

EFFECTS OF FLUOXETINE ON DEVELOPMENT AND METAMORPHOSIS
OF THE AFRICAN CLAWED FROG, *XENOPUS LAEVIS*

by

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(Under the Direction of Marsha Black)

ABSTRACT

Fluoxetine, a widely prescribed antidepressant, has been detected at low concentrations (ppt - ppb) in surface water. Mammalian studies indicate that fluoxetine may inhibit the thyroid axis. In larval frogs, increasing levels of thyroid hormones are necessary for metamorphosis to occur, and the presence of fluoxetine in aquatic habitats may have the potential to decrease thyroid hormone levels and delay the completion of metamorphosis. To test this hypothesis, we exposed *Xenopus laevis* embryos to fluoxetine until the completion of metamorphosis. Metamorphosis was significantly delayed at 50 ppb, a concentration that would not be expected to occur in the environment. Effects at environmentally relevant concentrations included significant reductions in size at metamorphosis, as well as limb malformations. Because decreased survival and reproductive success have been associated with malformations and reduced size at metamorphosis, our findings suggest that the presence of low levels of fluoxetine in aquatic habitats may adversely affect amphibian populations.

INDEX WORDS: pharmaceutical, fluoxetine, *Xenopus laevis*, amphibian, malformation, ammonium perchlorate

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Introduction

The recent detection of low concentrations of pharmaceutical compounds in surface waters has created a new area of research devoted to determining the fate of these compounds in the environment and their potential to affect aquatic organisms. Developing amphibians living in areas receiving pharmaceutical wastes may be affected by these compounds. The research presented herein focuses on one pharmaceutical compound, fluoxetine, and its effects on amphibian development and metamorphosis. The following literature review provides background information regarding sources, occurrence and environmental fate of pharmaceuticals in general and then focuses on fluoxetine. A review of the current literature on fluoxetine as it relates to aquatic toxicology and potential effects in amphibians is presented. The literature review is followed by a statement of the research hypothesis and a brief description of the experimental approach.

Literature Review

Susceptibility of Developing Amphibians to Aquatic Contaminants

Amphibians represent an important component of aquatic ecosystems and are considered good indicators of water quality due to physiological and morphological characteristics that

make them especially sensitive to aquatic contaminants. Most amphibians rely on aquatic habitats primarily for breeding and larval development, while others spend their entire lives in water. Poor water quality can therefore have significant effects during each stage of the life cycle. The vitelline membrane of the embryo is permeable to allow water and solute exchange, and contaminants may also cross the vitelline membrane and reach the embryo. Under certain conditions, contaminants can also prevent the vitelline membrane from expanding as the egg develops. As a result, the embryo becomes curled and malformations may appear upon hatching (Rowe et al. 2003). Larvae are also susceptible to prevailing conditions in the aquatic environment. After hatching, most gas and ion exchange takes place at the gills, and the integument is also permeable to water. Because the entire body surface is exposed, larval amphibians are extremely vulnerable to contaminants at this stage in development (Rowe et al. 2003).

Metamorphosis is also a sensitive period in amphibian development. Thyroid hormones play an important role in the regulation of metamorphosis. The thyroid gland develops during the embryo stage and is functional at the time of hatching (Shi 2000). Normally, the hypothalamus releases thyrotropin releasing hormone (TRH) from the hypothalamus, which results in the stimulation of the pituitary gland. The pituitary gland releases thyroid stimulating hormone (TSH). TSH then stimulates the thyroid gland to produce thyroxine (T_4) (Fig. 1.1). Finally, T_4 is converted to T_3 at the target tissue by deiodination, and the hormone becomes biologically active (Vander et al. 2001). During premetamorphosis, the stage immediately following hatching, the larvae grow rapidly, but do not undergo any major developmental changes. At this time, thyroid hormone levels are low. During the next stage in development, prometamorphosis, the hind limbs begin developing, and plasma thyroid hormone concentrations

increase. At metamorphic climax, thyroid hormones reach peak levels, and at the same time, the animal undergoes rapid changes in development. Within a short time period, the forelimbs emerge and the tail is resorbed. When the tail has been fully resorbed, metamorphosis is complete, and the animal becomes a juvenile frog (Shi 2000; Denver et al. 2002).

Developing amphibians are extremely sensitive to contaminants during metamorphosis, especially those that interfere with the production of thyroid hormones. Metamorphosis has been blocked experimentally by inhibiting the production of endogenous thyroid hormones in tadpoles. In *Xenopus laevis* tadpoles that were born without a thyroid gland, the tadpoles became abnormally large, but never metamorphosed. Two years after the completion of the experiment, the tadpoles were still developmentally in the premetamorphosis stage (Dodd and Dodd 1976). Metamorphosis has also been blocked by chemical inhibitors, including thiourea, thiouracil, sulfonamides, thiocyanate, potassium perchlorate, and methimazole (Shi 2000). Additionally, Goleman and colleagues (2002) demonstrated that aqueous exposure to ammonium perchlorate decreased whole body T₄ levels and inhibited metamorphosis in *Xenopus laevis*.

Although amphibians are highly susceptible to aquatic contaminants during early development and metamorphosis, they are only beginning to be studied by toxicologists, and most research to date has focused on the effects of metals, acidification, and pesticides (Sparling et al. 2000). Pharmaceuticals have recently been recognized as emerging environmental contaminants and may have the potential to affect developing amphibians.

Pharmaceuticals as Potential Aquatic Contaminants

Advances in the pharmaceutical industry over the past thirty years have dramatically improved the quality of life for millions of people living in developed countries. According to

Pharmaceutical Research and Manufacturers of America, annual pharmaceutical sales since 1980 have risen from \$22 billion to \$212 billion in 2004, and an average of 32 new medicines are approved by the FDA each year (PhRMA 2004). Because pharmaceuticals are able to alleviate symptoms of, and in some cases cure illnesses, their development and use will certainly continue to increase. Although intended for human and veterinary use, the release of pharmaceuticals to the environment has the potential to affect other organisms because many drugs target physiological mechanisms that are conserved across taxa. It is important, then, to consider the prevalence of pharmaceuticals in the environment and to evaluate the potential effects of these substances on non-target organisms.

Pharmaceuticals may be present in both surface and ground water. Disposal of unused pharmaceuticals by the manufacturer and the general public into domestic waste systems is one possible route of groundwater contamination. Pharmaceuticals have been detected in landfill leachate, but are presumed to be quickly broken down by microbes and are not considered to be a significant source of groundwater contamination (Daughton and Ternes 1999). Scientists are mainly concerned with the release of urine and feces containing pharmaceutical compounds and their metabolites directly into surface waters through runoff and via releases from wastewater treatment plants. Agriculture is a major source of direct surface water contamination by veterinary pharmaceuticals. Fecal matter containing pharmaceutical compounds given to livestock may enter streams and lakes during rain events. In addition, fields fertilized with sewage sludge from domestic sources also contribute to surface water runoff (Daughton and Ternes 1999). While runoff is an important source, the primary routes by which pharmaceuticals enter the aquatic environment are through human excretion, washing, and direct disposal of unused drugs into sewage systems (Daughton 2003a). Depending on the type and level of

sewage treatment available, pharmaceuticals may enter surface waters via effluent release.

Because municipal sewage is not treated to drinking water standards, most wastewater treatment plants do not use technologies such as ozonation and reverse osmosis that would remove many pharmaceuticals and other organic compounds from treated effluent (Daughton and Ternes 1999; Daughton 2003a,b). In areas of the world where sewage treatment does not exist or in places where malfunctioning septic systems are in use, untreated sewage that contains pharmaceuticals is directly released into water bodies (Daughton and Ternes 1999; Daughton 2003a). Regardless of the availability or type of wastewater treatment system employed, pharmaceuticals that are used by the general population are eventually released into the aquatic environment.

From the time that pharmaceuticals are taken by humans to their eventual release into surface waters, these compounds are subject to chemical alteration (Daughton and Ternes 1999). When drugs are taken by humans, the lipophilicity of the parent compound allows absorption by the target tissue. In order to be eliminated through urine or feces, the compound must become more water soluble. Most drugs undergo phase I and II metabolism, although the specific metabolic pathway employed depends upon the compound. First, phase I enzymes (cytochrome P450s, reductases, or hydrolases) catalyze the addition of reactive functional groups to the molecule. This functional group is then conjugated (glucuronidation, sulfation, methylation, acetylation, amino acid conjugation, or glutathione conjugation), increasing the overall hydrophilicity of the molecule (Parkinson 2001). The water-soluble pharmaceutical metabolite is finally excreted in feces or urine, making its way to the municipal wastewater treatment plant.

Wastewater treatment plants primarily receive pharmaceutical metabolites generated as previously described, but may also receive unmetabolized parent compounds from disposal of unused pharmaceuticals directly into domestic sewage systems (i.e. flushing) by the

manufacturer, hospitals, medical clinics, and consumers (Daughton and Ternes 1999; Daughton 2003a). When these compounds arrive at wastewater treatment plants, microbes are introduced to break them down into lower molecular weight molecules leading to complete mineralization to carbon dioxide and water. Pharmaceuticals and pharmaceutical metabolites, however, likely bypass microbial degradation because the concentrations of these compounds in sewage are so low that minimum substrate concentrations for degradative enzymes are not met (Daughton and Ternes 1999). Most drugs are resistant to hydrolysis during sewage treatment, and photolysis is the most likely method by which pharmaceuticals are broken down (Velagaleti 1997). Photolysis is limited, however, to compounds with an absorbance in the range of 290-800 nm and can only occur in the upper layer of sewage that receives direct sunlight (Velagaleti 1997). The degradation of pharmaceutical compounds in wastewater treatment systems is therefore minimal. Water soluble metabolites are discharged in aqueous effluent, while parent compounds, due to their relative lipophilicity, may partition to sludge (Velagaleti 1997) or bind to suspended particulate matter in effluent (Daughton and Ternes 1999). A study based on computer modeling indicates that many parent compounds and metabolites partition to particulate matter in effluent (Khan and Ongerth 2002). A recent field study shows that several pharmaceutical compounds resist breakdown by wastewater treatment and are detected in effluent (Stackelberg et al. in press). Taken together, research on the fate of pharmaceuticals during wastewater treatment indicates that both parent compounds and their metabolites enter sewage treatment plants, undergo minimal degradation, and are then discharged in treated effluent to nearby streams, lakes, and oceans.

The fate of pharmaceuticals in the aquatic environment is not well understood. Few studies on this topic exist, and studies to date have mainly focused on fugacity modeling and

extrapolation based on physical and chemical properties of pharmaceutical compounds.

Lipophilic parent compounds would likely bind to sediments and particulate matter, possibly leading to bioaccumulation (Velagaleti 1997; Brooks et al. 2003a). Unbound metabolites would be subject to microbial degradation, because microflora present in the environment are more diverse and include oligotrophic bacteria not present in wastewater treatment systems that would be capable of interacting with low concentrations of pharmaceutical metabolites (Daughton and Ternes 1999). While microbial degradation is possible, conversion of conjugated metabolites to the original parent form through hydrolysis may also occur in the aquatic environment (Daughton and Ternes 1999). Even though effluent released into surface waters likely contains a greater percentage of metabolites than parent compounds, hydrolysis of metabolites in the aquatic environment could result in environmental concentrations of parent drugs that equal or exceed the amount of parent compound originally released in effluent. The conversion of metabolites to the original compound is also important because, as stated previously, parent compounds may have more potential to bioaccumulate. Both parent compounds and metabolites would be subject to photolysis in the aquatic environment (Velagaleti 1997). The degree of photolysis depends on the depth of the body of water into which effluent is released and the amount of sunlight that it receives. Current knowledge of the fate of pharmaceuticals in the environment is largely speculative, and more laboratory simulations and field studies are needed.

Although pharmaceutical use and release to surface waters is not a new phenomenon, the presence of these compounds in the environment is a topic that has just begun to be studied. This is mainly due to recent advances in analytical chemistry techniques that now allow for the detection of low concentrations of organic compounds in water (Daughton and Ternes 1999). One of the first attempts to look for pharmaceuticals in the aquatic environment was conducted

by the United States Geological Survey (Kolpin et al. 2002). In the study, surface water samples were collected from sites across 30 U.S. states and were analyzed for over 40 pharmaceutical compounds. The USGS detected low concentrations of pharmaceuticals (ng/L- μ g/L) at many locations, indicating their presence in surface waters on a national scale. Subsequent investigations in the United States, Europe and Canada have reported similar concentrations of pharmaceuticals in surface waters (Metcalf et al. 2003; Boyd et al. in press; Kolpin et al. in press; Stackelberg et al. in press). These studies show that pharmaceuticals are ubiquitous aquatic contaminants.

The presence of pharmaceuticals in the environment has prompted scientists to question whether low levels of these compounds may cause effects in non-target aquatic organisms. Studies to date have reported that pharmaceuticals are detected in the environment at concentrations that are orders of magnitude below human therapeutic doses. Because pharmaceuticals are designed to be biologically active, they are likely to have some effect on aquatic organisms; however the major question is whether environmental concentrations are able to elicit these effects. The issue is further confounded by the fact that the number of studies devoted to detecting pharmaceuticals thus far has been few and that these studies mainly focus on reporting the concentrations of parent compounds. Because metabolites and other degradation products would also be present in the environment, current studies may underestimate the total amount of pharmaceutical compounds present. Some pharmaceutical metabolites may be more biologically active than the parent compound, thus having the potential to be more toxic (Daughton and Ternes 1999). Because pharmaceutical compounds and their metabolites occur at such low concentrations in the environment, toxicological studies with aquatic organisms should focus on effects from chronic, environmentally realistic exposure

concentrations. Conducting studies in this manner will allow scientists to determine whether pharmaceutical contamination of water bodies is harmful to aquatic life.

Amphibians as Potential Receptors of Pharmaceutical Compounds in the Environment

Pharmaceutical contamination is a ubiquitous problem, and any water body that receives wastewater treatment effluent could be affected. Amphibians are a diverse group of organisms that live in a variety of freshwater aquatic habitats, including streams, lakes, wetlands, and small ponds, for all or part of their life cycle. Many of these areas receive inputs of effluent from wastewater treatment plants that likely contain trace quantities of pharmaceutical compounds. For example, constructed wetlands that are designed to reduce effluent loadings from rivers and lakes also provide breeding habitat for amphibian species (James and Bogaert 1989). The dilution of effluent in constructed wetlands is less than would be expected in larger bodies of water, such as rivers and lakes. Because constructed wetlands create preferred habitat for breeding amphibians and likely contain higher concentrations of wastewater treatment effluent than traditional release sites, it is important that larval amphibians be considered in toxicity evaluations of pharmaceutical compounds.

Fluoxetine: Mechanism of Action and Environmental Occurrence

My research focuses on the effects of the pharmaceutical drug fluoxetine (Fig. 1.2). Fluoxetine is listed as the 31st most-prescribed drug in medicine (RxList 2001) and is a member of the class of anti-depressant drugs commonly referred to as selective serotonin reuptake inhibitors (SSRIs). SSRIs act on the central nervous system and reduce the reuptake of serotonin by transport proteins, thereby increasing the transmission of serotonergic neurons (Ni and Miledi

1997; Fong 1998). By artificially increasing serotonin transmission, serotonin reuptake inhibitors have been shown to improve mood in people experiencing depression and certain types of compulsive behavior disorders (Appleton 1997). In humans, fluoxetine is metabolized by cytochrome P-450 enzymes to produce the biologically active N-demethylated metabolite, norfluoxetine, which is then glucuronidated and excreted in urine. Urine contains less than 10% of the parent compound (Heimke and Hartter 2000).

Fluoxetine has been detected in the aquatic environment at low concentrations in the United States and Canada. The United States Geological Survey found fluoxetine concentrations of 0.012 µg/L in surface waters (Kolpin et al. 2002). Fluoxetine was also found in wastewater treatment effluent at concentrations as high as 0.540 µg/L (Weston et al. 2001). In the Great Lakes area of Canada, fluoxetine was detected in effluent (0.099 µg/L) as well as surface waters (0.046 µg/L) (Metcalf et al. 2003). In contrast, recent studies conducted in Iowa (Kolpin et al. in press), Louisiana (Boyd et al. in press), and at an undisclosed location (Stackelberg et al. in press) included fluoxetine in their analysis but did not detect the compound in water samples. Overall, few studies have attempted to detect fluoxetine, or pharmaceuticals in general for that matter, in surface waters. One would expect the occurrence of fluoxetine in the environment to mirror patterns of use, and it is possible that the drug may be found only in heavily populated areas. There are also many variables that may influence the detection of fluoxetine, such as site selection, timing of sampling, and flow conditions at the time of sampling. While the prevalence of fluoxetine in the environment is uncertain, the compound has been shown to occur in surface waters and further study of its effects on aquatic organisms is warranted.

Fluoxetine Ecotoxicology Research

The recent detection of fluoxetine in surface waters has prompted researchers to examine the effects of fluoxetine on non-target aquatic species. Most studies with fluoxetine have taken place within the last four years, and information is limited. Presented here is a review of the current literature on fluoxetine as it relates to aquatic toxicity, covering acute and chronic responses, as well as reproductive, developmental, and behavioral endpoints.

Because fluoxetine has been detected at such low concentrations (ng/L) in the environment, acute mortality from exposure to environmentally relevant concentrations is not expected to occur. Median lethal concentrations (LC₅₀) have been reported for fish, daphnids, a midge, and an amphipod (Table 1.1). Brooks et al. (2003b,c) reported a 48-h LC₅₀ of 705 µg/L in fathead minnows, and a similar value (614 µg/L) was reported for a 7-day exposure with western mosquitofish (Henry and Black 2004). LC₅₀s for daphnids ranged from 234 to 820 µg/L, with *Ceriodaphnia dubia* displaying more sensitivity to fluoxetine exposure than *Daphnia magna* (Brooks et al 2003b,c; Henry et al. 2004). The midge, *Chironomus tentans*, and the amphipod *Hyaella azteca* seemed to be the least sensitive of all organisms tested (Brooks et al. 2003c). These organisms were exposed through sediment, rather than water, and insensitivity may indicate reduced bioavailability of fluoxetine in sediment versus water. The species most sensitive to waterborne fluoxetine exposure was *Pseudokirchneriella subcapitata*, green algae. The median concentration to inhibit growth (EC₅₀) in algae was 24 µg/L (Brooks et al. 2003b,c). For all organisms tested, acute effects occurred at fluoxetine concentrations considerably higher than those reported in the environment.

A variety of effects have been observed with sub-acute to chronic exposures. In marine invertebrates, fluoxetine seems to stimulate reproduction. Exposure to fluoxetine accelerated the

development of the testes in fiddler crabs (Sarojini et al. 1993) and also increased the ovarian index and oocyte size in the red swamp crayfish (Kulkarni et al. 1992). Spawning in several bivalve species was induced by serotonin (Gibbons and Castagna 1984; Fong 1998) and by low concentrations of two serotonin reuptake inhibitors, fluoxetine and fluvoxamine (Fong 1998). In another study, Couper and Leise (1996) found that serotonin induced metamorphosis in a marine mollusk. This information has been applied in commercial aquaculture to facilitate bivalve culturing (Gibbons and Castagna 1984).

In contrast to marine studies that indicate that fluoxetine stimulates reproduction and development, research conducted with freshwater aquatic organisms in an environmental context implies that fluoxetine may inhibit reproduction and delay sexual maturation. Lowest observable effect concentrations (LOECs) of 112 µg/L (Brooks et al. 2003b) and 447 µg/L (Henry et al. 2004) reduced the number of offspring produced by *C. dubia* during 7-day, multigenerational exposures. Reproductive endpoints, including fecundity, rate of fertilization, egg hatching success, abnormal development, and circulating plasma steroids, were also assessed in Japanese medaka exposed to fluoxetine concentrations ranging from 0.1 to 5 µg/L (Brooks et al. 2003b). Fluoxetine exposure did not affect reproduction but did result in developmental abnormalities in offspring including edema, spinal curvature, incomplete development of pectoral fins, and reduced eyes. Henry and Black (personal communication) found that western mosquitofish exposed to 60 µg/L fluoxetine for 90 days experienced delayed development of adult sexual morphology, as evidenced by the presence of a gonopodium in males or a black spot in females. Taken together, the results of these studies indicate that fluoxetine may negatively affect reproduction, disrupt normal development, and delay sexual maturity in freshwater aquatic organisms.

Fluoxetine has also been shown to affect behavior in fish. Western mosquitofish exposed to fluoxetine did not respond to stimuli and were lethargic (Henry and Black personal communication). In an effort to prove that serotonin controls territorial aggression in coral reef fish, Perreault et al. (2003) injected bluehead wrasse with fluoxetine and found that treated individuals displayed fewer aggressive chases when an intruder fish was introduced to their territory. Decreased aggression in fish may affect mating and other behaviorally controlled processes in the wild.

Effects of Fluoxetine on the Thyroid Axis

Although there have been no direct toxicological studies on SSRIs and amphibians, research shows that thyroid function in mammals is affected by serotonin and by the serotonin reuptake inhibitor, fluoxetine. Mitsuma and Nogimori (1983) demonstrated that in rats, serotonin (5-HT) inhibits the release of TRH from the hypothalamus. They also showed that serotonin inhibited TRH release from the rat retina in a dose-dependent manner (Mitsuma et al. 1996). Saphier and colleagues (1994) also support the inhibitory effect of 5-HT on TRH release in humans. Because fluoxetine acts by increasing 5-HT transmission (Ni and Miledi 1997; Fong 1998), this drug should inhibit TRH release as well. In fact, TRH content of hypothalamic neuron cultures decreased in a dose-dependent manner following fluoxetine treatment (Jackson and Luo 1998). These studies indicate that serotonin and fluoxetine both inhibit the release of TRH from the hypothalamus, which may lead to a decrease in circulating levels of thyroid hormones (Fig. 1.3). Another study, conducted by Golstein et al. (1983), suggests that fluoxetine may inhibit the thyroid axis in mammals, but by another mechanism. They found that chronic administration of fluoxetine to rats resulted in a decrease in serum T_3

and T₄ levels. The authors also observed elevated TSH levels and concluded that fluoxetine directly influenced the decrease in T₃ and T₄ levels, leading to an inhibition of TH-mediated thyroid-pituitary feedback. Although a consensus has not been reached regarding the mechanism of action, all of these studies suggest that fluoxetine may have the ability to inhibit the thyroid axis and decrease circulating levels of thyroid hormones in mammals. Because mechanisms of thyroid hormone synthesis and release are highly conserved across species, fluoxetine may have the same effect in other organisms, including larval amphibians.

Objectives and Outline of Thesis Research

The purpose of this research was to evaluate the effects of fluoxetine on amphibian metamorphosis. The effect of this compound on amphibians is unknown; however, mammalian studies show that fluoxetine may inhibit the thyroid axis and decrease circulating levels of thyroid hormones. In larval amphibians, increasing levels of these hormones are necessary for metamorphosis to occur. I hypothesized that the presence of low concentrations of fluoxetine in aquatic habitats may decrease thyroid hormone levels in amphibian larvae and delay the completion of metamorphosis. I tested this hypothesis in a preliminary experiment by measuring the time to completion of metamorphosis in *Xenopus laevis*, the African clawed frog (Chapter 2). I confirmed these preliminary observations in a second experiment using increased numbers of replicates and a narrowed range of exposure concentrations (Chapter 3). Finally, the major findings of the two experiments are compared and the implications of environmental exposure of amphibians to fluoxetine are discussed (Chapter 4).

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Table 1.1. Summary of effects of fluoxetine exposure on freshwater aquatic organisms.

Organism	Duration	EC ₅₀	NOEC	LOEC	Medium	Source
<i>Pseudokirchneriella subcapitata</i>	120-h	24 µg/L ^b	--	13.6 µg/L ^d	aqueous	Brooks et al. 2003b,c
<i>Daphnia magna</i>	48-h	820 µg/L ^a	--	--	aqueous	Brooks et al. 2003b,c
<i>Ceriodaphnia dubia</i>	48-h	510 µg/L ^a	--	--	aqueous	Henry et al. 2004
<i>Ceriodaphnia dubia</i>	48-h	234 µg/L ^a	--	--	aqueous	Brooks et al. 2003b,c
<i>Ceriodaphnia dubia</i>	7-d	--	89 µg/L ^c	447 µg/L ^c	aqueous	Henry et al. 2004
<i>Ceriodaphnia dubia</i>	7-d	--	56 µg/L ^c	112 µg/L ^c	aqueous	Brooks et al. 2003b
<i>Pimphales promelas</i>	48-h	705 µg/L ^a	--	--	aqueous	Brooks et al. 2003b,c
<i>Gambusia affinis</i>	7-d	614 µg/L ^a	--	--	aqueous	Henry and Black 2004
<i>Gambusia affinis</i>	90-d	--	--	60 µg/L ^e	aqueous	Henry and Black 2004
<i>Oryzias latipes</i>	28-d			0.1 µg/L ^f	aqueous	Brooks et al. 2003b
<i>Chironomus tentans</i>	10-d	15.2 mg/kg ^a	--	1.3 mg/kg ^b	sediment	Brooks et al. 2003b,c
<i>Hyalella azteca</i>	10-d	>43 mg/kg ^a	--	5.4 mg/kg ^b	sediment	Brooks et al. 2003b,c

EC₅₀ = median effect concentration; NOEC = no observable effect concentration; LOEC = lowest observable effect concentration;

^asurvival (LC₅₀)

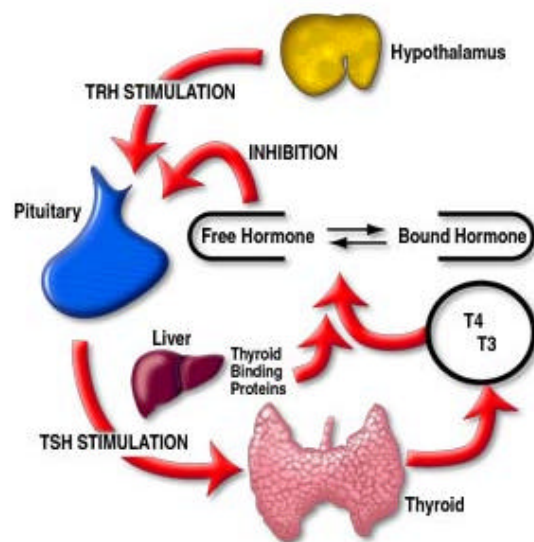
^bgrowth

^cnumber of neonates per female

^dcell deformities

^edelayed sexual maturation

^fdevelopmental abnormalities



www.dpcweb.com/images/medicalconditions/thyroid/thyroid%20illustration.jpg

Figure 1.1. Diagram of the vertebrate thyroid axis. Reproduced with permission. © 2000 Diagnostic Products Corporation.

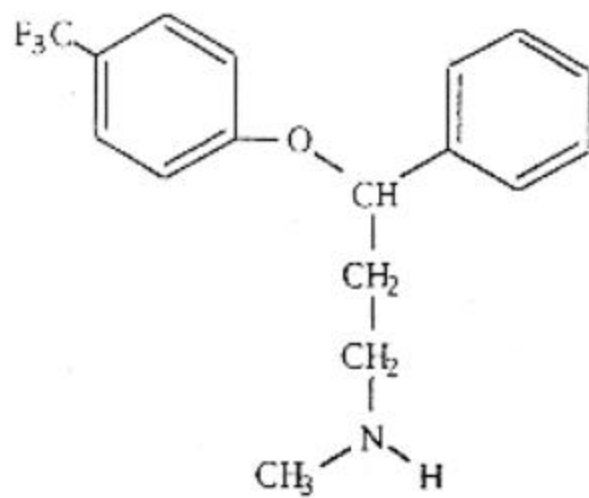


Figure 1.2. Molecular structure of fluoxetine.

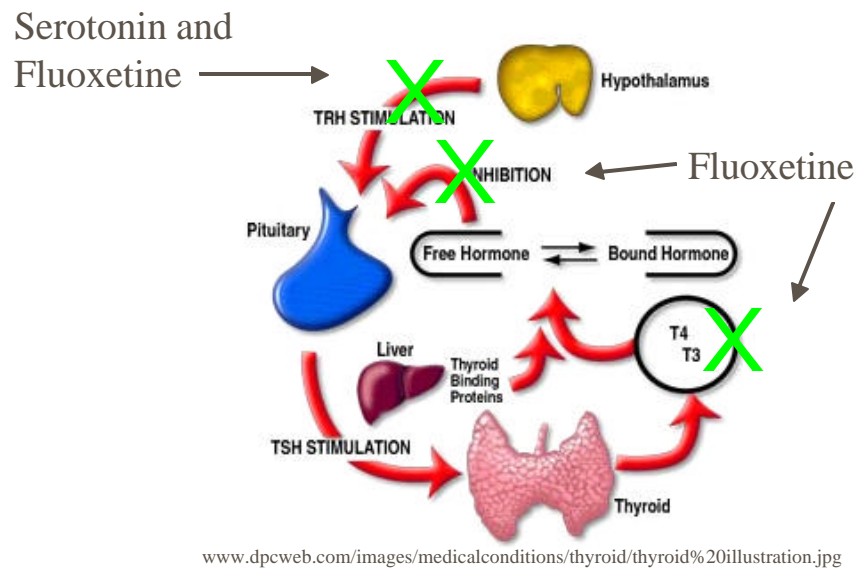


Figure 1.3. Proposed points of thyroid axis inhibition by serotonin and fluoxetine. Modified from Fig. 1.1. Original diagram reproduced with permission. © 2000 Diagnostic Products Corporation.

CHAPTER 2

EFFECTS OF FLUOXETINE ON DEVELOPMENT AND METAMORPHOSIS OF *XENOPUS* *LAEVIS*¹

¹ Rogers ED, Black MC. To be submitted to *Journal of Toxicology and Environmental Health*

Introduction

Pharmaceuticals have recently been recognized as potential trace contaminants in aquatic environments. These chemicals can enter aquatic ecosystems following their prescribed use in human or veterinary medicine after passing through wastewater treatment plants and entering surface waters. While environmental concentrations are generally less than one part per billion (ppb) (Kolpin et al. 2002; Metcalfe et al. 2003), chronic exposure may affect non-target aquatic organisms because pharmaceuticals are designed to have a biological effect, aquatic organisms are exposed throughout their development, and exposure may occur over multiple generations (Daughton and Ternes 1999).

Our research focuses on fluoxetine, a member of the class of anti-depressant drugs commonly referred to as selective serotonin reuptake inhibitors (SSRIs). These compounds act on the central nervous system and reduce the reuptake of serotonin by transport proteins, thereby increasing the transmission of serotonergic neurons (Ni and Miledi 1997; Fong 1998). Fluoxetine is listed among the 200 most-prescribed drugs in medicine (RxList 2001), and low concentrations (0.012 ppb – 0.099 ppb) have been reported in surface water samples from streams in the United States and Canada (Kolpin et al. 2002; Metcalfe et al. 2003).

Although there have been no direct toxicological studies on fluoxetine and amphibian metamorphosis, research shows that thyroid function in mammals is affected by serotonin and fluoxetine. Mitsuma and Nogimori (1983) demonstrated that in rats, serotonin (5-HT) inhibits the release of TRH from the hypothalamus. They also showed that serotonin inhibited TRH release from the rat retina in a dose-dependent manner (Mitsuma et al. 1996). Saphier and colleagues (1994) also support the inhibitory effect of 5-HT on TRH release in humans. Since fluoxetine acts by increasing 5-HT transmission (Ni and Miledi 1997; Fong 1998), this drug

should inhibit TRH release as well. In fact, TRH content of hypothalamic neuron cultures decreased in a dose-dependent manner following fluoxetine treatment (Jackson and Luo 1998). These studies indicate that serotonin and fluoxetine both inhibit the release of TRH from the hypothalamus, which may lead to a decrease in circulating levels of thyroid hormones. Another study, conducted by Golstein et al. (1983), suggests that fluoxetine may inhibit the thyroid axis in mammals, but by another mechanism. They found that chronic administration of fluoxetine to rats resulted in a decrease in serum T_3 and T_4 levels. The authors also observed elevated TSH levels and concluded that fluoxetine directly influenced the decrease in T_3 and T_4 levels, leading to an inhibition of TH-mediated thyroid-pituitary feedback. Although a consensus has not been reached regarding the mechanism of action, all of these studies suggest that fluoxetine has the ability to inhibit the thyroid axis.

The thyroid axis plays an important role in amphibian development. Increasing concentrations of thyroid hormones are necessary for metamorphosis to occur (Shi 2000; Denver et al. 2002), but if thyroid hormone levels are depressed metamorphosis may be delayed or totally inhibited. Metamorphosis has been blocked experimentally by inhibiting the production of endogenous thyroid hormones in tadpoles. Tadpoles whose thyroid glands were removed early in development became abnormally large, but never metamorphosed (Dodd and Dodd 1976). Metamorphosis has also been blocked by chemical thyroid inhibitors, including thiourea, thiouracil, sulfonamides, thiocyanate, potassium perchlorate, and methimazole (Shi 2000). Additionally, Goleman and colleagues (2002) demonstrated that aqueous exposure to ammonium perchlorate decreased whole body T_4 levels and inhibited metamorphosis in *Xenopus laevis*.

The objective of this study was to evaluate the effects of fluoxetine exposure on development and completion of metamorphosis in *Xenopus laevis*. Because mammalian studies

have shown that fluoxetine inhibits the thyroid axis, the drug is hypothesized to have a similar effect on the thyroid axis of amphibians. Inhibition of the thyroid axis by fluoxetine should decrease thyroid hormone levels in tadpoles, resulting in delayed metamorphosis.

Materials and Methods

Adult care and breeding

Laboratory reared, sexually mature *Xenopus laevis* frogs were obtained from a commercial supplier (Carolina Biological Supply Company, Burlington, NC, USA) and held in flow-through aquaria containing dechlorinated tap water. Adults were maintained according to ASTM standards for FETAX testing (ASTM 1999). Water temperature was regulated at $23 \pm 3^{\circ}$ C, and a natural light cycle was mimicked by adjusting the timing of fluorescent lights to reflect environmental conditions. Adults were fed frog brittle (Nasco, Ft. Atkinson, WI, USA) daily.

To stimulate breeding, two pairs of frogs were injected with human chorionic gonadotropin (hCG), with males and females receiving 500 and 1,000 I.U., respectively. Following injection, mating pairs were placed in separate breeding tanks with false bottoms containing FETAX medium (ASTM 1999). The pairs were kept in a dark incubator overnight to breed.

Exposure

After obtaining eggs, adults were removed from the breeding containers. Egg masses from each breeding pair were combined and placed in fresh FETAX solution. Viable eggs were then chosen at random from the pooled egg mass for use in the experiment. Eggs were placed in 8x8x6" polyethylene containers (1 egg per container) in one liter of FETAX, fluoxetine, or

ammonium perchlorate solution. Ammonium perchlorate served as a positive control because it is a known inhibitor of the thyroid axis and metamorphosis in *Xenopus laevis* (Goleman et al. 2002). Exposing embryos individually allowed us to avoid pseudoreplication and eliminate competition demonstrated in high-density larval exposures (Wilbur and Collins 1973; Semlitsch and Caldwell 1982). The following concentrations were used; fluoxetine - 0.05, 0.5, 5, 50, and 500 ppb; ammonium perchlorate - 10, 100, and 1,000 ppb. All dilutions were made in FETAX solution, and FETAX solution served as the negative control. There were five experimental replicates for the negative control and each concentration of fluoxetine and ammonium perchlorate. Tadpoles were fed tadpole formula frog brittle (NASCO) *ad libitum*. A 50% change of exposure solutions was performed two times per week. When each animal completed metamorphosis (determined by complete resorption of the tail), it was euthanized in MS-222.

Five exposure containers were chosen at random for weekly water quality measurements including temperature, dissolved oxygen, pH, ammonia, conductivity, hardness and alkalinity. In addition, composite water samples were collected at the beginning of the experiment and on day 40 and were sent to Mississippi State Chemical Laboratory (Mississippi State, MS, USA) for fluoxetine analysis. Water samples for analysis of ammonium perchlorate were taken at the beginning of the experiment and were analyzed by the Ecosystems Research Division of the United States Environmental Protection Agency (Athens, GA, USA).

The following data were recorded during the exposure: time to hatching, time to forelimb emergence, and time to tail resorption (metamorphosis). Developmental analyses were performed at two discrete time points- before (day 47) and after (day 61) forelimb emergence occurred in controls. On these dates, individuals were weighed, staged (Nieuwkoop and Faber 1994), and photographed with a digital camera (Magnafire SP, Olympus, Inc.) attached to a

dissecting microscope. Image analysis software (Sigma Scan Pro 5.0) was used to determine hind limb length from digital images. At the completion of metamorphosis, snout vent length was measured, and the animals were weighed. Throughout the experiment, individuals were observed daily for gross malformations and abnormal swimming behavior.

The experiment was terminated at 157 days, when the last tadpole in the 10 ppb ammonium perchlorate group completed metamorphosis. At that time there were 5 individuals remaining in the two higher ammonium perchlorate treatments that had not reached forelimb emergence. Since it was obvious that these animals would not complete metamorphosis within a reasonable amount of time, the experiment was terminated at 157 days.

Statistics

All data were analyzed by ANOVA followed by appropriate post-hoc tests. Before ANOVAs were executed, Shapiro-Wilk's test for normality and Bartlett's test for homogeneity of variance were performed. Following ANOVA, William's test was applied to data sets that showed monotonic trends, while t-tests with Bonferroni's adjustment were used for data sets that displayed oscillating trends. All analyses were performed using ToxStat Version 3.4 (WEST, Inc., Cheyenne, WY, USA).

Results

Water Quality

Ranges of water quality characteristics measured in exposure containers were as follows: temperature: 21.8 to 22.0 °C; dissolved oxygen: 7.01 to 7.32 mg/L; pH: 6.93 to 7.09; conductivity: 1105-1126 µS/cm; ammonia: < 0.2 mg/L; hardness: 106-109 mg/L as CaCO₃;

alkalinity: 63-64 mg/L as CaCO₃ (Table 2.1). All water quality values were within the optimum exposure conditions specified for *Xenopus laevis* tadpoles (ASTM 1999).

Measured Concentrations of Ammonium Perchlorate and Fluoxetine

Ammonium perchlorate solutions were analyzed at the beginning of the experiment and were 9.5, 91, and 944 ppb (nominal: 10, 100, and 1,000 ppb). Fluoxetine water samples from the 50 ppb tanks (nominal concentration) were taken at the beginning of the experiment and on day 40. The mean measured concentration at the beginning of the experiment was 51.4 ppb and declined to 37.5 ppb on day 40. We calculated the time-weighted average of the two measured concentrations to estimate the average fluoxetine concentration for the entire experiment, and the result was 38 ppb. We used the time-weighted average to extrapolate the measured values of all of the nominal concentrations of fluoxetine that were used. Measured concentrations based on the time-weighted average were as follows: 0.038, 0.38, 3.8, 38, and 380 ppb (nominal: 0.05, 0.5, 5, 50, and 500 ppb). Concentrations of fluoxetine and ammonium perchlorate referred to in text and figures are measured concentrations.

Mortality

Overall, 51% of the tadpoles died during the 157-day exposure. Two individuals, one in the control group and one in the 9.5 ppb ammonium perchlorate group, were accidentally siphoned during water changes and did not recover from their injuries. Because these deaths were not caused by treatment, they were not included in graphs or statistical analyses. No additional mortality occurred in controls. The concentration with the highest cumulative mortality was 380 ppb fluoxetine. All tadpoles in this treatment died within 20 days of exposure

(Fig 2.1). High cumulative mortality (80%) also occurred in the 0.38 ppb fluoxetine group (Fig. 2.2). Cumulative mortality in treatment groups did not increase monotonically with concentration, and there was a baseline mortality of 20% (1 tadpole per treatment) in all treatment groups (Fig. 2.2). Baseline mortality occurred during early development in all fluoxetine treatments, except 38 ppb. In general, the majority of deaths in all treatment groups occurred after forelimb emergence.

Development

Controls completed forelimb emergence in an average of 59.75 days, and tadpoles in 0.038, 0.38, and 3.8 ppb fluoxetine completed forelimb emergence at approximately the same time as the control (Fig. 2.3). Individuals exposed to 38 ppb fluoxetine completed forelimb emergence an average of four days later than controls, but the delay was not significant. Forelimb emergence was delayed by 8 days in the 9.5 ppb ammonium perchlorate treatment, and this delay was also not statistically significant. Individuals exposed to 91 and 944 ppb ammonium perchlorate had not completed forelimb emergence at the time that the experiment was terminated. Developmental parameters, including mass, hind limb length, and stage were measured immediately before (day 47) and after (day 61) forelimb emergence occurred in controls. There were no significant effects on mass among treatments at either time point. Hind limb length of tadpoles was significantly shorter than controls at 91 and 944 ppb ammonium perchlorate on day 47 and at all concentrations of ammonium perchlorate and 50 ppb fluoxetine on day 67 (Fig 2.4A). The same trends were observed with staging. Development was significantly delayed at day 47 by 91 and 944 ppb ammonium perchlorate, and at all concentrations of ammonium perchlorate as well as 38 ppb fluoxetine on day 61 (Fig 2.4B).

Both staging and hind limb length measurements indicated that tadpoles exposed to ammonium perchlorate and 38 ppb fluoxetine experienced developmental delays during the experiment.

Metamorphosis

Metamorphic success rates for surviving individuals were 100%, 80%, 20%, 60%, and 40% for control, 0.038, 0.38, 3.8, and 38 ppb fluoxetine treatments, respectively. Individuals exposed to 91 and 944 ppb ammonium perchlorate did not undergo metamorphosis before the termination of the experiment, and only one tadpole completed metamorphosis in the 9.5 ppb group (20% success rate). The 20% metamorphic success rates for 0.38 ppb fluoxetine and 9.5 ppb ammonium perchlorate treatments were each due to the metamorphosis of one individual (n=1). All other tadpoles in these treatments died before completing metamorphosis. Because both of these groups had only one replicate remaining, they were excluded from all statistical analyses pertaining to metamorphosis.

Controls completed metamorphosis in 70 days, and the individuals in 0.038 and 3.8 ppb fluoxetine completed metamorphosis shortly thereafter at 78 and 80 days, respectively (Fig 2.5). Metamorphosis was significantly delayed at 38 ppb fluoxetine, and these individuals completed metamorphosis 50 days later than controls. Individuals exposed to 3.8 and 38 ppb fluoxetine were significantly smaller than controls at metamorphosis (Fig. 2.6A), and the mean mass of froglets from 0.038, 3.8 and 38 ppb fluoxetine treatments was significantly reduced (Fig 2.6B).

Discussion

Because no amphibian toxicity data were available for fluoxetine, we designed this experiment as a range finding test in order to gain preliminary information on concentrations

causing lethality, as well as sub-lethal developmental effects. Because mortality was not expected to occur at low concentrations, we used a wide range of exposure concentrations with five replicates per treatment. In the 0.38 ppb fluoxetine and 9.5 ppb ammonium perchlorate groups, four out of five tadpoles died over the course of the experiment, leaving only one replicate remaining to complete metamorphosis. Due to a lack of replicates ($n = 1$ remaining in each group), both of these treatment groups were excluded from statistical analyses pertaining to metamorphosis. Mortality in remaining treatment groups was almost as high, weakening the overall statistical power of the study. Future experiments will be designed using fewer concentrations and more replicates per treatment.

Despite the statistical limitations of this study, we were able to determine lethal and sub-lethal concentrations of fluoxetine. Fluoxetine was acutely toxic to tadpoles at 380 ppb, causing 100% mortality within 20 days of exposure. This dose is considerably higher than concentrations that would be expected to occur in the environment, and our results suggest that acute lethality is probably not a concern. During early development, at least 20% of tadpoles in all treatment groups died. Interestingly, no mortality occurred during hind limb development, while the majority of tadpole deaths took place during metamorphic climax. Fluoxetine may cause death in later stages of development by interfering with structural and physiological changes that take place during metamorphic climax. While long-term exposure did result in tadpole mortality, the lowest concentration to achieve cumulative mortality above 20% was 0.38 ppb, which is still higher than environmentally relevant concentrations of fluoxetine. Our study indicates that the presence of low levels of fluoxetine in aquatic systems should not affect the short or long-term survival of developing frogs.

Environmentally relevant concentrations of fluoxetine had no effect on time to completion of metamorphosis or snout-vent length of metamorphs; however mass at metamorphosis was significantly reduced at concentrations that may be expected to occur in the environment. The lowest concentration of fluoxetine that produced metamorphs with significant mass reduction was 0.038 ppb. This concentration is well within the range of levels that have been reported in freshwater aquatic systems (0.012 – 0.099 ppb) (Kolpin et al. 2002; Metcalfe et al. 2003). Because snout-vent length and mass are both measures of size, significant reductions in mass at metamorphosis caused by exposure to environmentally relevant concentrations of fluoxetine may have serious fitness implications for amphibians. Smaller metamorphs are more vulnerable to predation by gape-limited predators (Caldwell et al. 1980). In some species, reduced size at metamorphosis has also been associated with increased times to reach sexual maturity and therefore longer times to first reproduction (Smith 1987; Semlitsch et al. 1988). Smaller metamorphs may have a lower probability of reaching sexual maturity and reproducing successfully.

Conclusion

Although the results of this study are preliminary, our findings suggest that fluoxetine delays metamorphosis in *Xenopus laevis*, but not at levels that have been reported in the environment. We believe that the delayed metamorphosis observed in this study was due to an inhibition of the thyroid axis by fluoxetine; however more research is necessary to establish a definitive link between fluoxetine exposure, depressed levels of thyroid hormones, and delayed metamorphosis. Mass at metamorphosis was, however, significantly reduced at environmentally relevant concentrations. Although the life cycle of *Xenopus laevis* differs from North American

species, our results suggest that the presence of fluoxetine in aquatic habitats may have the potential to affect the fitness of native anurans.

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Table 2.1. Summary of water quality parameters measured weekly during the 157-day exposure.

	Temperature (°C)	Dissolved Oxygen (mg/L)	pH	Conductivity (μ S/cm)	Ammonia (mg/L)	Hardness (mg/L as CaCO ₃)	Alkalinity (mg/L as CaCO ₃)
Control	22.0 \pm 0.07	7.32 \pm 0.01	6.93 \pm 0.04	1126 \pm 13	<0.2	106 \pm 2.29	63 \pm 0.59
Fluoxetine	22.0 \pm 0.06	7.01 \pm 0.04	7.09 \pm 0.01	1123 \pm 27	<0.2	109 \pm 1.03	64 \pm 0.02
Ammonium Perchlorate	21.8 \pm 0.14	7.21 \pm 0.03	6.95 \pm 0.08	1105 \pm 3	<0.2	107 \pm 1.12	63 \pm 0.37

Data are means \pm SD.

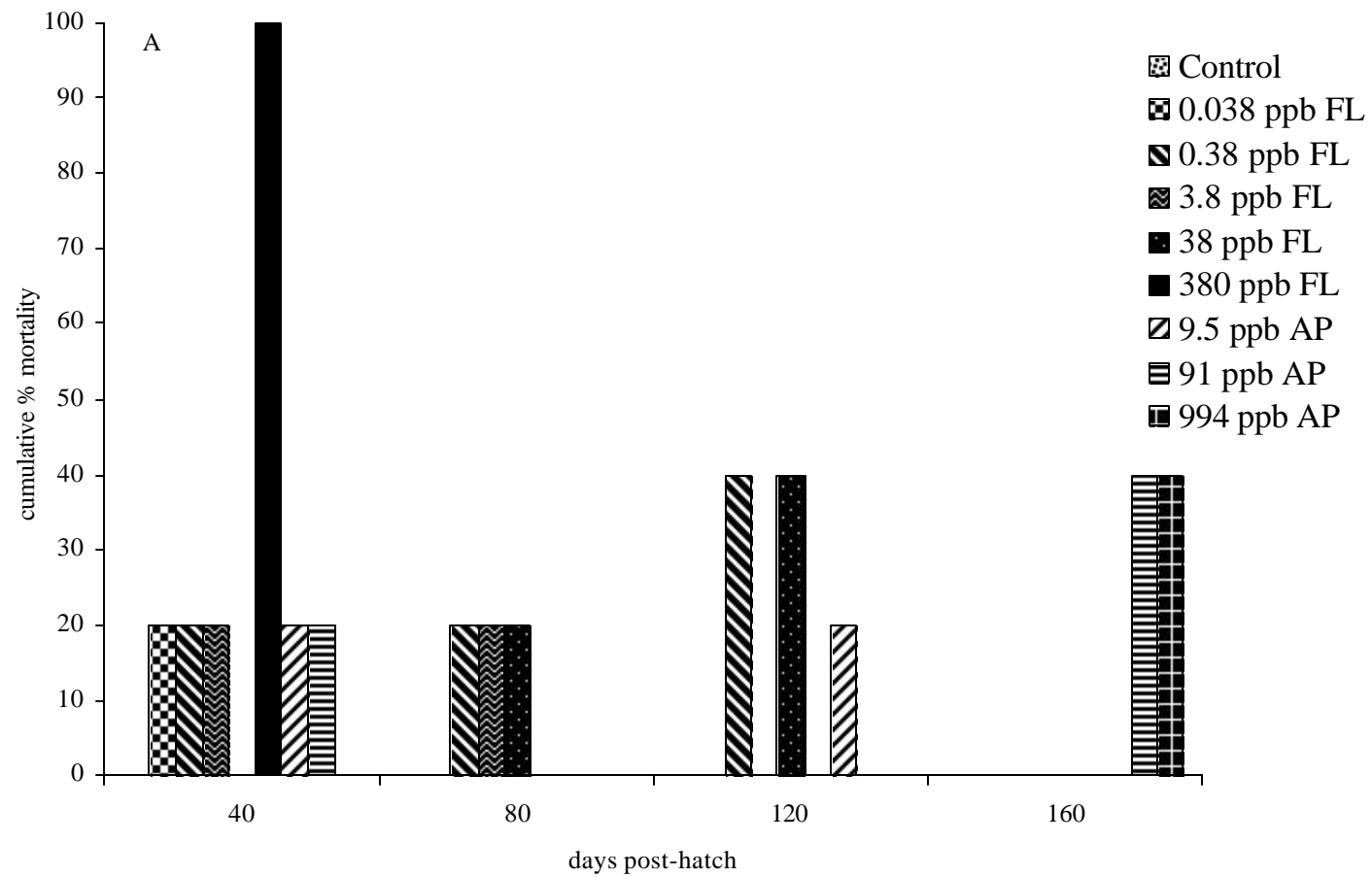


Figure 2.1. Plot of percent mortality over the course of the exposure. FL = fluoxetine, AP = ammonium perchlorate.

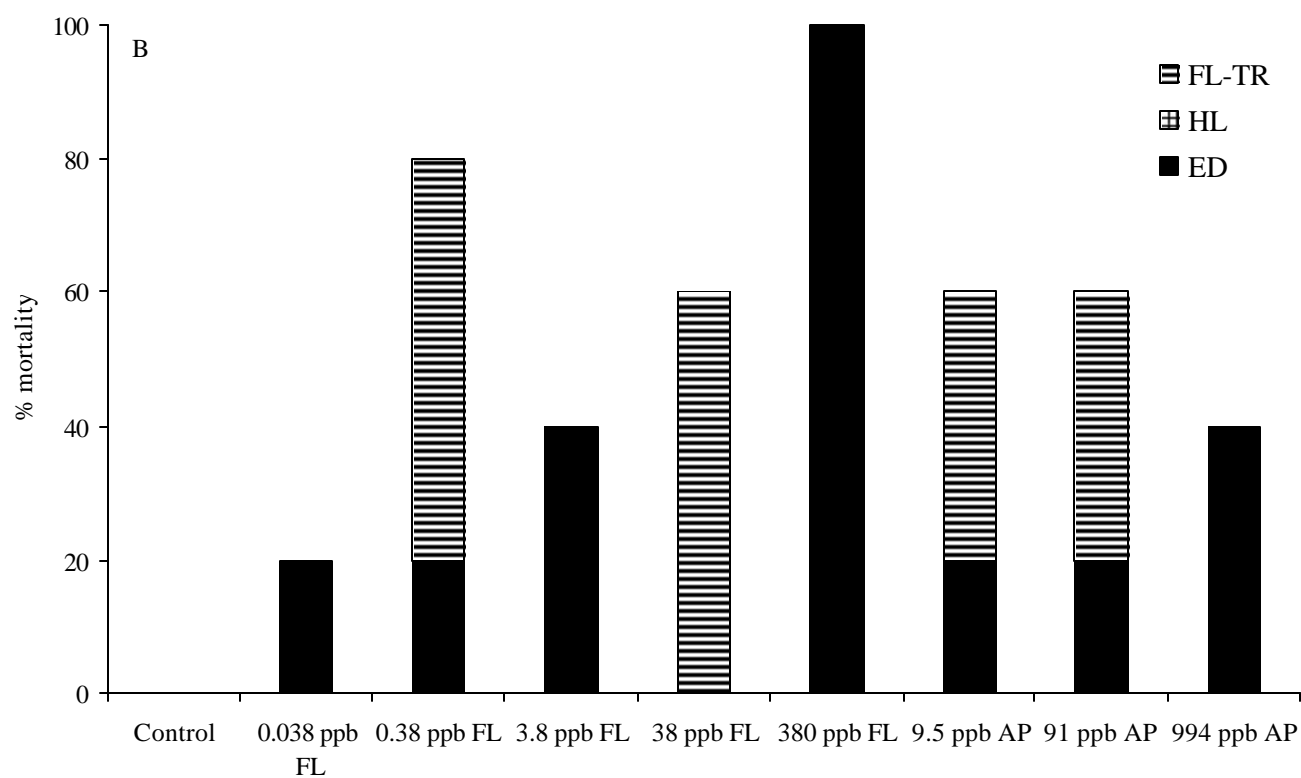


Figure 2.2. Plot of cumulative percent mortality. FL = fluoxetine, AP = ammonium perchlorate, FL-TR = % mortality occurring during the time between forelimb emergence and tail resorption, ED = % mortality occurring during early development. No mortality occurred during the time between early development and forelimb emergence (HL).

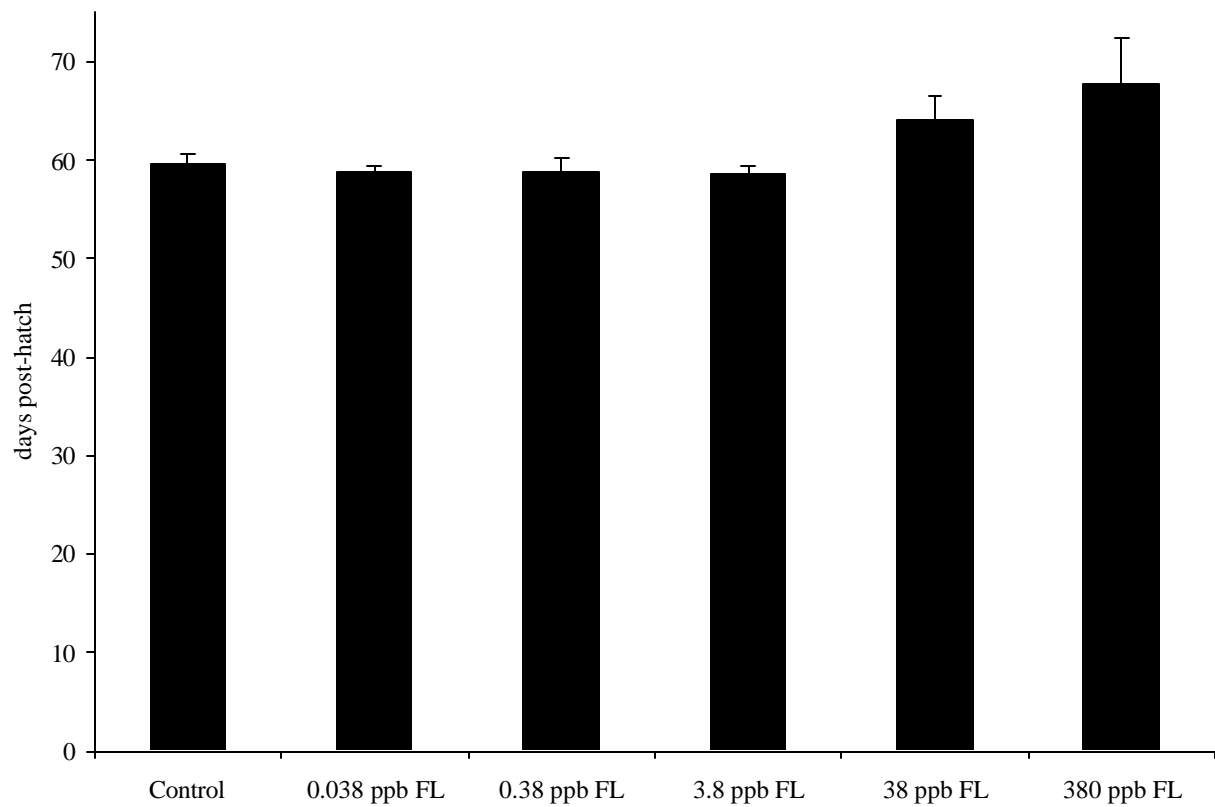


Fig. 2.3. Mean time to forelimb emergence (\pm SE) in days post-hatch. FL = fluoxetine, AP = ammonium perchlorate. No significant differences were observed among treatments at $\alpha = 0.05$.

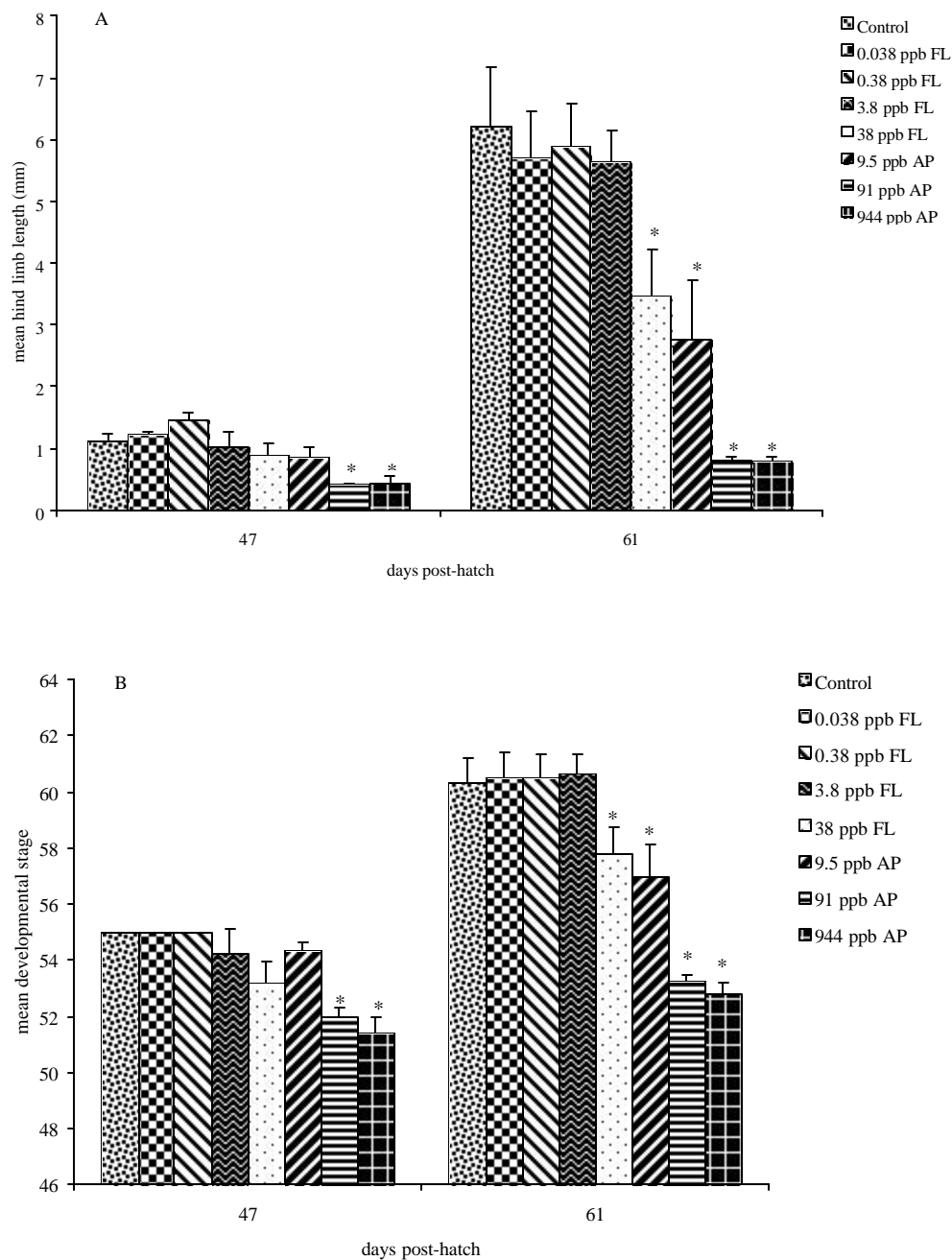


Fig. 2.4. Plots of mean hind limb length (\pm SE) (panel A) and mean developmental stage (\pm SE) (panel B) at 47 and 61 days post-hatch. Developmental stages were assigned according to Nieuwkoop and Faber (1994). FL = fluoxetine, AP = ammonium perchlorate.

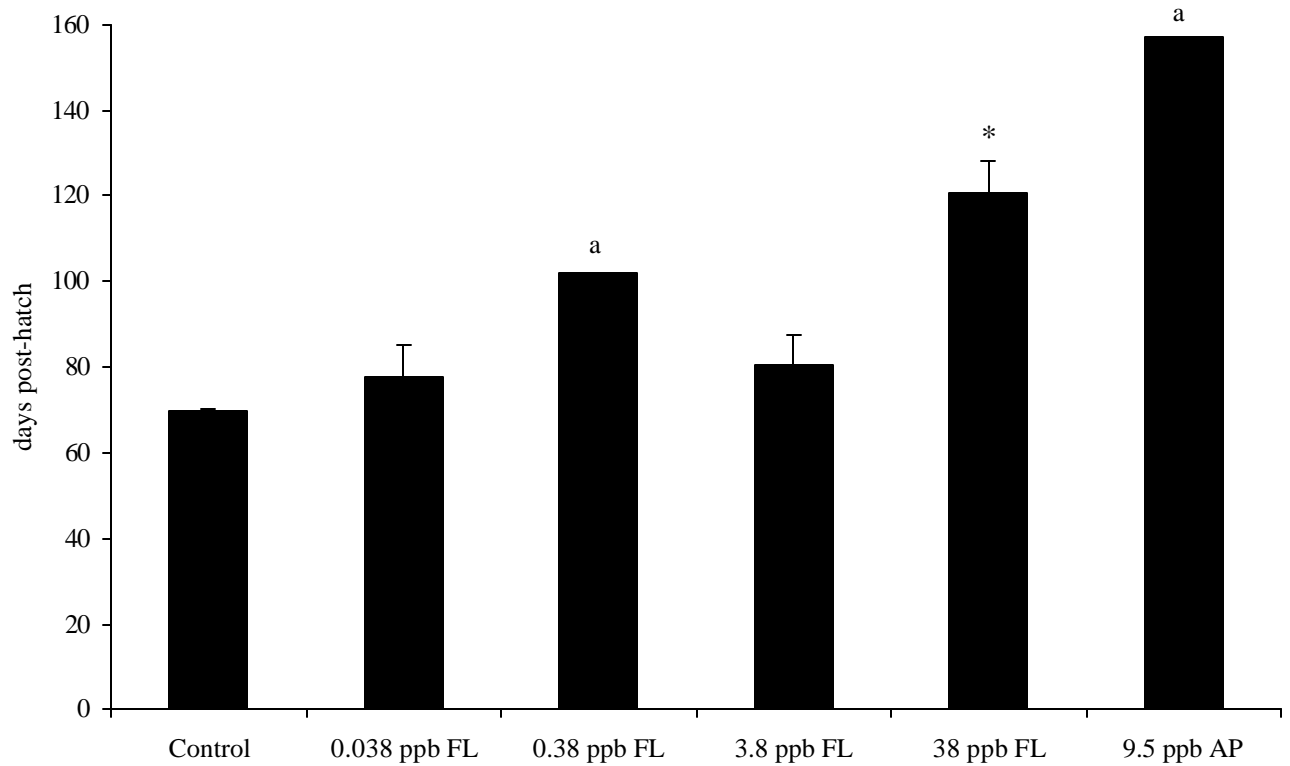


Fig. 2.5. Mean time to completion of metamorphosis (\pm SE) in days post-hatch. FL = fluoxetine, AP = ammonium perchlorate; * = significantly greater than control at $\alpha = 0.05$; a = data not included in statistical analysis ($n = 1$).

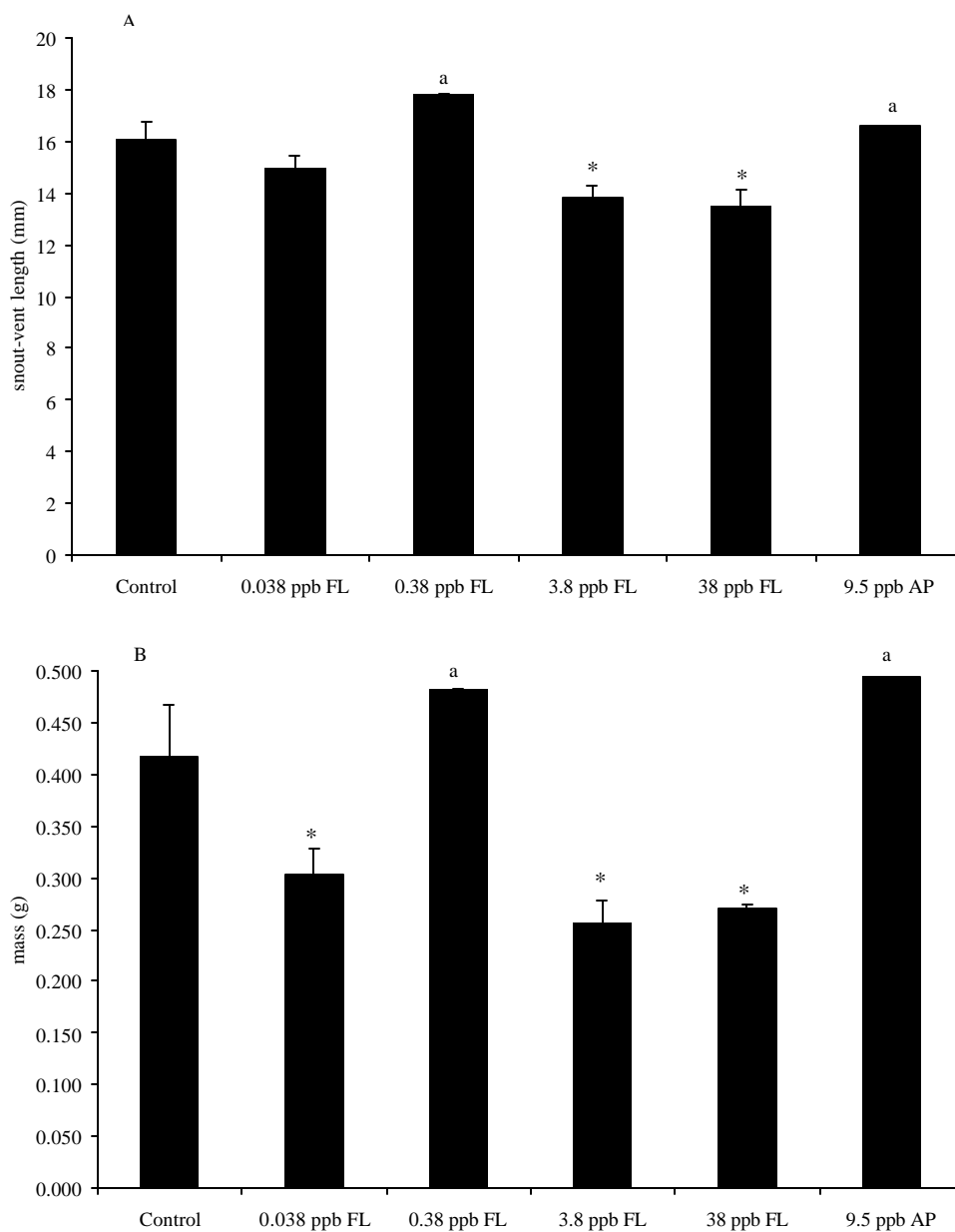


Fig. 2.6. Plots of mean snout-vent length (\pm SE) (panel A) and mean mass (\pm SE) (panel B) at metamorphosis. FL = fluoxetine, AP = ammonium perchlorate, * = significantly different from control ($\alpha = 0.05$); a = data not included in statistical analysis ($n = 1$).

CHAPTER 3

DEVELOPMENTAL ABNORMALITIES AND REDUCTIONS IN GROWTH IN TADPOLES (*XENOPUS LAEVIS*) EXPOSED TO FLUOXETINE¹

¹Rogers ED, Black MC. To be submitted to *Environmental Toxicology and Chemistry*

Introduction

Pharmaceuticals have recently been recognized as potential trace contaminants in aquatic environments. These chemicals can enter aquatic ecosystems following their prescribed use in human or veterinary medicine after passing through wastewater treatment plants and entering surface waters. Most municipal sewage treatment plants do not have the capability to remove pharmaceuticals from treated effluent before it is released into surface waters. While environmental concentrations are generally less than one part per billion (ppb) (Kolpin et al. 2002; Metcalfe et al. 2003), chronic exposure may affect non-target aquatic organisms because pharmaceuticals are designed to have a biological effect, aquatic organisms are exposed throughout their development, and exposure may occur over multiple generations (Daughton and Ternes 1999).

Our research focuses on fluoxetine, a member of the class of anti-depressant drugs commonly referred to as selective serotonin reuptake inhibitors (SSRIs). These compounds act on the central nervous system and reduce the reuptake of serotonin by transport proteins, thereby increasing the transmission of serotonergic neurons (Ni and Miledi 1997; Fong 1998). By artificially increasing serotonin transmission, serotonin reuptake inhibitors have been shown to improve mood in people experiencing depression and certain types of compulsive behavior disorders (Appleton 1997). Fluoxetine is listed among the 200 most-prescribed drugs in medicine (RxList 2001), and low concentrations (0.012 ppb – 0.099 ppb) have been reported in surface water samples from streams in the United States and Canada (Kolpin et al. 2002; Metcalfe et al. 2003).

A variety of effects have been observed with sub-acute to chronic exposures. In marine invertebrates, fluoxetine seems to stimulate reproduction. Exposure to exogenous serotonin

accelerated the development of the testes in fiddler crabs (Sarojini et al. 1993) and also increased the ovarian index and oocyte size in the red swamp crayfish (Kulkarni et al. 1992). Spawning in several bivalve species was induced by serotonin (Gibbons and Castagna 1984; Fong 1998) and by low concentrations of two serotonin reuptake inhibitors, fluoxetine and fluvoxamine (Fong 1998). In another study, Couper and Leise (1996) found that serotonin induced metamorphosis in a marine mollusk. This information has been applied in commercial aquaculture to facilitate bivalve culturing (Gibbons and Castagna 1984).

Reproductive and developmental toxicity information in freshwater organisms is limited, but suggests that they may respond differently to fluoxetine exposure. Lowest observable effect concentrations (LOECs) of 112 ppb (Brooks et al. 2003a,b) and 447 ppb (Henry et al. 2004) reduced the number of offspring produced by *Ceriodaphnia dubia* during 7-day multigenerational exposures. Henry and Black (personal communication) found that western mosquitofish (*Gambusia affinis*) exposed to 60 ppb fluoxetine for 90 days experienced delayed development of adult sexual morphology, as evidenced by the presence of a gonopodium in males or a black spot in females. In contrast to marine studies that indicate that fluoxetine stimulates reproduction and development, research conducted with freshwater aquatic organisms in an environmental context implies that fluoxetine may impair reproduction and delay development.

Amphibian development is regulated by thyroid hormones. The thyroid gland develops during the embryo stage and is functional at the time of hatching. During premetamorphosis, the stage immediately following hatching, the larvae grow rapidly, but do not undergo any major developmental changes. At this time, thyroid hormone levels are low. During the next stage in development, prometamorphosis, the hind limbs begin developing, and plasma thyroid hormone

concentrations increase. At metamorphic climax, thyroid hormones reach peak levels, and at the same time, the animal undergoes rapid changes in development. Within a short time period, the forelimbs emerge and the tail is resorbed. When the tail has been fully resorbed, metamorphosis is complete, and the animal becomes a juvenile frog (Shi 2000; Denver et al. 2002).

Metamorphosis has been blocked experimentally by inhibiting the production of endogenous thyroid hormones in tadpoles. Tadpoles whose thyroid glands were removed early in development became abnormally large, but never metamorphosed. Two years after the completion of the experiment, the tadpoles were still developmentally in the premetamorphosis stage (Dodd and Dodd 1976). Metamorphosis has also been blocked by chemical thyroid inhibitors, including thiourea, thiouracil, sulfonamides, thiocyanate, potassium perchlorate, and methimazole (Shi 2000). Additionally, Goleman and colleagues (2002) demonstrated that aqueous exposure to ammonium perchlorate decreased whole body T_4 levels and inhibited metamorphosis in *Xenopus laevis*.

Although there have been no direct toxicological studies on fluoxetine and amphibian metamorphosis, research shows that thyroid function in mammals is affected by serotonin and by the serotonin reuptake inhibitor, fluoxetine. Mitsuma and Nogimori (1983) demonstrated that in rats, serotonin (5-HT) inhibits the release of TRH from the hypothalamus. They also showed that serotonin inhibited TRH release from the rat retina in a dose-dependent manner (Mitsuma et al. 1996). Saphier and colleagues (1994) also support the inhibitory effect of 5-HT on TRH release in humans. Because fluoxetine acts by increasing 5-HT transmission (Ni and Miledi 1997; Fong 1998), this drug should inhibit TRH release as well. In fact, TRH content of hypothalamic neuron cultures decreased in a dose-dependent manner following fluoxetine treatment (Jackson and Luo 1998). These studies indicate that serotonin and fluoxetine both

inhibit the release of TRH from the hypothalamus, which may lead to a decrease circulating levels of thyroid hormones. Another study, conducted by Golstein et al. (1983), suggests that fluoxetine may inhibit the thyroid axis in mammals, but by another mechanism. They found that chronic administration of fluoxetine to rats resulted in a decrease in serum T₃ and T₄ levels. The authors also observed elevated TSH levels and concluded that fluoxetine directly influenced the decrease in T₃ and T₄ levels, leading to an inhibition of TH-mediated thyroid-pituitary feedback. Although a consensus has not been reached regarding the mechanism of action, all of these studies suggest that fluoxetine has the ability to inhibit the thyroid axis.

The objective of this study was to evaluate the effects of fluoxetine exposure on development and completion of metamorphosis in *Xenopus laevis*. Because mammalian studies have shown that fluoxetine inhibits the thyroid axis, the drug is hypothesized to have a similar effect on the thyroid axis of amphibians. Inhibition of the thyroid axis by fluoxetine should decrease thyroid hormone levels in tadpoles, resulting in delayed metamorphosis.

Materials and Methods

Tadpoles were exposed from early development through completion of metamorphosis. Stage 33/34 *Xenopus laevis* larvae (Carolina Biological Supply Co., Burlington, NC, USA) were placed in 8x8x6" polyethylene storage boxes (1 embryo per container) containing fluoxetine, ammonium perchlorate, or FETAX solution. Ammonium perchlorate was used as a positive control in this experiment because it is a known inhibitor of the thyroid axis and metamorphosis in *Xenopus laevis* (Goleman et al. 2002). All solutions were prepared in FETAX solution (ASTM 1999), and contaminant-free FETAX solution served as the negative control. The following nominal concentrations were used; fluoxetine - 0, 0.1, 0.5, 5, and 50 ppb; ammonium

perchlorate - 10 ppb. Twelve replicates were used for each treatment (overall number of individuals exposed = 72). Tadpoles were fed tadpole formula frog brittle (NASCO, Fort Atkinson, WI, USA) *ad libitum*. All solutions were changed weekly (100% change), and fluorescent lights in the laboratory were adjusted to mimic the natural light cycle at the time of the experiment.

All animals were observed daily for behavioral changes, gross malformations, forelimb emergence, and tail resorption. Malformations were assessed according to Bantle et al. (1991) and Meteyer (2000). In addition, all individuals were photographed and staged beginning on the 40th day of exposure and at 10-day intervals thereafter. Staging was performed by examining individuals with a compound dissecting scope (Olympus, Inc.), and developmental stages were assigned according to Nieuwkoop and Faber (1994). Organisms were photographed with a digital camera with macro capability (Cyber Shot DSC-P10, Sony, Inc.), and image analysis software (Sigma Scan Pro 5.0) was used to determine total body length and tail length from digital images. Individuals were weighed when they reached forelimb emergence and complete tail resorption. When metamorphosis was complete, froglets were euthanized by immersion in MS-222 and stored in 10% neutral buffered formalin.

Twelve exposure containers were chosen at random for bi-weekly water quality measurements including temperature, dissolved oxygen, pH, ammonia, conductivity, hardness and alkalinity. The ammonium perchlorate stock solution used to make the 10 ppb exposure solution was analyzed by ion chromatography (Dionex DX-500, Sunnyvale, CA, USA) by the USEPA Region IV Ecosystems Research Division (Athens, GA, USA). Composite samples from 50 ppb fluoxetine tanks were taken immediately following a weekly 100% water change, four days later, and immediately before the next water change in order to account for degradation

of the compound. Samples were analyzed by direct injection onto an HPLC (Waters 2695, Milford, MA, USA) by the Mississippi State Chemical Laboratory (Mississippi State, MS, USA).

All data were analyzed by analysis of variance followed by appropriate post-hoc tests. Before ANOVAs were executed, Shapiro-Wilk's test for normality and Bartlett's test for homogeneity of variance were performed and data were transformed when necessary to meet the assumptions for ANOVA. Following ANOVA, William's test was applied to data sets that showed monotonic trends, while t-tests with Bonferroni's adjustment were used for data sets that displayed oscillating trends. Analyses of variance and Bonferroni's post-hoc tests were performed using SYSTAT Version 9 (SPSS, Inc., Chicago, IL, USA). William's test was performed using ToxStat Version 3.4 (WEST, Inc., Cheyenne, WY, USA).

At the thesis publication deadline, this experiment was near completion, with one tadpole remaining in the ammonium perchlorate exposure that had not yet completed metamorphosis. Ammonium perchlorate was therefore excluded as a positive control from all analyses pertaining to metamorphosis. This modification was necessary to meet thesis publication and graduation deadlines. This experiment will be carried to completion and data for ammonium perchlorate will be included when the results of this study are published in a scientific journal.

Results

Water Quality Parameters

Ranges of water quality characteristics measured in exposure containers were as follows: temperature: 18.4 to 18.7 °C; dissolved oxygen: 7.41 to 7.53 mg/L; pH: 7.02 to 7.07; conductivity: 997 to 1066 µS/cm; ammonia: < 0.2 mg/L; hardness: 94 to 95 mg/L as CaCO₃;

alkalinity: 51-52 mg/L as CaCO₃ (Table 3.1). All water quality values, except temperature, were within the optimum exposure conditions specified for *Xenopus laevis* tadpoles (ASTM 1999).

Analyses of Ammonium Perchlorate and Fluoxetine Solutions

The measured concentration of the 1,000 ppm ammonium perchlorate stock solution used in this experiment was 990.9 ppm. This stock solution was diluted to a nominal concentration of 10 ppb. Based on the measured value of the stock solution, the actual concentration of the 10 ppb solution should be 9.9 ppb. Measured concentrations of fluoxetine from the 50 ppb (nominal concentration) tanks taken immediately following a 100% water change, halfway between, and before the next weekly water change were 51.8 ppb, 23.0 ppb, and 29.8 ppb respectively and reflect means of samples analyzed in triplicate. A time-weighted average of these concentrations was calculated to estimate the average actual concentrations to which the animals were exposed during each week of the exposure. The time-weighted average was 29.5 ppb. This value was used to extrapolate all other concentrations of fluoxetine used in this experiment. Based on the time-weighted average, measured concentrations corresponding to nominal concentrations of 0.1, 0.5, 5, and 50 ppb were 0.059, 0.295, 2.95, and 29.5 ppb respectively. Concentrations of fluoxetine and ammonium perchlorate referred to in text and figures are measured concentrations.

Mortality

High cumulative mortality was observed in tadpoles exposed to fluoxetine. Control mortality was 16.7%, and mortality of tadpoles exposed to fluoxetine was substantially higher (Fig 3.1 A). Mean times to death decreased with increasing fluoxetine concentration (Fig 3.1 B).

When mortality was examined with respect to developmental stage, the majority of the tadpoles died at stage 59 (forelimb emergence) or later (Fig. 3.2).

Development

Staging the tadpoles at 10-day intervals revealed that development was significantly delayed compared to controls at exposure concentrations of 29.5 ppb fluoxetine and 9.9 ppb ammonium perchlorate (Fig 3.3). Tadpoles exposed to ammonium perchlorate experienced developmental delays throughout the experiment, whereas tadpoles in the 29.5 ppb fluoxetine treatment were significantly underdeveloped until day 90, when they began to undergo metamorphosis.

Developmental delays that were detected by staging tadpoles at regular intervals were further reinforced by delays in observed times to reach forelimb emergence. Tadpoles exposed to 29.5 ppb fluoxetine ($p = 0.012$) and 9.9 ppb ammonium perchlorate ($p = 0.001$) took significantly longer to reach forelimb emergence than controls (Fig. 3.4). Mass at forelimb emergence was not affected in tadpoles exposed to fluoxetine, but tadpoles exposed to 9.9 ppb ammonium perchlorate were significantly more massive than controls ($p = 0.008$).

Delays in early development and forelimb emergence ultimately translated into longer times to complete metamorphosis. Tadpoles exposed to 29.5 ppb fluoxetine took significantly longer than controls to complete metamorphosis ($p = 0.022$) (Fig. 3.5) and had significantly longer tail resorption times ($p < 0.001$) (Fig. 3.6). Tail length as a percentage of total body length was significantly greater than controls at 29.5 ppb fluoxetine, also indicating delayed tail resorption (Fig 3.7). Additionally, mass at metamorphosis was significantly less than controls at all concentrations of fluoxetine tested ($p = 0.024$) (Fig. 3.8).

Malformations and Behavior

Malformations were observed at all concentrations of fluoxetine tested. In 0.059, 0.295, 2.95, and 29.5 ppb fluoxetine treatments, percentages of tadpoles with a malformation were 58, 58, 83, and 83, respectively. No malformations occurred in negative controls, while 58% of positive controls (ammonium perchlorate) were malformed. Mean times to the appearance of a malformation decreased with increasing fluoxetine concentration, and tadpoles exposed to 29.5 ppb fluoxetine took significantly less time to develop malformations than other treatments ($p < 0.001$) (Fig. 3.9). Of the tadpoles exposed to 29.5 ppb fluoxetine, 67% developed dorsal flexure of the tail within the first 25 days of exposure. These individuals grew and developed much slower than other tadpoles in the same treatment and displayed abnormal swimming behavior in which tadpoles floated upside down at the surface of the water and swam upside down in circles when prodded. Later in development, these tadpoles and other members of the 29.5 ppb fluoxetine treatment developed bilaterally symmetrical micromelia of the forelimbs. Malformations in lower concentrations of fluoxetine occurred during metamorphic climax and included bilaterally symmetrical micromelia of the forelimbs, as well as bilaterally symmetrical primary rotations of the hind limbs (Figs. 3.10, 3.11).

Discussion

Exposure to fluoxetine did not cause acute mortality, but did lead to significant mortality later in development at all concentrations tested. Most of the tadpoles died during metamorphic climax, a critical stage in development when significant physiological and anatomical changes take place within a very short period of time. Disruption of these processes due to serotonergic or purported anti-thyroidal properties of fluoxetine may have contributed to mortality in later

stages of development. It is also important to note that 80% cumulative mortality was observed at the lowest concentration of fluoxetine tested (0.059 ppb), which is an environmentally relevant concentration. In environments receiving inputs of fluoxetine, long-term survival of amphibian larvae may be affected.

The results of this experiment support our hypothesis that fluoxetine has the ability to delay metamorphosis in developing frogs. All animals, including controls, took longer to complete metamorphosis than in a preliminary exposure to fluoxetine (Chapter 2). The exposure took place during the winter months when temperatures in the laboratory were approximately four degrees cooler than they were in the summer (Chapter 2), and this likely explains why all of the tadpoles took longer to complete metamorphosis. Regardless of lower temperatures, metamorphosis of tadpoles exposed to 29.5 ppb fluoxetine was significantly delayed compared to negative controls. This concentration is considerably higher than would be expected to occur in the environment. During early development, tadpoles exposed to 29.5 ppb fluoxetine seemed to experience developmental delays comparable to the positive control, ammonium perchlorate. Tadpoles in these two groups completed forelimb emergence at approximately the same time, however tadpoles exposed to 29.5 ppb fluoxetine completed metamorphosis faster than those exposed to ammonium perchlorate. While both 29.5 ppb fluoxetine and 9.9 ppb ammonium perchlorate significantly delayed metamorphosis in this species, ammonium perchlorate was a stronger inhibitor. This is not surprising, considering that ammonium perchlorate prevents the synthesis of thyroid hormones by blocking iodide uptake by the thyroid gland (Wolff 1998). The mechanism by which fluoxetine inhibits the thyroid axis is not certain, but probably involves interference with TRH or TSH (Golstein et al. 1983; Jackson and Luo 1998), which control the release of thyroid hormones from the thyroid gland and do not influence iodination.

Nevertheless, our results indicate that fluoxetine does delay metamorphosis in *Xenopus laevis*. Additional studies with fluoxetine demonstrating delayed metamorphosis accompanied by indicators of depressed thyroid function are needed to confirm that fluoxetine delays metamorphosis by inhibiting the thyroid axis.

Fluoxetine also reduced mass at metamorphosis at all concentrations tested. The lowest concentration of fluoxetine that produced metamorphs with significant size reduction was 0.059 ppb, which is well within the range of concentrations that have been reported in freshwater aquatic systems (0.012 – 0.099 ppb) (Kolpin et al. 2002; Metcalfe et al. 2003). Reduced size and mass at metamorphosis may have serious fitness implications for some amphibians. Smaller metamorphs of some species are more vulnerable to predation by gape-limited predators (Caldwell et al. 1980). In some species, reduced size at metamorphosis has also been associated with increased times to reach sexual maturity and therefore longer times to first reproduction (Smith 1987; Semlitsch et al. 1988). Smaller metamorphs with lower mass may have a lower probability of reaching sexual maturity and reproducing successfully.

The high incidence of malformations observed in this experiment was unexpected. In a preliminary experiment using a similar range of nominal fluoxetine concentrations, malformations were not observed (Chapter 2; Rogers and Black 2003). In the preliminary experiment, we used embryos of adults bred in our laboratory, whereas in the current experiment, tadpoles were obtained from a commercial supplier. Genetic differences between test organisms and increased sensitivity of purchased larvae to additional stressors (i.e. fluoxetine exposure) may explain why malformations occurred. In addition, lower temperatures lengthened the exposure time and the number of replicates was increased from 5 to 12. Both of these factors may have enhanced the probability of observing malformations. Although there have been very

few studies conducted with fluoxetine, malformations have been also observed in Japanese medaka (Brooks et al. 2003a). Concentrations were similar those used in this study, but the incidence of malformations in fish was much lower (2.24% at 5 ppb). In our study, at least 58% of tadpoles at all concentrations of fluoxetine tested developed malformations.

The ability of fluoxetine to produce a high incidence of limb malformations in *Xenopus laevis* tadpoles may be related to the anti-thyroidal properties of this compound. Retinoids are necessary for normal limb development, and disruptions in retinoid metabolism and signaling pathways have been shown to cause malformations in amphibians (DeYoung et al. 1991; Johnson and Scadding 1991; Maden 1997). Heterodimers that influence transcription of thyroid-regulated genes are composed of thyroid hormone and retinoid-X- receptors (Denver 2002), and interactions between these two receptors have been linked to limb malformations (Maden and Corcoran 1996). Fort et al. (1999) found that sediment extracts from a contaminated site in Minnesota induced limb malformations in *X. laevis* and that addition of thyroxine in concurrent exposures significantly reduced the incidence of limb malformations. In addition, known thyroid antagonists, including PCB 126, dithiocarbamates, propylthiourea, and ammonium perchlorate have induced malformations in developing amphibians (Fort et al. 1999; Gutleb et al. 1999; Goleman et al. 2002). These studies provide strong evidence that thyroid inhibition is linked to the development of limb malformations. Fluoxetine-induced malformations observed in this study further support our hypothesis that fluoxetine inhibits the thyroid axis in developing amphibians.

Conclusions

Fluoxetine delayed metamorphosis in *Xenopus laevis*, but not at an environmentally relevant concentration. Delayed metamorphosis may have been caused by reductions in thyroid hormone levels produced by the purported anti-thyroidal properties of fluoxetine. Future studies aimed at measuring thyroid hormone levels in tadpoles during exposure will establish a definitive link between fluoxetine and thyroid inhibition in amphibians. Exposure to environmentally relevant concentrations of fluoxetine produced significant effects including chronic mortality, reduced mass at metamorphosis, and limb malformations. Although the life cycle of *Xenopus laevis* differs from North American species, our results suggest that the presence of fluoxetine in aquatic habitats may have the potential to affect the survival and fitness of native anurans. Further research using native species is therefore warranted.

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Table 3.1. Summary of water quality parameters measured bi-weekly during the exposure.

	Temperature (°C)	Dissolved Oxygen (mg/L)	pH	Conductivity (µS/cm)	Ammonia (mg/L)	Hardness (mg/L as CaCO ₃)	Alkalinity (mg/L as CaCO ₃)
Control	18.6 ±1.9	7.53 ±0.17	7.02 ±0.06	1029 ±26	<0.2	95 ±1.16	51 ±0.04
Fluoxetine	18.4 ±2.5	7.41 ±0.23	7.07±0.08	997 ±52	<0.2	94 ±1.21	52 ±0.18
Ammonium Perchlorate	18.7 ±2.1	7.46 ±0.09	7.04±0.04	1066 ±35	<0.2	95 ±1.07	51 ±0.67

Data are means ±SD.

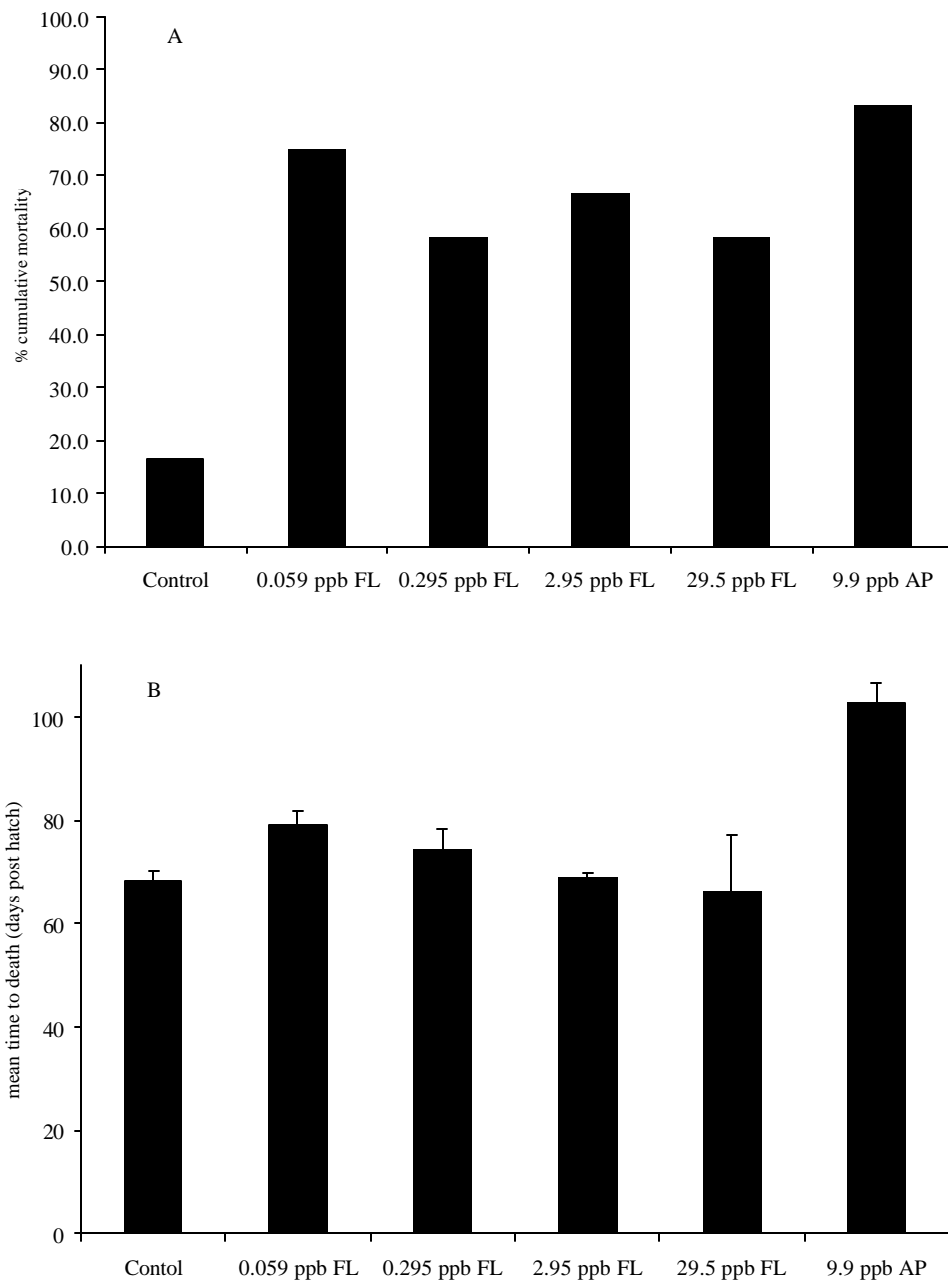


Figure 3.1. Plots of cumulative percent mortality (panel A) and mean time to death (in days post hatch) of tadpoles exposed to fluoxetine (FL) and ammonium perchlorate (AP) (panel B).

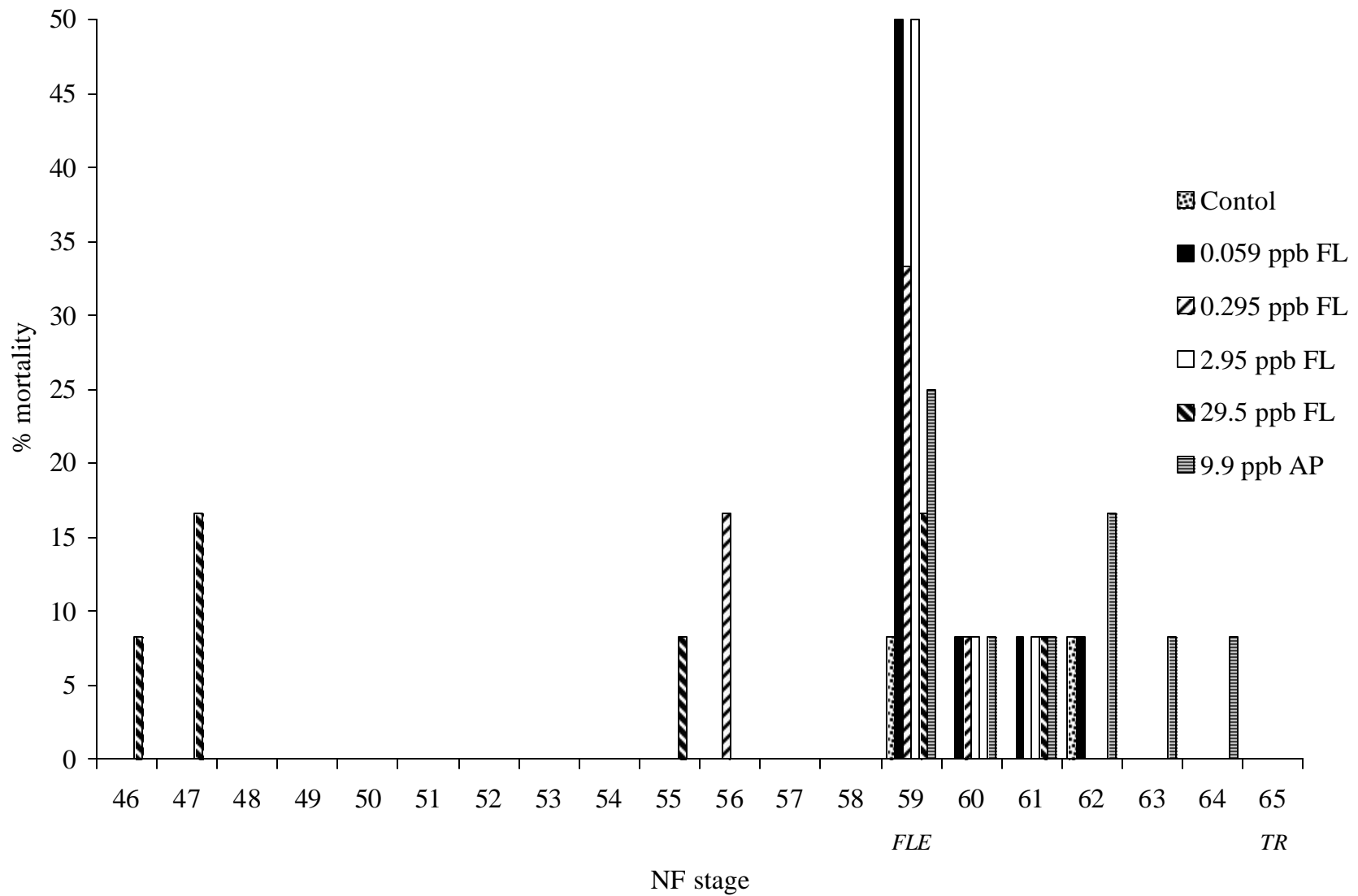


Figure 3.2. Percent mortality by developmental stage. FLE = forelimb emergence, TR = tail resorbtion, NF refers to the staging system of Nieuwkoop and Faber (1994).

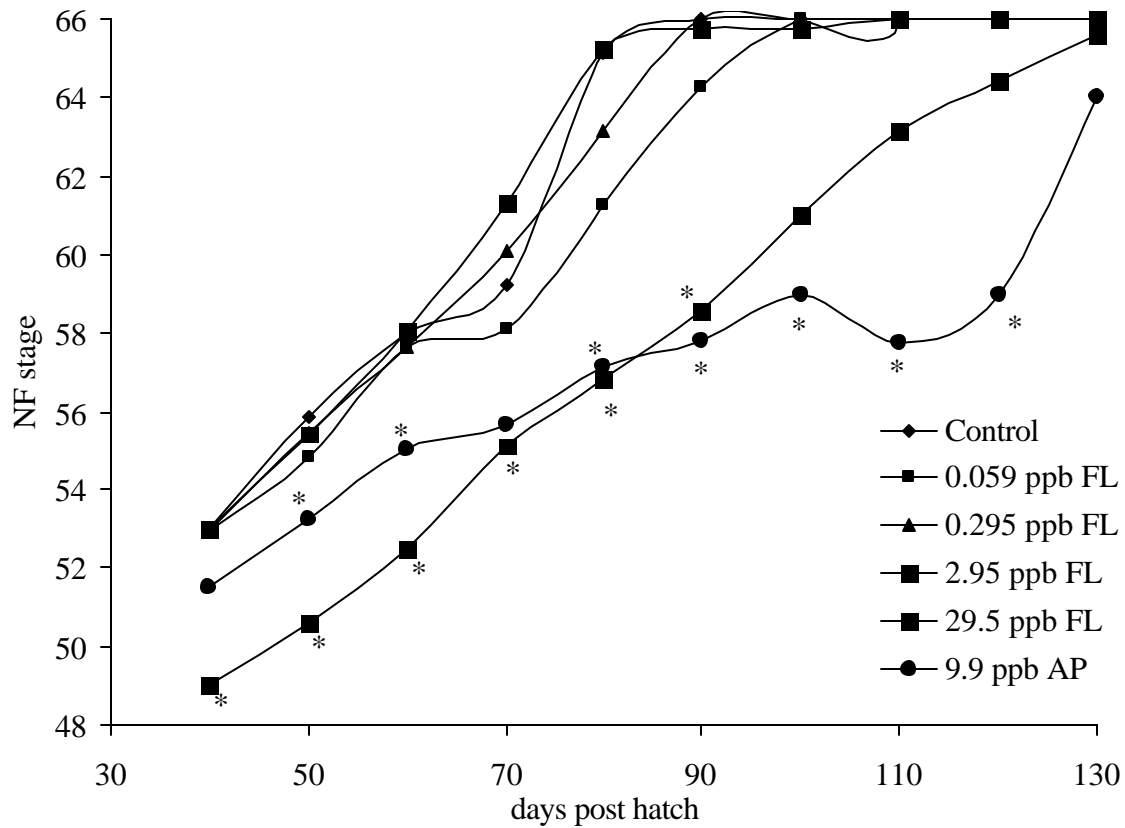


Figure 3.3. Developmental stages of tadpoles by days post hatch. FL = fluoxetine, AP = ammonium perchlorate, NF refers to the staging system of Nieuwkoop and Faber (1994), * = significantly different from control at $\alpha = 0.05$.

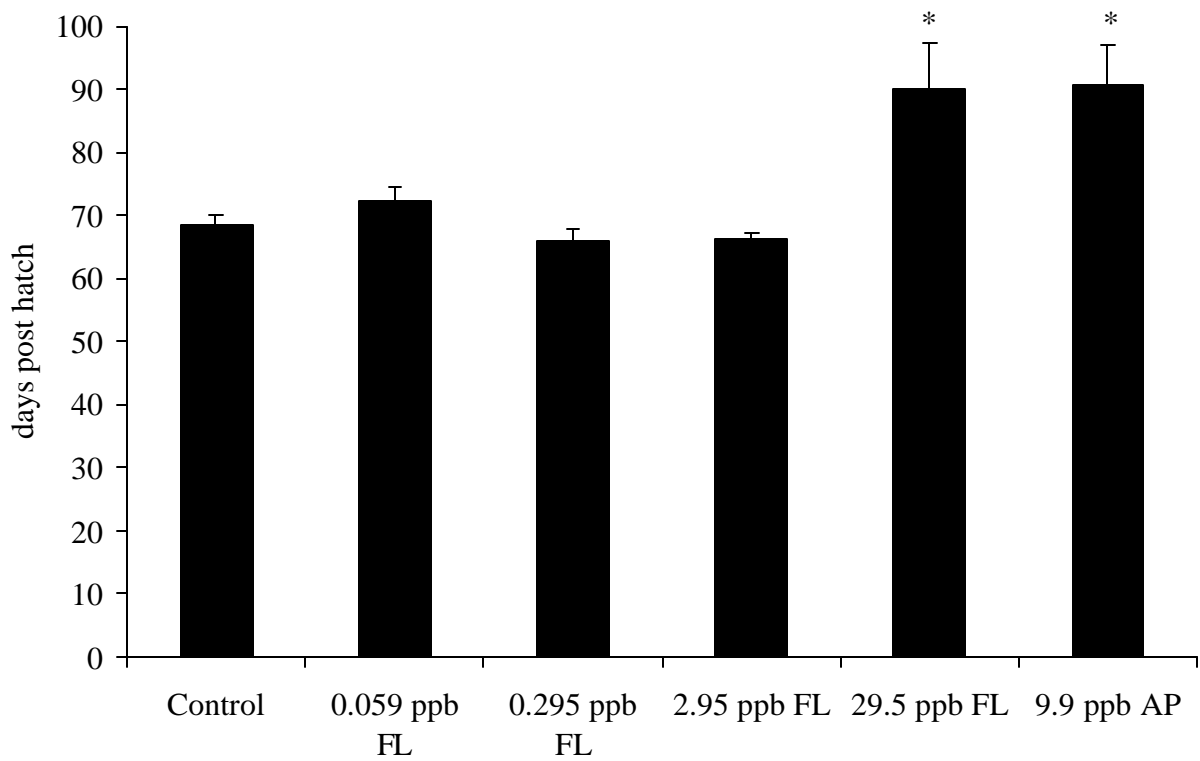


Figure 3.4. Mean times to forelimb emergence in days post hatch. FL = fluoxetine, AP = ammonium perchlorate, * = significant difference from control (FL $p = 0.012$) (AP $p = 0.001$).

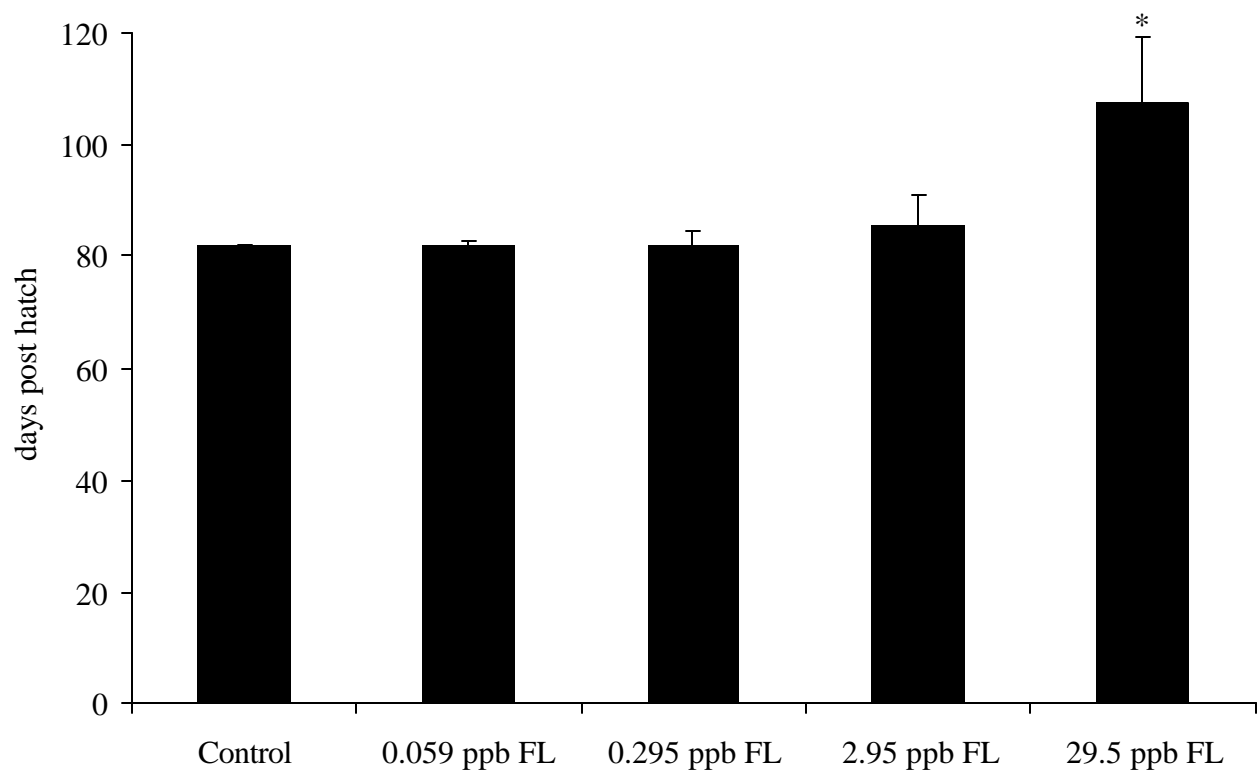


Figure 3.5. Mean time to completion of metamorphosis by days post hatch. FL = fluoxetine, * = significantly different from control ($p = 0.022$).

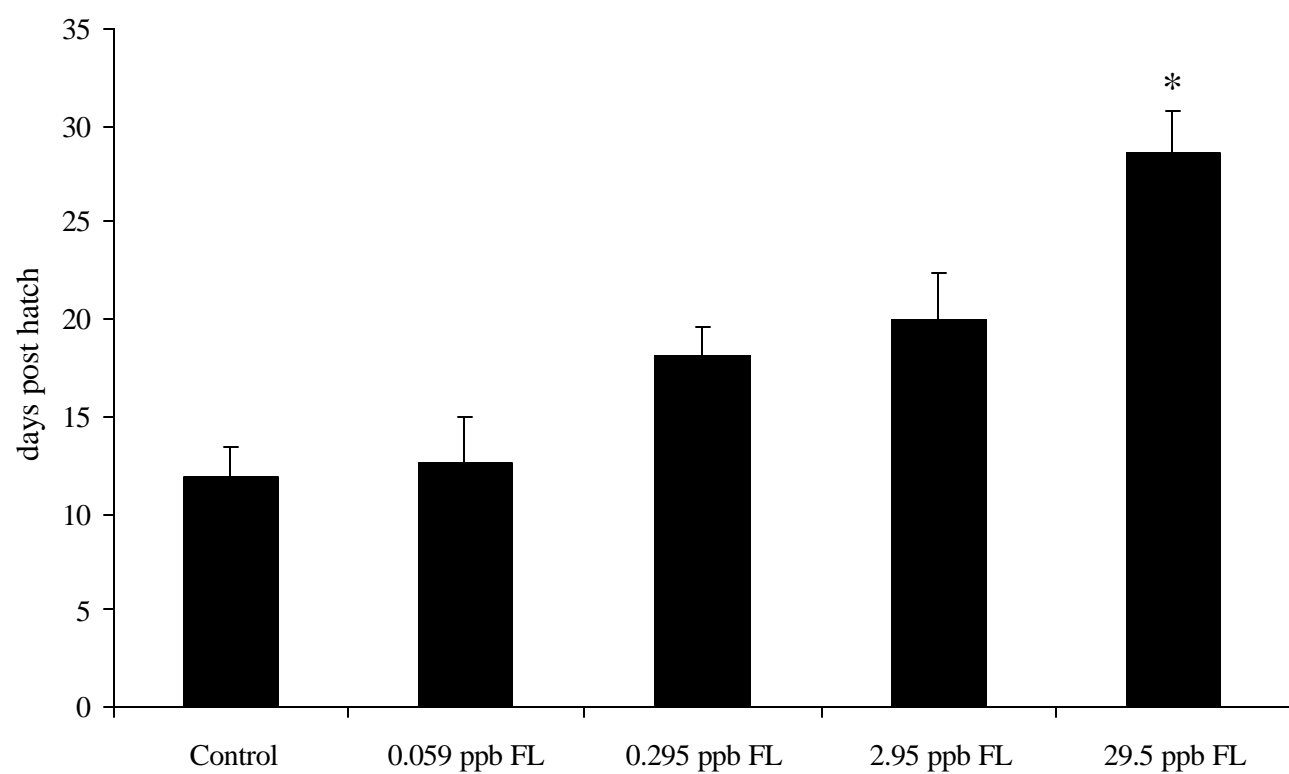


Figure 3.6. Mean tail resorption time in days post hatch. FL = fluoxetine, * = significantly different from control ($p < 0.001$).

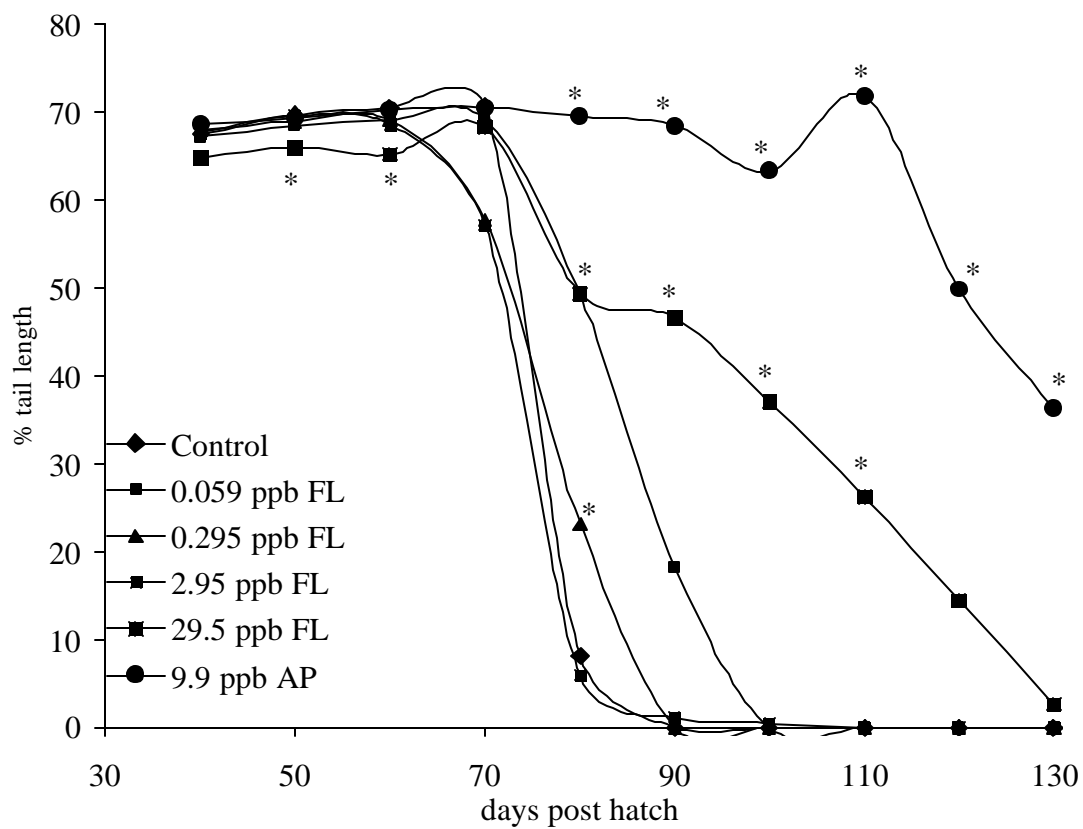


Figure 3.7. Changes in tail length as a percentage of total body length by days post hatch. FL = fluoxetine, AP = ammonium perchlorate, * = significantly different from control at $\alpha = 0.05$.

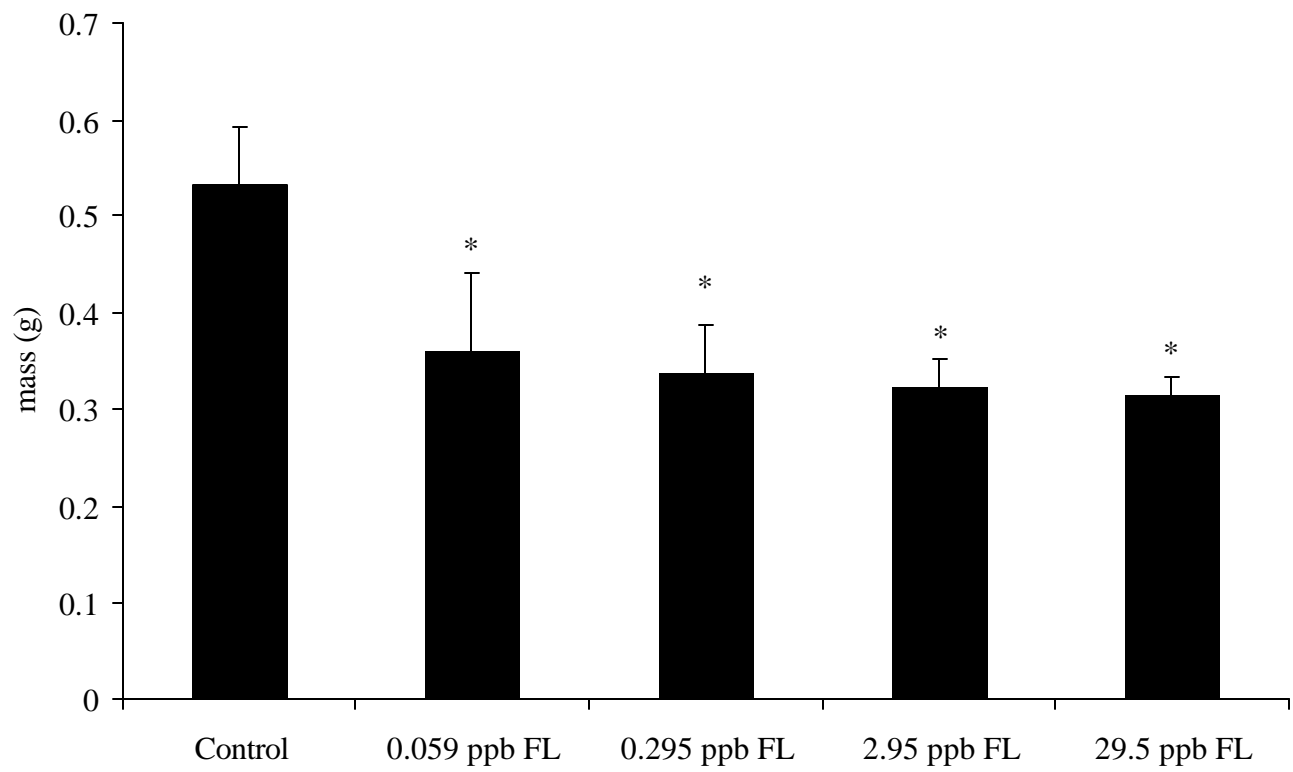


Figure 3.8. Mean mass of tadpoles upon completion of metamorphosis. FL = fluoxetine, * = significantly different from control at $\alpha = 0.05$.

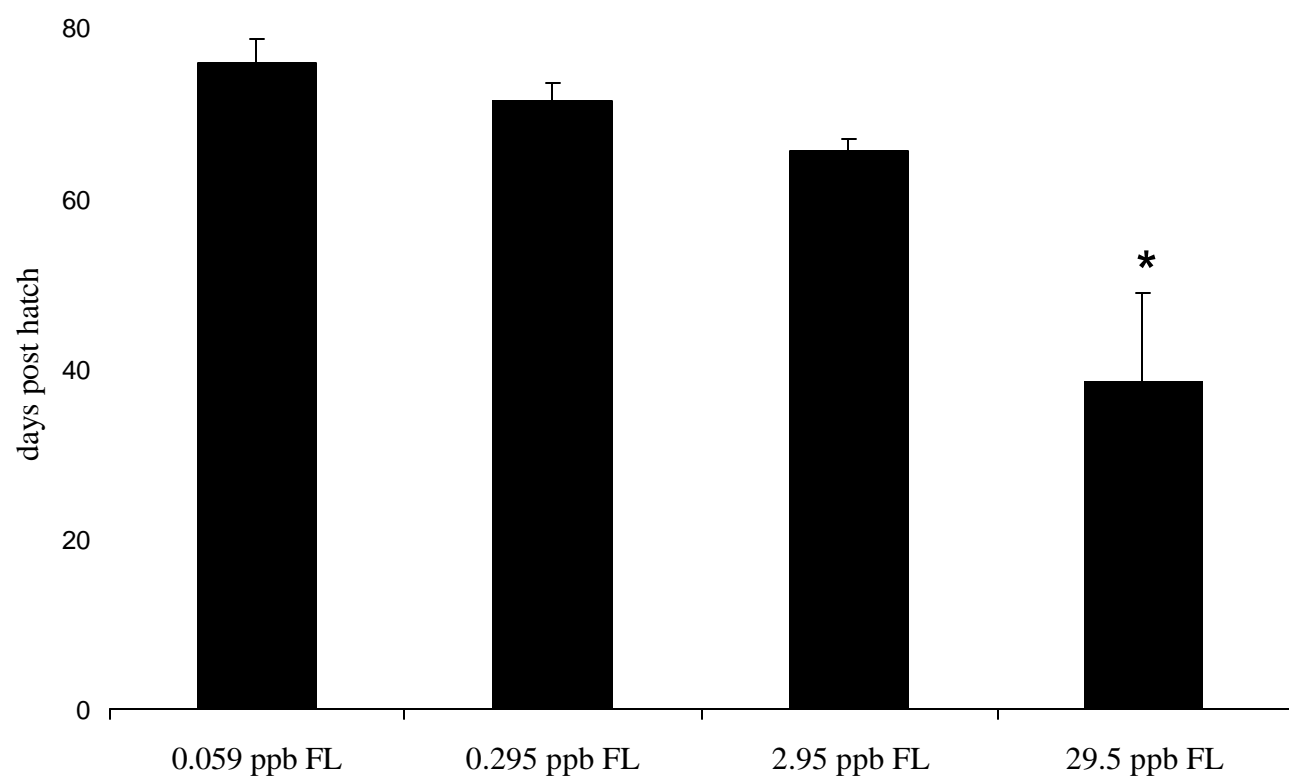


Figure 3.9. Mean times to development of malformations in days post hatch. FL = fluoxetine, * = significant difference ($p < 0.001$).

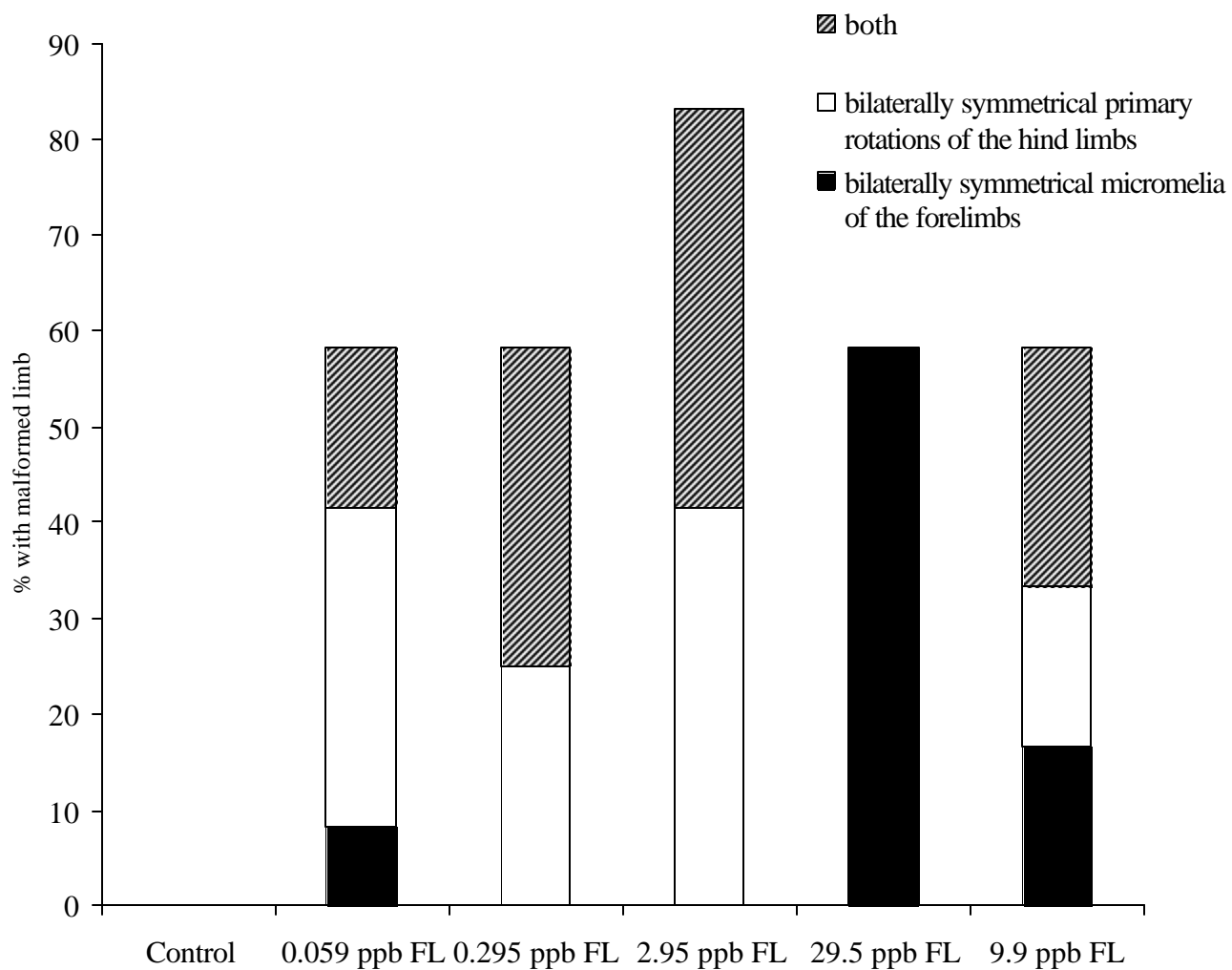


Figure 3.10. Percentages of tadpoles with limb malformations. FL = fluoxetine, AP = ammonium perchlorate.



Figure 3.11. Digital images of malformed *Xenopus laevis* larvae exposed to fluoxetine. Malformations (clockwise from upper left): bilaterally symmetrical primary rotations of the hind limbs; bilaterally symmetrical primary rotations of the hind limbs and bilaterally symmetrical micromelia of the forelimbs; dorsal flexure of the tail; bilaterally symmetrical micromelia of the forelimbs.

CHAPTER 4

CONCLUSION

This research indicates that fluoxetine affects survival, development, and metamorphosis in *Xenopus laevis*. In a preliminary range-finding experiment (Chapter 2), exposure to fluoxetine resulted in mortality and delayed metamorphosis of tadpoles. Mass at metamorphosis was significantly reduced as well. In a definitive experiment (Chapter 3), fluoxetine also delayed metamorphosis and reduced mass at metamorphosis; however higher mortality and malformations not observed in the preliminary experiment were also seen. Heightened effects in Chapter 3 may be explained by genetic differences in test organisms. Tadpoles obtained from a commercial supplier may have been more sensitive than lab-bred organisms, and transportation stress may have contributed to increased mortality, especially that observed at the beginning of the experiment. Increased sensitivity of purchased larvae to additional stressors (i.e. fluoxetine exposure) may also explain malformations, as no malformations were observed in negative controls. In addition, lower temperatures lengthened the exposure time and the number of replicates was increased. Both of these factors may have enhanced the probability of observing malformations.

The results of both experiments confirm our hypothesis that exposure to fluoxetine delays metamorphosis in *Xenopus laevis*. Delayed metamorphosis was likely due to reductions in thyroid hormone levels produced by the anti-thyroidal action of fluoxetine that has been

demonstrated in mammals (Golstein et al. 1983; Jackson and Luo 1998). Limb malformations observed in Chapter 3 may also provide evidence for thyroid axis inhibition by fluoxetine in amphibians, as malformations have been associated with other anti-thyroidal compounds (Fort et al. 1999; Gutleb et al. 1999; Goleman et al. 2002). Future research is necessary, however, to establish a definitive link between fluoxetine exposure and thyroid axis inhibition in amphibians.

In order to assess the potential risks to developing amphibians living in areas receiving effluent containing low concentrations of fluoxetine, it is necessary to evaluate the major findings of this research in an environmentally realistic context. Because fluoxetine has been found at such low levels in the environment (e.g. 0.012 – 0.099 ppb), delays in metamorphosis are unlikely to occur. However, effects that occurred at environmentally relevant concentrations (mortality, reduced mass at metamorphosis, and malformations) may adversely affect native amphibians. Because *Xenopus laevis* is not a native species and does not enter the terrestrial environment following metamorphosis, extrapolation of effects to native species can be made but should be interpreted with caution. Mortality resulting from chronic exposure to fluoxetine may reduce the number of individuals that complete metamorphosis and enter the terrestrial environment. Malformations may interfere with locomotion (Hopkins et al. 2000) and the ability to escape predators. Reduced mass at metamorphosis may also increase vulnerability to predation (Caldwell et al. 1980), and delay sexual maturation as well as reproduction (Smith 1987; Semlitsch et al. 1988). Our results indicate that exposure to environmentally relevant concentrations of fluoxetine may have the potential to reduce long term survival and fitness of native anurans.

This research provided the first evaluation of fluoxetine exposure on amphibian development and metamorphosis. Because effects were seen at environmentally relevant

concentrations, further study on this topic is warranted. Although delays in metamorphosis did not occur at environmentally relevant concentrations, future studies aimed at measuring thyroid hormone levels in tadpoles during exposure will attempt to establish a definitive link between fluoxetine and thyroid inhibition. This finding may have implications for other aquatic organisms whose development is also influenced by thyroid hormones. Because the life cycle of *Xenopus laevis* differs considerably from North American species, research using native species will be conducted to better characterize the effects of fluoxetine on native anurans.

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