transcript abundance in the goldfish. Evidence for potential interactions between the CART and NPY systems in fish stems from immunofluorescent labeling studies in the catfish *Clarias batrachus*, where co-localization of CART and NPY immunoreactivity was observed in the dorsal telencephalon (314).

Serotonin does modulate GH release from the goldfish pituitary (33) and GH does stimulate somatic growth and food-intake (284). However, FLX injections in the first experiment described in this chapter did not result in a change in serum GH or in *sst-2* mRNA abundance, providing evidence against an involvement of the central GH axis with regard to the

decreased feeding and weight loss observed in both experiments described in this chapter. This is further supported by the lack of changes in the GH-axis observed in waterborne FLX exposures investigating reproductive endpoints in male fish (see Chapter 5). Overall the FLX injection experiment supported potential roles for *npy*, *crf* and *cart-1* mRNA in the modulation of feeding and growth in goldfish, while no evidence for an involvement of the GH axis was noted. Therefore, for the second experiment using a waterborne exposure, the mRNA abundance of only these targets was investigated. The fact that paradoxical responses, such as the observed increase in *npy* mRNA abundance in the hypothalamus, were noted in the injection study led to the incorporation of a pair-feeding design to discern between fasting-induced changes and true FLX-mediated changes in the subsequent waterborne exposure.

6.4.3. Waterborne FLX elicits an anorexic response in goldfish and modulates neuropeptide mRNA abundance in the hypothalamus

The hypothalamic mRNA abundance of both *npy* and *crf* changed in FLX-exposed fish. The mRNA abundance of NPY in the group exposed to 0.54 µg/L FLX was significantly reduced compared with its pair-fed group, providing support that FLX suppressed the physiological increase in *npy* mRNA abundance of this feeding peptide. However, the reverse pattern was observed in fish exposed to 54 µg/L FLX. This indicates a complex regulation of *npy* mRNA abundance based on the severity of food restriction, as well as potentially non-linear dose-response effects of FLX on hypothalamic *npy* mRNA abundance. Therefore the waterborne exposure study provided no clear correlation between *npy* mRNA abundance and the observed changes in feeding rates. This may be partially attributed to the fact that *npy* mRNA abundance may have responded in an inverse manner to serum glucose concentrations, which increased in goldfish exposed to 0.54 µg/L but not 54 µg/L FLX (data not shown, ref. 315).

The mRNA abundance of *crf* is significantly increased in comparison to the pair-fed group in fish exposed to 54 µg/L FLX. The pair-fed group exhibited reduced *crf* mRNA abundance similar to the fasted group, indicating a potential adaptive decrease of the mRNA abundance in food-deprived fish. The action of FLX appears to prevent this reduction, and may therefore be involved in the anorexigenic effects in fish exposed to 54 µg/L FLX.

6.4.4. Are the anorectic FLX effects related to the observed disruptive effects of FLX on the reproductive axis?

Evidence from studies in mammalian models provided evidence for neuroendocrine pathways involved in a crosstalk between nutritional status and reproductive axis (297). Crosstalk between the the nutritional status and the reproductive axis ensures that the activation of the energetically demanding reproductive axis occurs only in the presence of sufficiently available energy. Briefly, the hormone leptin is released from peripheral adipose tissue in mammals and serves as a feedback loop to negatively regulate feeding behaviour in the hypothalamus (297). Additionally, leptin functions to stimulate the reproductive axis in the neuroendocrine brain, as indicated by its ability to restore LH pulsatile release in *lep* -/- animal models (316). Therefore, the hormone leptin represents a well-characterized pathway that links energy status and reproduction in the hypothalamus (317). Interestingly, reported evidence supports a permissive role for leptin in stimulating the reproductive axis via 5-HT neurons in the hypothalamus (318). In fish, leptin is not as well characterized as in mammals, but its role appears to be conserved as its acute administration reduces feeding and body weight in goldfish (319). A stimulatory effect of recombinant human leptin on LH/FSH secretion was reported in sexually mature rainbow trout pituitary cells *in vitro* (320). These results should however be interpreted with caution and need to be substantiated by using endogenous fish leptin ligands.

Therefore, this study investigated whether the anorectic effects of FLX might be coupled to decreased hepatic *lep* mRNA abundance, or to alterations in the abundance of hypothalamic *lepr* receptor mRNA. No changes were observed in either parameter, providing evidence that FLX does not influence leptin signalling at the level of mRNA abundance. This is in line with the fact that the feeding rate and growth endpoints are affected only at a waterborne FLX concentration of 54 µg/L while some reproductive endpoints, such as milt release (Chapter 5), are inhibited at FLX concentrations as low as 0.54 µg/L FLX. The only study that investigated a relation between these commonly disrupted physiological endpoints in reproduction and feeding.in rats used repeated i.p. injections of 10 mg FLX per kg for 21 d (91). At this pharmacological concentration, FLX effects on both systems were equivocal, as a correlation between both processes indicated by pair-feeding experiments appeared in the short term (days 1-14) but not in the long term (days 15-21). Additional pair-feeding experiments during the reproductive period, measuring reproductive endpoints such as milt, would be necessary to separate these effects further. In addition, the investigation of recently identified additional molecular targets such as kisspeptin, which may be involved in cross-talk of nutritional status and reproduction in the neuroendocrine brain (321), may be useful to further discard the hypothesis that FLX- induced disruption of reproduction is secondary to its anorectic properties.

6.4.5. Other potential mechanisms that may underlie the anorexigenic effects of FLX

Microarray analysis of FLX-treated goldfish hypothalami reported several changes in the mRNA abundance of genes belonging to the gene ontology category of metabolism (132). Among these targets were lactate dehydrogenase and dehydrolipidamide dehydrogenase, both of which are involved in lactate metabolism (322). Interestingly, lactate may be involved in glucose metabolism and the glucosensing mechanism in the rainbow trout hypothalamus (323). Glucose

is an inhibitory metabolic signal for food intake in fish (282). The mRNA abundance of glycolytic enzymes aldolase C and hydroxypyruvate isomerase were also found to be regulated by i.p. injected FLX using the microarray (132), implicating changes in these metabolic pathways within the brain may result in modulation of negative feed-back of glucose on food intake.

Several gene targets involved in lipid-metabolism were also identified in the same study, among them apolipoprotein E (*apoe*). Apolipoprotein E has emerged as an important regulator of food intake in rats (324) and the hypothalamic mRNA abundance of this protein is stimulated by feeding entrainment (325). Furthermore, the hypothalamus mRNA abundance of ApoE was stimulated by signals including leptin and E_2 (326), both known mediators between nutritional status and the reproductive axis in mammals. However, while dose and timing of FLX injections of the goldfish used in the microarray experiment were the same as used in these experiments, the experiments were conducted at a different time of the year and thus difference in the transcriptome would be expected. Furthermore, this earlier study (Chapter 3) investigated reproductive effects of FLX (132) and none of the metabolic target genes identified by the microarray were validated using real-time RT-PCR. Additional studies are necessary to test the hypothesis that these targets are involved in the observed anorexigenic effects of FLX in goldfish.

6.5. Conclusions

The disruptive effects of FLX and SSRIs in general with regard to 5-HT-regulated processes such as fish reproduction have been discussed previously (Chapters 3 and 5). The potential impact of SSRIs on feeding and growth, however, has received much less consideration in the literature. The two experiments reported here demonstrate that FLX acts as an anorectic

agent in goldfish, and that these changes correlate with changes in neuropeptide gene mRNA abundance involved in feeding, especially *crf*. However, FLX-induced changes in *crf* mRNA abundance were observed only in FLX-injected goldfish and those exposed to a concentration of 54 µg/L, indicating that more environmentally relevant concentrations of FLX (e.g. 0.54 µg/L) may not result in significant disruption of feeding rate or somatic growth. Further studies over longer periods of exposure, however, are necessary to substantiate this conclusion.

Chapter 7: General discussion and conclusions

Partially based on Mennigen et al., 2010. Pharmaceuticals as neuroendocrine disruptors: Lessons learned from fish on Prozac™. Journal of Toxicology and Environmental Health Part B: Critical Reviews, accepted.

7.1. Evidence for FLX-induced neuroendocrine disruption and wider implications for eco-toxicological research

Several excellent reviews provide a strong case for the need for eco-toxicological risk assessment of FLX (65,81) and SSRIs in general (88) in the aquatic environment. These reviews indicate that FLX does trigger a risk assessment under European Union guidelines (81) and that there is a need to investigate more sensitive sub-lethal endpoints in fish exposed to FLX in order to improve estimates of environmental effects. This thesis provides evidence in support of specific sub-lethal effects of FLX on physiological systems resulting from neuroendocrine disruption, particularly with respect to reproduction and feeding/growth. This chapter recapitulates key evidence for neuroendocrine disruption by FLX in the goldfish model presented in this thesis. Additionally, studies on other fish species are presented to complement the picture of neuroendocrine disrupting effects of FLX in fish. Wider implications of these results are discussed in the context of the numerous aquatic contaminants and conditions that potentially may disrupt the neuroendocrine 5-HT system in fish. Therefore, implications of the results and mechanisms presented in this thesis provide a framework for the investigation of the potential additive effects of FLX and other SSRIs detected in the aquatic environments (63) and in fish (86). The results are also useful for the study of potential additive effects of FLX and other aquatic factors known to alter the 5-HT system in the brain, including such factors as hypoxia (57), ammonia (56), metals (53,54,55) and PCBs (54).

7.2. The reproductive axis is the principal target for FLX-induced disruption

The most sensitive endpoint with regards to FLX-induced disruption in goldfish identified in this thesis is the reproductive axis. This action was especially prominent in male fish, where a significant reduction in milt release was observed following exposures to the high but environmentally-relevant FLX concentrations (0.54 µg/L). This is still well below the maximum detected combined SSRI concentration of 3 µg/L reported in some streams (2,63). While serum E_2 concentrations significantly decreased at a FLX concentration at 0.54µg/L, no other measured parameters related to the neuroendocrine brain were significantly changed in female fish (Appendix 1). Therefore, evidence from this thesis suggests that FLX-induced neuroendocrine disruption of the reproductive axis may be more pronounced in male than female fish at environmental concentrations. Studies investigating the effects of FLX in other fish report similar results (**Fig. 7.1**), with FLX-induced disruption of reproductive parameters at concentrations of environmental relevance and above, and a particular sensitivity to FLX in male fish. Sexually mature male fathead minnows exposed to 0.028 µg/L FLX had a significant increase in plasma vitellogenin (H. Schoenfuss, personal communication[14]).Vitellogenin is not usually produced in males. It is an E_2-regulated egg-yolk protein normally only produced in sexually mature female fish. Developing male mosquitofish (*Gambusia affinis*) exposed to 71 µg/L FLX for 100 d had delayed appearance (approximately 10 d) of the gonadopodium. A larger number of studies have been conducted on FLX-induced disruption of the reproductive axis in female fish. Female zebrafish showed significantly decreased egg production (86%) and a concurrent decrease in ovarian E_2 concentration by (68%) when exposed to 34 µg/L FLX for 7 d (256). Exposure of mosquitofish to 71 µg/L FLX for 100 d delayed sexual development in

[14] This information was provided in an email correspondence with Dr. H. Schoenfuss, Director of the Aquatic Toxicology Laboratory at St. Cloud State University, MN, U.S.A.

female fish as expressed by a delayed onset puberty (approximately 10 d) and an overall decrease in the development of secondary sex characteristics by 15% (327).

A key challenge in eco-toxicological research is to link changes at the molecular or physiological levels in fish to meaningful endpoints at the population level. Using a model proposed by Ankley and colleagues (328), a 75% decrease in serum E_2 as reported by Lister *et al*. (256), is predicted to result in the complete decline of a fish population after 6 years. This detrimental effect was also associated with a concurrent decrease in egg release in zebrafish exposed to FLX (256). My own studies in goldfish confirmed the observed decrease in serum E_2 at various FLX concentrations tested, ranging from 75% inhibition in fish repeatedly injected with 5 µg/g FLX (Chapter 3) to an approximately 50% inhibition of serum E_2 concentration in female goldfish exposed to 0.54 µg/L and 54 µg/L FLX (Appendix 1). Therefore, it is predicted that the observed effects of FLX in goldfish are ecologically relevant as regards potential population effects.

While this model represents a useful tool to extrapolate the commonly measured blood sex steroids to higher levels of biological organization (i.e. populations), the true endpoints that ultimately mediate reproductive success, such as sperm release and egg production, may represent a more direct approach. In this regard, male cyprinid fish appear to be more sensitive than female fish, as established by this thesis (Chapter 5) and by Lister and colleagues (256).

7.3. FLX affects other neuroendocrine processes in fish

Experiments investigating effects of waterborne FLX also reveal that FLX affects several other neuroendocrine-modulated processes, including feeding and growth, which I also studied (**Fig. 7.1**). The results showed a significant inhibition of feeding and body mass only at a FLX concentration of 54 µg/L, which was higher than the lowest concentration at which reproductive effects were observed. Other studies investigating the effects of waterborne SSRIs on feeding and growth in fish reported disrupting effects at similar concentrations. For example, the feeding rate was reduced in fathead minnows exposed to FLX with a lowest observed effect concentration (LOEC) of 106 µg/L (303). Fathead minnows exposed to 15 µg/L of the SSRI sertraline showed a statistically significant reduction in feeding rate by 11% at pH 8.5 (304). In comparison, a significant effect (50% reduction in feeding rate) was only observed at a concentration of 250 µg/ L at pH 7 in this same study.

Nutritional status is a key factor in the stimulation of the reproductive axis, ensuring that the costly energetic requirements of reproduction can be met by the organism (329). Therefore, the observation that FLX-induced disruption of feeding rate and growth occurs at higher waterborne FLX concentrations (µg/L) than FLX-induced disruption of reproductive parameters (ng/L), argues against a primary disruption of the nutritional status by waterborne FLX. Rather, FLX induced inhibition of the reproductive axis appears to be specific and independent of the anorexigenic properties of FLX in goldfish. This is further supported at the molecular level by the lack of a FLX-induced disruption of the leptin system which represents a link between nutritional status and reproductive axis in the neuroendocrine brain (Chapter 6).

With regards to the potential consequences of FLX-induced inhibition of feeding and growth at the population level, extrapolation is difficult. Growth itself represents a complex

parameter in fitness and selection in the early life of fishes, with a selection for larger body size that is approximately 5-times stronger than for mammalian taxa (330). However, the FLX concentrations needed to induce significant reductions in goldfish mass are well above concentrations likely encountered by fish in their aquatic environments.

Only a few studies have estimated stress parameters in FLX-exposed fish. Plasma cortisol concentrations significantly increased after 24 h by 245% in toadfish containing a FLX implant at a dose of 50 µg/g bw (133). Plasma cortisol significantly increased by 118% in rainbow trout exposed to 54 µg/L FLX for 14 d 30 min after exposure to handling stress, when compared with unexposed control fish (P. Stroud, personal communication[15]). Adult zebrafish exposed to 100 µg/L FLX for 14 d displayed a decrease in whole body cortisol concentration by approximately 66% (331). Given that 5-HT acts to stimulate the neuroendocrine stress axis in fish (37), and that the components of the stress axis are capable of inhibiting both the reproductive axis (332) as well as the feeding and growth axes in fish (36), future experiments investigating low concentration FLX exposures on the stress axis in relation to reproduction, growth and feeding are warranted.

Studies where fish were exposed to waterborne FLX have also described several behaviours associated with reproduction, feeding/growth, aggression and locomotion. The following examples serve to illustrate this point. Reproductive behaviours were significantly reduced in male fathead minnows exposed to 0.1 µg/L FLX (R. Klaper, personal communication[16]). Prey-catching behaviours are inhibited in a dose-dependent manner in hybrid striped bass, with significant effects observed at the lowest concentration (23.2 µg/L) tested

[15] These unpublished data were provided by Pamela Stroud, MSc candidate in Dr. Moon's lab at the University of Ottawa, ON, Canada.
[16] This unpublished data was provided via email correspondence by Dr. Rebecca Klaper of the Great Lakes WATER Institute in Milwaukee, WI, U.S.A.

(130). Aggressive behaviour was decreased in the male bluehead wrasse when repeatedly injected with 5 µg/g bw FLX (150). Similarly, aggressive behaviours are decreased in Siamese fighting fish (*Betta splendens*) exposed to 3 µg/L waterborne FLX (333). Locomotion was decreased in zebrafish larvae exposed to 1.5 mg/L FLX for 24 h (334), as are erratic movements in adult zebrafish exposed to 100 µg/L FLX (331). Locomotor activity was decreased by approximately 2.5-fold in Chinook salmon (*Oncorhynchus tshawytscha*) injected with 2.5 mg/kg FLX for 20 d (335). Mosquitofish exposed to FLX concentrations of 0.5-53.4 µg/L displayed lethargic and less responsive behaviours which appeared to be dose-dependent (327). Predator avoidance behaviour decreased in fathead minnows exposed to FLX (0.025 µg/L) in combination with other SSRIs, reaching a total concentration of 0.75 µg/L (336). Behavioural effects of FLX in exposed fish are largely inhibitory and appear to be consistent across fish species, with the lowest concentrations eliciting observable effects in a range that lies below 1 µg/L.

These results as well as results of my own studies are summarized in **Fig. 7.1**. This figure demonstrates that the most sensitive endpoints which appear in the environmentally relevant range of FLX and SSRI concentrations are related to reproduction and behaviour, while other processes, such as feeding/growth and stress, appear only at higher waterborne FLX concentrations. As discussed in the case of the stress axis, however, experiments of FLX exposures are sometimes lacking with respect to environmentally relevant concentrations. Therefore, studies of FLX effects on the stress axis at environmentally relevant concentrations are warranted to complement the known concentration-dependent effects of FLX on the neuroendocrine system. However, data from all studies may prove useful in the larger context of potential synergistic action of aquatic contaminants, as several other compounds in addition to

SSRIs act on the 5-HT system and may therefore potentially disrupt the endpoints described. This idea is discussed in the next section.

Fig. 7.1 *Schematic overview of identified endpoints in fish and associated changes in the neuroendocrine axis. See text for explanation.*

7.4. The role of the 5-HT system in neuroendocrine disruption in fish

The approach taken in this thesis, from initial transcriptomic screening to the investigation of specific identified targets such as IT (Chapters 4 and 5), supports the usefulness of a mechanism of action (MOA)-based approach in eco-toxicological research (337). This molecular approach confirmed that the 5-HT system is highly conserved in fish (10), and ample literature links chronic FLX effects on the 5-HT system to subsequent decreases in oxytocin in mammalian models (211,212,146). Indeed, this described mechanism was hypothesized to mediate the widely reported sexual dysfunctions in human patients using SSRIs (90). Evidence for an involvement of NPY and CRF in FLX-induced anorexia was similarly postulated in rats (338).

However, a MOA-based approach needs to be complemented by knowledge of specific differences between the models usually used for pharmacological testing, such as rats, and non-target species such as fish. For example, disruption of specific neuroendocrine pathways at environmentally relevant concentrations of FLX, such as the IT pathway, occurred only in conjunction with pheromone exposure in male fish. In goldfish, pheromone effects are species-specific and more pronounced in male fish, when compared to females. This particular example also points to the concept that multiple stressors and/or signals which challenge homeostasis are important in eco-toxicological research (339). This concept pertains critically to the neuroendocrine brain, as the main function of the hypothalamus is considered to be the regulation of homeostasis (340). Regulation occurs through the integration of environmental (external) and internal signals in order to coordinate neuronal and hormonal responses, subsequently altering physiology and behaviour (340). In this thesis for example, neuroendocrine and physiological effects at environmentally relevant concentrations of FLX (0.54 µg/L) were

observed when the homeostasis of the system was challenged, for example by pheromone exposure.

Serotonin may play a key role in this allostatic process as it is considered to "hold a pivotal role in the homeostasis of the brain, which is consistent with the function of 5-HT throughout evolution and explains the large number of biological systems and behavioural activities associated with serotonergic function" (176; p. 33). Therefore, this thesis will not only allow for a better risk assessment of effects of FLX or SSRIs on fish, but will be useful for the characterization of potential mixtures of aquatic contaminants and effects that are known to modulate the 5-HT system (see Chapter 1, **Fig. 1.3)**.

For example, while several SSRIs, all sharing the 5-HT transporter as their principal MOA, are detected in the brain of wild-caught fish (86), only a single study has investigated the potentially additive effects of mixtures more consistent with environmental samples (336). Several other aquatic conditions and contaminants including hypoxia (57), ammonia (56), metals (53,54,55) and PCBs (54), are reported to decrease 5-HT concentrations in the fish brain. Some of these conditions, including hypoxia (57), lead (54) and PCBs (54) inhibited the 5-HT system, which furthermore was causally linked to observed decreases in the neuroendocrine regulation of the reproductive axis in the Atlantic Croaker. Therefore, both the characterized seasonality of the neuroendocrine 5-HT system (Chapter 2), as well as the subsequent mRNAs altered by FLX, may serve to provide the mechanistic framework for the study of contaminant mixtures which act on the 5-HT system. A characterization of mixture effects is often identified as a future research need in eco-toxicological review papers, as it represents environmentally more relevant scenarios (67). Regarding the 5-HT system in the neuroendocrine brain, a study of potential sexual dimorphisms would be interesting as my study clearly demonstrated that male goldfish have a

lower threshold with regard to neuroendocrine disruption of mRNA abundance in FLX exposures than females (Chapter 5, Appendix 1). While true sexual dimorphisms in the brain are very difficult to establish (141), some evidence exists in both mammals (341) and fish (342) for dimorphisms in the hypothalamic 5-HT system. Sex-dependent effects of SSRIs, which target the 5-HT system, have been described in the endocrine stress axis of sheep. For example, a sertraline-induced increased ACTH and cortisol secretion occured in females, but not in males (343).

Therefore, further studies should fully elucidate potential sex differences of neuroendocrine FLX effects in fish.

7.5. Future directions for research

Based on the findings presented in this thesis, future research directions include studies investigating the mechanism of action of FLX in the neuroendocrine brain, as well as studies investigating the potential for interactions of FLX with other contaminants in the environment. With regard to mechanistic studies, a complementary investigation of FLX on protein concentrations of identified targets in the neuroendocrine brain is warranted. This is of importance, as measurement of mRNA abundance does not necessarily reflect actual translation into protein, because of mechanisms such as mRNA half-life, mRNA transport, interfering miRNAs, translational control, or protein stability (344). The example of the identified FLX-induced decrease of $PGF_{2\alpha}$ stimulated *it* mRNA abundance in the neuroendocrine brain illustrates this point. While the several fold mRNA regulation of *it* mRNA of this abundant neuropeptide supports an involvement of IT in pheromone signalling and its disruption by FLX, measurement of IT protein concentration, will strengthen this point. Measurement of IT protein concentration in the brain and the serum of goldfish, for example by ELISA (enzyme linked

immuno-sorbent assay) or HPLC, already validated in fish (214,217), will further help to separate mechanisms involved: isotocin could potentially act on the neuroendocrine brain to regulate sexual behaviour, and/or it can stimulate gonadal ITR to regulate milt release and steroidogenesis. Increased protein concentrations of IT in the brain or the blood would be indicative of a contribution of the respective pathways. Utilization of an i.c.v. injected IT antagonist validated in fish (148) would be useful to further support involvement of IT in $PGF_{2\alpha}$ -stimulated behaviour and subsequent milt release.

Another important point for future mechanistic studies is the fact that the hypothalamus contains a high density of specific nuclei involved in neuroendocrine processes (234). While dissection of the hypothalamus and telencephalon provides a degree of specificity in gene expression responses that would certainly be masked by investigations of the whole brain, more specific characterization of responses in mRNA abundance using in situ hybridization may be warranted. This is exemplified in studies in the rat: when investigating FLX-induced changes in hypothalamic *npy* mRNA abundance in rats, directionally inverse changes were measured in two hypothalamic nuclei, specifically a decrease in *npy* mRNA abundance in the PVN and an increase *npy* mRNA abundance in the arcuate nucleus (345). Therefore, while dissection of the goldfish brain in telencephalon and hypothalamus can reveal differential regulation of mRNA abundance, as I observed for *npy* mRNA abundance in FLX injected fish (Chapter 6), *in-situ* mRNA staining or laser guided micro-dissection of nuclei and subsequent measurement of mRNA abundance would provide further specificity regarding FLX action in the hypothalamus. This is especially important because 5-HT, the target system modulated by FLX, displays a dense net-like innervation pattern in the neuroendocrine brain of goldfish (Chapter 4; 15) which potentially targets several hypothalamic nuclei.

Mechanistic studies would also benefit from the investigation of neuroendocrine systems other than reproduction, feeding and growth. The 5-HT system is involved in regulating multiple neuroendocrine axis, among them the stress axis (37), a finding that was supported by the positive regulation of *crf* mRNA by FLX in goldfish (Chapter 3). The stress axis negatively regulates both reproduction (346,347) and feeding (290,348,349) in cyprinid fish; some of these effects are at least partially mediated through action in the neuroendocrine brain, such as CRF and cortisol mediated decrease of feeding (348,349) and cortisol induced reduction of mRNA abundance of *sgnrh* in the brain and *lh-ß* and *fsh-ß* mRNA abundance in the pituitary (347). Peripheral interaction between the stress axis and the reproductive axis has also been demonstrated, for example cortisol induced suppression of gonadotropin-stimulated sex steroid synthesis in the ovary of zebrafish (346). Therefore, the involvement of the stress axis in FLX induced disruption of reproduction and feeding warrants further research, especially given that studies investigating FLX effects on stress parameters in fish are lacking at environmentally relevant FLX concentrations.

The range of neuroendocrine systems affected by FLX has also been hypothesized to be related to FLX effects on targets other than the 5-HT system in the rat (149). This hypothesis is supported by the finding that the DA metabolite HVA increases in the hypothalamus of FLX injected goldfish concurrent with a decrease in hypothalamic dopamine transporter mRNA (Chapter 3). The neurotransmitter DA is of particular interest, because of its prominent inhibitory role in the neuroendocrine control of the reproductive axis in teleost fish (350). Interestingly, the pheromone 17,20P stimulates the reproductive axis by decreasing pituitary dopamine turnover in male goldfish. Because waterborne FLX exposures at waterborne concentrations between 0.1 and 0.5 µg/L have been shown to increase whole brain DA concentration in male Japanese

medaka[17], the possibility for an involvement of DA in the FLX induced disruption of 17,20P stimulated milt release exists and warrants further research.

Another target of FLX that may not be related to its serotonergic action is its effect on neuroplasticity (182), which was initially described in mammals, but appears to be conserved in fish, as indicated by several FLX-regulated transcripts involved in neuroplasticity in the hypothalamus of FLX injected goldfish (Chapter 3). Neuroplasticity has been identified as a specific target for endocrine disrupting chemicals in mammals (351), and comparative studies in fish are warranted, especially given the high potential for neuroplasticity in the adult teleost brain (352).

In the larger context of environmental impact of aquatic contaminants, the identified endpoints should be investigated in SSRIs in conjunction with other aquatic contaminants that affect the 5-HT system. As multiple SSRIs are detected in streams and fish tissue, environmentally relevant SSRI mixtures should be investigated to characterize potentially additive or synergistic effects. As well, other contaminants, such as metals and PCBs, which have been shown to disrupt the neuroendocrine 5-HT system (54), should be investigated with regard to potential interactive effects with FLX. Lastly pharmaceuticals of environmental relevance, such as FLX and the estrogenic compound EE_2, may synergistically affect reproductive endpoints in fish. For example, milt release in male goldfish has been shown to be decreased with both E_2 (279) and FLX (Chapter 5), and investigations of environmentally relevant mixtures of these compounds will address the need for studying interactive effects of pharmaceuticals in the environment.

[17] This information was provided in an email correspondence with Dr. Bryan Brooks, Associate Professor of Environmental Science at Baylor University, TX, USA.

7.6. Conclusions

The thesis provides evidence in support of the hypothesis that FLX disrupts neuroendocrine signalling in goldfish, in particular with regard to reproduction and feeding. The underlying molecular mechanisms identified are partially conserved with regard to effects in mammals, providing evidence for the usefulness of a mode-of-action approach in ecotoxicology. Importantly, the results provide evidence for FLX disruption of critical reproductive parameters, such as milt release, at environmental concentrations. These endpoints represent useful tools for the characterization of sublethal effects of FLX and should contribute towards a better understanding of eco-toxicological risk associated with FLX and SSRIs. The mechanistic framework will facilitate and focus mode of action based research of a variety of compounds acting on the 5-HT system in fish, and therefore contribute to studies investigating effects of complex mixtures in the future.

Appendix 1: Effect of waterborne FLX exposures on the reproductive axis of sexually mature female goldfish

A1.1 Introduction

Following the evidence that pharmacological concentrations of FLX injected in female goldfish severely impair the reproductive axis, as indicated by a decrease in circulating E_2 as well as the significant decrease in the abundance of *it* mRNA in hypothalamus and telencephalon (Chapter 3), I further tested the hypothesis that waterborne FLX can disrupt reproductive in a similar fashion through modulation of the identified IT pathway. While studies investigating low concentration effects of FLX and other SSRIs in mammals are almost completely lacking, studies in female fish indicate the potential of low FLX concentrations to alter circulating E_2 concentrations in female fish (256,353). For example, Foran and colleagues (353) described an increase in circulating E_2 concentrations in Japanese medaka exposed to 0.5 µg/L FLX for 4 weeks, an effect that was not observed at higher concentration of FLX. Conversely, Lister and colleagues (256) reported a decrease of ovary E_2 concentration in zebrafish exposed to FLX concentrations between 0.32-32 µg/L FLX. In spite of the fact that FLX concentrations as low as 0.32 µg/L resulted in decreases of ovary E_2 content by 50% , a significant difference from control values was only reported at a FLX concentration of 32 µg/L FLX, which reduced ovary E_2 concentration by 70%. In the latter study, Lister and colleagues further investigated potential mechanisms at the level of the ovary, and suggested that the dose-dependent decrease of *lhr*, *fshr* and *cyp19a* mRNA abundance may be involved in the decrease in ovary content of E_2. Functionally, the significant decrease in ovary E_2 concentration in zebrafish exposed was correlated with a significant decrease (-8%) in cumulative egg production over a 14 d period. Therefore a waterborne FLX exposure was conducted to firstly validate the directionality of

FLX-induced serum E_2 concentration at environmental concentrations, and secondly to investigate whether correlating changes in mRNA abundance, especially *it*, occur in the neuroendocrine brain and pituitary (as suggested by my initial pharmacological study in Chapter 3, see ref. 132) and/or at the level of the ovary (as suggested by Lister and colleagues, see ref. 256). Given the lack of identified changes in serum LH in the pharmacological study, I included the analysis of pituitary mRNA abundance of *fsh-ß* in order to complement measurements of serum LH and GH for which RIA methods exists in goldfish (220). As well, given the observed changes in *it* mRNA abundance in FLX-injected goldfish (132), I included the related neuropeptide *vt* as well, since its mRNA abundance had previously also been described to be decreased in FLX injected fish (150), and a role for VT on the reproductive axis in female fish, including stimulatory effects on ovary E_2 release (354), is emerging.

A1.2 Materials and Methods

For materials and methods please refer to Chapter 5, as the same exposure protocol and analysis as utilised for male fish were used for female fish. A schematic overview of the exposure is provided in **Fig.A1.1**.

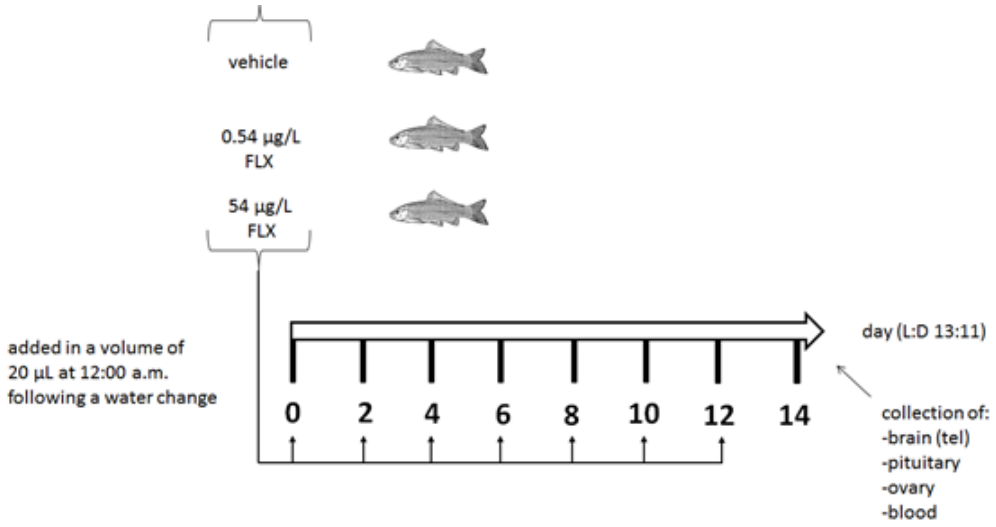


Fig. A1.1. *Experimental design of sexually mature female goldfish exposed to waterborne FLX.*

A1.3. Results

A1.3.1. Waterborne FLX significantly decreases serum E_2 concentration

Serum concentration of E_2 were transformed to fit normal distribution and analyzed in a one-way ANOVA ($df=2$; $F=4.95$; $p \leq 0.05$), followed by Bonferroni-adjusted post-hoc tests. Serum concentration of E_2 was significantly decreased compared to control in fish exposed to 0.54 µg/L FLX. Serum concentrations of T were normally distributed and analyzed in a one-way ANOVA, but did not reveal any statistically significant differences ($df=2$, $F=2.41$; $p > 0.05$).

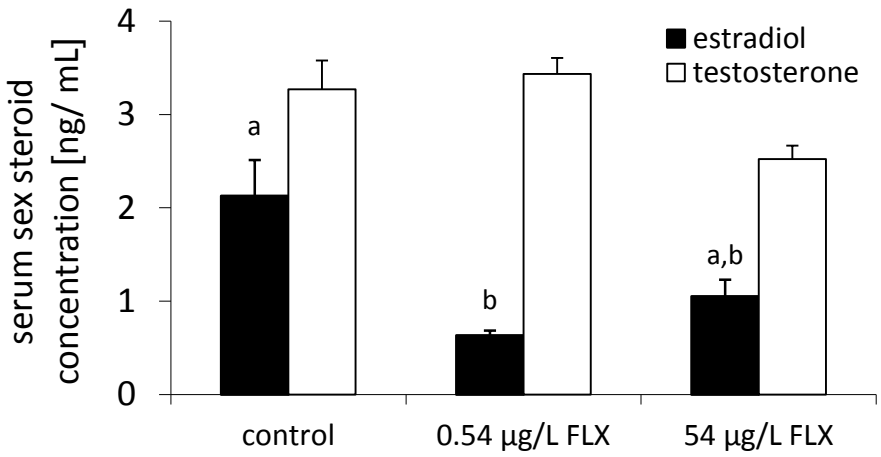
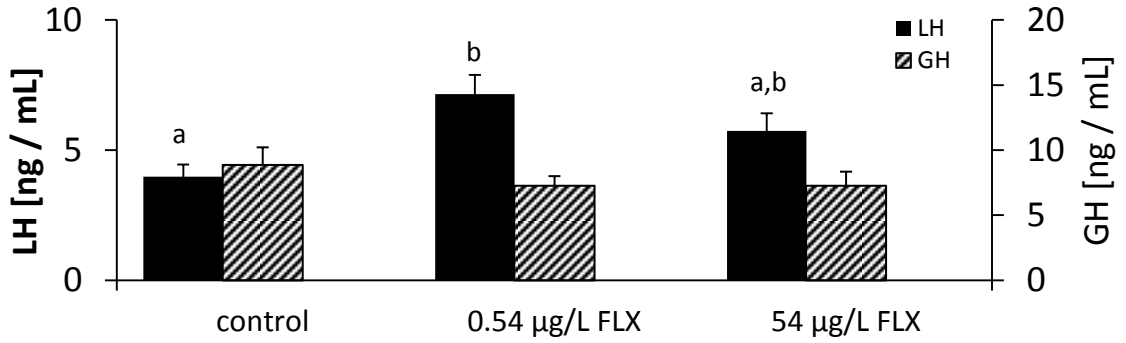


Fig. A1.2 *Relative serum concentration (±S.E.) of E_2 and T in female goldfish of the control group and groups exposed to 0.54 µg/L FLX and 54 µg/L FLX respectively. Data (n=6-8 per treatment group) were normally distributed or transformed to fit normal distribution and analyzed using a one-way ANOVA with Bonferroni-adjusted post-hoc tests.*

A.1.3.2. Fluoxetine increases serum LH at a concentration of 0.54 µg/L but does not alter parameters of FSH or GH.

Data for the serum concentration of LH was not normally distributed and analyzed using the Kruskal-Wallis test (df=2; H=6.27; $p \leq 0.05$). Serum LH of fish exposed to 0.54 µg/L FLX was significantly elevated in Bonferroni-adjusted Mann-Whitney U comparison to control fish (**Fig. A1.3A**; $p \leq 0.05$). No difference in serum GH was measured using a one-way ANOVA (**Fig. A1.3A**; df=2; F=0.14; $p > 0.05$). The mRNA abundance of *lh-ß* (df=2; F=0.31; $p > 0.05$), *fsh-ß* (df=2; F=1.47; $p > 0.05$) and *gh* (df=2; F=0.19; $p > 0.05$) in the pituitary was analyzed by one-way ANOVA and did not change significantly in FLX exposed fish compared to control (**Fig. A1.3B**).

A



B

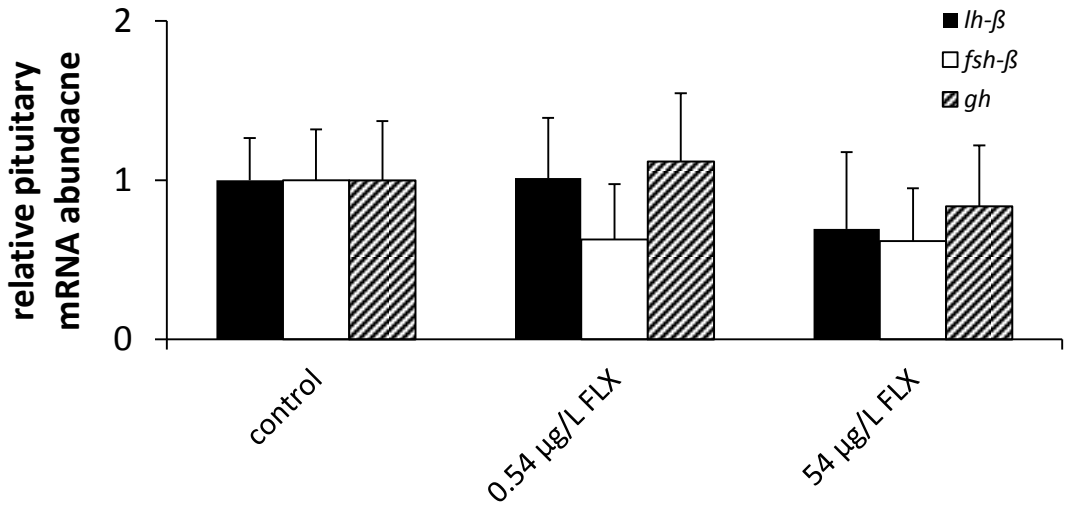


Fig. A1.3. *Assessment of the effect of FLX on serum hormone concentrations: Relative serum concentration (±S.E.) of LH and GH in female goldfish of the control group and groups exposed to 0.54 µg/L FLX and 54 µg/L FLX respectively (A). Relative mRNA abundance (±S.E.) of lh-ß, fsh-ß and gh in pituitaries of female goldfish of the control group and groups exposed to 0.54 µg/L FLX and 54 µg/L FLX respectively. An n=7 pituitaries were used for each group. Data for LH (n=14-15 per group) was non-parametric and analyzed using a Kruskal-Wallis test with Bonferroni adjusted Mann-Whitney U test for post-hoc analysis. Data were normally distributed and analysed using a one-way ANOVA.*

A.1.3.3. Waterborne FLX does not significantly alter mRNA abundance of the nonapeptides it and vt in the telencephalon

The mRNA abundance of *it* (df=2; F=0.06; p >0.05) and *vt* (df=2; F= 0.13; p >0.05) does not significantly change in female fish exposed to waterborne FLX at either 0.54 µg/L or 54µg/L.

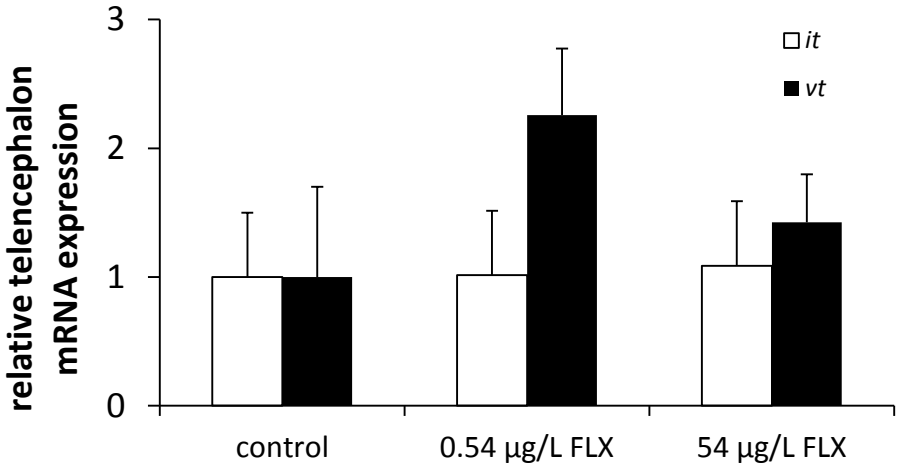


Fig. A1.4. *Relative mRNA abundance (±S.E .) of it and vt in the telencephalon of female goldfish of control group and groups exposed to 0.54 µg/L FLX and 54 µg/L FLX respectively. Data (n=6-7 per group) was transformed to fit a normal distribution and analysed using a one-way ANOVA.*

A.1.3.4. Waterborne FLX does not significantly alter mRNA abundance of hormone receptors in the ovary, but increases cyp19a mRNA abundance

The mRNA abundance of the hormone receptors *lhr* (df=2; F=0.04; p >0.05) and *ghr* (df=2, F=0.38 p >0.05) did not reveal significant changes in the ovary of female fish exposed to waterborne FLX concentrations of 0.54 µg/L and 54 µg/L when compared to control using a one-way ANOVA (**Fig. A1.5A**). The mRNA abundance of *fshr* (df=2; F=4.24; p <0.05) did not reveal a significant difference between control and FLX exposed fish (**Fig.A1.5A**). The

abundance of *cyp19a* in the ovary was not normally distributed and hence analyzed using a Kruskal-Wallis test (df=2; H 6.274; $p \leq 0.05$). The mRNA abundance of *cyp19a* increased significantly in ovaries of female fish exposed to 0.54 µg/L compared to control ($p < 0.05$).

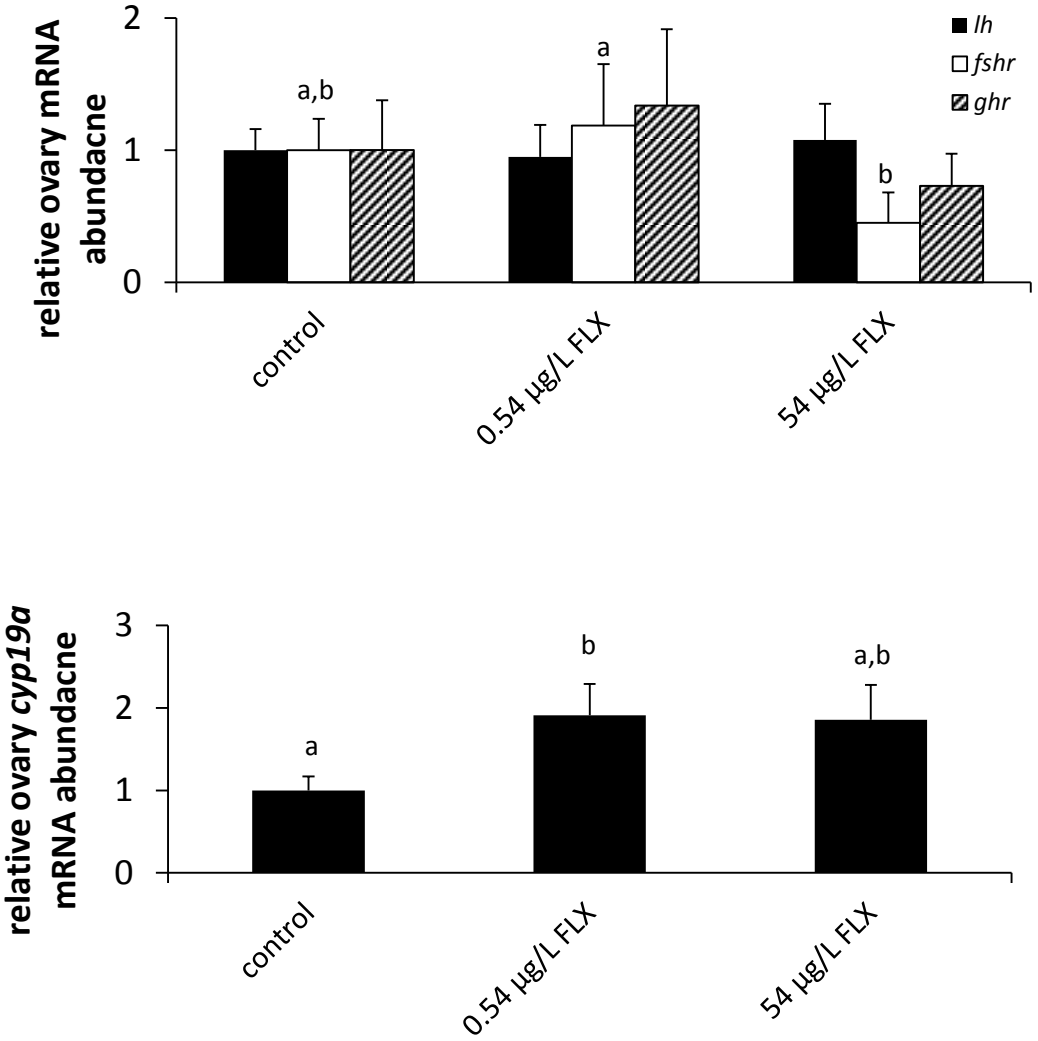
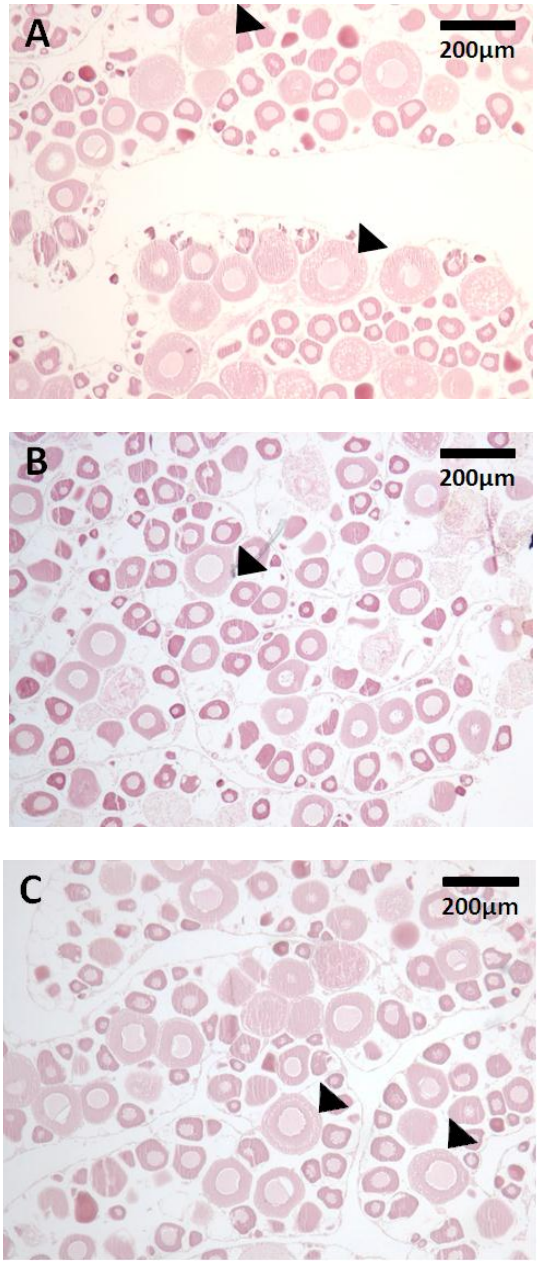


Fig. A1.5. *Relative mRNA abundance (±S.E.) of lhr, fshr and ghr (A) and cyp19a (B) in ovaries of female goldfish of the control group and groups exposed to 0.54 µg/L FLX and 54 µg/L FLX respectively. Data for lhr, fshr and ghr (n=7-8 per group) were transformed to fit a normal distribution and analysed using a one way ANOVA. A Bonferroni-adjusted post-hoc test was used for further analysis. Data for cyp19a (n=7-8 per group) was nonparametric and analysed using a Kruskal-Wallis test with Bonferroni-adjusted Mann-Whitney U post-hoc tests. Different letters indicate a significant difference at p ≤0.05.*

A.1.3.5. Waterborne FLX does not significantly alter histology or size of the ovary

Female goldfish did not reveal any significant changes in ovarian histology (**Fig. A1.6A-C**) or GSI (**Fig. A1.6D**; df=2; H=1.28; p >0.05). Presence of early vitellogenic follicles was detected in ovaries of control fish (**Fig. A1.6A**), as well as fish exposed to 0.54 µg/L FLX (**Fig. A1.6B**) and 54 µg/L FLX (**Fig. A1.6C**).



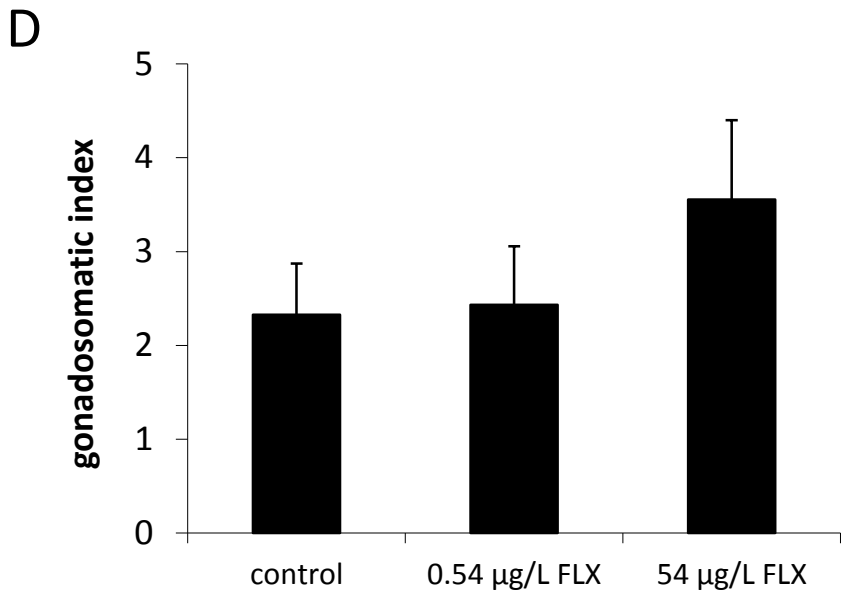


Fig. A1.6. *Investigation of ovaries (n=5 per group) development in female goldfish at the end of the exposure after 14 d: eosin stained 5µm slices of ovaries of control fish (A), fish exposed to 0.54 µg/L FLX (B) and 54 µg/L FLX (C), respectively. Black arrows indicate the presence of early vitellogenic follicles. The GSI (n=10) per group in female goldfish of the control group and groups exposed to 0.54 µg/L FLX and 54 µg/L FLX, respectively (D).GSI data was not normally distributed and analysed using a Kruskal-Wallis test.*

A1.4 Discussion

The results confirm the hypothesis that waterborne FLX decreases serum E_2 concentration in female fish. While this decrease is less severe than the observed decrease in female fish injected with FLX (chapter 3, 132) the observed decrease in fish exposed to 0.54 µg/L is significant and quantitatively similar to waterborne FLX induced decreases in another cyprinid species, the zebrafish (256). With regard to the proposed role of central IT the decrease of circulating E_2, it appears that at least at the mRNA abundance level, *it* is not correlated with the decrease in serum E_2. Likewise, assessment of hormone status does not implicate a centrally mediated effect, as no decreases were observed in either *lh-ß, fsh-ß or gh* mRNA abundance in the pituitary. Evidence indeed indicated evidence to the contrary, as serum LH was significantly increased in fish which revealed significantly decreased serum E_2 concentration. This may

indicate a disruption in feedback control, as decreases in E_2 are often correlated with uncoupling of LH release, as it is under negative feedback control from serum E_2 (355). The fact that ovarian *cyp19a* mRNA abundance was equally increased in this group may further argue for a disruption of this feedback mechanism, as LH is known to stimulate *cyp19a* mRNA abundance and activity in fish. Therefore the increase in ovarian mRNA abundance of *cyp19a* may be a response to restore homeostasis. The question of the exact level of the HPG axis at which waterborne FLX induces interference with this axis is still elusive, as this study failed to provide evidence that could integrate the proposed neuroendocrine disruption via the IT pathway (chapter 3, ref. 132) and disruption at the level of the ovaries (256). Given the observed increase in serum LH concentration, a peripheral effect may be plausible, which would subsequently result in reduced negative feedback and increased LH release, as observed in this study. In agreement with the idea of a peripheral effect of FLX, SSRIs have been shown to modulate circulating 5-HT concentrations in fish (134). At least in higher vertebrates a role for 5-HT in oocyte physiology has been proposed (356), and the possibility for potentially similar mechanisms in fish should be elucidated. With regards to eco-toxicology it is important to note that the effects of pharmaceuticals on non-target species in the environment may not follow linear dose-response relationships, as equally determined by Foran and colleagues (353).

Appendix 2: The physiological role of IT in feeding and growth

A2.1 Introduction

In the course of the investigation of the physiological role of IT in the goldfish, especially with regard to reproduction (chapter 4), I also investigated a potential involvement of IT in the regulation of feeding and growth in goldfish. This was due to the fact that the mammalian homologue oxytocin has been characterized not only as a potent stimulator of reproductive behaviour, but also as a potent anorexigenic satiety factor in the rat (357). Indeed, the mammalian nonapeptide hormone oxytocin has been proposed to serve as a switch between mutually exclusive feeding and reproductive behaviours in the rat (29). As not only reproductive (chapter 3,5,A1) but also feeding and growth parameters (chapter 6) were disrupted in FLX-injected and exposed goldfish, I further investigated the functional role of IT in teleost fish in the regulation of feeding and growth. The general hypothesis was that the function of IT would be anorexigenic, especially since other physiological effects, such as the regulation of reproductive behaviour, are known to be conserved in the oxytocin hormone lineage (358).

To specifically test this hypothesis, I designed two *in vivo* experiments in order to assess whether IT acts as an anorexigenic factor in fish. In the first experiment, goldfish were entrained to a feeding regiment and sacrificed at different time points around the scheduled feeding time. The specific prediction was that an increase in hypothalamic *it* mRNA abundance should occur in post-prandially sacrificed fish, in agreement with its putative role a satiety factor. In a second experiment fish were fed or fasted for 2 and seven days respectively. The prediction was that fasting status would reduce *it* mRNA abundance in the hypothalamus to stimulate feeding behaviour.

A2.2 Materials and methods

A2.2.1 Animals and in vivo experiments

Two different *in vivo* experiments were conducted to investigate an involvement of IT in the regulation of feeding in goldfish. In the first experiment, periprandial changes of *it* mRNA were investigated in goldfish entrained to a specific feeding regiment. For this experiment, sexually regressed male and female goldfish were purchased from a commercial supplier in July (Aleong's International Inc., Mississauga, ON, Canada) and maintained under natural simulated photoperiod. After 2 weeks of acclimatization, goldfish were separated into 4 groups of n=15 fish. Fish were maintained on a daily feeding regimen of 2% body weight of food pellets (Martin Mills, Elmira, ON, Canada) and were fed at 12:00 daily for 15 days. On day 16, goldfish were sacrificed 2 hours prior to scheduled feeding time, at scheduled feeding time and 2h past feeding. Hypothalamic tissue was collected, pooled (final n=7 per treatment) and stored at -80 °C until further processing.

In a second experiment using sexually regressed male and female fish, I investigated the effect of nutritional status on *it* mRNA abundance in the hypothalamus. Sexually regressed male and female goldfish were purchased from a commercial supplier in July (Aleong's International Inc., Mississauga, ON, Canada) and maintained under natural simulated photoperiod. After 2 weeks of acclimatization, goldfish were separated into 4 groups of n=15 fish. Fed fish were maintained on a daily feeding regimen of 2% body weight of food pellets (Martin Mills, Elmira, ON, Canada), while fasted fish did not receive any food. Fish from one fed and fasted fish group were sacrificed after 2 d and 7 d. Hypothalami were removed and pooled (final n=7 per treatment) and stored at -80 °C until further processing.

A2.2.2 Total RNA isolation, cDNA synthesis and real-time RT-PCR assays

The extraction of mRNA, synthesis of cDNA and real-time RT-PCR assay for the measurement of it mRNA were all conducted as described in chapter 3.

A2.3 Results

A2.3.1 Postprandial increase in hypothalamic it mRNA abundance

The mRNA abundance of hypothalamic *it* (df=2; F=3.99; $p \leq 0.05$) revealed a postprandial increase, that was significantly higher ($p \leq 0.05$) than the pre-prandial (-2h) *it* mRNA abundance (**Fig. A2.1**).

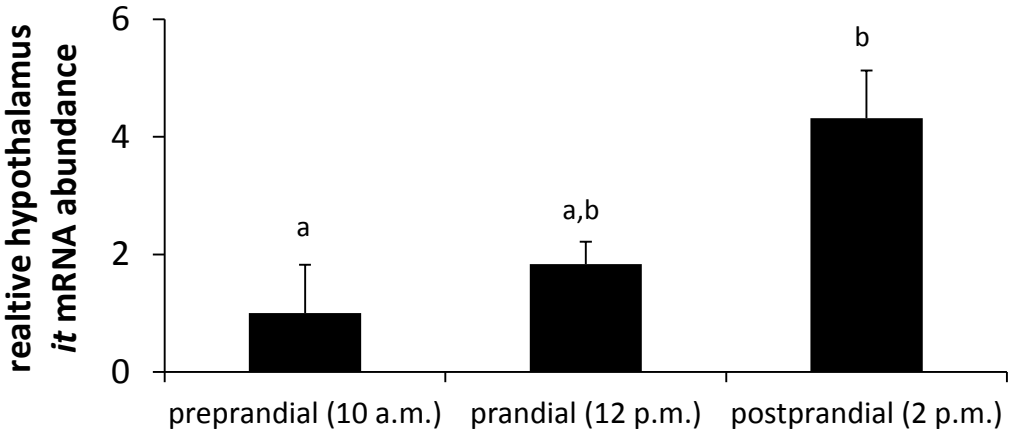


Fig. A2.1. *Periprandial relative mRNA abundance (±S.E.) of it in the hypothalamus in goldfish maintained on a regular feeding schedule for 2 weeks. Data (n=7 per group) was transformed to fit a normnal distribution and analysed using a one-way ANOVA, followed by Bonferroni –adjusted post-hoc tests. Different letters indicate a significant difference at $p \leq 0.05$.*

A2.3.2 Short term fasting decreases mRNA abundance of hypothalamic it

Hypothalamic *it* mRNA abundance was analysed using a two-way ANOVA. Abundance of *it* mRNA changed with treatment (df=1; F=7.128; $p \leq 0.05$), but neither time (df=1, F=1.722; $p > 0.05$) or an interaction between time and treatment (df=1; F=1.62; $p > 0.05$) were detected. Using a Bonferroni-adjusted post-hoc analysis, a significant decrease ($p \leq 0.05$) in goldfish fasted for 2 d but not 7 d when compared to normally fed controls was found (**Fig. A2.2**).

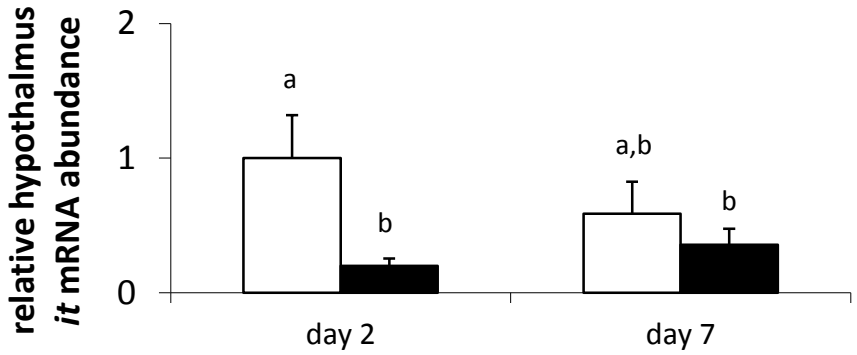


Fig. A2.2. *Relative mRNA abundance (±S.E.) of it in the hypothalamus of daily-fed and fasted fish for 2 d and 7 d. Data (n=7 per group) was normally distributed and analyzed using a two-way ANOVA, followed by Bonferroni-adjusted post-hoc tests. Different letters indicate a significant difference at p ≤0.05.*

A2.4. Discussion

A2.4.1 Isotocin inhibits food intake and may act as a satiety factor

The results support the hypothesis of a conserved inhibitory effect of IT on food intake in goldfish when compared to oxytocin in mammals (357). The observed inhibitory effect of IT on food intake is supported by the pattern observed for periprandial *it* mRNA abundance in the

hypothalamus. The postprandial increase in hypothalamic *it* mRNA expression supports a role for IT as a short term satiety factor. To my knowledge no direct periprandial measurement of oxytocin mRNA has been shown in mammals, but oxytocinergic neurons in the POA are activated post-prandially as determined by *c-fos* staining in the rat (359). In my experiment goldfish fasted for 2 d revealed a significant decrease in hypothalamic *it* mRNA abundance, implicating a promotion of feeding behaviour through the reduction of *it* mRNA. Conversely, increases in mRNA abundance of orexigenic factors such as *npy* have been described in the hypothalamus of fasted goldfish (307). In microarray studies using fasted rats (360) and fasted mice (361), respectively, a decrease in oxytocin mRNA abundance was measured in the subfornical organ and PVN, both hypothalamic areas that are believed to be important in food intake control.

Overall, this study provides evidence for an involvement of IT in the network regulating feeding in teleost fish, however more research is required to fully establish how IT is integrated in the network of peptides exerting control over food-intake and energy metabolism.

This is in line with the conservation of other physiological effects elicited by the conserved oxytocin-like peptides in vertebrates (204), such as osmoregulation (208) and reproductive behaviour (358). With regard to the observed FLX-induced inhibition of feeding by FLX in goldfish (described in chapter 6) evidence does not suggest an involvement of IT as an anorectic agent, as *it* mRNA abundance is decreased in FLX injections and waterborne exposure experiments.

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