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**The Effects of Fluoxetine Exposure on Growth and Metabolism in the Goldfish
*Carassius Auratus***

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The effects of fluoxetine exposure on growth and metabolism in the goldfish,

Carassius auratus

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Abstract

Fluoxetine (FLX), the active ingredient of Prozac™, is a selective serotonin re-uptake inhibitor (SSRI) and has been detected in sewage treatment plant effluents, surface waters, and in multiple fish species. One large gap in the literature exploring the effects of FLX is its effects on food intake and metabolism. The objectives of this study are to assess the role of FLX (540 ng FLX/L of tank water (high environmental level) and 54 µg/L) on food intake and whether FLX can cause effects on glycolytic metabolism unrelated to feeding in goldfish.

FLX did elicit food deprivation and weight change in the high dose group and this resulted in changes in glycolytic parameters including plasma glucose and hexokinase activity. FLX also directly affected several parameters irrespective of food intake including regulating plasma glucose, presumably through CRF, cholesterol concentrations, hepatic glycogen concentration and muscle hexokinase activity.

Résumé

Prozac™, ou la fluoxétine (FLX), est un inhibiteur sélectif de la recapture de la sérotonine, qui commence à apparaître dans les effluents des usines de traitement des eaux usées, dans les eaux de surface, et peut être détectée dans plusieurs types de poissons. Un trou dans la littérature sur les effets de la FLX apparaît au niveau des effets sur le métabolisme et l'apport alimentaire.

Les objectifs de cette étude sont d'étudier le rôle de la FLX (540ng/L [niveau environnemental élevé] et 54 µg/L) sur l'apport alimentaire et voir si la FLX peut causer des effets sur le métabolisme indépendamment de l'apport alimentaire sur les cyprins dorés.

L'apport alimentaire, le gain de poids, et la concentration du plasma en glucose et l'activité d'enzymes glycolytiques dans les muscles squelettiques furent tous affectés par le manque de nourriture causé par la FLX dans le groupe d'essai dose élevée. FLX affecta aussi directement plusieurs paramètres indépendamment de l'apport alimentaire. FLX joua un rôle dans la régulation de la concentration de cholestérol et de glucose dans le plasma, la concentration de glycogène hépatique, et l'activité musculaire de l'hexokinase.

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List of Abbreviations

5-HT	5-Hydroxytryptamine
CART	Cocaine-and amphetamine-regulated transcript
CRF	Corticotropin releasing factor
CS	Citrate synthase
E2	Estradiol
ER	Estrogen receptor
F-1,6-BP	Fructose 1,6 bisphosphatase
FLX	Fluoxetine
GCK	Glucokinase
GH	Growth hormone
GSI	Gonadosomatic index
HD	High dose
HK	Hexokinase
HSI	Hepatosomatic index
LD	Low dose
nFLX	Norfluoxetine
NPY	Neuropeptide Y
PAH	Polycyclic aromatic hydrocarbons
PCB	Polychlorinated bisphenols
PEPCK	Phospho <i>enol</i> pyruvate carboxykinase
pHD	Pair-fed high dose
PK	Pyruvate kinase
pLD	Pair-fed low dose
POMC	Pro-opiomelanocortin
PPCP	Pharmaceuticals and personal care products
QPCR	Real time - RT PCR
SERT	Serotonin transporter
SSRI	Selective serotonin reuptake inhibitor
STP	Sewage treatment plant

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Ottawa, September 2009

Jeanette Sassine

Chapter 1: Introduction

1.1 Rationale

Prozac™ is one of the most common prescriptions to treat depression worldwide. It has been widely distributed since 1986 and its use is prevalent in over 90 countries. Like many pharmaceuticals, some of it is excreted in the urine as a result of our inability to fully metabolize and utilize the drug. As a result it does arrive at urban sewage treatment plants (STP) where it is not fully removed leading to its release within the post-STP effluent. The active component of Prozac™ is fluoxetine (FLX), a synthetic chemical partially resistant to degradation, therefore current STP methods are unable to fully degrade this compound (along with many others). Since FLX is flushing through the STPs, it is beginning to appear in our aquatic environments and although FLX does degrade over time, our constant consumption and continuous release leads to its pseudopersistence in the environment.

FLX is a Selective Serotonin Re-uptake Inhibitor (SSRI) and its effects on serotonin and subsequent physiological and biological responses are well described in humans; however its effects, if any, are just now beginning to be described in aquatic organisms, including fish. It is well known that FLX in humans alters serotonin levels which cause a shift in emotions, decreases sexual drive and alterations in appetite. All three of these characteristics are normally tightly regulated in all organisms including aquatic organisms to achieve fitness and success in their environment.

In very recent years, research has examined the direct effects of FLX exposure on fish and other aquatic organisms. Results are not encouraging as most research shows that fish respond physiologically not unlike humans taking therapeutic doses of FLX. One significant gap

in research to date is the effects on growth and metabolism. My research focuses on examining the effects of FLX on these two factors in the common goldfish, *Carassius auratus*. Goldfish are a representative of the Cyprinidae, or the carp and minnow family, many of which are abundant in Canadian natural aquatic systems.

1.2 Pharmaceuticals in the Environment

Pharmaceuticals and personal care products (PPCPs) are considered to be emerging environment contaminants. A variety of PPCPs are found ubiquitously distributed in the aquatic environment (Daughton and Ternes, 1999; Kolpin *et al.*, 2002; Lange and Deitrich, 2002; Metcalfe *et al.*, 2003; Sanderson *et al.*, 2004; Mimeault *et al.*, 2005; Brooks *et al.*, 2005; Fent, 2006; Vasskog *et al.*, 2006). PPCPs are flushed down drains and pass through the sewage treatment plants without being fully degraded or removed. Human pharmaceuticals are specifically manufactured to produce a specific response in humans. For example, FLX, is used to treat depression by blocking the re-uptake of serotonin (Altamura *et al.*, 1994); gemfibrozil is used to lower lipids by increasing peroxisomal fatty acid oxidation by activating the peroxisome proliferator-activated receptor- α : and, ciprofloxacin is an antibiotic that inhibits DNA gyrase (an enzyme that supercoils DNA) in both gram positive and gram negative bacteria. Given these pharmaceuticals, along with many others are now in the environment, it is probable that some may affect non-target organisms. It is possible that drugs may affect fish as in humans or effects could be completely different from the desired response in target organisms. Some drugs affect fish similarly to humans because they are designed to affect specific cellular receptors/effectors and many of these are highly conserved between species (Fent, 2006). In other cases, receptors are not conserved and so responses from drug exposure may not be similar. In both cases there is

reason for concern and this has caused a surge of research in the past few years to evaluate the risks and concerns of the growing pharmaceutical concentrations found especially within the aquatic environments.

The average age of the world population is increasing and according to Statistics Canada (www.statscan.ca) the average age in Canada in 2001 was 37.6 and in 2008 was 39.4. As the population ages, logically the numbers of prescriptions will increase. According to IMS Health Canada (www.gdsourcing.com/works/IMSHealth.htm) the number of total retail prescriptions (including new and refills) in 2006 was approximately 380 million and in 2008 this number increased to 450 million. As a result of this increase, we are seeing higher levels and more pharmaceuticals appearing in the environment.

Pharmaceuticals primarily enter the environment by passing through the STP (Glassmeyer *et al.*, 2005). Pharmaceuticals are ingested and then excreted into the municipal sewage system arriving at STPs where they are not fully degraded and they either become attached to the sewage sludge and in some provinces spread on agricultural lands, or become part of the effluent stream and thus diluted into receiving streams (Jones-Lepp *et al.*, 2001; Weston *et al.*, 2001; Kolpin *et al.*, 2002; Vanderford *et al.*, 2003; Metcalfe *et al.*, 2003; Furlong *et al.*, 2004; Brooks *et al.*, 2004; Hua *et al.*, 2006). Other routes of exposure include improper disposal, veterinary use, aquaculture, industry and livestock (Daughton, 2004).

1.3 Chemical and Physiological Properties of Fluoxetine

Fluoxetine inhibits the re-uptake of serotonin from the synaptic cleft. Without the presence of FLX, serotonin or 5-HT (5-hydroxy-tryptamine) is released into the synaptic cleft by a pre-synaptic neuron. It then binds to 5-HT receptors found on the membranes of pre- and post-

synaptic neurons. Serotonin is able to bind to a variety of 5-HT receptors which can then initiate a series of responses. After serotonin is released it is taken-back into the pre-synaptic neuron by a serotonin transporter (SERT). Since serotonin is involved in many biological and physiological systems including regulating emotions, appetite, sexuality and behaviour, changes in serotonin levels or its binding potential may result in a wide degree of problems. Many such as depression and bulimia, occur because there is inadequate interaction between the released serotonin and the serotonin receptors. To overcome this problem SSRIs (such as FLX) were designed to block the SERT, thus blocking serotonin re-uptake back into the pre-synaptic neuron from the synaptic cleft (Ranganathan *et al.*, 2001).

The human pharmaceutical dose for Prozac™ ranges between 20 to 60 mg per day, depending on the person, condition and circumstances. FLX is metabolised in the liver by cytochrome P450 into its demethylated metabolite norfluoxetine (nFLX) (Fig. 1.1) (Rhang *et al.*, 1995; Hiemke and Hartter, 2000). Its structural similarity enables it to act as FLX; therefore, nFLX is an active metabolite (Hartke and Mutschler, 1993). It has been reported that 2-11% of the ingested dose of FLX is excreted in urine (Altamura *et al.*, 1994). Between excretion and improper medication disposal, FLX, along with many other PPCPs, are appearing in inflow waters to STPs. These plants use a variety of techniques to break-down waste; unfortunately FLX is resistant to hydrolysis and photolysis, therefore at least some of it along with nFLX passes into the effluent stream (Glassmeyer *et al.*, 2005).

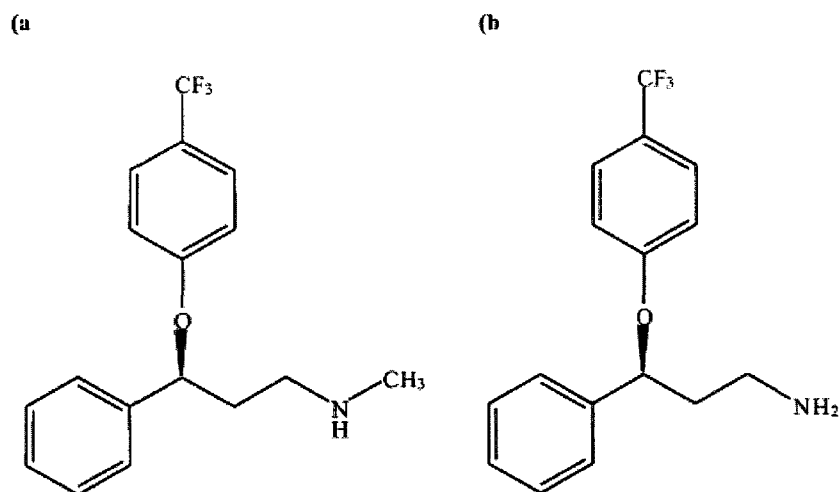


Figure 1.1. Chemical structures of a) fluoxetine and b) norfluoxetine (from Brooks *et al.*, 2003a)

Kolpin *et al.* (2002) analyzed water samples from 139 streams across 30 states within the United States of America between 1999 and 2000. The streams chosen were biased based on those downstream from intense urbanization and livestock production. They found FLX concentrations as high as 0.012 $\mu\text{g/L}$. Several aquatic locations were tested for a variety of SSRIs in Tromsø, Norway (Vasskog *et al.*, 2006). A pump station had a FLX concentration of 2.3 ng/L, three STP influents had concentrations of 0.4 ng/L, 1.3 ng/L and 2.4 ng/L, and two STP effluents had FLX concentrations of 1.2 ng/L and 1.3 ng/L; a third effluent had undetectable concentrations. Sewage from a variety of hospitals passed through the chosen STPs. Sampling was also done across the Great Lakes and rivers and streams in Canada where concentrations of FLX ranged from 13 to 540 ng/L (Weston *et al.*, 2001; Metcalfe *et al.*, 2003).

Physiochemical and environmental fate parameters of FLX and its metabolite nFLX were described by Brooks *et al.* (2003a). The chemical formula for FLX is $\text{C}_{17}\text{H}_{18}\text{F}_3\text{NO}$, and nFLX is $\text{C}_{16}\text{H}_{16}\text{F}_3\text{NO}$, with molecular masses of 309.33 and 295.3 g/mol, respectively. However, when

FLX is present in the environment, typically it is in the acid form increasing its molecular mass to 345.79 (Stanley *et al.*, 2007). Brooks *et al.* (2003a) also provide a range of bioconcentration factors (BCFs) based on pH. For FLX and a pH range of 2.0 – 11.0 the BCF range is ~1 – 1072, while for nFLX it is ~1-716. The K_{ow} of FLX is 4.05 and its solubility is 38.4 mg/L (Christensen *et al.*, 2007). Because of the physiochemical properties of FLX and its constant release into the environment, it does show pseudopersistence (Daughton and Ternes, 1999).

Many environmental contaminants including polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs) and a variety of PPCPs are chiral compounds and are distributed in the environment as racemic mixtures (Stanley *et al.*, 2007). FLX is one of these chiral compounds. Recently, Stanley *et al.* (2007) established the enantiospecific effects of S- and R-FLX (see Fig. 1.2). Previous evidence suggested a reason for concern because Wong *et al.* (1993) showed that R-nFLX, the demethylated metabolite of R-FLX, was 20-times less potent than S-FLX in rat brains. As a result, Stanley *et al.* (2007) hypothesized that S-FLX would be more toxic in *Daphnia magna* and the fathead minnow, *Pimephales promelas*. The endpoints assessed were immobilization, grazing rate and reproduction (*D. magna*), and survival, feeding and growth rates (*P. promelas*). As expected, S-FLX was more toxic to sublethal standardized and behavioural endpoints in the minnow. This is most likely due to the fact that the metabolite, S-nFLX was shown earlier (Wong *et al.*, 1993) to be more toxic. Similar results were not seen with *D. magna*. Stanley *et al.* (2007) propose that this differential response could be due to closer homology between the minnow (a fish) and the rat (a mammal) than rat and *D. magna* (a crustacean).

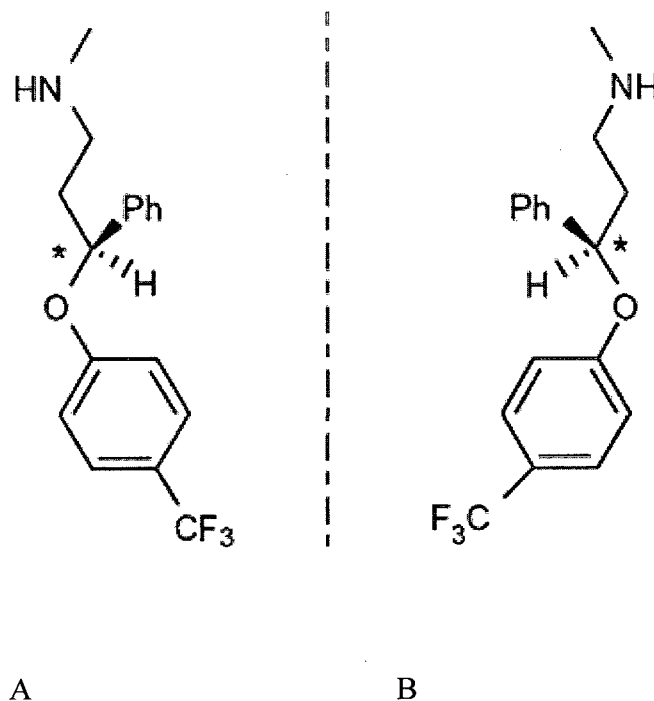


Figure 1.2. Structures of chiral compounds, S (A) and R (B) – fluoxetine (Stanley *et al.*, 2007)

1.4 Fluoxetine Effects in Aquatic Organisms

FLX is not only found in STPs and receiving streams, but it is shown to accumulate in aquatic vertebrates. Due to increasing evidence for the widespread occurrence of PPCPs in municipal effluent discharges and surface water, Brooks *et al.* (2005) monitored levels of several antidepressants in three wild fish species; *Ictalurus punctatus* (channel catfish), *Pomoxis nigromaculatus* (black crappie) and *Lepomis macrochirus* (bluegill). Two of the compounds measured were FLX and nFLX and the highest accumulation appeared in brain tissues, with concentrations of 1.58 ± 0.74 ng/g and 8.86 ± 5.9 ng/g, respectively. In liver, FLX and nFLX had concentrations of 1.34 ± 0.65 ng/g and 10.27 ± 5.73 ng/g, respectively and the final tissue

measured, skeletal muscle, had concentrations of 0.11 ± 0.03 ng/g and 1.07 ± 0.41 ng/g, respectively. These studies were done on fish taken from streams dominated by urban STPs. Brooks *et al.* (2003) presented a summary table of standardized toxicity tests for a variety of test organisms. The organisms presented were *Pseudokirchneriella subcapitata* (algae), *Ceriodaphnia dubia* (daphnid), *Daphnia magna* (crustacean), *Pimephales promelas* (fathead minnow), *Hyalella azteca* (amphipod) and *Chironomus tentans* (midge). Half-maximal effective concentrations (EC50), no observed effect concentrations (NOEC) and lowest observed effect concentrations (LOEC) were reported in this summary. EC50 values for the organisms listed were 24 µg/L, 234 µg/L, 820 µg/L, 705 µg/L, >43 mg/kg (in sediment) and 15.2 mg/kg (in sediment), respectively. The only NOEC value reported was for *C. dubia* at 56 µg/L. LOEC values were given for *P. subcapitata* and *C. dubia* at 13.6 µg/L and 112 µg/L, respectively and *H. azteca* and *C. tentans* at 5.4 mg/kg and 1.3 mg/kg, respectively. All of these numbers indicate that any sub-lethal or lethal effects would only be seen at concentrations which are higher to much higher than any reported in the environment.

FLX is found in STP effluents and accumulates in aquatic organisms, and so it is not surprising that significant research looking at the variety of effects reported in exposed organisms is published. Serotonin is involved in regulating many biological functions including emotions, aggression, appetite and sexuality and since its effects are so widespread, the presence of FLX may result in a multitude of problems for organisms.

1.4.1 Reproduction

It is known that serotonin affects reproduction in mammals (Hindberg and Neish, 1992; Cerda, 1998; Hernandez-Rauda *et al.*, 1999; Arcand-Hoy and Benson, 2001) and as a result, one

could predict that FLX exposure may affect reproduction in aquatic animals exposed to this drug. FLX regulates serotonin activity, and serotonin in return is implicated in stimulating the release of gonadotropins which in turn stimulate sex steroid synthesis, oogenesis development and vitellogenesis (Arcand-Hoy and Benson, 2001). FLX creates increased synaptic serotonin levels that could then induce stimulation in fecundity. Evidence does suggest that variations in serotonin levels correlate with female reproductive phases in humans (Hindberg and Naesh, 1992). Evidence also exists for this in other animals including fish (Somoza and Peter, 1991; Khan and Thomas, 1992; Senthikumaran *et al.*, 2001). This is especially true in seasonally reproductive animals because serotonin levels vary with reproductive potential and gonadal recrudescence (Hernandez-Rauda *et al.*, 1999).

Flaherty and Dodson (2005) looked at the acute and chronic toxicity of FLX using *Daphnia*. Specifically they examined a variety of toxicity factors including resting egg production, brood size and proportion of male broods produced (indication of stress and sexual reproduction). Results showed that chronic FLX exposure at 36 µg/L significantly increased *Daphnia* fecundity or brood size. They also found that a mixture of FLX at 36 µg/L with clofibrac acid (a fibrate drug) at 100 µg/L resulted in significant mortality.

Reproduction was also examined in the marine bivalve *Macoma balthica* by Honkoop *et al.* (1999) with FLX-exposure. In this species there is significant variation in the timing of reproduction which is observed from several aspects. At the microhabitat level, reproduction occurs within a few weeks; however across microhabitats and between geographical sites, reproduction varies significantly more and can occur as early as early February and as late as the end of August. For males, the ideal spawning temperature is between 7 and 14 °C. In order to test the effects of waterborne FLX on spawning, Honkoop *et al.* (1999) exposed the bivalves to the

drug more than one month after their normal spawning season. In 4 of the 5 replicates a significantly higher percentage of bivalves spawned than in the controls. Thus in the presence of FLX, the bivalves were able to reproduce much later in the season than those not exposed. Disruption of the timing of the reproductive period can affect fitness and survival of any organism particularly if the two sexes are not equally affected.

As noted above, serotonin induces the release of gonadotropins. A consequence of this is an increase in gonadal steroidogenesis and higher concentrations of circulating steroid hormones (Foran *et al.*, 2004). Serotonin also induces oocyte maturation in teleosts, including Japanese medaka (*Oryzias latipes*). Together with increased steroid levels, changes in gametogenesis may occur. To test this hypothesis, Foran *et al.* (2004) exposed Japanese medaka to FLX over a four-week period. They measured several aspects of reproductive competence including fecundity, fertility and hatching success. They also examined several physiological parameters including gonadosomatic index (GSI) and endocrine function including circulating steroid concentrations, gonadal steroidogenesis and hepatic vitellogenin concentrations. Their results demonstrated that environmental concentrations of FLX of 0.1, 0.5, 1 and 5 $\mu\text{g/L}$ had little effect on the physiological or reproductive parameters studied.

Recently Mennigen *et al.* (2008) reported that injections of a pharmacological dose of FLX (5 $\mu\text{g/g}$) into goldfish yielded a 35% increase in hypothalamic serotonin levels, in addition to a significant decrease in plasma E2 (estradiol). Examining changes in mRNA levels of estrogen receptors, ER α and ER β 1 in the hypothalamus and the telencephalon they found an expected decrease in ER β 1 in both the telencephalon and hypothalamus and a decrease in ER α in the telencephalon. Microarray data that was confirmed by real-time RT PCR also showed a decrease in the neuropeptide isotocin (Mennigen *et al.*, 2008), a key player in regulating sexual

behaviour in mammals (Goodson 2000 and Thompson, 2004) and reproduction in fish (Gozdowska *et al.*, 2006).

1.4.2 Aggression

Serotonin plays a role in regulating emotions and subsequent behaviour which is why SSRIs are designed to be used as antidepressants. Recently, several studies examined aggression in fish exposed to FLX. It is known that serotonin plays an inhibitory role in expression of aggression (Edwards and Kravitz, 1997, Nelson and Chiavegatto, 2001; Summers *et al.* 2005). Clotfelter *et al.* (2007) examined the effects of serotonin on aggression in the Siamese fighting fish *Betta splendens*. With the addition of serotonin (5-HT) and with the 5-HT_{1A} receptor agonist 8-OH-DPAT, the fish showed a decrease in aggressive behaviour. However they also used the 5-HT_{1A} receptor antagonist, WAY-100635, and did not see the expected increase in aggression. Although they showed that the addition of FLX did result in an increase in 5-HT, it did not yield the expected decrease in aggression; instead no change was observed. These conflicting results suggest that the role of 5-HT in aggression may be more complex than initially thought.

Perrault *et al.* (2003) examined aggression in the male coral reef fish, the bluehead wrasse *Thalassoma bifasciatum*. Typically, males aggressively defend their spawning territories and chase away intruders. In a lab setting, male fish were injected with FLX over several weeks and aggressive chases monitored in response to the introduction of an intruder fish. Results found that fish injected with FLX had the expected fewer aggressive chases than those injected with saline. The males injected with FLX had lower activity prior to intruder invasion. Prior activity did not correlate with aggressive chasing behaviour indicating that changes in aggression

were not solely based on reduction in behavioural display but with a direct aggression response. A similar experiment was simulated in the environment and, again, there was a decrease in aggression in those injected with FLX.

1.4.3 Feeding

Serotonin has long been thought to be involved in neural feeding pathways; indeed Prozac™ is often used to treat food intake disorders including bulimia. Research on the effects on feeding in aquatic animals exposed to FLX are surprisingly limited seeing how feeding is so important to ecological fitness. Hybrid striped bass showed a decrease ability to capture prey when exposed the FLX versus those not exposed (Gaworecki and Klaine, 2008). This decrease in ability (estimated as an increase in time to capture prey) is both concentration and duration dependent. Serotonin levels also correlated with this trend, and even after a six day recovery period (after a six day exposure period), exposed fish did not return to values found for control fish (Gaworecki and Klaine, 2008). Similarly milnacipran, a serotonin and norepinephrine re-uptake inhibitor was also shown to be an appetite suppressor in mice (Nonogaki *et al.*, 2007). Goldfish receiving multiple pharmacological injections of FLX showed a reduction in food intake after five days, and after 13 days had a 40% reduction in weight gain (Mennigen *et al.*, 2009).

1.4.4 Microcosm studies

Although single species toxicity tests yield significant information, a microcosm design can provide a more realistic overview of effects. In microcosm studies multiple species are placed in a very large tank with the surrounding biota resembling that of the natural habitat.

When measuring toxicity of pharmaceuticals, namely FLX, it is very difficult to conclude effects because in the natural environment there are many chemicals and pharmaceuticals present in the environment at one time and the ecosystem by definition is complex. Interactions can occur to create additive, synergistic or contrary effects, or possibly even more complex reactions. In a natural habitat there would also be a variety of species, including micro-organisms, which could alter the parent form of the chemical to create a more or less potent product. Also, it is entirely possible that although the presence of these drugs may not have a direct effect on fish, it may affect other levels within the hierarchy or the food web which would then affect fish in an indirect manner. All of these factors are important in evaluating the toxicity and the apparent risk of pharmaceuticals and chemicals in the aquatic environment.

Microcosm studies done with a variety of drugs in combination with FLX showed both lethal and sub-lethal effects at many hierarchical levels (Richards *et al.*, 2004; Johnson *et al.*, 2007). Species included in the microcosm by Richards *et al.* (2004) were juvenile sunfish (*Lepomis gibbosus*), phytoplankton, zooplankton, bacteria and a variety of plants including *Myriophyllum spicatum*, *M. sibiricum* and *Lemma gibba*. They used three different concentration-treatment groups based on environmental findings: low, medium and high. The doses in each treatment level for ibuprofen, ciprofloxacin and FLX were: 6 µg/L, 10 µg/L, 10 µg/L, respectively in the low treatment; 60 µg/L, 100 µg/L and 100 µg/L, respectively in the medium treatment; and, 600 µg/L, 1000 µg/L and 1000 µg/L, respectively in the high treatment. At the time of this study environmental concentrations for FLX and ciprofloxacin were unknown and so concentrations were estimated based on concentrations of similar pharmaceutical-types. Results varied across species, but sunfish appeared to be the most sensitive with 100% mortality in the high treatment group and nearly 50% mortality in the medium dose group.

Death was not an expected result. Richards *et al.* (2004) proposed that elevated synaptic serotonin levels may lead to constricted arterioles and this may lead to impaired gas exchange across the gills; hypoxia may ensue ultimately leading to fish death. It is also possible that synergistic effects occur between the pharmaceuticals. Richards *et al.* (2004) were unable to conclusive state this because they did not test each drug separately, one design problem with microcosm studies. A strong reaction was not observed in any of the other organisms within the microcosm. There was, however some mortality within the plant species and a significant decrease in zooplankton diversity.

A follow up microcosm study done by Laird *et al.* (2007) examined the effects of a mixture of SSRIs on zooplankton communities. Although there are hundreds of detected, and probably many more undetected, pharmaceuticals in the environment, SSRIs are ranked within the top 10 most toxic classes to algae (Sanderson *et al.*, 2004). The SSRIs tested by Laird *et al.* (2007) were sertraline, FLX, and fluvoxamine. Results did not show any significant variation in diversity or abundance among the zooplankton. Based on their results, they concluded that it is unlikely these pharmaceuticals would be significantly toxic to aquatic ecosystems.

1.5 The Role of Serotonin in Appetite

Serotonin is synthesized in neurons within the central nervous system and within the gastrointestinal tract and in particular the enterochromaffin cells (Foran *et al.*, 2004). The chemical and 3D-structure of 5-HT are presented in Figure 1.3.

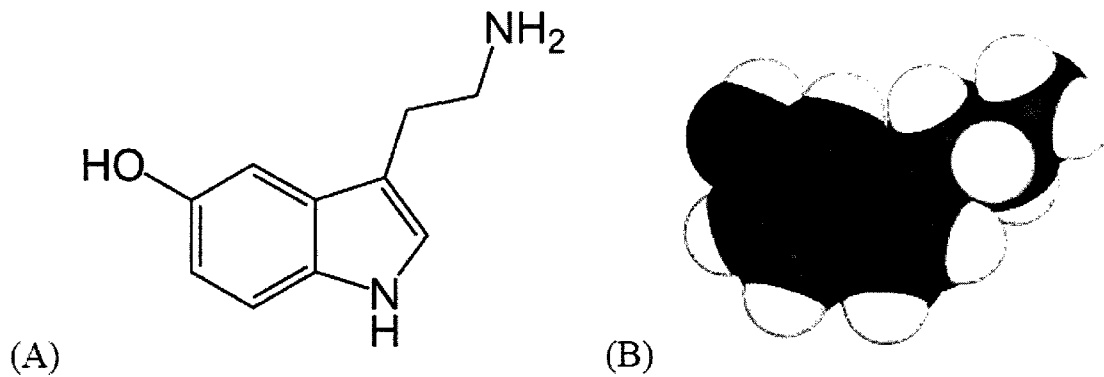


Figure 1.3. The chemical structure (A) and 3D-structure (B) of 5-HT

The hypophagic effects of FLX in humans are well known and the drug is prescribed for the treatment of obesity and bulimia. Not only is serotonin implicated in appetite control by increasing levels of 5-HT, but also injections of 5-HT receptor antagonists show an increase in food consumption as well as an increase in body weight (Leibowitz and Alexander, 1998). There are a variety of medial nuclei in the hypothalamus, including paraventricular, ventromedial and supra-chiasmatic, which are known to control normal eating patterns. Injections of serotonergic agents into these hypothalamic regions drastically shift eating behaviours (Leibowitz and Alexander, 1998). FLX injections also enhance energy metabolism by increasing lipid oxidation and decreasing carbohydrate oxidation thus repartitioning body energy reserves (Sakaguchi and Bray, 1989; Boschmann *et al.*, 1996). Significant evidence exists that show 5-HT injection into the paraventricular nucleus results in a decrease in carbohydrate consumption (Leibowitz and Alexander, 1998).

Serotonin does not act alone in modulating selective food consumption and appetite. Many components exist in this complex of central and peripheral controls of appetite. dePedro *et al.* (1998) examined the role of serotonin and corticotropin-releasing factor (CRF; also referred

to as corticotrophin-releasing hormone, CRH) in feeding patterns in goldfish (Fig 1.4). They demonstrated that increased neural 5-HT levels lead to increased CRF levels. To test the role of CRF in goldfish eating behaviours they employed a CRF antagonist, α -helical CRF; injecting this antagonist lead to the inhibition of anorexigenic behaviours supporting a role for CRF in modulating appetite. By creating a hypophagic response, CRF is known as an anorexic factor. The anorexic effect was not completely blocked which suggested other components are at play in addition to the involvement of selective 5-HT receptors (dePedro *et al.*, 1998). Using a serotonin and norepinephrine re-uptake inhibitor, milnacipran, Nonogaki *et al.* (2007) showed a decrease in hypothalamic transcripts for pro-opiomelanocortin (POMC) and cocaine-and amphetamine-regulated transcript (CART), key feeding factors in this part of the brain.

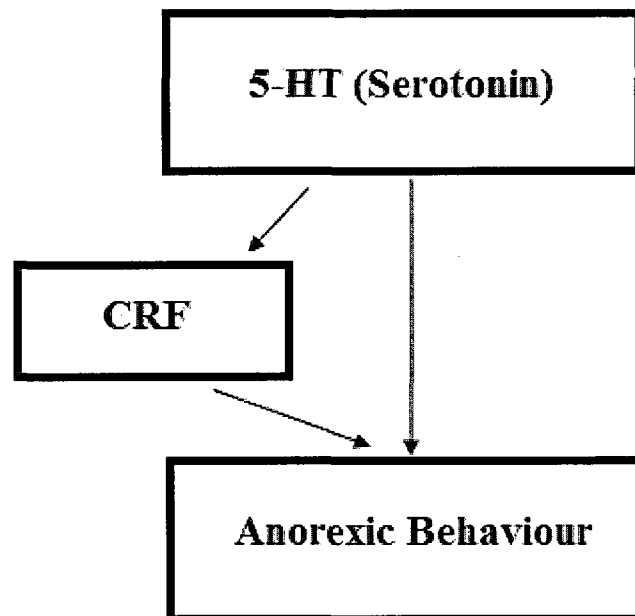


Figure 1.4. Serotonin leads to anorexic behaviour via anorexic factor CRF as described in dePedro *et al.* (1998).

Along with anorexic factors which decrease appetite creating a hypophagic response, there are also orexigenic factors which increase appetite creating a hyperphagic response (Fig. 1.5). It is thought that serotonin depletes these factors, thereby inhibiting appetite (Miura *et al.*, 2007). Ghrelin and orexin are two peptides in this category. Miura *et al.* (2007) reported that ghrelin and orexin are both implicated in eating behaviours, specifically in goldfish. Ghrelin is known to stimulate the release of growth hormone (GH) and gonadotropins (Miura *et al.*, 2007), resulting in an orexigenic effect. However, an increased concentration of synaptic serotonin inhibited ghrelin (via neuropeptides Y or NPY inhibition), thus inhibiting GH. However, Somoza *et al.* (1991) reported that increased serotonin levels stimulated gonadotropin release from the pituitary gland by the 5-HT_{2C} receptor subtype.

There are thirteen known serotonin receptors in mammals; 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-HT_{1E}, 5-HT_{1F}, 5-HT_{2A}, 5-HT_{2B}, 5-HT_{2C}, 5-HT₃, 5-HT₄, 5-HT_{5A}, 5-HT₆ and 5-HT₇. These receptors are found in blood vessels, the central nervous system, the gastrointestinal tract, platelets, the peripheral nervous system and smooth muscle. 5-HT receptors are G-protein coupled receptors (GPCRs) and ligand-gated ion channels. These receptors are seven transmembrane domain receptors that sense an outside signal to induce a signal transduction pathway via secondary messengers. This results in a cellular response which differs depending on which receptors were occupied. Many serotonin receptors are involved in feeding. To date 5-HT_{1A} (Carvalho *et al.*, 2004), 5-HT_{1B} (Ledonne *et al.*, 2009), 5-HT_{2A} (Carvalho *et al.*, 2004; Iizuka *et al.*, 2009), 5-HT_{2B} (Carvalho *et al.*, 2004) and 5-HT_{2C} (Nonogaki *et al.*, 2009) are all implicated in a feeding response.

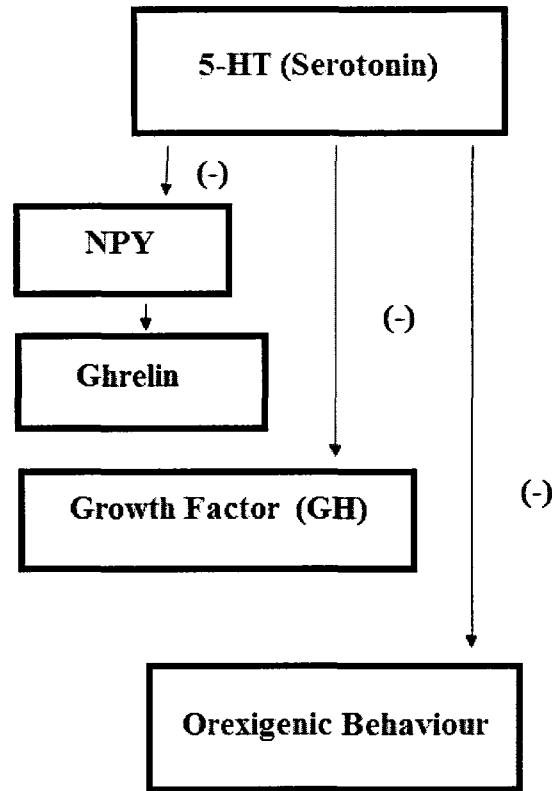


Figure 1.5. Serotonin inhibits orexigenic behaviours through the orexigenic factors NPY, ghrelin and growth factors.

1.6 Thesis Objectives and Hypotheses

There is significant evidence reporting the presence of FLX in the aquatic environment and that it can accumulate and possibly lead to increased mortality in aquatic organisms. Specifically, serotonin is known to play a vital role in modulating appetite through the control of orexigenic and anorexigenic factors and thus FLX is used to treat over-eating disorders such as bulimia and obesity by regulating serotonin levels. However, little research has been done exploring the effect of FLX on feeding behaviours of aquatic vertebrates. My research goals are to evaluate the effects of environmentally relevant levels of FLX on a common fish species, the

goldfish *Carassius auratus*. The first objective is to determine whether an exposure to FLX will limit food intake and growth in goldfish over a 28 day period. Brooks *et al.* (2005) confirmed the uptake of FLX by fish from the environment and since then Gaworecki and Klaine (2008) showed that hybrid striped bass show an inhibition of food intake when exposed to FLX and Mennigen *et al.* (2009) showed that goldfish food intake is also inhibited by FLX injection. I would predict that exposing goldfish to FLX will affect food intake, and ultimately re-organize metabolism and in particular glucose metabolism in order to compensate for reduced intake.

When food intake is restricted energy balance is disrupted and the organism including fish must compensate by mobilizing resources and adjusting metabolic enzymes. Given literature on fasted fish (Moon *et al.*, 1989; Cui and Wang 2007), I would predict the mobilization of glycogen and lipid stores and the breakdown of muscle protein. Muscle protein breakdown results in the release of amino acids so I would also predict an increase in plasma amino acids. Because of food restriction I would also predict a decrease of blood glucose and an activation of key glycolytic and gluconeogenic enzymes in order to maintain glycemia.

The second objective of this thesis is to determine whether or not FLX is directly acting upon food intake and metabolic parameters, or whether or not the metabolic changes are merely a reflection of food deprivation. In order to determine this I will set up pair-fed groups to either relate metabolic changes to food restrictions or directly to FLX exposure. FLX is known to directly affect serotonin and subsequently feeding peptides in fish (Lebowitz and Alexander, 1998; dePedro *et al.* 1998), and this could enhance specific metabolic endpoints involved with maintaining glycemia. At this point, the literature is extremely limited in regards to direct effects of FLX on metabolic parameters and it is unknown whether FLX will directly affect metabolic parameters irrespective of food intake.

Chapter 2: Materials & Methods

2.1 Fish

Male and female common goldfish, *Carassius auratus* were purchased from Aelong's International Inc (Mississauga, ON, Canada) in August 2008 and were allowed to acclimate in the University of Ottawa Aquatic Care Facility for more than one month. All tanks received dechloraminated City of Ottawa tap water at 18 °C and fish were held under a natural simulated photoperiod. Fish were fed commercial trout feed (details below). Goldfish were anesthetized using 3-aminobenzoic acid ethylester (MS-222; Aqua Life, Syndel Laboratories Ltd., Vancouver, BC, Canada) at a lethal dose for dissection (excess of 200 mg/L) and a lower dose for all handling procedures (150 mg/L). All experiments were conducted under a protocol approved by the University of Ottawa Animal Care Protocol Review Committee and adhere to the guidelines established by the Canadian Council on Animal Care for the use of animals in teaching and research.

2.2 Experimental design

Prior to experimentation fish were randomly distributed into six, 70 L tanks. In order to keep track of fish throughout the experiment, each fish was tagged by differential cutting of the dorsal, pelvic and caudal fins. Flow from all six tanks was shut off during a dosing experiment but aeration was achieved using an air stone in each tank. Tank 1, the control tank, was fed *ad libitum* daily with Martin Proficient Classic Floating Trout Grower 3 mm pellets (Martin Mills Inc., Travistock, ON, Canada) containing 44% crude protein, 0.45% sodium, 10% crude fat, 3.5% crude fiber, 0.9% calcium, 0.97% phosphorous, 6800 IU/Kg Vitamin A, 2100 IU/Kg

Vitamin D3, 320 IU/Kg Vitamin E. 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, this continued for 30 min or until the fish no longer ate the pellets. Two tanks, the low dose (LD) and high dose (HD) tanks were also fed *ad libitum* and intake was determined as described above. A 2 mg/mL stock Fluoxetine-HCl (FLX; Sigma Aldrich) in methanol was prepared according to Gaworecki and Klaine (2008) and suitable dilutions used to achieve the LD and HD tanks at a nominal concentration of 540 ng/L and 54 µg/L, respectively. The volume of total methanol in each tank (70L) was 1.89 mL in the HD tank and 19 µL in the LD tank which is well below toxic levels (Kaviraj *et al.*, 2004). The two doses of FLX were based upon the highest reported FLX environmental concentration (540 ng/L; LD) in STP effluents (reported by Weston *et al.*, 2001 in Brooks *et al.*, 2003b) and 100-times that concentration (HD), comparable to doses used in researching other aquatic organisms (Flaherty *et al.*, 2001). In addition to the LD and HD groups, there were also pair-fed groups which were fed the same amount as the LD and HD groups but these pair-fed groups were not exposed to the drug giving rise to pair-fed low (pLD) and pair-fed high (pHD) groups. A sixth group, the fasted group received no food and no FLX for the duration of the 28 day experiment. All tanks had twelve fish (20 ± 1.7 g) and based on half-life data of FLX in water (Paterson and Metcalfe, 2008), 50% of the water from each tank was renewed every 48 h. Half-life of FLX determined by Paterson and Metcalfe (2008) during the depuration phase using Japanese medaka was 9.4 ± 1.1 days. However, because fish could not be contained in a tank without water flow for more than 48 h due to build-up of waste products, renewal was done every 48 h. After 28 days all fish were harvested using an overdose of MS-222 (an excess of 200 mg/L). Blood was collected from the caudal vessels using a 1 mL heparinized syringe. Red blood cells were separated from plasma by centrifugation at 10,000 xg

(Beckman-Coulter microfuge R centrifuge) for 2 min. Liver and gonad mass were recorded.

Liver and skeletal muscle samples were removed and frozen on dry ice and stored at -80°C until used.

Muscle moisture content was determined by weighing a sample before and after dehydration by placing the muscle piece in an oven at 55°C for 48 h or until its weight did not change. Hepatosomatic index (HSI) was estimated by mass of whole liver divided by total body mass x100; gonadosomatic index (GSI) was estimated by mass of the whole gonad divided by total body mass x100. Weight gain was calculated by subtracting the initial weight from the final weight. These data were estimated for each individual fish used in the experiment.

2.3 Metabolite analyses

2.3.1 Amino acids

Amino acid levels in skeletal muscle and plasma were estimated according to Troll and Cannan (1953) by homogenizing muscle tissue in 50 mM imidazole buffer (1:10 wt/vol) using beads and a mixer mill (Retsch MM301, Newtown, PA. USA). Muscle homogenates were centrifuged at 10,000 xg at 4 °C for 10 min using a Beckman-Coulter microfuge R centrifuge; the pellet was discarded and the supernatant used to assess amino acid levels. The assay consisted of adding to individual test tubes either 250 µL leucine standard (0-100 µg/mL), 50 µL muscle supernatant + 200 µL water, or 10 µL plasma + 240 µL water. Samples were combined with 500 µL pyridine (in 0.01 M KCN), 500 µL phenol (42 mM phenol in absolute ethanol) and 100 µL ninhydrin (280 mM ninhydrin in absolute ethanol) in a boiling water bath (tubes capped with a marble) for 5 min followed by cooling to room temperature and diluting the mixture to 5

mL with 60% ethanol. Samples were pipetted into individual wells of a 96 well microtitre plate and read using a Spectramax Plus 384 Spectrophotometer (Molecular Devices, Sunnydale, CA, USA) at 570 nm. Concentrations were determined by generating a standard curve using the various concentrations of the amino acid leucine.

2.3.2 *BCA protein determination*

Tissue samples were homogenized in 50 mM imidazole buffer (1:10 wt/vol) using beads and the mixer mill and centrifuged as above. Samples were analyzed using the BCA (bicinchoninic acid) method according to Smith *et al.* (1985). Specifically, 10 μ L plasma, or 10 μ L of tissue homogenate was added to the wells of a 96 well microtitre plate followed by 200 μ L BCA-copper solution. The BCA-copper solution was prepared by mixing 10 mL BCA (Sigma) with 200 μ L copper(II)-sulphate solution (Sigma, C2284). The plate was incubated at 37 °C for 20 min and analyzed with a microplate spectrophotometer (see 2.3.1) at 550 nm. Concentrations were estimated from a standard curve using bovine serum albumin (BSA).

2.3.3 *Bradford protein determination*

Kinetic analyses (see 2.4.1, below) were undertaken on homogenates containing β -mercaptoethanol; this reagent interferes with the BCA protocol so protein determinations for these assays used the Bradford method with BioRad reagents. Ten μ L homogenates were added to a 96 well plate followed by 200 μ L of the prepared BioRad dye reagent solution (Hercules, CA, USA). The BioRad dye reagent solution was prepared by diluting the BioRad concentrate 4-times in stopping buffer (see 2.4.1, below). The plate was briefly incubated at room temperature and analyzed using a spectrophotometer (see 2.3.1) at 595 nm. Again, sample concentrations

were estimated against a BSA standard curve. The standard curve for this particular assay could not exceed 1 mg/mL because the BioRad protocol cannot read outside this range; this required significant and several sample serial dilutions prior to analysis.

2.3.4 Glucose

Blood plasma was tested for glucose content. Specifically, 10 μ L plasma was added to a 96 well microtitre plate followed 200 μ L of a solution cocktail containing 40 mM Tris-HCl, 60 mM Trizma base, 1 mM MgSO₄, 1 mM ATP, 2 mM NAD⁺, and 0.1 U/mL G6PDH (*Leuconostoc* G6PDH) and assessed spectrophotometrically at 340 nm (see 2.3.1) after a 20 min incubation; this initial reading served as the blank. To each well was added 10 μ L hexokinase (6 units of hexokinase to each well), and the plate was re-read after an additional 20 min incubation. Glucose concentration of each sample was determined against a D-glucose standard curve.

2.3.5 Glycogen

Liver samples were homogenized frozen in 6% perchloric acid (PCA) at 1:4 wt/vol on ice using a teflon pestle attached to a Wheaton Overhead Spinner. The homogenate was centrifuged as above at 11,000 xg for 5 min and the pellet discarded. One-hundred μ L of the acidified supernatant was placed in a tube containing 50 μ L 3 M NaHCO₃ and 1 mL amyloglucosidase solution (1 mg amyloglucosidase (Sigma) per mL in acetate buffer, pH 4.8). Tubes were placed in a shaking 37 °C water bath for 2 h and then terminated with 25 μ L 70% PCA. The samples were centrifuged briefly at approximately 7 000 rpm, and assayed using the above glucose protocol (see 2.3.4). Bovine glycogen was used to create a glycogen standard curve and values are reported as glycosyl units per g tissue.

2.3.6 Triglyceride

A kit (TECO Diagnostics, San Jose, CA USA) was used to determine plasma triglyceride concentration. The kit contained a prepared liquid reagent, which initiated a colorimetric reaction, and a triglyceride standard (200 mg/dL). Samples were assayed by plating 2 μ L plasma + 200 μ L of the liquid reagent in a 96 well microtitre plate. Samples were incubated at 37 °C for 10 min and read using a spectrophotometer (see 2.3.1) at 520 nm. Values were compared with a standard curve generated by the provided standard reagent.

2.3.7 Cholesterol

A kit (TECO Diagnostics) was used to estimate plasma cholesterol concentrations. The kit contained a prepared liquid reagent, which initiated a colorimetric reaction, and a cholesterol standard (200 mg/dL). Samples were assayed by plating 2 μ L plasma + 200 μ L of the liquid reagent in a 96 well microtitre plate. Samples were incubated at 37 °C for 10 min and read using a spectrophotometer (see 2.3.1) at 520 nm. Values were compared with a standard curve generated by the provided standard reagent.

2.4 Enzyme kinetic analyses

2.4.1 Sample Preparation

All liver samples (approximately 100 mg) were homogenized (1:10 wt/vol) with a stopping buffer consisting of 50 mM imidazole-HCl, 25 mM β -mercaptoethanol, 5 mM EDTA, 5 mM EGTA, 100 mM potassium fluoride, bovine glycogen (0.5 mg/mL buffer) and aprotinin

(0.02 mg/mL buffer), a protease inhibitor at pH 7.4 with the Wheaton Overhead Spinner on ice. Muscle samples were homogenized (1:5 wt/vol) with the same stopping buffer, with beads and a mixer mill (see 2.3.1). Sample homogenates were centrifuged at 10,000 xg for 10 min at 2 °C; the pellet was discarded. Supernatants were applied to a Sephadex G25 column (2 mL Sephadex pre-swollen in stopping buffer placed in a 3 mL plastic syringe with a cotton plug) at 100 µL/mL of column matrix to remove small molecular weight activators and/or inhibitors. Once the supernatant was added to the column, the column was centrifuged at 500 rpm using a Beckman CS-6 Centrifuge for 5 min at room temperature. Pre-column supernatants were used to determine activities of hexokinase (HK, EC 2.7.1.1), glucokinase (GCK, EC 2.7.1.2), citrate synthase (CS, EC 2.3.3.1) and phosphoenolpyruvate carboxykinase (PEPCK, EC 4.1.1.32); post-column effluents were used to determine pyruvate kinase (PK, EC 2.7.1.40). Activities were assessed using a microplate spectrophotometer (see 2.3.1) and SOFTmax Pro software was used to calculate individual activities. Activities were based either on tissue wet weight or protein content determined by the BioRad protocol (see 2.3.3). Activities from liver were expressed on a per protein basis because HSI varied between groups but protein concentration did not, and muscle activities were expressed on a per tissue basis because protein concentration varied between groups but moisture content in muscle did not.

2.4.2 Hexokinase/Glucokinase/Glucose-1-dehydrogenase

Samples were assayed for hexokinase (HK) and glucose-1-dehydrogenase (G1DH), and then calculated for glucokinase (GCK) according to the procedure of Tranulis *et al.* (1996) as modified by Panserat *et al.* (2000a). This assay is based on the reduction of NADP⁺ by glucose-6-phosphate dehydrogenase (G6PDH) and 6-phosphogluconate dehydrogenase (6PGDH). Since

two NADPHs are produced per mole of glucose utilized, the production of NADPH is related to glucose by dividing absorbancy by two. The assay was performed in 200 μ l of a buffered salt medium consisting of 8 mM $MgCl_2$, 1 mM KCl, 15 mM KH_2PO_4 , 20 mM $NaHCO_3$, 80 mM Tris, 4 mM EDTA, 2.6 mM dithiothreitol and 2 mM $NADP^+$, adjusted to pH 8.0, to which was added 0.1 U/mL G6PDH and 6PGDH just prior to the assay. Twenty μ l D-glucose was added to the assay mix in concentrations of 10 mM (final concentration = 1 mM) or 1.2 M (final concentration = 120 mM) to measure the activities of the low K_m HK and total HK, respectively. The reaction was initiated by adding 10 μ l tissue extract to the assay mix in a microplate well, and the assay allowed to proceed for 20 min at 37 $^{\circ}C$. Activities of G1DH were also measured by performing the assay as described above, but without adding G6PDH or 6PGDH to the assay mixture. Absorbancy (OD) values were measured using a microplate spectrophotometer (see 2.3.1) at 340 nm and 37 $^{\circ}C$ over the 20 min period. Glucokinase (GCK) activity was calculated by the difference in activities of the low K_m HK and 1/3 G1DH activity from total HK activity according to Panserat *et al.* (2000a).

2.4.3 Pyruvate kinase

Samples were assayed in a 50 mM HEPES buffer with 10 mM $MgCl_2$, 2.5 mM ADP, 1.2 mM NADH and 30 mM KCl, pH 7.2; assays were initiated by adding the substrate phosphoenolpyruvate (PEP) to the well. Activity ratios were assessed to determine whether differential regulation of PK had occurred as a result of the experimental conditions (Moon *et al.*, 1989; Perry *et al.*, 1988). Ratios were calculated as the activity at low substrate concentration [0.05 mM PEP] versus high substrate concentration [2.5 mM PEP]. Activity ratios were also assessed with the modulators alanine (inhibitor of PK; final 2 mM) and fructose-1,6-

bisphosphate (activator of PK; final 10 μ M). Samples were assayed with a spectrophotometer at 340 nm (see 2.3.1) at room temperature for 20 min.

2.4.4 *Phosphoenolpyruvate carboxykinase*

Samples were assayed with a 50 mM imidazole-HCl buffer containing 20 mM NaHCO₃, 0.5 mM phosphoenolpyruvate (PEP), 1 mM MnCl₂ and 0.12 mM NADH, pH 7.4. Activities were initiated with deoxyguanosine diphosphate (dGDP) in excess. Activities were measured using a 96 well plate and the spectrophotometer at 340 nm (see 2.3.1) at 37 °C for 20 min.

2.4.5 *Citrate Synthase*

Homogenates were prepared as described above (see 2.4.1) except using a 75 mM Tris-HCl buffer with 10 μ L/mL of 100X-diluted Protease Inhibitor Cocktail (Sigma, P2714). Blanks were assayed with a 75 mM Tris-HCl buffer (pH 8.0) with 0.1 mM DTNB and 0.5 mM oxaloacetate (OXA). Ten μ L homogenate and 200 μ L Tris-HCl buffer was added to each well and read at 412 nm (see 2.3.1) to determine basal activity. Activities were then assayed with the addition of 10 μ L acetyl-CoA (final concentration 0.3 mM in 50 mM Tris-HCl) at 412 nm at room temperature for 20 min.

2.5 Gene expression

Expression of genes was measured using real-time RT-PCR (QPCR). Total RNA was prepared using Trizol according to the manufacture (Invitrogen, Burlington, ON); the extracted RNA was DNase treated using the RNeasy® Micro Kit (Qiagen, Mississauga, ON). Total RNA quantity was assessed using the Nanodrop 1000 (Thermo Scientific, Wilmington, DE, USA) and

only RNA with a 260/280 ratio >1.9 was used for cDNA synthesis. First strand cDNA synthesis was prepared from 2 µg total RNA and 200 ng random hexamer primers (Invitrogen) using Superscript II RNaseH reverse transcriptase (SSII) as described by the manufacturer. Individual liver samples of five randomly selected fish per treatment group were used. Each QPCR reaction contained the following final concentrations: 20 ng first-strand cDNA template, 1X QPCR buffer, 3 mM MgCl₂, 150–600 nM gene specific primer, 0.5X SYBR green (Invitrogen), 200 µM dNTPs, 1.25 units HotStartTaq (Invitrogen), and 20 µM ROX reference dye, in a 25 µl reaction volume.. The forward and reverse primers were designed using Primer3 (<http://frodo.wi.mit.edu/>) software and are listed on Table 2.1.

Table 2.1 Primer sequences

Gene	Forward primer	Reverse Primer	Amplicon size (bp)
Phosphoenolpyruvate Carboxykinase	ACCTGCACCTGGAATC AAAC	CTCGTACACCAAAGGCA CAC	191
Glucokinase	GAACGACACAGTAGC CACCA	AGTTCACCATTGTCCCC AAA	187
Fructose-1,6-bisphosphatase	CTCTGTTGCGTTTGTG TGCT	TTGTTCAAGAGCGGTGA GTG	160
Hexokinase	GTAATGCAAGGAACC GGAGA	CACTACGGCTACAACGT CCA	250
Citrate synthase	TGCCAGAAATGCCAG CGCAT	AAGCTGCTCTCGCTGTT CAGAGC	451
18S	AAACGGCTACCACATC CAAG	CACCAGATTTGCCCTCC A	409

The thermal cycling program used was an initial 1 cycle Taq activation at 95 °C for 15 min, followed by 40 cycles of 94 °C for 15 s, 58–60 °C for 5 s, 72 °C for 30 s, and a detection step at 80 °C for 8 s. Dilutions of cDNA pooled from a subset of individual samples of different

treatment groups were used to construct a relative standard curve for each primer set. After the reaction was complete, a dissociation curve was generated starting at 55 °C (+1 °C/30 s). PCR reactions were performed using the MX4000 Multiplex Quantitative PCR system (Stratagene, La Jolla, CA, USA) and data were analyzed using the MX4000 Software Package. Standard curves relating initial template copy number to fluorescence and amplification cycle were generated using the amplified QPCR product from a pool of all individual samples (n=2 from each treatment, n=12 in total) as a template, and were used to calculate mRNA copy number in each sample. In all cases, only standard curves with slopes between -3.2 and -3.5 with R^2 values of >0.98 , were used. After each run, a random subset of QPCR products (total n=4) from control and treatment groups were pooled and purified using a Minelute Reaction Cleanup kit (Qiagen, Mississauga, ON) and sequenced with a CEQ™8000 Genetic Analysis System (Beckman Coulter, Mississauga, ON) to confirm specificity of the assays. Results were normalized to 18S, and then normalized to the control group set to 1.

2.6 Statistical analysis

Experimental results in most figures are presented as mean + standard error of the mean (SEM). Statistics were performed using SigmaPlot 11.0 software (Systat Software Inc., Chicago, IL, USA). In order to compare all six groups to each other, statistical significance was tested with one-way ANOVA, and in cases where the normality tests failed, one-way ANOVA on ranks was performed. The individual p-values considered to be significant are indicated in each figure legend. The appropriate post-hoc test was used for each data set; where sample sizes were not equal, Dunn's Method was used and in all other circumstances the Student-Newman-Keuls procedure was used.

Chapter 3 – Results

There were two primary objectives to this thesis. The first objective was to determine whether there is an effect of FLX on feeding, weight gain and glycolytic metabolism, and the second was to determine if any metabolic effects were specific to FLX, *per se*, or because of food restriction. Therefore, I have separated this section into two parts, the first evaluating whether exposure to a low (LD; 0.54 µg/L) or high (HD; 54 µg/L) doses of FLX elicits a change in growth and metabolism (section 3.1), and the second to determine if metabolic effects are due to FLX exposure *per se* and not simply an outcome of eating less food (section 3.2).

3.1 The effects of FLX on growth and metabolism

FLX administration results in an inhibition of food intake by altering serotonin levels and subsequently levels of feeding peptides (see Introduction). The regulation of serotonin during feeding and the impact of FLX on serotonin are well documented in humans and recently also in the goldfish (Mennigen *et al.*, 2009). To test whether FLX affects growth and metabolism, this section will only examine results seen in the control, fasted and dosed (LD, HD) groups.

Weight gain/loss was estimated by taking the original mass and final mass of each individual fish and expressing this final mass as a per cent change from the original. Results presented in Figure 3.1 demonstrate a significant decrease between the control (4% weight gain) and the high dose (HD) groups (7% weight loss). The low dose (LD) group was unchanged from the control group (no weight gain or loss). Finally, the fasted group showed a significant weight loss of 14% which was significantly different from all groups.

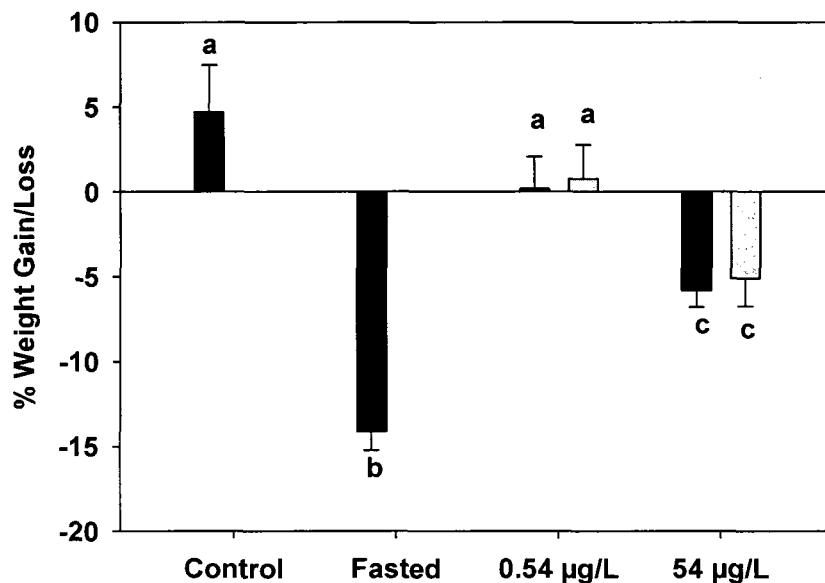


Figure 3.1. Per cent weight gain or loss of goldfish following a day 28 day exposure to FLX compared with control (*ad libitum* feeding) and fasted groups. The dark bars represent the four treatment groups and the grey bars represent the pair-fed groups. In this situation, 0 represents the weight on day 1; goldfish mass at day 1 was 20 ± 1.7 g. Data represent mean + SEM (n=12). Letters that differ indicate significant difference by one-way ANOVA on ranks ($p < 0.001$, followed by Student-Newman-Keuls procedure).

Food intake was measured each day for the control and the dosed groups. The measured percent food intake (compared to body weight) from the dosed groups was used to determine how much to give to their respective pair-fed groups. The LD group ate similarly to the control group but as noted in Figure 3.2 the HD group ate less than the control. Thus food intake at least in the HD group was reduced which at least in part supports the weight loss data for this group.

3.1.1 Morphometric data

On harvest day, day 28 of FLX exposure, several morphometric parameters were collected. Average skeletal muscle moisture content across the groups was unchanged at approximately 78% (Fig. 3.3A). The calculated hepatosomatic index (HSI) increased in the LD and HD groups (and their respective pair fed groups) compared with the control group (Fig. 3.3B), while HSI significantly decreased in the fasted group. The calculated gonadosomatic index (GSI) was unchanged within males and females across treatment groups (Fig. 3.4).

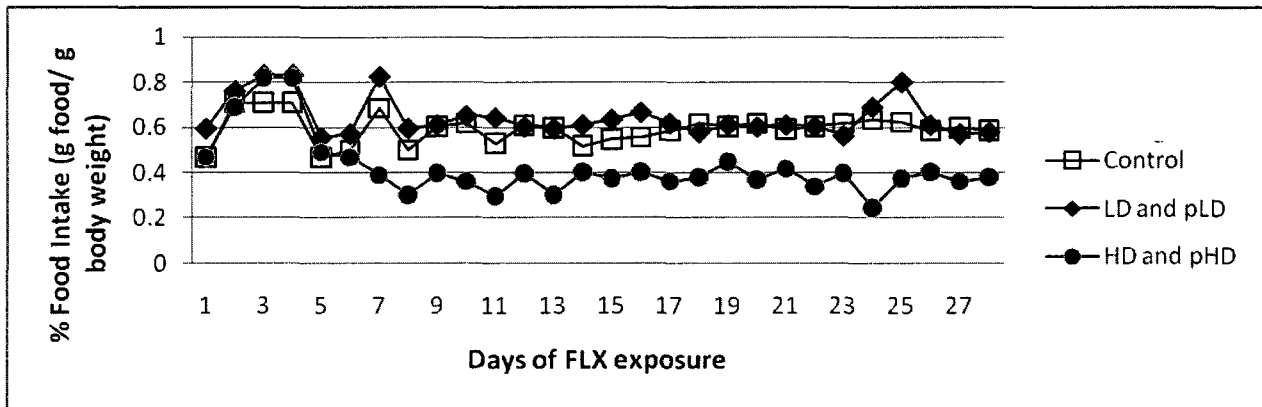
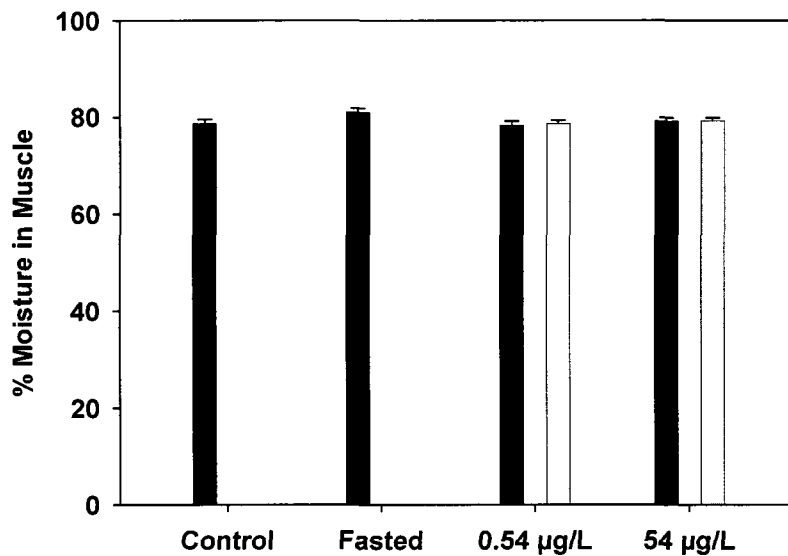
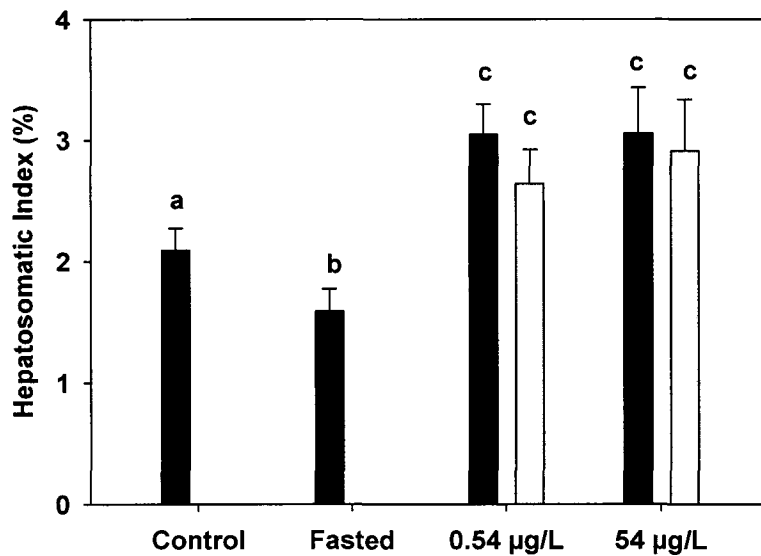


Figure 3.2. Daily measurements of food intake of Control (open squares), the low dose (LD; diamond), and the high dose (HD; circle) groups over the 28 day experiment. Percentage of food intake was measured in the dosed groups and then given to their respective pair-fed groups (pLD, pHLD).



A



B

Figure 3.3. Muscle moisture content (A) and hepatosomatic index (B) taken from male and female goldfish after a 28 day exposure to 0 (Control), 0.54 or 54 µg FLX per L of tank water or fasted. The dark bars represent the four treatment groups and the grey bars the pair-fed groups for each FLX exposure. Data represent mean + SEM (n=12). Letters that differ indicate significance differences by one-way ANOVA on ranks ($p < 0.001$, using the Student-Newman-Keuls procedure).

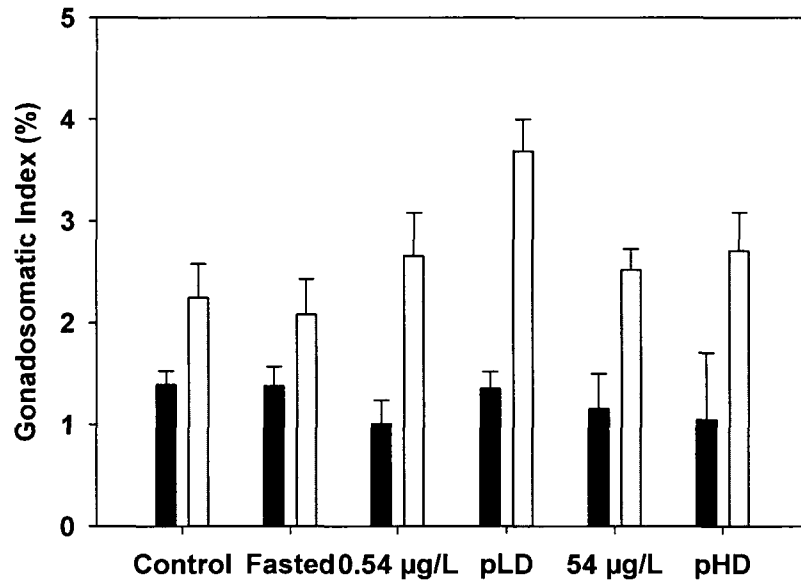
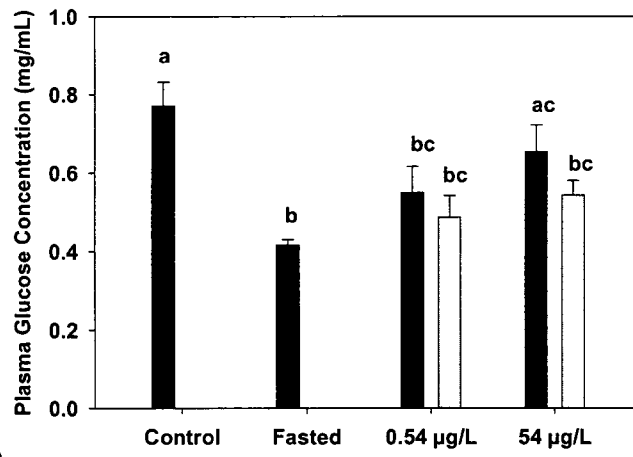


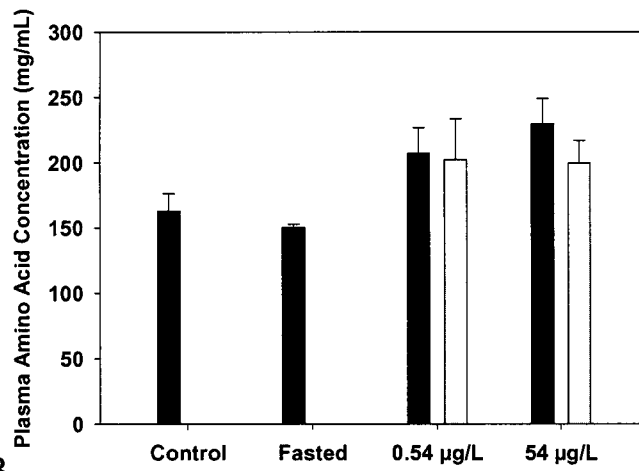
Figure 3.4 Gonadosomatic index (GSI) of male and female goldfish following a 28 day FLX exposure of the six different treatment groups. Dark bars represent male fish and grey bars represent female fish in each group; pLD and pHD represent pair-fed dose groups. Data represent mean + SEM (n=12). No significant differences were found within males or females across treatments using one-way ANOVA.

3.1.2 Metabolite data

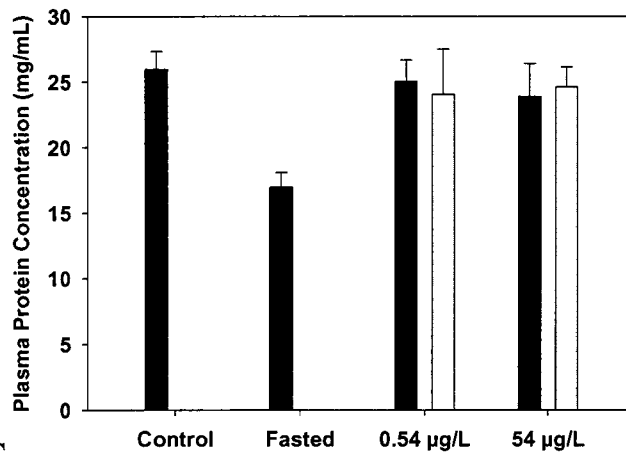
Plasma samples were analysed for glucose, protein, amino acid, cholesterol and triglyceride concentrations. Glucose concentrations were significantly decreased in the LD and fasted groups compared with the control group; the HD group was unchanged from the control (Fig. 3.5A). Plasma amino acids increased in the HD group (Fig. 3.5B) and protein (Fig. 3.5C) concentrations were not significantly changed in any group. Few significant changes occurred in plasma cholesterol or triglycerides with the exception of a higher cholesterol value in the HD group (Fig. 3.6A) and a significant decrease in plasma triglycerides in the fasted group compared with all other treatment groups (Fig. 3.6B). Protein content was unchanged by treatment in the liver of goldfish (Fig. 3.7A). Hepatic glycogen concentration was estimated and converted to glycogen content per total liver based upon changes noted in HSI (see Fig. 3.3B). The LD and HD groups had significantly higher glycogen content than the fasted but not the control groups, but no differences were seen in glycogen concentration across the groups (Fig. 3.7B). Muscle samples demonstrated non-significant increases in amino acid content in the dosed groups (Fig. 3.8A) while protein content decreased significantly only in the fasted group (Fig. 3.8B).



A

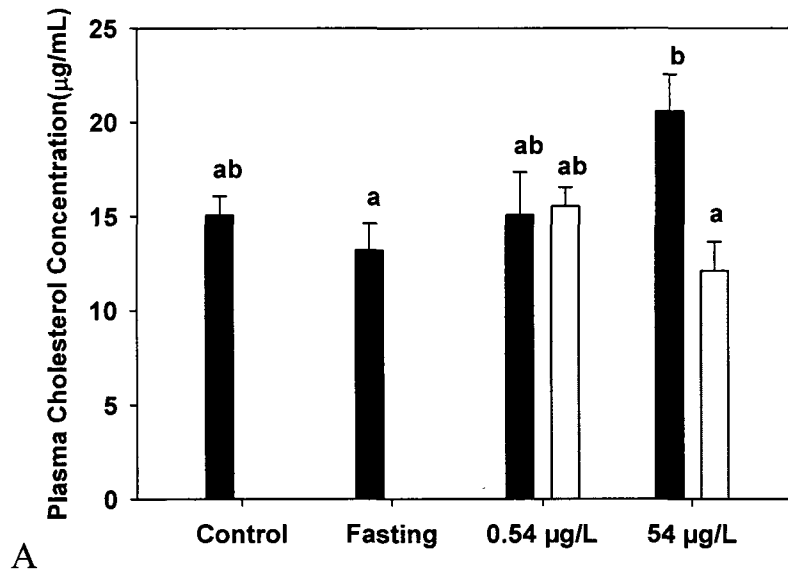


B

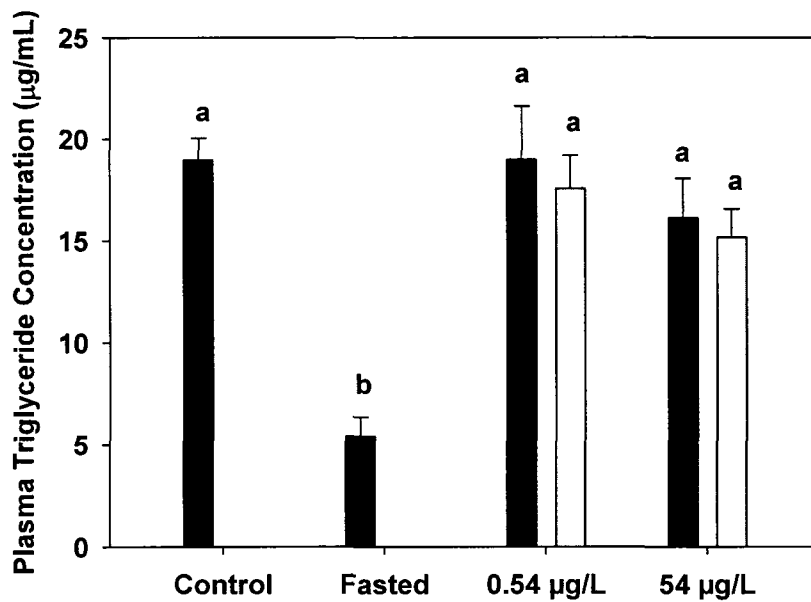


C

Figure 3.5. Male and female goldfish plasma glucose (A), amino acids (B) and protein (C) concentrations following a 28 day exposure to FLX compared with control (*ad libitum* feeding) and fasted groups. Dark bars represent the four treatment groups and grey bars represent the pair-fed groups. Data represent means + SEM (n=7, 5 and 5, respectively). Letters that differ indicate significant difference using a one-way ANOVA on ranks ($p < 0.001$, followed by Student-Newman-Keuls procedure).



A



B

Figure 3.6. Male and female goldfish plasma cholesterol (A) and triglyceride (B) concentrations following a 28 day exposure to FLX compared with control (*ad libitum* feeding) and fasted groups. Dark bars represent the four treatment groups and grey bars represent the pair-fed groups. Data represent mean + SEM (n= 6-11 for triglycerides; n= 7-11 for cholesterol). Letters that differ indicate significant difference using one-way ANOVA ($p < 0.001$ for triglycerides; $p = 0.006$ for cholesterol; using Dunn's post-hoc test).

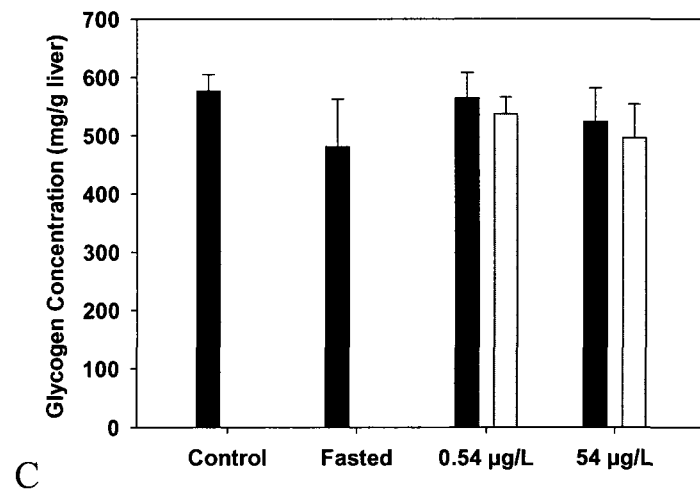
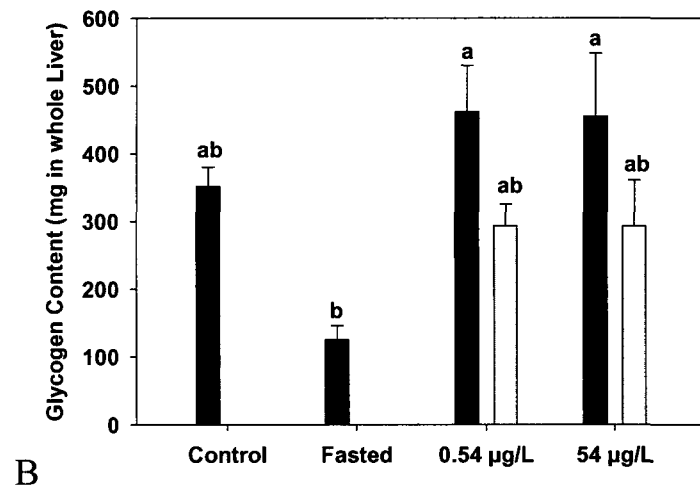
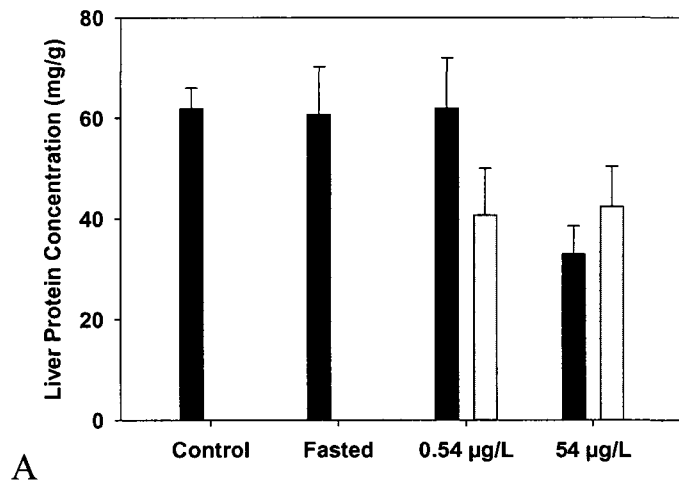


Figure 3.7. Male and female goldfish liver protein (A), glycogen content (B) and glycogen concentration (C) following a 28 day exposure to FLX compared with control (*ad libitum* feeding) and fasted groups. Dark bars represent the four treatment groups and the grey bars represent the pair-fed groups. Data represent mean + SEM (n=8 for protein; n=6 for glycogen). Letters that differ indicate significant difference using one-way ANOVA ($p \leq 0.013$, followed by Student-Newman-Keuls procedure).

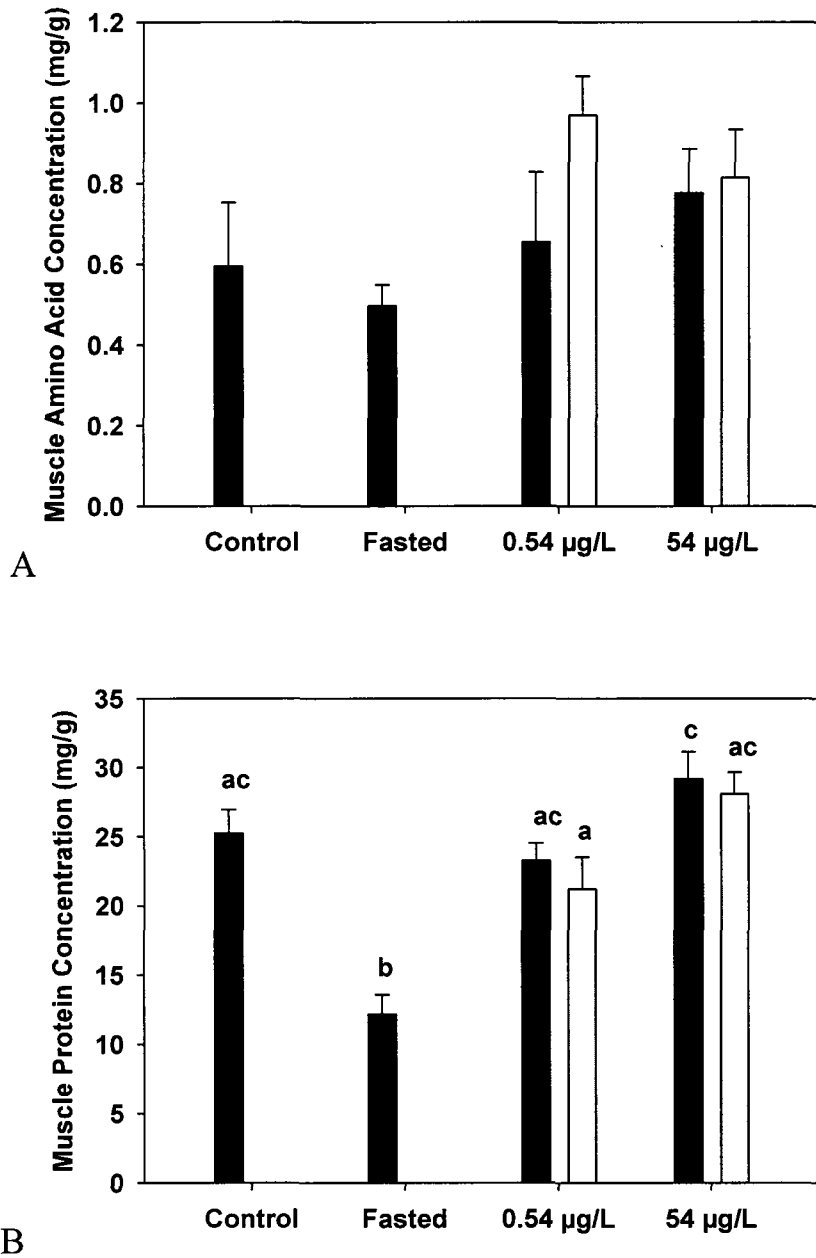


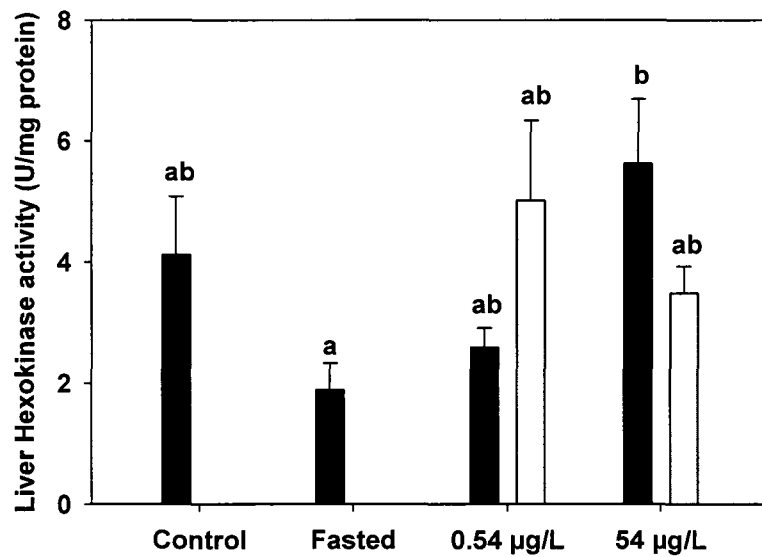
Figure 3.8. Male and female goldfish muscle amino acid (A) and protein (B) concentrations following a 28 day exposure to FLX compared with control (*ad libitum* feeding) and fasted groups. Dark bars represent the four treatment groups and the grey bars represent the pair-fed groups (see Fig. 3.1). Data represent mean + SEM (n=8). Letters that differ indicate significant difference using one-way ANOVA on ranks ($p < 0.001$, followed by Student-Newman-Keuls procedure).

3.1.3 Enzyme activities

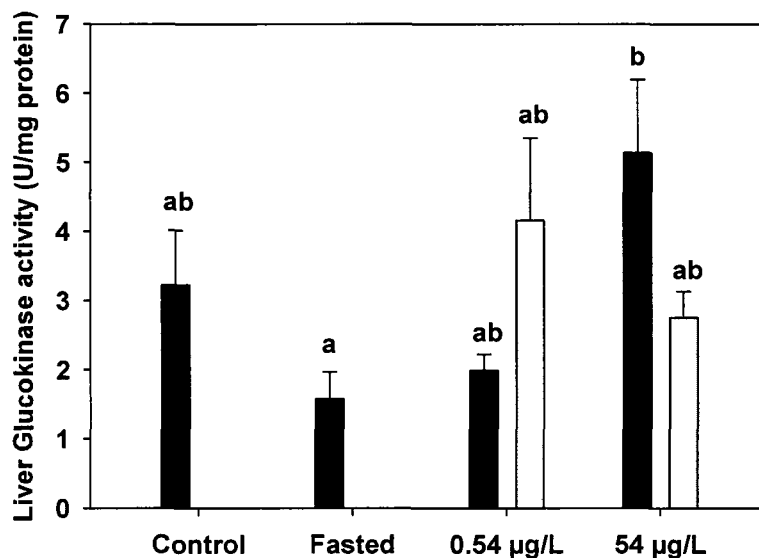
HSI changed between groups so enzyme activities from liver samples are presented as units per mg protein (U/mg protein) as protein did not significantly change. Muscle tissue protein concentration changed between groups but moisture content did not so enzyme activities are described as units per mg wet weight (U/mg wet wt).

Hexokinase (HK) and glucokinase (GCK) activities were relatively constant across groups in the liver with the exception of a decrease in the fasted group (Fig. 3.9). PEPCK and citrate synthase (CS) activities did not change between groups (Fig. 3.10). Pyruvate kinase (PK) activity was measure at optimal activities and to determine whether the various treatments changed the regulation of PK activity ratios between low and high concentrations of PEP were also calculated (Table 3.1). These ratios were also calculated in the presence of two modulators, alanine a negative modulator, and fructose-1,6-bisphosphate (F-1,6-P2) a positive modulator of PK. No statistical differences were noted in either liver PK activities or activity ratios between groups.

Optimal activities of skeletal muscle PK decreased in the fasted group and all activity ratios whether with alanine or F-1,6-P2 or neither increased compared to the other groups (Table 3.1). Muscle tissue HK activities were significantly changed by the various treatments (Fig. 3.11A). Activities increased in the LD and fasted groups compared with the control while activities decreased in the HD group again compared with the control. Glucokinase expression and activities are typically not found in muscle tissue (Blin et al., 1999; Panserat et al., 2000a) and was not assayed and no PEPCK activity was detected in muscle. Citrate synthase activities showed a significant decrease in the muscle of the fasted compared to all other groups (Figure 3.11B).

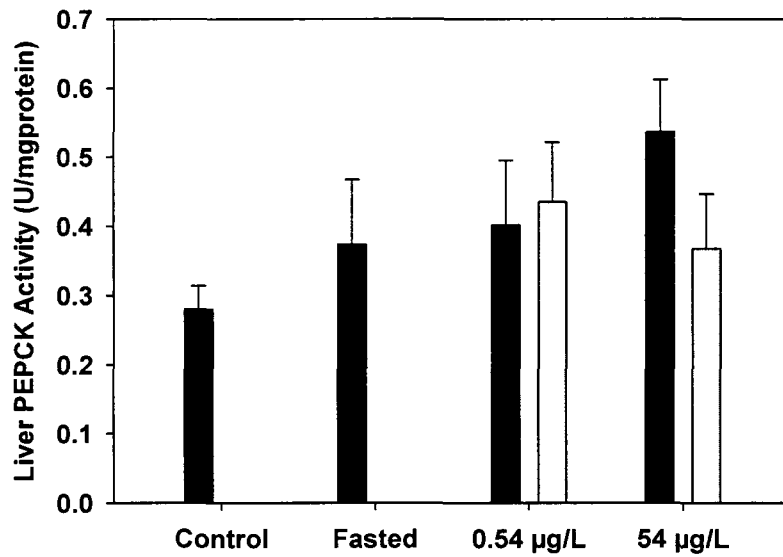


A

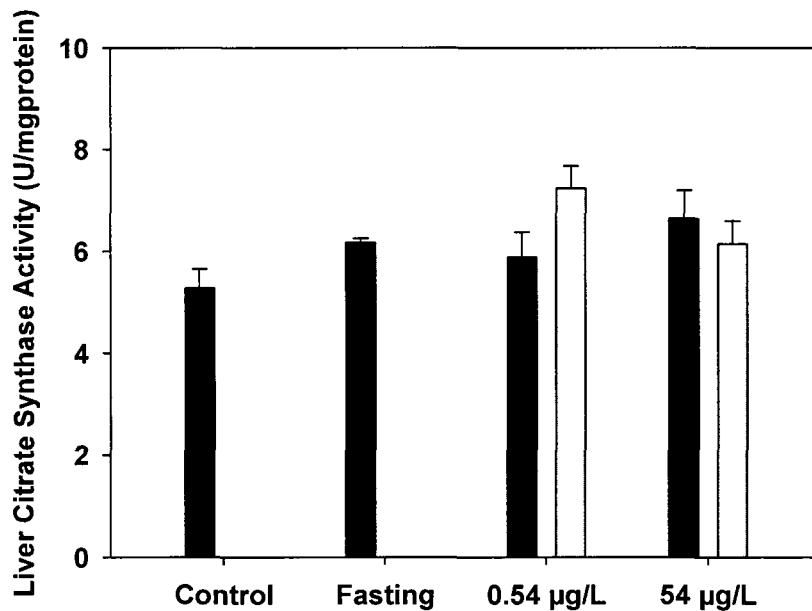


B

Figure 3.9. Hepatic hexokinase (A) and glucokinase (B) activities following a 28 day exposure to FLX compared with control (*ad libitum* feeding) and fasted groups in male and female goldfish. Dark bars represent the four treatment groups and grey bars represent pair-fed groups (see Fig. 3.1). Data represents mean + SEM (n=7-8). Letters that differ indicate significant differences by one-way ANOVA on ranks ($p < 0.05$, using the Dunn's Method).



A

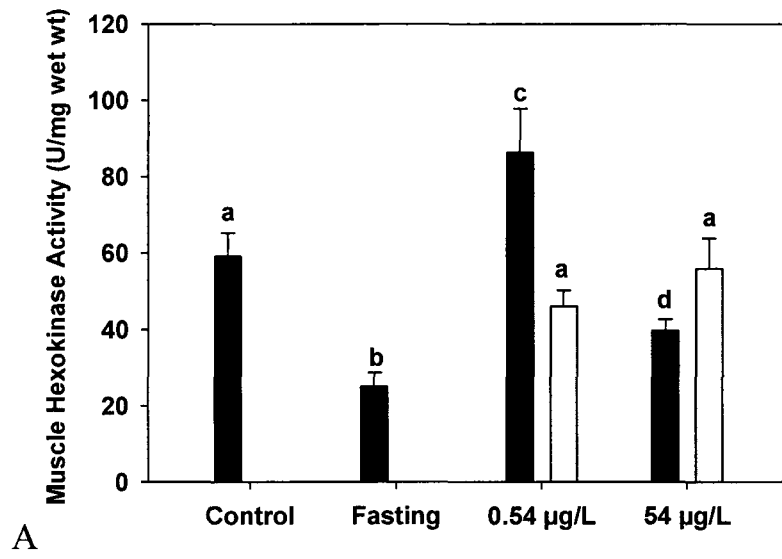


B

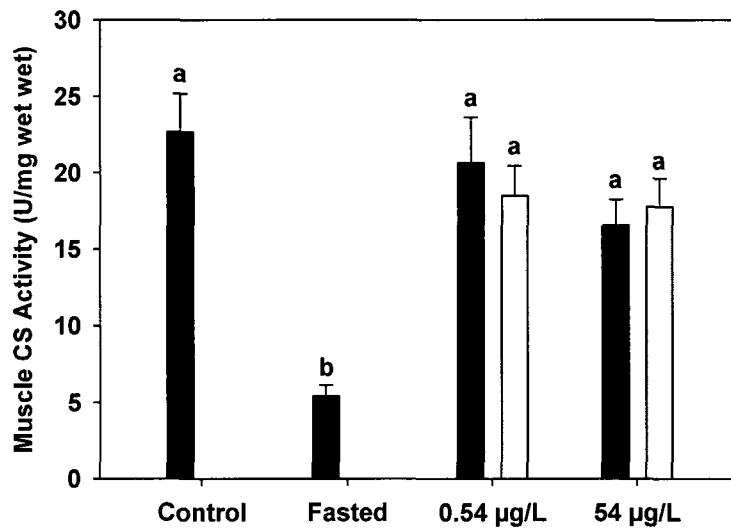
Figure 3.10. Liver phosphoenolpyruvate carboxykinase (PEPCK) (A) and citrate synthase (B) activities following a 28 day exposure to FLX compared with control (*ad libitum* feeding) and fasted groups in male and female goldfish. Dark bars represent the four treatment groups and grey bars represent pair-fed groups (see Fig. 3.1). Data represents mean + SEM (n=8). Letters that differ indicate significant differences by one-way ANOVA on ranks (p=0.001 in muscle, p=0.003 in liver, using the Student-Newman-Keuls procedure).

Table 3.1 Pyruvate Kinase activity and activity ratios (see 2.4.3) in liver and muscle tissues following a 28 day exposure to FLX compared with control (*ad libitum* feeding) and fasted groups in male and female goldfish. Letters that differ indicate significant differences based on one-way ANOVA [(6 separate ANOVAs were performed, one for each activity ratio), (n=7-8), $p < 0.05$ followed by Dunn's Method].

	Control	Low	Pair Low	High	Pair High	Fasting
Liver Tissue						
Optimal Activity (U/mg protein)	3.26 ± 0.90	4.26 ± 1.21	2.92 ± 1.22	4.86 ± 1.21	2.17 ± 1.00	3.57 ± 0.90
Activity Ratio	0.37 ± 0.04	0.32 ± 0.03	0.39 ± 0.1	0.28 ± 0.04	0.26 ± 0.03	0.49 ± 0.10
Activity with Alanine	0.34 ± 0.05	0.32 ± 0.03	0.39 ± 0.06	0.28 ± 0.05	0.29 ± 0.05	0.46 ± 0.05
Activity with F-1,6-BP	0.56 ± 0.03	0.49 ± 0.04	0.48 ± 0.04	0.56 ± 0.06	0.56 ± 0.07	0.52 ± 0.06
Muscle Tissue						
Optimal Activity (U/g wet wt)	0.13 ± 0.03 ^a	0.17 ± 0.07 ^a	0.12 ± 0.03 ^a	0.09 ± 0.02 ^{ab}	0.14 ± 0.05 ^a	0.04 ± 0.02 ^b
Activity Ratio	0.33 ± 0.11 ^a	0.35 ± 0.07 ^a	0.40 ± 0.03 ^a	0.40 ± 0.07 ^{ab}	0.34 ± 0.05 ^{ab}	0.74 ± 0.10 ^b
Activity with Alanine	0.25 ± 0.08 ^a	0.30 ± 0.04 ^a	0.43 ± 0.05 ^a	0.41 ± 0.08 ^{ab}	0.35 ± 0.05 ^{ab}	0.67 ± 0.10 ^b
Activity with F-1,6-BP	0.37 ± 0.09 ^a	0.37 ± 0.03 ^a	0.49 ± 0.04 ^a	0.52 ± 0.05 ^{ab}	0.43 ± 0.05 ^{ab}	0.65 ± 0.06 ^b



A



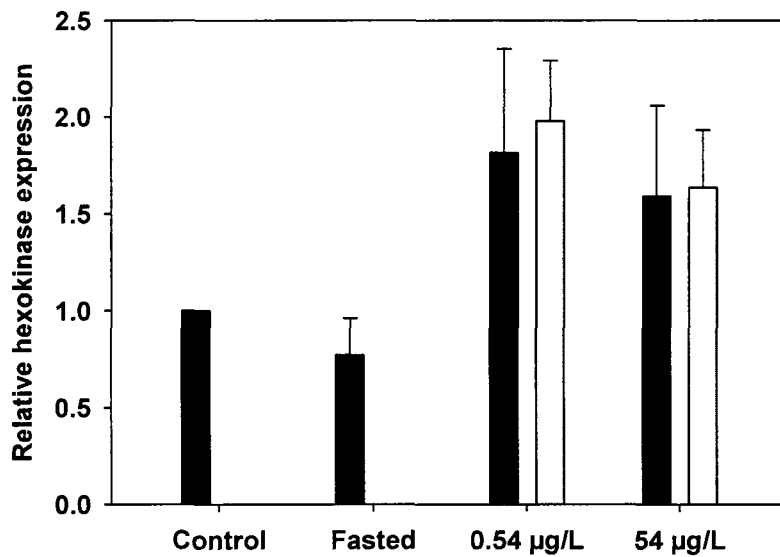
B

Figure 3.11. Male and female goldfish muscle hexokinase (A) and citrate synthase (B) activities following a 28 day exposure to FLX compared with control (*ad libitum* feeding) and fasted groups. Dark bars represent the four treatment groups and grey bars represent pair-fed groups (see Fig. 3.1). Data represents mean + SEM (n=8). Letters that differ indicate significant differences by one-way ANOVA ($p=0.003$, using the Student-Newman-Keuls procedure).

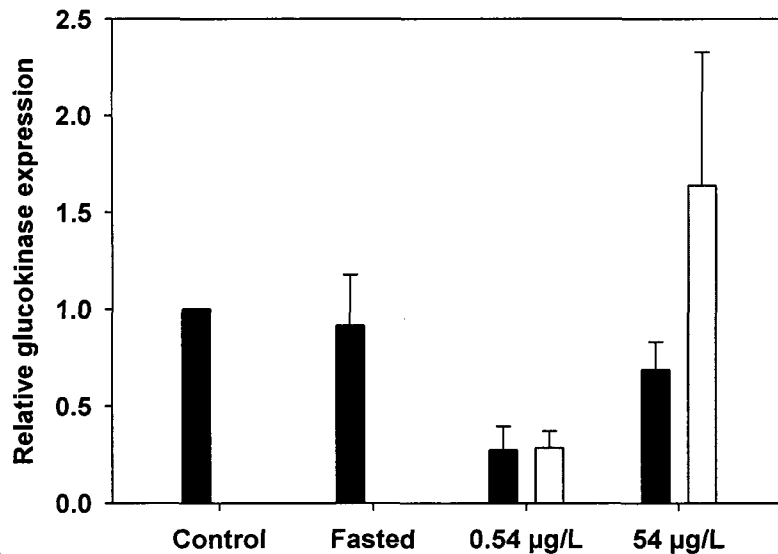
3.1.4 Enzyme transcripts

To evaluate whether the kinetic changes observed are due to protein modification or may be directed by changes in mRNA expression, real-time RT-PCR (QPCR) was conducted on several of the key enzymes for which activity estimates were undertaken. Although hepatic HK mRNA transcripts appeared to increase, the large variation in values masked any significant differences in transcript levels amongst the various groups (Fig. 3.12A). Similarly GCK mRNA transcripts appeared to decrease in the LD groups but not the HD group but again none of these changes were significant (Fig. 3.12B).

Two hepatic gluconeogenic enzyme transcripts were also estimated but none of these showed any significant differences between the groups (Fig. 3.13).



A



B

Figure 3.12. Relative hepatic hexokinase (A) and glucokinase (B) transcript levels (normalized to 18S) following a 28 day exposure to FLX compared with control (*ad libitum* feeding) and fasted groups in male and female goldfish. Dark bars represent the four treatment groups and grey bars represent the pair-fed groups (see Fig. 3.1). Data were normalized to 18S and then control transcripts were adjusted to one and all other groups compared with the normalized control. Data represent means + SEM (n= 5). No statistical differences using one way ANOVA.

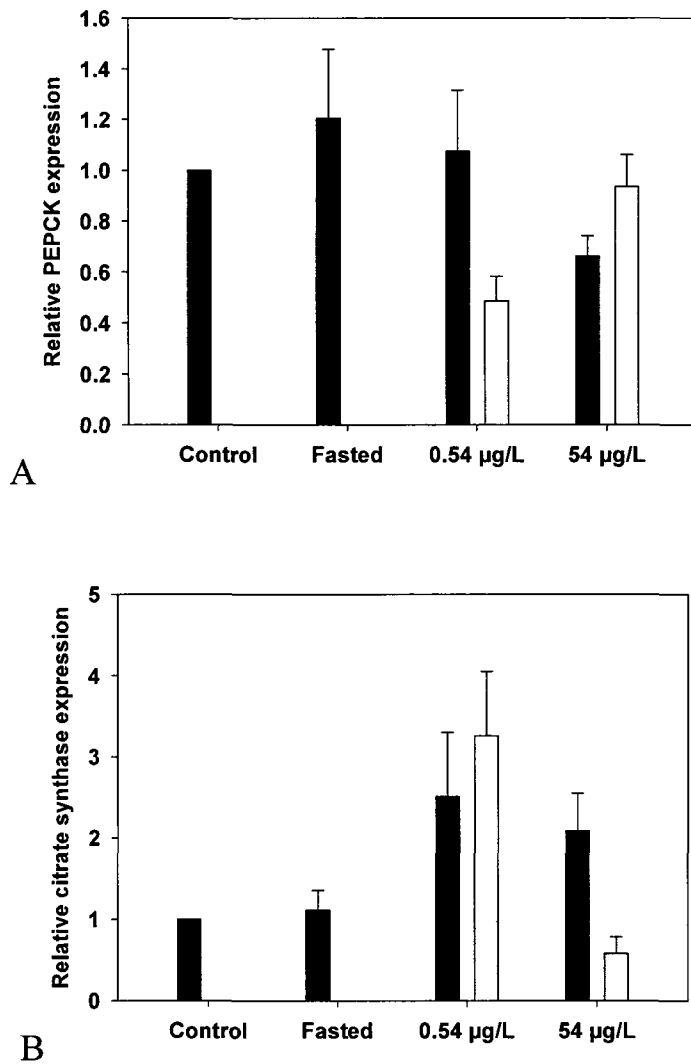


Figure 3.13. Relative phosphoenolpyruvate carboxykinase (PEPCK) (A) and citrate synthase (B) transcript levels (normalized to 18S) following a 28 day exposure to FLX compared with control (*ad libitum* feeding) and fasted groups. Dark bars represent the four treatment groups and grey bars represent the pair-fed groups (see Fig. 3.1). Data is normalized to 18S and then again to control. Data represent means + SEM (n= 5); no statistical differences using one way ANOVA.

3.2 The effects on FLX on metabolism irrespective of feeding

Results in section 3.1 demonstrated that there were mass and metabolic changes in goldfish exposed to FLX but some of the major effects seen were found comparing the fasted with the control or dosed groups. In order to determine whether or not FLX elicits direct effects rather than simply a result of fasting, I compared my results between the dosed groups, low dose (LD) and high dose (HD), with their respective pair-fed groups (pLD and pHD).

3.2.1 Morphometric data

Morphometric parameters including muscle moisture content (Fig. 3.3A), hepatosomatic index (HSI; Fig. 3.3B), gonadosomatic index (GSI; Fig. 3.4) and body weight change (Fig. 3.1) did not differ between the dosed groups and their pair-fed groups. Given these values were unchanged, comparing the FLX dosed groups to control and fasting should provide an indication of a specific FLX effect. No differences were noted in either moisture content or GSI across groups, so FLX does not affect either parameter. The dosed groups and the pair fed groups HSI was elevated significantly above both the control and fasted groups, suggesting FLX is indirectly affect the liver by food restriction. Similarly, the LD FLX treatment resulted in no change in weight but the HD treated goldfish lost mass compared with the LD and control but not as much as the fasted group (Fig. 3.1).

3.2.2 Metabolite data

The only plasma metabolites between groups to change were glucose (Fig. 3.4A), cholesterol (Fig. 3.5A) and triglycerides (Fig. 3.5B). All groups, except the HD group, had significant decreases in glucose levels from the control. Since the LD group, and its pair-fed

group mirrored the fasted group, it suggests that the decrease in glucose is a food deprivation response. The HD group glucose concentration did not decrease from control as the other groups, and so FLX may also be eliciting a direct effect on glucose on top of a simple fasting effect. With plasma cholesterol, effects were only seen in the HD group which was significantly increased above all other groups again suggesting a specific effect. Triglycerides were unchanged between dosed and the pair-fed group, so FLX appears to have no specific effect on this parameter.

Liver (Fig. 3.7) and skeletal muscle (Fig. 3.8) metabolites were unchanged between dosed and pair-fed groups and the only difference across groups was seen in the fasted group. It is apparent that FLX does not directly affect the content of liver or muscle carbohydrate or protein.

3.2.3 Enzyme activities

The large variation in hepatic enzyme activities within and between groups resulted in few differences between the dosed and pair-fed groups. Hepatic HK and GCK showed similar trends with only differences seen between the HD and the fasted groups (Fig. 3.9); thus FLX did not affect either enzyme. This is true as well for hepatic PEPCK and CS (Fig. 3.10) and PK (Table 3.1).

Skeletal muscle CS (Fig. 3.11B) and PK (Table 3.1) activities were unchanged between the dosed and pair-fed groups; again the only changes were related to the fasted group not to the dosed groups. Muscle HK (Fig. 3.11A) is more complex as differences do exist within the dosed-pair-fed groups and between these and the fasted group. The LD group activity is significantly elevated and this does appear to be a specific FLX effect.

3.2.3 *Enzyme transcripts*

Again, the large within group variance seen in transcript levels masks any effect of treatment. Certainly there is no evidence for FLX affecting any of these transcript levels directly.

Chapter 4: Discussion

In this study I established that exposing adult goldfish to fluoxetine (FLX) reduced food intake and ultimately led to a number of direct changes in metabolic endpoints. In addition, FLX also had a direct effect on specific metabolic parameters including the regulation of plasma glucose and cholesterol concentrations, liver glycogen concentration, and hexokinase (HK) activities. For the purpose of clarity I will discuss these two sets of findings separately.

4.1 Fluoxetine affects food intake and glycolytic parameters

Similar to previous studies (Nonogaki *et al.*, 2007; Gaworecki and Klaine, 2008; Mennigen *et al.*, 2009), goldfish exposed to FLX reduced their food intake (Fig. 3.2) and ultimately decreased weight gain (Fig. 3.1) and thus growth. This experiment was conducted in late September through to early October when goldfish generally experience a slow but linear somatic growth rate and little difference between male and female growth patterns (Marchant and Peter, 1986). Control fish followed this growth pattern while fish exposed to the high dose (HD) FLX treatment (54 $\mu\text{g/L}$) experienced a drop in weight similar to those in the study of Cui and Wang (2007) that reported on body parameters in food-deprived goldfish. The low dose (LD) FLX treatment (0.54 $\mu\text{g/L}$) did not significantly affect food intake or growth, although there was a trend towards a decreased weight gain compared with the control group. The Mennigen *et al.* (2009) study on goldfish and FLX reported that when injected intraperitoneally every three days with a pharmacological dose (5 $\mu\text{g FLX/g}$ body weight) of FLX, goldfish decreased food intake after 5 days and experienced a 40% decrease in weight gain after 13 days compared with the control non-dosed group. Mennigen *et al.* (2009) suggested that CRF (an

anorexic neuropeptide) could be at least partly responsible for this response as they reported a 2.3-fold increase in CRF mRNA transcripts in the hypothalamus. The levels of CRF and NPY (orexigenic factor) mRNA transcripts were estimated in the hypothalamus of the six groups of goldfish used in my study (J. Mennigen, personal communication). CRF transcripts significantly increased in the HD group and NPY transcripts significantly decreased in the LD and HD groups supporting the anorexic response FLX elicits in these goldfish. Although the change in body weight of the LD group was not statistically different from the control group, the decreased NPY mRNA transcript levels implies FLX may be exerting a minor inhibitory effect at this high environmental level of FLX.

Other selective serotonin reuptake inhibitors (SSRIs) demonstrated similar effects on food intake. Nonogaki *et al.* (2007) used milnacipran and reported a decrease in food intake after administering the drug to mice for 1, 2 and 3 days. Gaworecki and Klaine (2008) found that hybrid striped bass exposed to a range of FLX concentrations (0, 35, 75 and 150 $\mu\text{g/L}$ of tank water) for 6 days did not capture prey at the same rate, or ultimately reach the same level of food intake as those that were not exposed. They also found that this response was both duration of exposure and FLX concentration dependent. Much like fish from my study, a LD of FLX did not elicit food deprivation (no change in weight). The findings by Gaworecki and Klaine (2008) ultimately support the findings presented here in that the high dose elicited a fasting response, and the lower dose did not.

Unexpectedly, many metabolic parameters that are considered to be indicators of food deprivation remained statistically unchanged in my experiment. Plasma amino acid levels (Fig. 3.5B) did not statistically change between groups. Cui and Wang (2007) found that food deprived goldfish decreased plasma protein concentrations after 42 days of food deprivation.

Plasma protein concentrations were not statistically different between groups in my experiment although there was a slight decrease in the fasted group (Fig. 3.5C) suggesting the period of food deprivation, 28 days versus the 42 day fast in the Cui and Wang trial was inadequate to elicit an effect.

Hepatosomatic index (HSI) was affected by food restriction and FLX (Fig. 3.3B).

Typically liver mass is expected to increase with drug exposure as the liver is the primary site for metabolism and detoxification of contaminants including drugs (Treinen and Moslen, 2001). It was reported that Japanese medaka exposed to ibuprofen had a higher HSI (Flippin *et al.*, 2007) and brown trout exposed to sewage effluent experienced liver damage (Bucher and Hofer, 1992). In my experiment goldfish exposed to both LD and HD FLX showed a significant increase in HSI. However, this increase does not appear to be a direct effect of FLX because the pair-fed groups, which were not exposed to FLX but received a similar food ratio as eaten by the FLX fish, also experienced an increased HSI. These results were unexpected and imply that HSI is being regulated by food intake, and not by drug exposure. Dietary intake can affect HSI (Singh *et al.*, 2009), although usually the opposite of what is seen here. An increase in dietary protein significantly increased HSI in the Asian catfish *Clarias batrachus*. Similarly, Pascual *et al.* (2003) found that a decrease in food intake over 46 days resulted in a decrease HSI in the gilthead (sea) bream *Sparus aurata*. The situation with the FLX and pair-fed groups, however is the opposite. However, true to the trend reported by Singh *et al.* (2009) and Pascual *et al.* (2003), the fasted group in my study experienced a drop in HSI. Most likely the variation in HSI is due to the fish losing total body weight, but not liver weight, resulting in a higher HSI. The differences noted in HSI between groups were important and had to be considered when liver metabolism was examined.

Fish maintain homeostasis during a fast by mobilizing energy reserves, including hepatic glycogen, and by activation of hepatic gluconeogenesis and by reduction of glycolysis (Sheridan and Mommsen, 1991; Moon and Foster, 1995; Navarro and Gutiérrez, 1995). Since fish exposed to HD FLX did not eat as much as the other groups and lost weight over the experimental period I examined a variety of enzyme activities. Enzymes involved in regulating the glycolytic pathway include hexokinase (HK), glucokinase (GCK) and pyruvate kinase (PK) (Enes *et al.*, 2009). Liver protein levels were not statistically different between groups (Fig. 3.7A) and thus enzyme activities are expressed per unit protein rather than per liver weight that did change (Fig. 3B).

Hexokinase is the enzyme responsible for the first step of glycolysis, the phosphorylation of glucose into glucose-6-phosphate. Fish liver HK activities are not thought to be regulated by nutritional intake (Panserat *et al.*, 2000b; Kirchner *et al.*, 2005; Moreira *et al.*, 2008; Enes *et al.*, 2009) although some studies do find activity changes (Soengas *et al.*, 2006). Hepatic HK activities were generally unchanged although there was a difference between the HD group and the fasted group (Fig. 3.9A) but not between any of the other groups; transcript levels were also unchanged (Fig. 3.12A). This does suggest a FLX effect that is independent of a food deprivation effect. HK phosphorylates glucose, and thus could account for the slight increase noted in hepatic glycogen content observed in the FLX-dosed groups (Fig. 3.7B).

Glucokinase (GCK) or HK IV is a member of the HK-family but has a molecular weight 50% that of the other HKs (50kDa rather than 100kDa for HK1-3), a low affinity for glucose but is sensitive to nutritional intake (Enes *et al.*, 2009). Unlike HK, GCK (activity and expression) typically decreases with food restriction (Caseras *et al.*, 2000; Kirchner *et al.*, 2003, Metón *et al.*, 2004; Soengas *et al.*, 2006). Similar results are seen in this study in the fasted group. GCK

activity, and transcript levels, remained unchanged in fish exposed to FLX even the HD group that did eat less food (Fig. 3.9B; 3.12B). Presumably the fasting experienced by fish exposed to HD FLX was not great enough to induce a response.

Pyruvate kinase catalyzes the final step of glycolysis by converting phosphoenolpyruvate (PEP) to pyruvate with the production of energy as ATP. Gene expression and activity typically decrease during starvation (Pilkis and Granner, 1992) but did not in liver of any group in my experiment (Table 3.1). In addition to monitoring optimal activity, activity ratios (activity at a low vs high PEP concentration) were assessed with and without the presence of modulators (alanine and fructose-1,6-bisphosphate [F-1,6-BP]). These activity ratios are determined to indicate whether changes in affinity constants occur and whether modulators affect these constants. For example, Moon *et al.* (1989) reported decreased hepatic PK activity and values of the PEP activity ratio with a 6 week fast in rainbow trout suggesting carbon flux through PK and glycolysis is reduced in favour of gluconeogenic flux. The activity ratios with alanine (an inhibitor) were decreased and with F-1,6-BP (an activator) were increased suggesting enhanced sensitivities of this enzyme to these modulators. However no changes were seen in any of these parameters in my experiment suggesting glycolysis was not a target for FLX and that the fasting period again was insufficient to trigger changes in this enzyme.

Gluconeogenesis, and enzymes involved in this process, primarily occurs in the liver and kidney (Knox *et al.*, 1980; Moon and Foster, 1995; Suarez and Mommsen, 1987).

Phosphoenolpyruvate carboxykinase is the enzyme that overcomes the irreversible nature of PK and is thought to be a rate-limiting step in gluconeogenesis. Although not significant, slight increases in activity were seen in the exposed and fasted groups compared with the control (Fig. 3.10A). Moon *et al.* (1989) and Kirchner *et al.* (2008) reported increased PEPCK activities and

expression in fasted rainbow trout although most recent studies found that activities and expression of PEPCK in rainbow trout are relatively high and unaffected by nutritional state (Enes *et al.*, 2009). Increased gluconeogenic activity, including activity of PEPCK, was also found in 21 day fasted mudskippers, *Boleophthalmus boddarti* (Lim and Ip, 1989). PEPCK transcript levels were also unchanged in my study (Fig. 3.13B).

Citrate synthase activities are an indicator of mitochondrial oxidative tricarboxylic acid cycle activities and consistent with the lack of significant changes in the other hepatic enzymes studied in my thesis, this enzyme did not change. Previous studies with rainbow trout found an increased mitochondrial oxidative potential, which was thought to be involved with providing the essential fuels for the enhanced gluconeogenic activity required during food deprivation (Moon *et al.*, 1989).

In general, hepatic glycolytic and gluconeogenic enzymes are unaffected by FLX exposure in goldfish. There are few enzyme studies done in goldfish liver and the only other nutrient study done (Cui and Wang, 2007) did not look at enzyme activities. All that can be said at this point is either the goldfish is less sensitive to fasting than for example a rainbow trout where there are many nutrient related studies, or the exposure times to FLX and the food-deprivation were not great enough to cause subsequent metabolic changes.

The moisture content in muscle tissue did not change between FLX exposed and control groups (Fig. 3.3A) and values were close to that reported in Cui and Wang (2007). These negligible changes in moisture content allowed me to use weight of muscle as a good indicator of changes in metabolites and enzymes across the groups.

Muscle protein (Fig. 3.8B) and amino acid (Fig. 3.8A) concentrations did not change between groups with the exception of protein concentration in the fasted group. Cui and Wang

(2007) found changes in total and muscle protein after 35 days of food deprivation in the gibel carp (subspecies of the goldfish) but not after 28 days which was the duration of exposure used in my study. During food deprivation protein synthesis decreases and protein catabolism is accelerated (Smith, 1981; Lounghna and Goldspink, 1984; Moon and Foster, 1995). At 28 days in my study the fasted group did demonstrate a significant decrease in protein, but no changes were seen in any of the other groups suggesting this was not a significant fasting stress. A longer fasting period, or less food intake, would be required to see any protein response, and thus any amino acid changes.

Since gluconeogenesis does not occur in muscle tissue, only glycolytic enzymes were measured in this tissue. In addition, GCK expression and activity are typically not found in muscle tissue (Blin *et al.*, 1999; Panserat *et al.*, 2000a) so it was not measured. Glycolysis in fish muscle is sensitive to changes in food intake (de Pedro *et al.*, 2006) and hypoxia (van den Thillart and Smit, 1984; Martínez *et al.*, 2006). Specifically de Pedro *et al.* (2006) reported that goldfish stores carbohydrates in muscle tissue due to decreases in food intake caused by leptin injections. Hypoxia also induces changes in muscle tissue by suppressing metabolism (van den Thillart and Smit, 1984; Martínez *et al.*, 2006). Generally PK activities fall with fasting in teleost fishes (DiBattista *et al.*, 2006) and in fact in Atlantic cod (*Gadus morhua*) there is a positive correlation between activities of muscle PK and growth rates (Pelletier *et al.*, 1994). This decrease with fasting was also observed in the goldfish fasted for 28 days in this study (Table 3.1). Unexpectedly PK activity ratios for PEP and alanine and F-1,6-BP all increased with fasting. Such increases suggest that kinetic curves for this enzyme are shifting towards lower [PEP]_s meaning that the affinity for PEP is increasing. What is responsible for this is not known as such estimates are not found in the literature but it is clear that muscle PK is significantly

being altered by fasting. Exposure to LD FLX did not change these values while HD FLX did increase these values to the point where they were not different from the fasted group. Such changes would imply an increased potential glycolytic flux in both the fasted as well as the HD FLX groups.

Muscle HK activities varied between groups (Fig. 3.11), but appeared to be directly affected by FLX exposure and thus will be discussed in the following section.

4.2 Fluoxetine exerts direct effects on metabolic parameters

Pair-fed groups for each of the FLX treatments were set-up in an attempt to establish whether FLX acts directly on metabolism irrespective of food intake. Since the dosed groups gained/lost the same percentage of weight as their respective pair-fed groups I was able to examine any differences between these groups to separate feeding effects from FLX effects. I found a number of metabolic parameters directly affected by FLX exposure.

Glucose levels are regulated by dietary intake and feeding schedule in fish (Sanchez *et al.*, 2009; López-Olmeda *et al.*, 2009) so it would be expected that groups experiencing fasting would see a decline in plasma glucose concentration. Declines in plasma glucose are reported in fasting periods as short as 3 days and as much as 30 days in tilapia (*Oreochromis mossambicus*) (Rogers *et al.*, 1992; Fox *et al.*, 2009; Riley *et al.*, 2008) and after 6 weeks in rainbow trout (Moon *et al.*, 1989). In my study changes in glucose were noted not only related to dietary intake but due to direct FLX exposure (Fig. 3.5A). Plasma glucose levels were significantly different between the HD group and the fasted group but not its respective pair-fed group. Serotonin was found to stimulate hyperglycemia at least in mammals (Carvalho *et al.*, 2002). Carvalho *et al.* (2004) injected FLX into the 3rd ventricle of the rat brain and found that this injection resulted in

hyperglycemia. Stimulation of 5-HT1A, 5-HT2A and 5-HT2B/2C receptors also induced hyperglycemia. Injections with FLX and a CRF antagonist blunted the increase in blood glucose suggesting an increase in CRF may be responsible for the spike. Although hyperglycemia was not noted in goldfish exposed to FLX in my study, the extent of decline in plasma glucose concentrations noted in the fasted group did not occur in the HD FLX suggesting some protection by FLX of plasma glucose concentrations. As mentioned above, Jan Mennigen (personal communication) found a spike of CRF in the HD group only, which in this case could have negated the drop in glucose found in the fasted group.

Cholesterol was another metabolic parameter directly affected by FLX exposure (Fig. 3.6A). The concentration of cholesterol increased in the HD group only and not in its respective pair-fed group or in any of the other groups suggesting a direct FLX effect. High cholesterol is a rare side effect of FLX treatment and no research has examined the effects of FLX on cholesterol and cholesterol pathways in fish. FLX treatment is reported to increase cholesterol levels in some patients (Thase *et al.*, 2007; Trivedi *et al.*, 2009). Although the change noted in the HD FLX group was not large the result supports some FLX effects in the goldfish. Teleost fish are generally considered to be hypercholesterolemic (Wallaert and Babin, 1992) so whether such a small change is significant is not known at this time. Further studies on fish cholesterol metabolism are needed as this is an under-studied area of fish nutrition.

There are reports where glycogen is both affected (Soengas *et al.*, 2006) and unaffected (Luo *et al.*, 2009) by food deprivation. Surprisingly, glycogen concentration did not change between groups when represented as concentration (mg glycosyl units/g of liver); however as a result of the statistical change in HSI between groups (Fig. 3.3B), glycogen data were also presented as total glycogen per liver (Fig. 3.7A/C). There was a statistical difference between the

two FLX-dosed groups and the fasted group based strictly upon the relative changes noted in liver mass compared with body mass. This effect is FLX exerting a direct effect on glycogen since similar statistical differences were not observed between the pair-fed and the fasted groups. Unfortunately the activities of glycogen phosphorylase, the enzyme responsible for the breakdown of glycogen, were not studied here and given the multiple controls over this particular enzyme it is possible that FLX is affecting other circulating hormones that could be affecting this particular enzyme. My study also found that hepatic glycogen content was independent of blood glucose as these parameters vary independently of one another (compare Figs 3.7B with 3.5A). Thus it does appear that FLX may play a role in hepatic glycogen homeostasis in the goldfish.

The final parameter that showed a direct effect of FLX exposure was liver (Fig. 3.9A) and muscle (Fig. 3.11A) hexokinase (HK) activities. In skeletal muscle both dosed groups are significantly different from their pair-fed groups; in both situations HK activities in the pair-fed groups were similar to that of controls. The LD HK activity was higher than its pair-fed and the HD group was lower than its pair-fed group. Thus FLX elicited a biphasic response with respect to HK activities. The reduction in activity was not as large as found in the fasted group, but since no reduction is seen in its pair-fed group, it can be assumed that this reduction is not due to food intake but a direct FLX exposure effect. Research to date looking at the effects of FLX on glycolysis and glycolytic enzymes is limited. Tassini *et al.* (2002) monitored glycolysis in human blood platelets with and without FLX exposure. Typically human blood platelets have a relatively active glycolytic and oxidative metabolism while storing a large amount of serotonin in secretory organelles. Coinciding with my findings Tassini *et al.* (2002) found that FLX altered glycolysis and inhibited lactate production at high doses (above pharmacological doses) but not

at low doses. The precise mode of action of FLX in this HBP study was not deduced. The impact of FLX dose on HK activities in the liver was contrary to that seen in the muscle (compare Figs 3.11A and 3.9A) with LD decreasing and HD FLX increasing activities. This result implies a modified glycolysis in these two organs modulated by FLX.

4.3 General conclusions and future directions

Over the past few years one finds in the literature an increased discussion of the presence, prevalence and effects of FLX in the environment. FLX is widely detected across many water systems stemming from any variety of sewage and waste water treatment plants (Jones-Lepp *et al.*, 2001; Weston *et al.*, 2001; Kolpin *et al.*, 2002; Vanderford *et al.*, 2003; Metcalfe *et al.*, 2003; Furlong *et al.*, 2004; Brooks *et al.*, 2004; Hua *et al.*, 2006). Recently, Brooks *et al.* (2005) detected several antidepressants in tissues of a variety of wild-caught fish species. Research assessing the risks and dangers associated with FLX on aquatic organisms has increased and the findings of my thesis contribute to the concerns raised in other studies.

In general my studies supplement previous studies (Mennigen *et al.*, 2009) looking at food deprivation caused by FLX and confirms that FLX is indeed a stimulator of anorexia. However, it appears that the level of food deprivation goldfish experienced in this study even at the HD and especially at the LD FLX exposure, was not sufficient to elicit a significant fasting response. I would predict that higher FLX concentrations would elicit greater affects on food intake and subsequent metabolic endpoints. However, since the high dose group was exposed to a concentration of FLX 100 times greater than the concentration in the environment, exposing to higher concentrations would ultimately be irrelevant. FLX did limit food intake at least in the HD group probably by changing the availability of CRF and NPY as Mennigen *et al.* (2009)

previously reported and also found using the experimental groups studied here and some metabolic parameters but it is also directly affected certain aspects of metabolism including plasma glucose, cholesterol, and hepatic glycogen and HK activities.

Findings from a laboratory setting can only help us speculate what might occur in the natural environment. In this case, the environmental setting may elicit a similar, and even exaggerate, scenario given the complexities of a natural setting. This exposure was for 28 days and a longer exposure, and therefore a longer period of food intake reduction, could result in more prominent metabolic changes. Fish live their entire life in polluted waters, and such long term exposure could most definitely elicit metabolic consequences. In the environment an entire cocktail of pharmaceuticals and personal care products exist which could alter metabolic state, and specifically other SSRIs that are proven to elicit reduced food intake (Nonogaki *et al.*, 2007).

My studies suggested that the FLX effects in goldfish were primarily driven by changes in food intake although I did see a number of changes that appear to be independent of food intake. Thus, future work assessing the role of FLX on metabolism requires an in depth look at the role of FLX in regulating glucose, glycogen, HK and glycolysis in general, and the role of FLX on cholesterol dynamics, an area that has received limited study in fish. Understanding the path FLX takes to elicit these changes could further the knowledge of the dangers of FLX in the environment, and on physiology and metabolism in the goldfish. I think it is also important to consider the possibility of synergistic or combined effects of multiple drugs and pollutant exposures that fish would encounter in their natural environment.

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